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# Lytic switch protein (ORF50) response element in the Kaposi's sarcoma-associated herpesvirus K8 promoter is located within but does not require a palindromic structure

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**Abstract**

Kaposi's sarcoma-associated virus (KSHV) ORF50 protein induces lytic replication and activates the K8 promoter. We show that ORF50-induced and tetradecanoyl phorbol acetate (TPA) induced K8 transcripts initiated from the same start site. A newly identified palindrome (PAL2), containing a 12-bp response region required for ORF50-induced activation in lymphoid cells, was identified in the K8 promoter. Specific DNA binding of bacterially expressed ORF50 was not seen with the K8 promoter despite specific binding to the PAN promoter. The new palindrome shared homology with a previously described ORF50 response element ($50RE_{K8}$ and $50RE_{57}$). We demonstrate that the new $50RE_{K8}$ ($50RE_{K8\text{-}PAL2}$) is not the palindrome per se. Instead, the response element is buried within the right arm of the palindrome. We propose that the complexity of the K8 response elements reflects the complexity of mechanisms used by ORF50 during viral reactivation.


*Keywords:* Kaposi's sarcoma-associated herpesvirus; Lytic switch protein; Virus replication; Gene expression regulation; Viral transcription activator; ORF50; K8 promoter; Response element; DNA-binding protein; Palindrome

## Introduction

Human herpesvirus 8 (Kaposi's sarcoma-associated virus, KSHV) is found within Kaposi's sarcoma cells (KS) (Chang et al., 1994; Zhong et al., 1996) and is now considered its etiologically agent. KS is an angiogenic proliferative disorder that is prevalent in individuals with AIDS and other forms of immune suppression. It is endemic in some Mediterranean countries and certain regions of Africa. KSHV is also associated with primary effusion lymphoma (PEL) (Cesarman et al., 1995) and multicentric Castleman's disease (Gessain et al., 1996; Soulier et al., 1995). Sequence analysis indicates that KSHV is related to primate γ-herpesviruses, Epstein–Barr virus (EBV), and herpesvirus saimiri (HSV) (Russo et al., 1996).

γ-Herpesviruses establish a latent infection characterized by limited viral gene expression. EBV is the prototypical γ-herpesvirus and reactivation of the EBV lytic cascade has been extensively studied (Adamson et al., 2000; Flemington, 2001; Hardwick et al., 1988; Holley-Guthrie et al., 1990; Jenkins et al., 2000; Kenney et al., 1989; Miller, 1989; Quinlivan et al., 1993; Ragoczy and Miller, 1999a; Segouffin et al., 1996; Sinclair et al., 1991; Zalani et al., 1996). The lytic gene cascade is initiated after the expression of the EBV R and Z proteins encoded by the BRLF1 and BZLF1 genes, respectively. Both of these proteins are transactivators and each one is necessary for full activation of EBV early gene expression. The Z protein transactivates the BRLF1 promoter by binding to specific Z-responsive elements (Adamson and Kenney, 1998; Flemington and Speck, 1990; Quinlivan et al., 1990; Rooney et al., 1989; Sinclair et al., 1991). While R has been shown to bind to GC-rich regions in several EBV genes (Gruffat et al., 1992; Quinlivan et al., 1993), activation of the BZLF1 promoter is mediated through activation of MAP-kinase pathways rather than direct binding of R to this promoter (Adamson et al., 2000).

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The ORF50 gene of KSHV is a positional homologue of the EBV BRLF1 and HVS ORF50 genes (Russo et al., 1996). The K8 gene of KSHV is a positional homologue of the EBV BZLF1 gene (Lin et al., 1999; Seaman et al., 1999). Transfection of latently infected PEL cell lines with ORF50 expression vectors is sufficient to activate the lytic cascade of gene expression from transcription of early genes to the production of viral particles. KSHV genes which have been examined for ORF50 responsiveness include the following: ORF50 (Sakakibara et al., 2001); thymidine kinase (Zhang et al., 1998); PAN (Lukac et al., 1998; Song et al., 2001); ORF57 (Duan et al., 2001; Lukac et al., 1998, 2001) and K8 (Lukac et al., 1998; Seaman et al., 1999). At least some of the ORF50 induced-gene expression is mediated by binding to specific DNA sequences (Rta response elements, RREs, ORF50 response element in *n* promoter, $50RE_n$) in several of these promoters.

We previously examined gene expression patterns and gene expression controls in the ORF50 region (Seaman et al., 1999). K8 expression is one of the earliest genes expressed following ORF50 expression. We identified two K8 transcripts and characterized the delayed-early expression pattern of these transcripts. ORF50 expression activated both ORF50 and K8 promoter sequences. Domain mapping experiments revealed a 100-aa C-terminal ORF50 activation domain. K8 promoter elements contributing to ORF50 activation have only been partially characterized. To better define ORF50 induction of K8 expression, we examined ORF50 binding to the promoter and performed reporter assays and mapping experiments. In this article, we show that expression of ORF50 resulted in transactivation of the K8 promoter in the absence of additional KSHV gene products, and the transcriptional initiation site was identical to the site used during TPA-induced viral reactivation. A 12-bp element contained the major response region required for ORF50-induced activation in lymphoid cells. We expressed ORF50 as a bacterial fusion protein and demonstrated that this protein is able to bind to the response element in the PAN promoter. Despite this, specific binding was not seen with the K8 promoter. Sequence analysis revealed that the 12-bp response element resided within an inverted repeat or palindrome (PAL2). Homology between PAL2 to a previously identified palindrome response element ($50RE_{K8}$ and $50RE_{57}$ and designated PAL1 in this article) was seen (Lukac et al., 2001; Wang et al., 2001a). We demonstrated that the response element ($50RE_{K8\text{-}PAL2}$) did not require preservation of the palindrome.

## Results

### *Transfection of cells with pCMV-50 results in ORF50 protein production*

To examine the effect of isolated ORF50 expression on gene expression, we expressed full-length ORF50 protein from a cDNA insert directed by the CMV immediate-early promoter. In western blot analysis a 120-kDa band was detected in cytoplasmic and nuclear extracts of TPA-treated BCBL-1 cells and pCMV-ORF50 transfected Cos-7 cells using polyclonal antibody (gift of D. Ganem) (Lukac et al., 1998). These bands had a higher molecular weight compared to in vitro translated ORF50, demonstrating posttranslational processing (data not shown).

### *Activation of K8 promoter by ORF50 does not require additional KSHV gene products in lymphoid cells*

To determine the K8 transcription initiation site utilized by ORF50, we performed primer extension assays. The template clone of K8 (pK8GEN) contained a KSHV genomic 503-bp DNA fragment from 74,348 to 76,738, which included 503-bp upstream of the transcription start site (74,845) used during phorpol ester induction of BCBL cells (Fig. 1A). The transcript initiation site during TPA induction of lytic replication from latent KSHV in BCBL-1 cells served as the positive control reaction (Fig. 1B). The primer K8PRXT (complement of 74,932–74,950) detected a single band in pK8GEN- and pCMV-50-transfected DG-75 cells (Fig. 1C), based on a DNA sequence ladder generated using pK8GEN as a template and K8PRXT as a sequencing primer. The bands seen in TPA-treated BCBL-1 cells and pCMV-50-transfected DG-75 cells corresponded to identical transcript start sites (nucleotide 74,845). The transcription induced by ORF 50 occurred in the absence of other viral gene products.

### *K8 promoter elements responsive to ORF50 span −110 to −100 nt from the transcription start site*

Serial deletions of the K8 promoter region used in the primer extension experiments and linked to a luciferase reporter gene were used to identify the region of the K8 promoter involved in ORF50-dependent transactivation (Fig. 2A). β-Galactoside expression from transfected expression vector was used to normalize the results for variations in transfection efficiency. Cotransfection of HeLa cells with pCMV-50 and pK8Luc-532 resulted in a 76-fold increase in luciferase activity. A 143-fold increase in luciferase activity was observed in cells cotransfected with pCMV-50 and pK8Luc-187. The construct with 106 bp of upstream sequence was completely unresponsive to transactivation by ORF50. Removal of −187 to −146 significantly reduced responsiveness. To confirm the significance of the −146 to −106 region of the promoter, fragments of the K8 promoter were placed upstream of the minimal adenovirus E1b promoter and chloramphenicol acetyltransferase (CAT) gene. Use of the −187 to −35 promoter region conferred responsiveness to ORF50. However the region identified as significant in the 5′ deletion experiments (−187/−106) did not convey ORF50 responsiveness

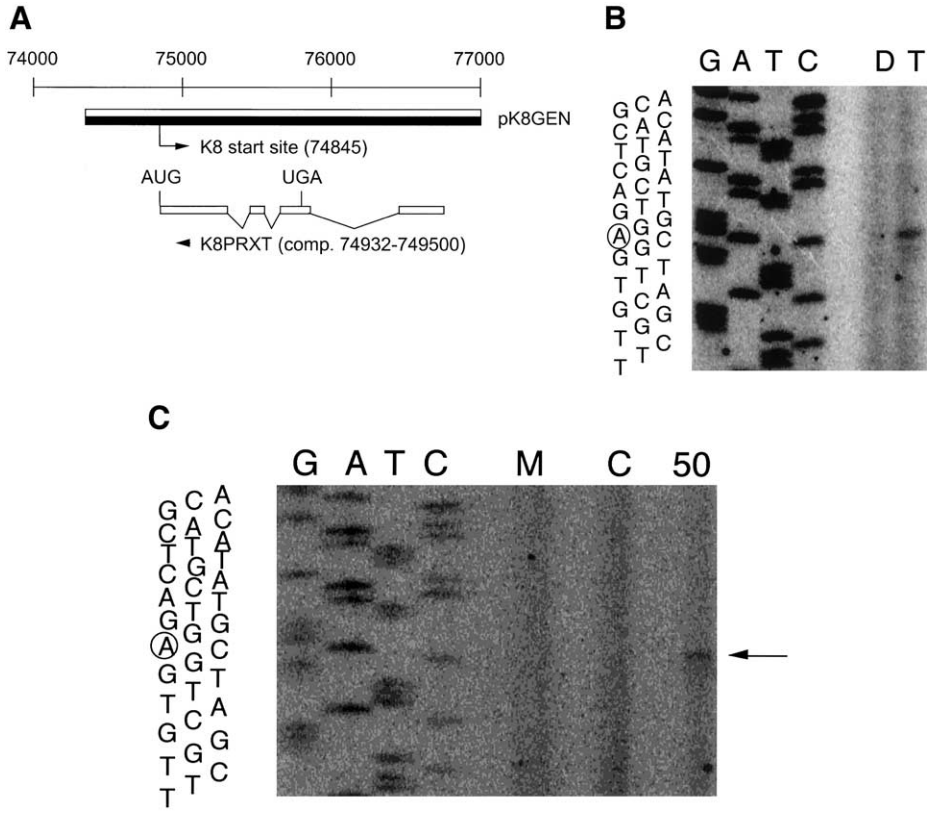



Fig. 1. ORF50 protein activates the K8 promoter in the absence of other viral proteins. (A) Schematic diagram of K8 genomic clone, pK8GEN. The top line indicates the region of the KSHV genome contained in pK8GEN. The arrow indicates the transcription start site. The structure of the major K8 transcript is shown below. The arrowhead indicates the primer (K8PRXT, complement of 74,932–74,950) used for the primer extension experiments shown in (B) and (C). (B) Identification of K8 mRNA in BCBL-1 by primer extension. BCBL-1 cells were treated with either DMSO (D) or TPA (T). Labeled K8PRXT primer hybridized with a transcript with a start site at 74,845 bp, as indicated by the circled codon. (C) DG-75 cells were mock transfected (M) or cotransfected with pK8GEN and either pcDNA3 (C) or pCMV-50 (50). Labeled K8PRXT again hybridized to RNA initiating at 74,845 (circled codon).

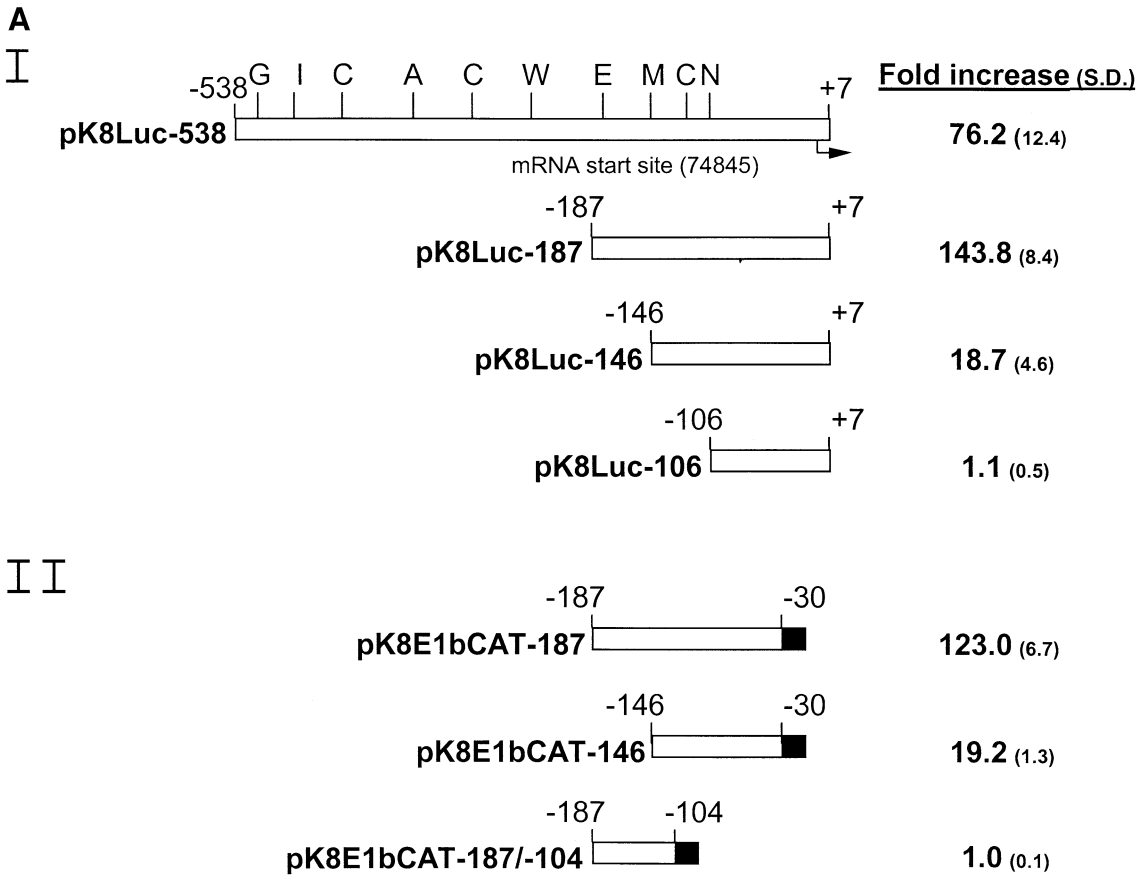
to the reporter gene construct. The (−146/−35) fragment was able to convey partial ORF50 responsiveness (19-fold).

To further our deletion analysis, small internal deletions were constructed using pK8Luc-187 (Fig. 2B). An 18-bp deletion corresponding to a potential E2F transcription-binding site (pK8LucΔ1) failed to reduce K8 promoter responsiveness to ORF50. Neither a 5-bp substitution (pK8LucMA) nor a 10-bp deletion in a potential NF-κB site (pK8LucΔ2) significantly decreased luciferase activity. Deletions, which removed both the E2F and the NF-κB sites (pK8LucΔ3), abrogated 80% of ORF50 responsiveness in HeLa cells. Only a modest reduction was seen in cotransfected DG-75 cells (50% of wild-type). A palindrome sequence (PAL1) that acts as an ORF50 responsive element (50RE) has been identified in the K8 promoter (Lukac et al., 2001; Wang et al., 2001a). Deletion of this region in pK8LucΔ4 resulted in a 50% decrease in luciferase activity in HeLa cells when compared to pK8Luc-187-transfected cells but no decrease was observed in DG-75 cells. A second palindrome sequence (PAL2) was identified upstream of the PAL1 site (−137/−98). Deletion of 10 bp at the 3′ end of this palindrome (pK8LucΔ5) resulted in a reduction in luciferase activity in both HeLa and DG-75 cells (≤20%).

### *Bacterially expressed ORF50 aa 1–377 binds to PAN promoter*

To determine whether ORF50 protein bound to the K8 promoter, ORF50 cDNA encoding amino acids 1–377 was cloned into the pGEX-2T (Pharmacia) (Fig. 3A). Cultures of *Escherichia coli* containing either pGEX-2T or pGEX50ΔC were induced with IPTG to express recombinant protein. Crude bacterial lysates were obtained and subjected to immunoblotting with antibody to GST (Fig. 3B). A 68-kDa band was detected in crude lysates obtained from IPTG-treated *E. coli* containing pGEX50ΔC. A 27-kDa band corresponding to GST protein was detected in lysates from *E. coli* containing pGEX-2T.

We used a 30-bp probe corresponding to the PAN RRE (PAN2) (Song et al., 2001) to determine if the fusion protein, GST50ΔC, had DNA-binding activity. Two bands were detected when lysate containing GST50ΔC protein was used in DNA-binding reactions (Fig. 3C). Preincuba-




**B**

| | % pK8Luc-196 activation (S.D) | |
|---|---|---|
| | HeLa | DG-75 |
| pK8Luc-187 (-187 E M C N +7) | 100 | 100 |
| pK8LucΔ1 (-187 -157 -139 +7) | 111 (39.3) | 82 (17.3) |
| pK8LucΔ2 (-187 -59 -48 +7) | 73 (13.5) | 108 (29.3) |
| pK8LucΔ3 (-187 -157 -139 -59 -48 +7) | 18 (9.0) | 55 (15.1) |
| pK8LucΔ4 (-187 -76 -60 +7) | 50 (2.3) | 151 (94.7) |
| pK8LucΔ5 (-187 -110 -100 +7) | 13 (9.7) | 20 (1.1) |
| pK8LucMA (-187 -59tccGa-55 +7) | 53 (9.7) | 114 (20.7) |
| pK8LucMB (-187 -68tccga-64 +7) | 72 (1.4) | 132 (43.7) |

tion of GST50ΔC with unlabeled PAN2 oligomer resulted in reduced binding by GST50ΔC to PAN RRE. In contrast, preincubation of GST50ΔC with the irrelevant oligomer, GBL, failed to inhibit the formation of the GST50ΔC/PAN2 complexes. Preincubation of the GST50ΔC-containing lysate with anti-GST antibody before addition of PAN2 probe resulted in the disappearance of the major and minor band but anti-E2F antibody had no effect on the binding of the GST50ΔC to the PAN2 probe (Fig. 3D).

*Bacterial ORF50 does not bind to K8 promoter fragments*

Three overlapping segments of the K8 promoter (Fig. 4A) were used as probes in electrophoretic mobility shift assays (EMSAs) to determine if ORF50 bound the K8 promoter elements. No bands were detected by EMSA when either GST or GST50ΔC lysate were incubated with K8 A/B or K8 H/C probes (Fig. 4B). Five bands were detected when GST50ΔC lysate was incubated with K8 G/I probe. These bands were also present when GST lysate was used in the DNA-binding reaction. Binding reactions with the $50RE_{PAN}$ showed specific binding to GST50ΔC in each experiment.

*ORF50 responsiveness is regulated by positive and negative cis-acting elements located in PAL2*

To more completely characterize ORF50 activation, we performed fine mapping experiments in the ORF50 response region(s) (Fig. 5A). Again, an increase in luciferase activity was seen in DG-75 cells when the PAL1 was altered (132% with pK8LucMB). PAL2 consisted of a 39-bp sequence with four distinct regions. The external regions, I (−131 to −137) and IV (−98 to −104), are 7 bp in length and complementary to each other. The internal regions II (−121 to −125) and III (−111 to −115) are 5 bp in length and complementary to each other. There are 5-bp intervening sequences that separate each of the four regions. Transversion of nt −100 to −104 in region IV of the palindrome (pK8LucMC) decreased ORF50-dependent transactivation in both HeLa and DG-75 cells (6 and 18% of the intact promoter, respectively). Finer mapping of this 5-bp region was done by making sequential 2-bp mutations. In addition complimentary mutations were made in region I. The 2-bp substitution made at −103 and −104 in region IV (pK8LucMD) resulted in a significant decrease in transfected HeLa cells (16%) but only a moderate decrease in DG-75 cells (57%). In contrast, the complementary mutation made at −131 and −132 in region I of the palindrome (pK8LucME) increased activity in DG-75 cells. The 2-bp mutation at −102 and −101 in region IV (pK8LucMF) and the complimentary mutation at −133 and −134 increased in both HeLa and DG-75 cells (165–213%). While the mutations at −99 and −100 in region I (pK8LucMH) resulted in a decrease in activity in HeLa cells (46%), an increase in activity was again seen in DG-75 cells (135%). Removal of a potential MEF2-binding site (pK8LucMJ) in region II (−125 to −121) produced an increase in luciferase activity in both HeLa and DG-75 cells. Mutation of the complementary region (region III) of the palindrome at −111 to −114 (pK8LucMI) resulted in a decrease in activity in transfected DG-75 cells (37%).

The K8 promoter fragment, which spanned the PAL2 region in the EMSAs, described above, interacted with a least five bacterial proteins. To determine if ORF50-specific binding was present but masked by the bacterial proteins, we constructed smaller oligomer probes for additional binding experiments. Again, GST-ORF50 bound efficiently to the PAN promoter response element (Fig. 5B). Neither the PAL1 nor the PAL2 probes demonstrated specific binding activity. It was possible that the promoter fragments disrupted the binding site and the oligomer probes were not large enough to include the entire ORF50-binding site. To address this possibility, we examined ORF50 binding to a probe with the entire K8 promoter (K8-200). A similar probe containing the PAN promoter was also constructed (PAN-200). Binding activity was easily seen with the PAN probe but not with the K8 probe (Fig. 5C). We concluded that ORF50 activation of the K8 promoter did not require direct ORF50 binding and was regulated positively by the right arm of the PAL2 palindrome.

## Discussion

The ORF50 gene of KSHV is a homologue of the EBV BRLF1 and the HVS ORF50 genes. The encoded protein is

Fig. 2. Mapping ORF50 response regions in the K8 promoter. Deletion analysis of the K8 promoter. (A) Various regions of the K8 promoter were PCR amplified and cloned into the reporter plasmids, pGL2-Basic or pE1bCAT. The map positions for each K8 promoter construct were determined relative to the K8 transcription start site. HeLa cells were cotransfected with pCMV-50 and each of the K8 promoter constructs. The position of potential cellular transcription factor-binding sites shown above the pK8Luc-538 construct were identified with MatInspector computer program. Transcription factor abbreviations: A, AP-1; C, CREB; E, E2F; M, MEF2; N, NF-κB; W, winged helix protein. I: ORF50-induced fold increase in luciferase activity was determined relative to activity in cells cotransfected with each of the luciferase K8 promoter constructs and pcDNA3 and is indicated to the right of each construct. The−187/−106 region contained elements responsive to ORF50. β-Galactosidase expression from a transfection expression vector was used to control for variations in transfection efficiency. II: The region identified in (I) was transferred to a adenovirus-minimal promoter construct. Fold increase in CAT activity was determined relative to activity in cells cotransfected with each of the K8 CAT constructs and pcDNA3. The K8 promoter regions required for ORF50 activation did not transfer ORF50 responsiveness to an Elb adenovirus minimal promoter construct. (B) Small deletions were generated by PCR using pK8Luc-187 as a template. The stippled box indicates the ORF57-like palindrome (PAL1). The black box indicates a newly identified palindrome. The location of potential CREB, E2F, MEF2, and NF-κB binding sites in pK8Luc-187 are also shown. Deleted regions of each promoter construct are shown on the left half of the figure. Luciferase activity relative to the wild-type promoter was determined in HeLa and DG-75 cells and the results are shown to the right of each construct. Disruption of the region−110/−100 produced the greatest disruption in ORF50 responsiveness.

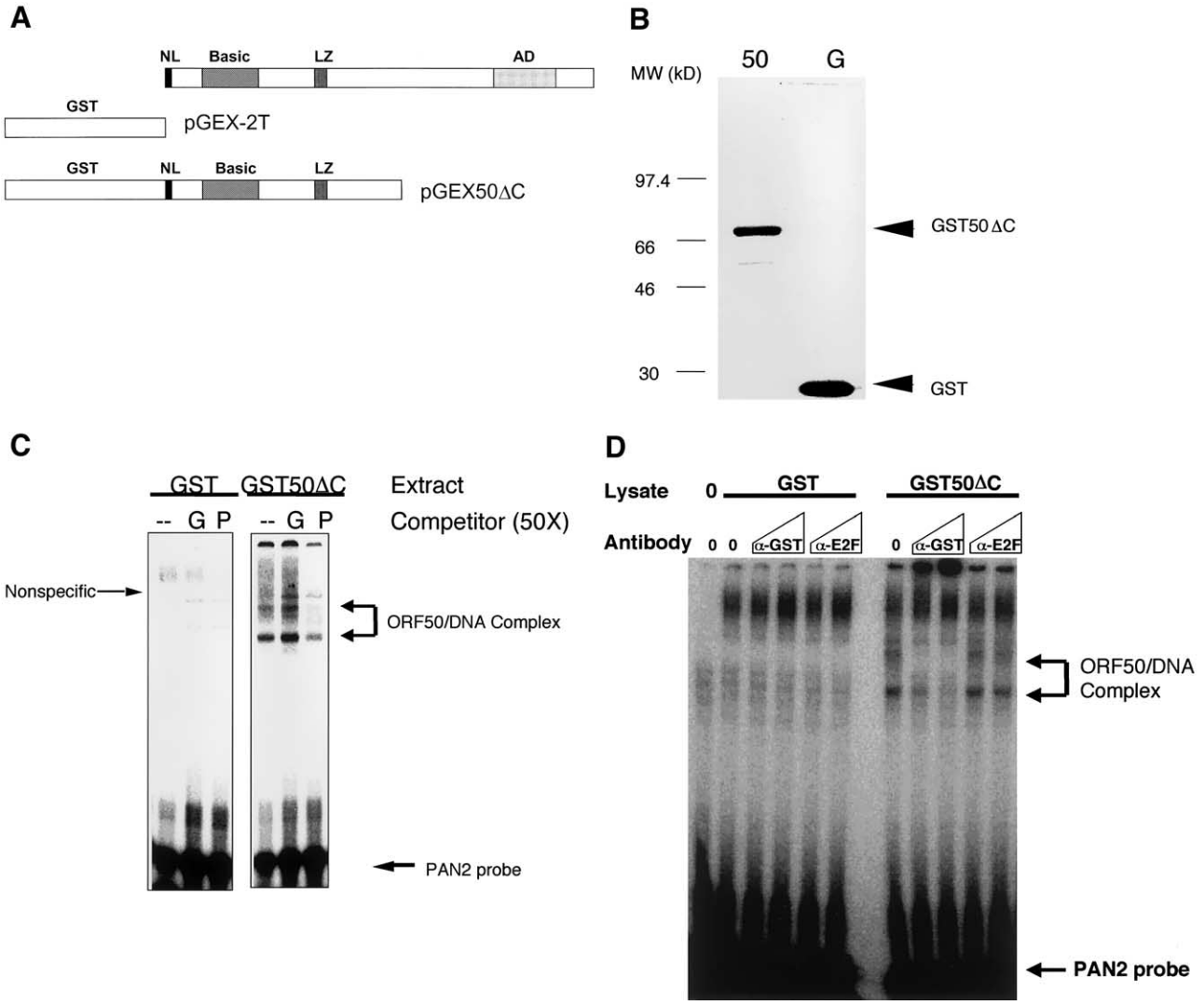



Fig. 3. Recombinant GST50ΔC protein binds to the ORF50 RRE present in the PAN promoter. (A) Cartoon of the construction of the ORF50-GST fusion protein expression vector. NL: Nuclear localization; Basic: DNA-binding domain; LZ; Leucine zipper; AD: activation domain. (B) Immunoblot of GST-ORF50 and GST using GST-specific antibody. Bacterial lysates are indicated above each lane. Predicted size of the protein is shown on the right (MW: molecular weight). (C) PAN oligomer competes for GST50ΔC binding. Bacteria extracts were incubated with 50X unlabeled oligomers for 30 min prior to the addition of labeled PAN2 probe. Competitors are as follows: G: oligomer corresponding to a region of pGL2-Basic (vector); P: PAN2. Binding reactions were subjected to polyacrylamide gel electrophoresis and binding complexes were identified by phosphoimaging. Arrows indicate GST ORF50-specific DNA complexes. (D) Bacterial lysates containing either GST or GST50ΔC were incubated with labeled PAN2 oligomer. For supershift assays lysates were preincubated with either anti-GST or anti-E2F polyclonal antibody for 30 min before addition of the PAN2 probe.

a transactivator of KSHV lytic gene expression and activates the lytic cascade of this virus (Gradoville et al., 2000; Lukac et al., 1998, 1999; Miller et al., 1997; Seaman et al., 1999). In this article we examined ORF50 activation of the K8 gene and identify novel sequences in the K8 promoter that are necessary for ORF50-dependent transactivation. ORF50 expression in KSHV-negative cells (DG-75 cells) initiated K8 mRNA transcription in the absence of other viral gene products. We identified a second K8 50RE, the PAL2 palindrome, which conveyed the majority of ORF50 responsiveness rather than the previously described $50RE_{K8}$. In contrast to the findings of others (Lukac et al., 2001; Wang et al., 2001b), we did not observe binding to K8 promoter sequences despite demonstrating sequence-specific DNA binding in the same experiments.

The discrepancy between our results and those of others regarding ORF50 binding to the K8 promoter may have been due to the systems used to produce the recombinant ORF50 proteins. Previously, a partially purified His-tagged ORF50 protein expressed by baculovirus was used to perform gel shifts. The protein used in this article was a GST/ORF50 fusion protein in crude bacterial extracts. We examined many sources of ORF50 for DNA-binding activity. In our hands, his-tagged bacterial ORF 50 and eukaryotic sources of ORF50 (transfected or transduced ORF50-expressing adenovirus) showed less binding activity than GST-50.

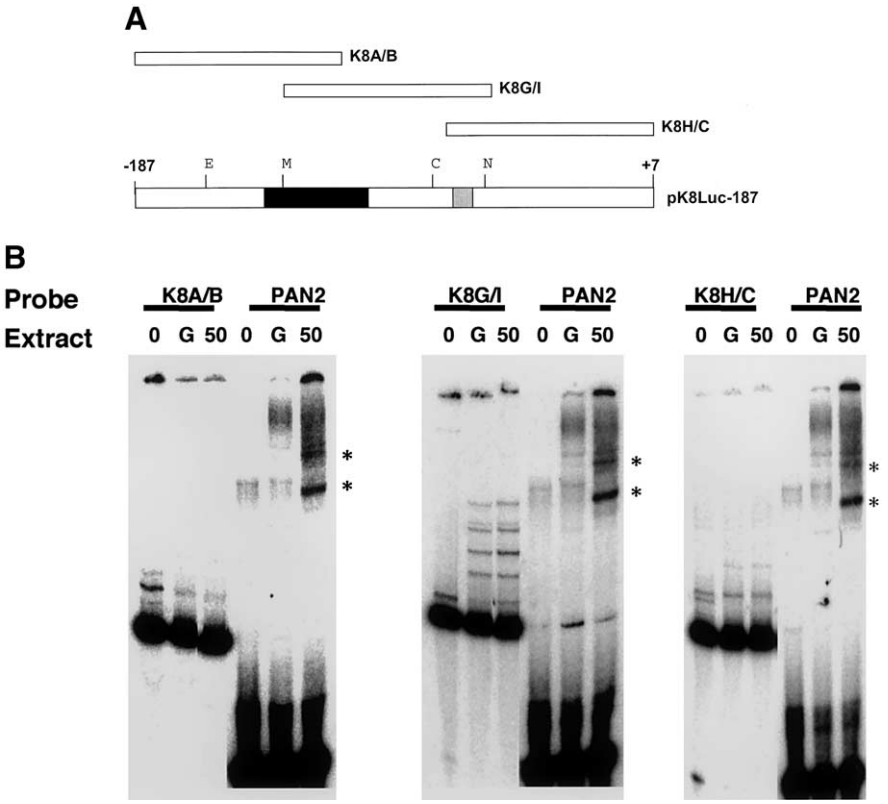



Fig. 4. GST50ΔC does not bind to the K8 promoter. (A) The top three lines indicate the position of K8 probes used for DNA-binding reactions. The fourth line is a schematic representation of pK8Luc-187. The stippled box indicates the position of the ORF57-like palindrome (PALI). The black box indicates the position of the upstream palindrome (PAL2) (E: E2F; M: MEF2; C: CREB; N: NFKB). (B) Either no extract (0), GST (G), or GST50ΔC (50) lysates were incubated with the K8A/B, K8GI, or K8H/C probes using PAN2-binding conditions. ORF50/DNA complexes are indicated (*) and are present within the PAN2 probe in each experiment. GST50/DNA complexes are not seen with K8 promoter fragments.

However we did establish that the slower mobility complexes represented specific binding by the GST-ORF50 protein. The ORF50/PAN-specific bands were competed by an oligomer containing the PAN ORF50 recognition site but were not competed by an unrelated oligomer. Additionally, antibody to the GST portion of the fusion protein but not antibody to E2F interfered with the formation of ORF50–DNA complexes. We concluded that complex formation between GST-ORF50ΔC and PAN involved direct protein–DNA binding, but ORF50 interactions with the K8 promoter are indirect.

Other sources of ORF50 protein may contain a cofactor(s) which facilitated K8 promoter and ORF50 binding. Sp1 and Oct-1 sites convey ORF50 responsiveness in the TK and ORF50 promoters, respectively (Sakakibara et al., 2001; Zhang et al., 1998). An MEF-2 (serum response factor family) DNA-binding site in the EBV BZLF-1 promoter is required for calcium-mediated activation (Liu et al., 1997). Sequence analysis of the K8 promoter identified many sites of potential interaction with known cellular transactivators. Deletion of either the E2F or the NF-κB sites failed to reduce ORF50-dependent transactivation regardless of cell type, suggesting that these sites, alone, are not sufficient for ORF50-dependent transactivation. The E2F site located between −187 and −146 nt of the promoter may explain the loss of promoter activity when this region was removed. It does not explain all of the decrease seen with the −146-nt construct since specific deletion of the E2F site reduced activity only when the downstream NF-κB site was also removed. Mutation of the potential MEF2 site identified in PAL2 of the K8 promoter resulted in an increase in ORF50-dependent transactivation. Here, the MEF2 site functioned as a repressor element and underscored the complex nature of K8 gene regulation.

Interactions between ORF50 and cellular factors are known to occur and may explain our findings. Interactions between both ORF50 and CREB-binding protein (CBP) and histone deacetylase (HDAC) have been reported (Gwack et al., 2001). CBP is a coactivator that forms a bridge between specific transcription factors and the basal transcription machinery and HDAC is an enzyme that deacetylates histones resulting in repression of transcription (Arias et al., 1994; Bannister and Kouzarides, 1996; Chrivia et al., 1993; Kamei et al., 1996; Ogryzko et al., 1996). These interactions may alter recruitment of promoter-specific transcription factors, basal transcription factors, or histone-controlled access to promoters. The activation domains of EBV BRLF1 and HVS ORF50 interacted with TATA binding protein (TBP)

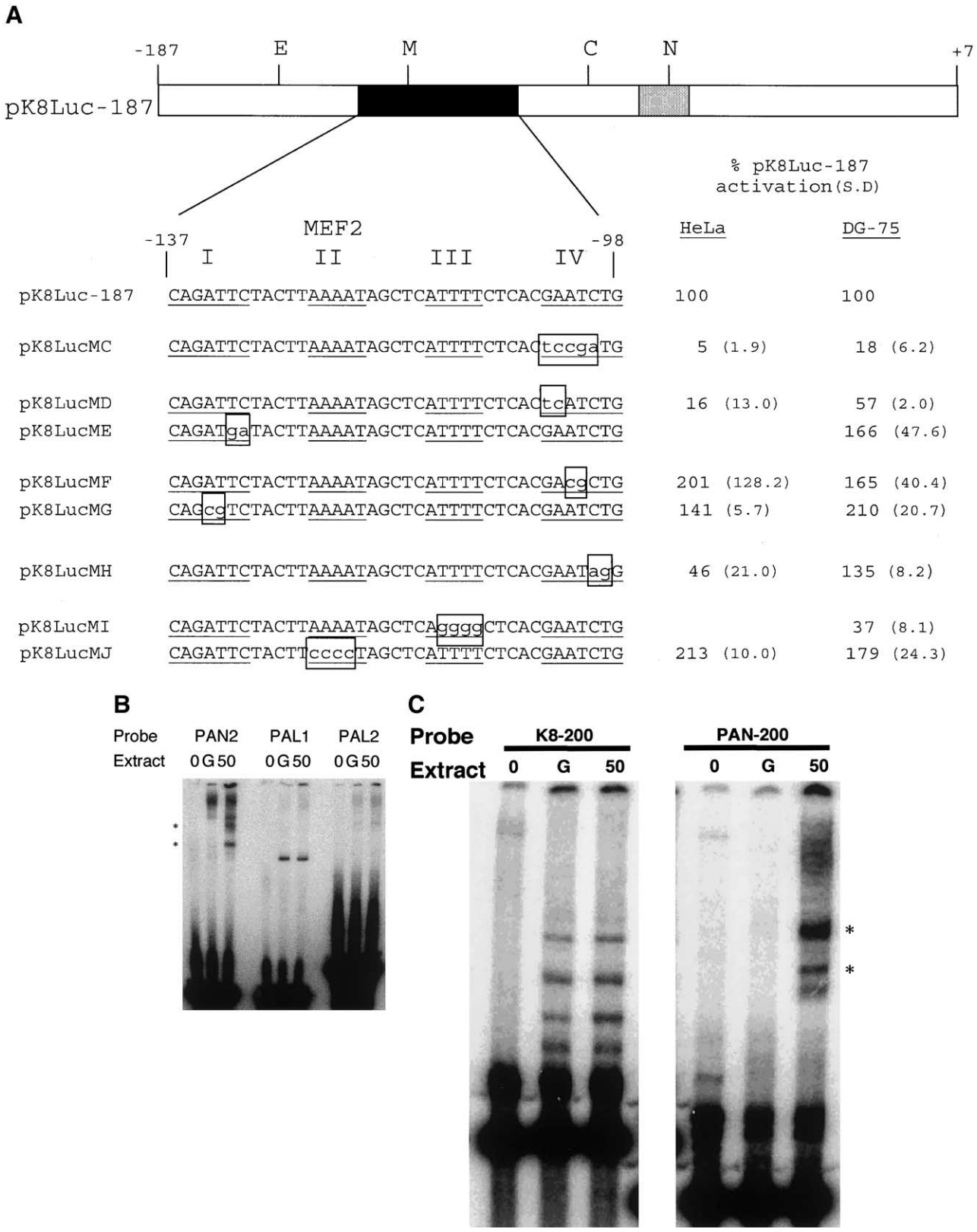



Fig. 5. Fine mapping of ORF50-responsive sequences in the K8 promoter. (A) Site-specific changes were made in the newly identified palindrome. The top line is a schematic representation of pK8Luc-187. The stippled box represents the ORF57-like palindrome (PAL1). The black box indicating the newly identified palindrome (PAL2) has been expanded to show the sequence. Underlined sequences representing regions I–IV show the position of PAL2. The potential CREB (C), E2F (E), MEF2 (M), and NF-κB (N) binding sites identified by MatInspector are shown above the sequence. Lowercase letters in boxes indicate nucleotide changes relative to pK8Luc-187. Luciferase activity is shown to the right of each construct and is expressed relative to pK8Luc-187 activity. Recombinant GST50ΔC does not bind either the PAL1 (P1) or the PAL2 (P2) present in the K8 promoter. No protein (0), GST (G), or GST50ΔC (50) protein was incubated with a labeled K8 probe corresponding to either K8 PAL1, K8 PAL2, or $50RE_{PAN}$ (PAN2) shown in (B) or a probe spanning 200 bp of the K8 (K8-200) or PAN (PAN-200) promoters shown in (C). GST ORF50/DNA complexes were only seen with PAN2.

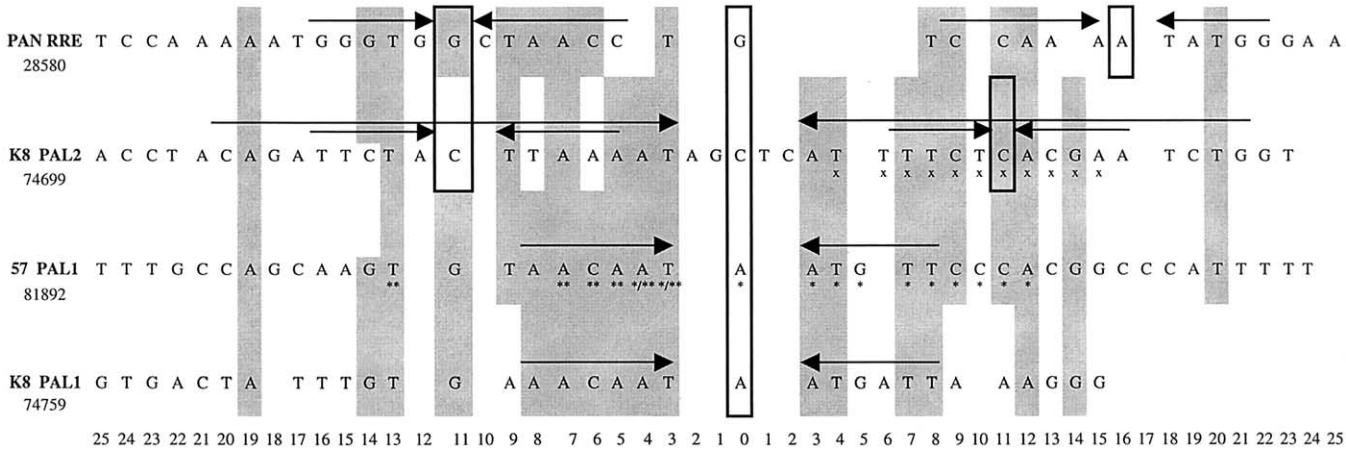



Fig. 6. Sequence comparison of various ORF50 response elements. The ORF50 response regions for K8 PAL2, K8 PAL1, ORF57 PAL1, PAN RRE. The genome location of each sequence is indicated below the name. Arrows = the location and direction of repeats; boxes = the center position of inverted repeats. Sequences required for ORF50 responsiveness previously identified by fine mapping are indicated by *(Lukac et al., 2001), **(Duan et al., 2001), and X (this article); and shading = sequence conserved in three of the four elements. Numbers at the bottom of the figure indicate position in the K8 PAL2 palindrome.

(Hall and Whitehouse, 1999; Manet et al., 1993). A novel cellular protein, K-RBP, was identified by specific interactions with ORF50 (Wang et al., 2001b). K-RBP is a member of the KRAB-zinc finger protein family, which uses the zinc finger motif to bind DNA. More recently, ORF50 has also been shown to interact with the RBP-J$\kappa$ protein and induce transcription through the Notch signaling pathway (Liang et al., 2002). Interactions with one or more of these factors may provide an indirect means of K8 activation.

Some sequence homology was identified when PAL2 was compared with PAL1 or $50RE_{57}$ (Fig. 6). The PAL2 sequence has not been previously identified as a K8 response element. Despite the homology, one notable difference between PAL2 and the other 50REs is the number of nucleotides between the palindrome arms. Divergence from the perfect palindrome sequence is known to affect the binding affinity of transcription factors and alter promoter strength (Klinger, 2001). The number of nucleotides between arms of the estrogen receptor element (ERE) has been shown to be a critical determinant of ERE responsiveness. The arms of PAL2 are separated by five nucleotides rather than the three nucleotides present in PAL1. We demonstrate a different pattern of responsiveness with PAL1 and ORF50 as compared to PAL2, possibly explained by the sequence structure of the 50REs.

Despite the occurrence of palindromic sequence in the 50RE, disruption of the right and left arms of PAL2 did not affect K8 responsiveness equally. An element spanning $-103$ to $-113$ in the 3′ end of PAL2 resulted in a decrease in ORF50-dependent transactivation. Constructs with the complementary mutations in the 5′ end did not have this effect. The second ORF50 response element is not the palindrome in which it is located but the response element is hidden within this structure. The MEF-2 site located within the left arm of the PAL2 site may explain the different effect of the two arms of the palindrome. Mutations in the 5′ end of $PAL1_{57}$ were also less effective in disrupting ORF50 responsiveness than those in the 3′ end. These observations provide evidence that the 5′ and 3′ portions of the repeated elements in ORF50 response elements have divergent roles.

While some homology between PAL1 and PAL2 50REs exists, we describe a number of differences. These include placement of cellular response elements in flanking regions and spacing of the two arms of the palindromes. Functional differences, as reflected by divergence in cell-line specific activity and the function of the arms of the palindromes, were present as well. Primary sequence and functional differences were also seen between the RE in the PAN promoter and those in K8 (sequence and variability in ORF50-binding activity). The structural and functional variability in ORF50 response elements indicates that ORF50 utilizes multiple mechanisms to maintain a tight control over KSHV lytic replication.

## Materials and methods

### *Cell lines*

BCBL-1 (KSHV-positive B-cell lymphoma) (Renne et al., 1996), DG-75 (EBV-negative, KSHV-negative Burkitt's lymphoma) cells were grown in RPMI. HeLa (cervical carcinoma) and Cos-7 (monkey kidney) cells were grown in Dulbecco's modified Eagle's medium (DMEM). All cells were grown with 10% fetal calf serum at 37°C in 5% $CO_2$.

### *Plasmids*

The construction of pCMV-50 was described previously (Seaman et al., 1999). The series of 5′ deletion K8 promoter constructs was created by inserting PCR-amplified regions

(Vent Polymerase, New England BioLabs) into pGL2-Basic (Promega) using the *Sma*I and *Sac*I sites: pK8Luc-538 (74,313 to 74,851) was created with HHV8.40 (5′ATCAGAGTCTATTCGCCC3′) and PK8C (5′GCGGAGCTCAATTTGGCAGGGTTACACGT3′); pK8Luc-187 (74,653 to 74,851) with PK8A (5′GCGCCCCGGGTT AACTTCCCAGGCAGTT3′) and PK8C; pK8Luc-146 (74,707 to 74,851) was constructed using a 153-bp *Hpa*I/*Sac*I fragment from pK8Luc-187; pK8Luc-106 (74,745 to 74,851) was constructed using a 113-bp blunt-ended *Hinf*I/*Sac*I fragment from pK8Luc-187. The fragments 74,667–74,810, 74,707–74,810, and 74,667–74,734 were inserted upstream of the adenovirus E1b minimal promoter element in the pGAL4E1bCAT reporter plasmid after removing the GAL4 DNA-binding elements, creating pK8E1bCAT-187, pK8E1bCAT-146, and pK8E1bCAT−187/−104 (Martin et al., 1990).

Small internal deletions were made by overlapping PCR according to the methods of Horton (Horton et al., 1989). For each mutant, two primary PCRs were performed. Mutant internal forward primers were used in forward primary PCRs with PK8C. Mutant internal reverse primers were used in reverse primary PCRs with PK8A. Each mutant internal forward primer was complementary to its respective reverse internal primer. Primary PCR products were gel purified, mixed, and used for secondary PCR. Since internal mutant primers were complementary, the 3′ end of single-stranded forward primary PCR products were capable of annealing to the 3′ end of single-stranded reverse primary PCR products at internal primer sequences. This annealing generated internal deletion mutant K8 promoter fragments after secondary PCR. All primary forward PCR products were generated with PK8C as the external primer. All reverse primary PCR were performed with PK8A as the external primer. Secondary PCR products were digested with *Sma*I and *Sac*I and inserted into pGL2-Basic. Except where indicated, pK8Luc-187 was used as the template in all primary PCRs. All mutants were sequenced: pK8LucΔ1 (deletion 74,687–74,696) was created with PK8LucΔ1F (5′GCAGTTTATTTTTAACAGAACCTACAGATTCTACTT3′, internal forward primer, InFP) and PK8LucΔ1R (5′AAGTAGAATCTGTAGGTTCTGTTAAAAATAAACTGA3′, internal reverse primer, InRP); pK8LucΔ2 (deletion 74,687–74,696): PK8LucΔ2F (5′TGTGAAACAATAATGATTATTTCCTCCGTTGTCGAC3′, InFP) and PK8LucΔ2R (5′GTCGACAACGGAGGAAATAATCATTATTGTTTCACA3′, InRP); pK8LucΔ3 (deletion 74,687–74,696 and 74,786–74,797) was made essentially as pK8LucΔ2 except that pK8LucΔ1 was used as the original template in the primary PCRs; pK8LucΔ4 (deletion 74,687–74,696): with PK8LucΔ4F (5′GGTTGATTGTGACTATTTAAAGGGGGTGGTATTTCC3′, InFP) and PK8LucΔ4R (5′GGAAATACCACCCCCTTTAAATAGTCACAATCAACC3′, RF); pK8LucΔ5 (deletion 74,687–74,696); PK8LucΔ5F (5′CTTAAAATAGCTCATTTTTGGTTGATTGTGACTATT3′, InFP) and PK8LucΔ5R (5′AATAGTCACAATCAACCAAAAATGAGCTATTTTAAG3′, InRP).

Site-specific base changes were made using pK8Luc-187 as a template according to the method of Ho et al. (1989). Mutant plasmids were generated by PCR with the following internal oligomers: pK8LucMA: PK8LucMAF (5′TGTGAAACAATAATGATTtccGaGGGTGGTATTTCC3′, forward primer, FP) and PK8LucMAR (5′GGAAATACCACCCtCggaAATCATTATTGTTTCACA3′, reverse primer, RP); pK8LucMB: PK8LucMBF (5′TTGTGAAACAtccgaGATTAAAGGG3′, FP) and PK8LucMBR (5′CCCTTTAATCtcggaTGTTTCACAA3′, RP); pK8LucMC: PK8LucMCF (5′ATTTTCTCACtccgaTGGTTGATTG3′, FP) and PK8LucMCR (5′CAATCAACCAtcggaGTGAGAAAAT3′, RP); pK8LucMD: PK8LucMDF (5′ATTTTCTCACtcATCTGGTTGA3′, FP) and PK8LucMDR (5′TCAACCAGATcaGTGAGAAAAT3′, RP); pK8LucME: PK8LucMEF (5′ACAGATgaTACTTAAAATAG3′, FP) and PK8LucMER (5′CTATTTTAAGTATcaTCTGT3′, RP); pK8LucMF: PK8LucMFF (5′TTTCTCACGAcgCTGGTTGATT3′, FP) and PK8LucMFR (5′AATCAACCAGcgTCGTGAGAAA3′, RP); pK8LucMG: PK8LucMGF (5′CTACAGcgTCTACTTAAAAT3′, FP) and PK8LucMGR (5′ATTTTAAGTAGAcgCTGTAG3′, RP); pK8LucMH: PK8LucMHF (5′TCTCACGAATagGGTTGATTGT3′, FP) and PK8LucMHR (5′ACAATCAACCctATTCGTGAGA3′, RP); pK8LucMI: PK8LucMIF (5′ATAGCTCAccccCTCACGAA3′, FP) and PK8LucMIR (5′TTCGTGAGggggTGAGCTAT3′, RP); pK8LucMJ: PK8LucMJF (5′CAGATTCTACTTccccTAGCTCATTTTC3′, FP) and PK8LucMJR (5′GAAAATGAGCTAggggAAGTAGAATCTG3′, RP). All primary forward PCR products were generated with PK8C as the external primer. All reverse primary PCR were performed with PK8A as the external primer. Primary PCR products were gel purified and used in secondary PCR. All secondary PCR products were digested with *Sma*I and *Sac*I and inserted into the *Sma*I/*Sac*I sites of pGL2-Basic.

pK8GEN was constructed by inserting a genomic region of KSHV from into pBluescript SKII. Primers HHVV8.40 and HHV8.49 (5′CGTGGAACGCACAGGTAAAG3′) were used to amplify K8 genomic DNA from BCBL-1 cells. This product was digested with *Apa*I (74,348) and *Eco*RI (76,469) and inserted into the *Apa*I/*Eco*RI sites of pBluescript SKII to create pK8GENΔPA. A 260-bp *Eco*RI/*Xba*I fragment corresponding to 76,470–76,738 was removed from pCMV-K8 and inserted into the *Eco*RI/*Xba*I site of pK8GENΔPA to obtain pK8GEN.

pGEX-50ΔC was constructed by inserting a *Bam*HI/blunt-ended *Acc*65I fragment from pCMV-50 into the *Bam*HI/blunt-ended *Eco*RI sites of pGEX-2T. Maintenance of the correct reading frame at the GST/ORF50 junction was confirmed by DNA sequencing.

### *DNA transfection*

BCL-1 cells were treated with tetradecanoyl phorbol acetate or vehicle alone. HeLa cells were seeded into six well plates at $2 \times 10^5$ cells/well 24 h before transfection.

Cells were transfected with 0.25 mg pCMV$\beta$ (Clontech), 1 $\mu$g of reporter plasmid, and either 1 $\mu$g of pcDNA3 or pCMV-50 using Superfect (Qiagen) according to the manufacturer's instructions. DG-75 cells were transfected by electroporation using a Bio-Rad gene pulser as previously described (Seaman et al., 1999).

*Primer extension*

DG-75 cells were cotransfected with pK8GEN and either pcDNA3 or pCMV-50. The primer, K8PRXT (5′TGCCCGTTGAGGCTTAGA3′), corresponding to the complement of 74,932–74,950, was mixed with 10 $\mu$g of total RNA (isolated at 48 h) and 0.5 mM deoxynucleotide mix, incubated at 75°C for 5 min, and slowly cooled to 42°C. Primer extension was performed at 42°C using Superscript II reverse transcriptase (Gibco-BRL). The DNA was precipitated with ethanol and resuspended in running buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.02% xylene cyanol), heated to 95°C for 10 min, and run on a 6% polyacrylamide sequencing gel containing urea. A DNA ladder was generated by sequencing pK8GEN with K8PRXT.

*Reporter gene assays*

CAT assays were performed as previously described (Seaman et al., 1999). Luciferase activity was determined 48 h after transfection, by adding 50 $\mu$l of cell lysate to 10 $\mu$l of luciferase substrate and determining luminescence using the luminometer of a Wallac VICTOR$^2$ multilabel counter (Luciferase assay kit, Promega). $\beta$-Galactosidase activity was determined with a Gal-Screen chemiluminescent reporter gene kit (Tropix) and used to normalize luciferase assay results.

*Electrophoretic mobility shift assay*

*E. coli* BL21 bacteria were transformed with pGEX-2T or pGEX-50ΔC. Bacteria were grown in LB medium containing 50 $\mu$g/$\mu$l ampicillin and grown at 37°C to an OD $A_{600}$ of 0.6–0.8. IPTG was added to a final concentration of 1 mM and cells were incubated at 30°C for 3 h. Bacterial lysates, prepared by sonication, were analyzed by SDS–PAGE, Coomassie blue staining, and immunoblotting with an anti-GST antibody (Sigma). EMSA was performed as described by Song (Song et al., 2001). Ten micrograms of crude bacteria extract was incubated with $^{32}$P-labeled probe in 1X binding buffer (10 mM Tris, pH, 150 mM KCl, pH 7.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 50 mM $\beta$-mercaptoethanol, 5 $\mu$g bovine serum albumin, 4 $\mu$g salmon sperm DNA) on ice for 30 min. For competition and supershift assays, lysates were incubated with cold competitor oligonucleotides or anti-GST antibody, respectively, in binding buffer on ice for 30 min prior to the addition of the $^{32}$P probe. Binding reactions were run on a 1X TGE, 5% polyacrylamide gel containing 5% glycerol at 4°C. Gels were dried and bands were detected by phosphorimaging. Oligomer used to construct probes were as follows: PAN2F-, 5′GGGAAATGGGTGGCTAACCTGTCCAAAATATG3′; PAN2R-, 5′GGGCATATTTTGGACAGGTTAGCCACCCATTT3′; K8PAL1F-, 5′GGGATTTGTGAAACAATAATGATTAAA3′; K8PAL1R-, 5′GGGCCCCTTTAATCATTATTGTTTCA3′; K8PAL2F-, 5′GGGAACCTACAGATTCTACTTAAAATAGCTCATTTTCTCACGAATCTG3′; K8PAL2R-, 5′GGGATCAACCAGATTCGTGAGAAAATGAGCTATTTTAAGTAGAATCTG3′.

*Western blot analysis of bacteria lysates GST50 C protein*

One hundred nanograms of crude bacteria was subjected to SDS–PAGE and proteins were blotted to nitrocellulose filter paper. The membrane was blocked by incubating with 1% blocking reagent (Roche) in 1X TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. The membrane was incubated with rabbit anti-GST antibody (Sigma) diluted 1:1000 in TBST for 1 h at room temperature. The membrane was washed with 1X TBST and incubated with a 1:1000 dilution of mouse anti-rabbit antibody conjugated to horseradish peroxide for 1 h at room temperature. The membrane was washed with 1X TBS (50 mM Tris, pH 7.5, 150 mM NaCl) and proteins were detected with an ECL western blotting detection kit (Amersham).

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