UCSF
UC San Francisco Electronic Theses and Dissertations

Title
Regulation of lytic gene expression in the Kaposi's sarcoma-associated herpesvirus

Permalink
https://escholarship.org/uc/item/4km84172

Author
Chang, Jean L.

Publication Date
1999

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

Regulation of lytic gene expression in the Kaposi's sarcoma-associated herpesvirus

by

Jean L. Chang

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Genetics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO



Date

University Librarian

Degree Conferred: .....

Copyright © 1999
by
Jean L. Chang

To Joe,

for his patience, good-humor, and understanding.

Acknowledgments

While experiments and results shape a graduate career, the personal and memorable experiences of graduate school are shaped by the day-to-day interactions with people. There are numerous people I must thank, without whom this thesis would not be possible. Sadly, I dare not try to create an exhaustive list else this thesis will never be completed. My apologies to those who are overlooked - the sieve-like qualities of my mind pre-date graduate school and I have yet to find a suitable remedy.

First and foremost, I must thank my graduate advisor, Don Ganem, for his unfailing support and encouragement. The breadth and depth of his knowledge, his scientific acumen, and his mastery of public speaking all raise a standard that I will always aspire to.

I would like to thank the members of my thesis committee, Carol Gross and Dave Morgan, for their insight and advice. To Patsy Babbitt, I would like to express my sincere appreciation for thoughtful conversations and the opportunity to broaden my horizons. My thanks to Sue Adams, Tom Porter, Nancy Biles and members of the Microbiology office for their help in simplifying my bureaucratic obligations.

I am indebted to Ganem lab members past and present for the delightful, stimulating, and productive environment that I have had the pleasure to work in over the years. Special thanks to: Dirk Dittmer for verbal skirmishes and all-

around pleasant company; Don Macrae for a wicked good sense of humor and a willing ear for sorrowful tales; the Mah Jong gang (aka. Shu-Chi Hsu, Joe Lin, and Dave Wang) for their friendship; Michael Lagunoff for advice and always having an answer; Andy Polson & Mallika Singh for letting me be their neighbor; Jessica Kirshner and Dave Lukac for sharing in the trials and tribulations of ORF50; Rob Brazas for boisterous fun and bad science movies; Delicia Caballero for her patience and good-nature; Laurent Coscoy for his help whenever I needed it; and Rolf Renne for his generosity and passion for good science. In remembrance of Rob Sadler, who was taken from us before his time.

Life at UCSF would have been dreary without the companionship of some wonderful friends - Tracy Ware, a true friend in weather fair or foul. Louise, Marc, and Clara Laurent, guiding the way by doing the impossible - juggling school, work, and family without breaking a sweat. Hernan Espinoza, a stalwart example that adults can be a kids too. Sean Bell, reminding me that graduate school can be FUN. Janet Molinari, every fiber a fast friend.

Although not physically at UCSF, my mother, father, and brother have been tangibly present in their love and support. Without them, I would be a lesser person. I am fortunate that I have had so many people to help me find my way but most importantly I must thank my husband, Joe Chou - my sustaining strength and inspiration.

Abstract

Regulation of lytic gene expression in the Kaposi's sarcoma-associated herpesvirus

by Jean L. Chang

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) plays an essential role in the development of KS. Several lines of evidence suggest that the lytic component of the viral life cycle is important for disease progression.

Using a B cell line, BCBL-1, which harbors KSHV in a latent state, a panel of cytokines was screened for their efficacy in viral reactivation. Certain cytokines were able to influence activation from viral latency. Specifically, interferon gamma was found to be a positive signal for induction of the lytic cycle while interferon alpha inhibited reactivation.

Reactivation involves the expression of immediate early (IE) proteins that direct the transcription of downstream targets. A genetic approach was used to identify two distinct types of motifs that are utilized by ORF50, a KSHV IE gene product, for the regulation of delayed early (DE) gene expression.

DE gene expression includes the synthesis of proteins involved in viral replication. In herpesviruses, viral DNA synthesis is a prerequisite for late gene expression. KSHV late promoters taken out of the viral context, however, were no longer

dependent upon viral replication for their expression. These results suggest that *in-cis* replication of the viral genome may be required for proper regulation of KSHV late gene expression.



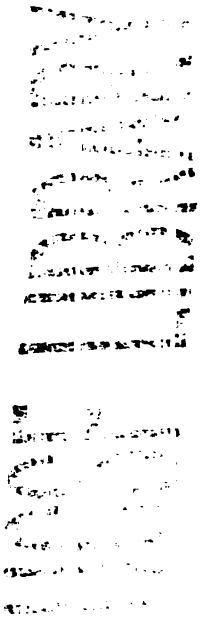


Table of Contents

Introduction 1

Kaposi's sarcoma - History 1

Early efforts to understand KS biology 2

Molecular Identification of KSHV 4

A model system for viral reactivation: BCBL-1 6

The herpesviral lytic life cycle - operational definitions 7

The latent to lytic switch 9

Late gene expression and viral replication 13

Overview of the thesis 14

Chapter 1 - Inflammatory cytokines and the reactivation of Kaposi's sarcoma-associated herpesvirus (KSHV) lytic replication 29

A quantitative assay system for lytic reactivation. 33

Screening of inflammatory cytokines for ability to induce KSHV 35

Effects of interferons on KSHV 36

Chapter 2 - KSHV ORF50 activation of delayed early promoters 62

Linker scanning mutagenesis reveals an ORF50 responsive element (50RE-1) in the Kaposin B promoter 66

The ORF50 responsive element of the kaposin promoter is also present and necessary for ORF50 activation of the nut-1 promoter 68

Additional 50RE-1 variants are present in the KSHV genome 68

The TK promoter also possesses an ORF50 responsive element 69

TK RRE confers ORF50 dependence upon heterologous promoters 70

Chapter 3 - Regulation of late gene expression 110

Northern analysis of assembly protein (AP) transcript. 113

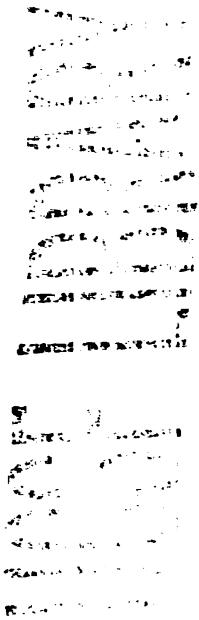
The 5′ end of the AP mRNA 114

KSHV late promoters taken out of the viral context are not dependent upon viral replication 115

Minimal promoter constructs are also insufficient to direct gene expression with late gene kinetics 118

A directed search for the KSHV lytic origin by transient and stable replication assay 118

Conclusion 173



List of Figures and Tables

Chapter 1

Table 1 Screening of inflammatory cytokines and growth factors for KSHV induction in BCBL-1 cells 44

Figure 1 Lytic reactivation of BCBL-1 by chemical induction 52

Figure 2 Kinetics of induction. BCBL-1 cells were induced with TPA, Ionomycin or both chemical inducers 54

Figure 3a IFNγ and Ionomycin induction of KSHV 56

Figure 3b Real-time RT-PCR quantitation of lytic induction by IFNγ and TPA 58

Figure 4 IFNα inhibits induction of KSHV late gene (K8.1) expression by ionomycin. 60

Chapter 2

Table 1 Additional 50RE-1 variants in the KSHV genome 81

Figure 1a Analysis of the kaposin promoter using linker-scanning mutants 88

Figure 1b Effect of linker scanning mutants on ORF50 activation of the Kaposin promoter in SLK cells 90

Figure 1c Effect of linker scanning mutants on ORF50 activation of the Kaposin promoter in CV-1 92

Figure 1d Effect of linker scanning mutants on ORF50 activation of the Kaposin promoter in BJAB cells 94

Figure 2a Diagram of the nut-1 deletion and linker mutants 96

Figure 2b Effect of deletion and linker mutants on ORF50 activation of the nut-1 promoter in SLK cells 98

Figure 3a Schematic of the TK promoter deletion series 100

Figure 3b Effect of deletion and linker mutants on ORF50 activation of the TK promoter in SLK cells 102

Figure 4a Schematic of TK ORF50-responsive regions placed in front of the minimal E1B or fos promoters 104

Figure 4b 117 bp region of the TK promoter confers ORF50 sensitivity to the minimal E1B promoter in SLK cells 106

Figure 4c 90 and 45 bp regions of the TK promoter increases ORF50-dependent activation of the minimal fos promoter in SLK cells 108

Chapter 3

Figure 1 Schematic of ORF17.5 locus, mRNA and gene product, Assembly Protein 135

Figure 2 Northern analysis of AP expression 137

Figure 3 AP cDNA sequences 139

Figure 4 RNase protection analysis of the AP transcript 141

Figure 5 Primer extension analysis of the start site of the AP transcript 143

Figure 6 Late gene promoter constructs 145

Figure 7a The 1.3kb and 700bp promoter regions of AP are both able to drive expression of a reporter gene 147

Figure 7b AP promoter regions are not responsive to TPA in 293 cells 149

Figure 7c AP promoter regions are mildly responsive to TPA in BCBL-1 cells 151

Figure 7d Ablation of ATG in AP promoter region increases reporter activity in response to TPA 153

Figure 8a pGL3 AP TTG is responsive to both TPA and ionomycin but insensitive to ganciclovir 155

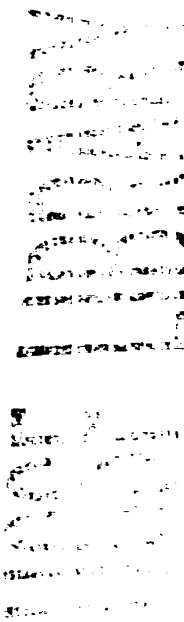


Figure 8b pGL3 AP TTG is responsive to both TPA and ionomycin in BJAB cells 157

Figure 8c gB and MCP promoter regions are unresponsive to ganciclovir in BCBL-1 159

Figure 8d gB and MCP promoter regions are also inducible in BJAB cells. 161

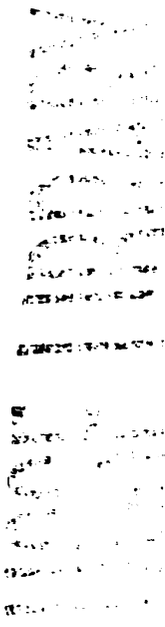
Figure 9 Minimal late gene promoter in BCBL-1 163

Figure 10 Genomic locus of candidate sites for the viral lytic origin 165

Figure 11a Assay for the KSHV lytic origin by transient transfection assay 167

Figure 11b Replicated DNA can be detected by transient assay in BCBL-1 169

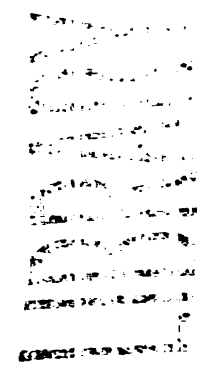
Figure 12 Assay for the viral lytic origin by stable transfection 171

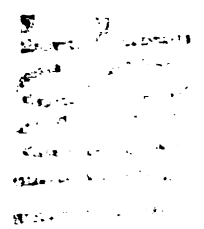


Introduction

Kaposi's sarcoma - History

In 1872, Moriz Kaposi reported five case studies which formed the basis for his classification of a new sarcoma of the skin, idiopathic multiple pigmented sarcoma (Kaposi, 1872). General characteristics of this disease include the formation of brown-red to blue-red nodules, usually forming on the feet and hands with eventual inward spread of nodule development to include the mucous linings of the larynx, stomach, liver and other internal organs. Kaposi also noted that although some nodules tend to regress or atrophy, the disseminated disease state was rapidly lethal. The appearance of nodules on both feet and hands led Kaposi to comment on the multifocal nature of this newly described disease, eponymously designated Kaposi's sarcoma (KS).

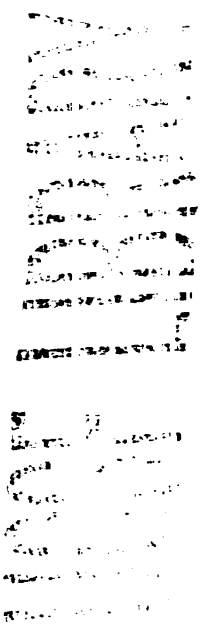




In the US, classical KS is rare and often benign. The disease is typically seen in elderly men of Mediterranean descent, of which only a subpopulation develop disseminated disease. In 1960, Dutz and Stout reported high incidence of KS disease in several African countries. African endemic KS occurs in all age groups, is generally more aggressive and includes general lymph node involvement not seen in classical KS (Dutz and Stout, 1960). Iatrogenic Kaposi's sarcoma has also been observed in patients receiving immunosuppresive treatment, including transplant recipients (Safai and Good, 1981).

In 1981, the Centers for Disease Control received reports of increase incidence of KS and *Pneumocystis carinii* pneumonia (PCP) in homosexual men (Haverkos and Curran, 1982). Both diseases were exceedingly rare in the US at that time and, in 27 cases, both diseases were occuring in the same patient. The connection between the two diseases was immunosuppression. KS heralded the onset of the AIDS epidemic.

Early efforts to understand KS biology



The inability to maintain KS cells in culture severely hampered scientists' ability to determine the nature and origin of KS. Histochemical and ultrastructural analysis yielded controversial results regarding the origin of the KS spindle cell, the presumed tumor cell within the lesions (Safai and Good, 1981). At the same time, several exogenous agents were postulated to be the etiological agent of KS, including CMV (Giraldo et al., 1972; Giraldo et al., 1978; Giraldo et al., 1975), however none of these propositions withstood rigorous epidemiologic scrutiny.

In 1988, Nakamura et al reported the culturing of KS cells using medium conditioned by HTLV-II infected T cell lines (TCM) (Nakamura et al., 1988). TCM was unable to support the growth of normal endothelial cells, suggesting a unique factor requirement for the stimulation and maintanance of KS cells, distinct from known endothelial cell growth factors. Conditioned media from KS cells cultured in TCM could stimulate both KS cells and normal endothelial cells (Salahuddin et al., 1988). Such media contained factors such as interleukin-1 and basic fibroblast growth factor (bFGF)-like molecules, which could have provided the stimulatory

impetus for vascularization and angiogenesis seen in chorioallantoic membrane assays and after subcutaneous injection of KS cells into nude mice (Salahuddin et al., 1988). The fact that the proliferating cells in these nude mice were of murine origin strongly implicates a paracrine activity in the pathogenesis of KS. Further studies have shown that several cytokines and growth factors are central to the autocrine and paracrine growth effects of KS cells (Barillari et al., 1992; Ensoli et al., 1989).

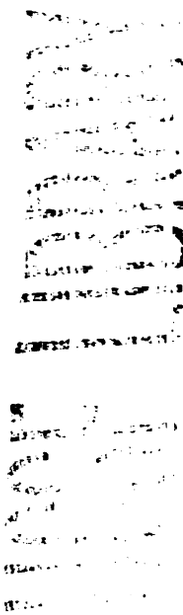
The initial trigger for the de-regulation of cytokine and growth factor expression was still unknown. HIV-tat was one potential candidate. Tat had been shown to synergize with bFGF to produce angiogenic lesions in mice (Ensoli et al., 1994). Additionally, incubation of AIDS-KS cells with conditioned media from tat-transfected COS-1 cells induced cell proliferation which could be specifically inhibited by anti-Tat antibodies (Ensoli et al., 1990). However, epidemiological evidence suggested that tat could not be the cause of KS. Although HIV was highly associated with AIDS-KS in homosexual men, there was no such association with other classes of KS (classical, African endemic, and iatrogenic) . Furthermore, the incidence of KS in other AIDS groups, hemophiliacs, other transfusion recipients, and children who are exposed to HIV through vertical transmission, is far lower than an HIV-associated model would predict (Beral et al., 1990).

Molecular Identification of KSHV

In 1994, evidence of a novel herpesvirus detected specifically in AIDS-KS tissue was reported (Chang et al., 1994). Using representational difference analysis (Lisitsyn et al., 1993), two fragments of DNA with homology to gammaherpesvirus structural genes were identified. Estimates by pulsed-field and Gardella gel electrophoresis placed the genome size at roughly 160 - 170 kb (Renne et al., 1996). The subsequent cloning and sequencing of the 165 kb viral genome revealed a 104.5 kb coding region flanked by large blocks of GC-rich terminal repeats on either side (Moore et al., 1996; Neipel et al., 1997; Nicholas et al., 1998; Russo et al., 1996). Initial computer characterization of the genome described 81 ORFs, 15 of which are unique to KSHV (Russo et al., 1996). KSHV encodes many proteins involved in the process of virion production, including proteins for viral nucleic acid metabolism, viral replication, and assembly of progeny virions. Additionally, several KSHV ORFs contain cellular homologs of genes that modulate cellular proliferation including vBcl-2 (ORF16) (Sarid et al., 1997), vIL-6 (ORF K2) (Moore et al., 1996; Neipel et al., 1997; Nicholas et al., 1997), v-cyclin (ORF72) (Cesarman et al., 1996; Chang et al., 1996), a G-protein coupled receptor homolog, v-GCR (ORF74) (Arvanitakis et al., 1997; Bais et al., 1998; Cesarman et al., 1996; Kirshner et al., 1999) and a homolog to cellular interferon regulatory factors, vIRF-1 (ORF K9) (Gao et al., 1997; Li et al., 1998; Moore et al., 1996). The exact contribution of these molecules to disease progression is yet to be elucidated.

KS lesions are characterized by a proliferation of spindle-shaped cells, endothelial-lined vascular slits, and occasional infiltrating lymphocytes (Safai and Good,

1981). Their bluish appearance is attributed to the presence of extravasated erythrocytes and hemosiderin-laden macrophages. KSHV DNA is found in virtually all KS tumors (Ambroziak et al., 1995; Boshoff et al., 1995; Chuck et al., 1996; Huang et al., 1995; Schalling et al., 1995). *In situ* hybridization with probes for latency-associated messages reveals that the vast majority of spindle cells are latently infected (Staskus et al., 1997; Sturzl et al., 1997). A subset of spindle cells are also positive for expression of lytic messages (Staskus et al., 1997), suggestive of spontaneous viral reactivation. The observation of nucleocapsids with electron-dense cores within endothelial and spindle shaped cells of KS lesions is consistent with reactivation (Ioachim, 1995; Orenstein et al., 1997; Said et al., 1997). It is most likely that these KSHV capsids were the viral inclusions orginally seen (Giraldo et al., 1972) which led to the investigation of CMV as an etiologic agent for KS. In recent studies, detailed transmission electron microscopy (TEM) reveals distinguishing differences between CMV and KSHV nucleocapsid particles (Said et al., 1997).



In addition to infection of endothelial and spindle cells, *in situ* hybridization shows that monocytes within the lesion are also infected (Blasig et al., 1997). While most infected monocytes express only latent message, a small population also expressed lytic markers, indicative of productive infection. In addition, KSHV can also be detected in peripheral blood mononuclear cells of KS patients, specifically $CD19^+$ B cells (Ambroziak et al., 1995). Moreover, Mesri et al. demonstrated that incubation of viral particles with umbilical cord blood mononuclear cells resulted

in viral transmission in $CD19^+$ B cells (Mesri et al., 1996), lending credence to the notion of the B cell as a site of primary KSHV infection.

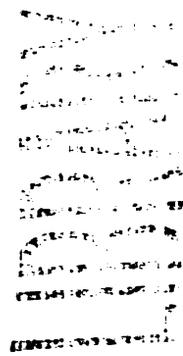
A model system for viral reactivation: BCBL-1

True to its classification as a lymphotropic herpesvirus, KSHV has been associated with two lymphoproliferative disorders: primary effusion lymphoma (PEL) (Cesarman et al., 1995; Cesarman et al., 1996) and multicentric Castleman's disease (Gessain et al., 1997; Soulier et al., 1995). Cells from PEL, also known as body cavity based lymphoma (BCBL), carry a viral load about 60-fold greater than that of a KS lesion as determined by Southern blot analysis (Cesarman et al., 1995). In our laboratory, we utilized a cell line derived from a PEL which is latently infected with KSHV (Renne et al., 1996). Limited gene expression is observed in BCBL-1 unless treated with inducers of lytic reactivation such as the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA). Forty-eight hours after chemical stimulation, genome-wide transcription can be detected and viral DNA increases 15-fold. Subsequently, electron-dense capsids can be seen in the nucleus and mature, enveloped virons are detected in the cytoplasm by TEM. Lytic reactivation in BCBL-1 is more robust than reactivation in other PEL-derived cell lines, making BCBL-1 our cell line of choice for studies of reactivation and lytic gene expression.

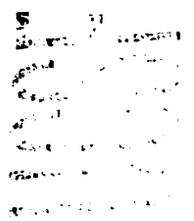
The herpesviral lytic life cycle - operational definitions

Immediate early genes (IE)

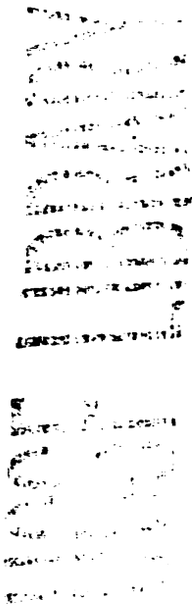
Herpesviral lytic genes fall into three classes that can be defined by their ability to be expressed in the presence of various chemical inhibitors (Honess and Roizman, 1974). IE genes are the first to be expressed in infection or reactivation. Operationally, they are defined as those genes which are transcribed after infection in the absence of *de novo* protein syntheis, as shown by Northern analysis of cells infected or reactivated in the presence of cycloheximide. Many IE gene products are activators of gene expression, including the herpes simplex virus (HSV) protein ICP4 and Epstein-Barr virus (EBV) proteins, R and Z.



Kinetic studies of KSHV lytic gene expression reveal several messages which appear within six hours of TPA induction: ORF50, ORF57, K-bZIP, K3 and K5. (Lukac et al., 1999; Sun et al., 1999). ORF50 is the earliest to be expressed and the only transcript of these candidate genes that can be induced in the presence of cycloheximide at early time points (Sun et al., 1999; Zhu et al., 1999, Seaman, 1999 #986). Three other IE genes, whose gene products are of unknown function, have been identified in a screen for cycloheximide resistant transcripts, ORF45, ORF K4.2 and a 4.5 kb mRNA complimentary to part of ORF29 (Zhu et al., 1999). Two caveats must be noted in the designation of these four genes as the primary IE genes of KSHV: First, inhibitors of protein synthesis are highly toxic to the B cell lines in which these experiments are performed, thus only robustly expressed IE genes will be detected as the toxic effects of cycloheximide prevent the



experimentor from looking for the accumulation of weakly expressed, but biologically significant, IE genes at later time points than 4 hours post-induction. Second, these experiments are performed in latently infected cells due to the low infectivity of KSHV in readily available cell lines (Renne et al., 1998) and the difficulty in obtaining primary cells (bone marrow microvascular endothelial cells or dermal microvascular endothelial cells) which are permissive for infection by KSHV (Flore et al., 1998; Moses et al., 1999). Reactivation from latency may invoke a very different cascade of gene expression and key IE genes involved in primary infection may not be identified by the systems currently used for analysis.



Delayed early genes (DE)

DE genes are defined as genes whose messages are cycloheximide sensitive, while insensitive to inhibitors of the viral polymerase. DE gene expression depends upon the presence of early gene products, hence the cycloheximide sensitivity of DE mRNA production. A number of KSHV ORFs are homologs to HVS and EBV DE genes (Russo et al., 1996). Most notable is the presence of homologs to the genes required for DNA replication of HSV (Knipe, 1989; Wu et al., 1988) and EBV (Fixman et al., 1995; Fixman et al., 1992), including ORF9 (DNA polymerase), ORF59 (polymerase processivity factor), ORF6 (ssDNA binding protein), ORF 56 (primase), ORF44 (helicase), and ORF 40/41 (helicase-primase associated factor). Other DE genes homologs are similar to proteins involved in viral nucleic acid metabolism and post-translational modification of viral proteins. It is presumed that most, if not all, of these genes will behave as DE genes.

Late genes

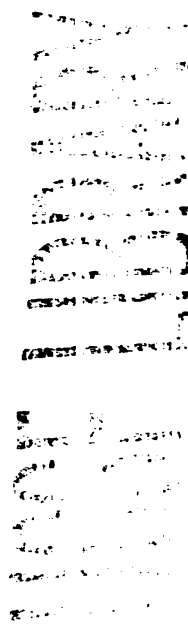
Sensitivity to both cycloheximide and viral polymerase inhibitors is the operational definition of herpesvirus late genes. These sensitivities reflect the need for IE gene product activation of DE genes, and DE gene products for replication of the viral genome, respectively. For all late genes, maximal expression is dependent upon viral DNA synthesis. How viral DNA synthesis contributes to late gene activation is not well understood. In HSV, viral DNA replication is an *in cis* requirement (Johnson and Everett, 1986; Mavromara-Nazos and Roizman, 1987). It is thought that late gene promoters might exist in a repressed state which is alleviated during replication due to a *cis*-modification of the promoter region, creating a more accessible template (Johnson and Everett, 1986).

The latent to lytic switch

Infection of B lymphocytes usually results in a state of latent infection characterized by a small program of gene expression (Zhong et al., 1996), low genome number and lack of infectious virions. The development of multifocal endothelial-derived lesions typical of KS disease suggests multiple independent infection events occuring in endothelium. B cells and monocytes stimulated to lytic replication may be the source of infectious virions.

In vitro studies show that KSHV lytic reactivation is repressed by foscarnet, ganciclovir, and cidofovir (Kedes and Ganem, 1997). After the identification of KSHV, several retrospective studies were initiated to study the effects of such herpesviral polymerase inhibitors on KS risk. Several studies showed that

foscarnet (phosphonoacetic acid) or ganciclovir reduced the relative risk of developing KS in at-risk AIDS patients (Glesby et al., 1996; Jones et al., 1995; Mocroft et al., 1996). In a recent prospective study, KS risk was reduced by 93% when patients were treated with intravenous ganciclovir (Martin et al., 1999). Taken together, these data suggest that lytic replication plays an important part in disease progression. Hence, determining the molecular mechanisms of initiation and regulation of the lytic cycle is central to our understanding of the pathogenesis of KS.



HSV reactivation from latency

Latent HSV has been shown to reside in sensory neurons (Cook et al., 1974). Lytic reactivation in these cells occurs in response to exogenous stressors such as cyclophosphamide plus dexamethasone treatment (Cook et al., 1991), UV irradiation (Blyth et al., 1976; Laycock et al., 1991), neurectomy (Tenser et al., 1988), and hyperthermia (Sawtell and Thompson, 1992). The molecular mechanism of lytic reactivation in neuronal cells is unclear; however, viral DNA synthesis is implicated as a necessary step for maximal viral gene expression (Nichol et al., 1996).

A key regulator of HSV lytic gene expression is ICP4. Many essential viral genes are activated by ICP4 (DeLuca and Schaffer, 1985; Everett, 1984; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985). The domain organization of ICP4 includes: a nuclear localization signal (Mullen et al., 1994), DNA binding and dimerization (Everett et al., 1990; Gallinari et al., 1994; Wu and Wilcox, 1990), and

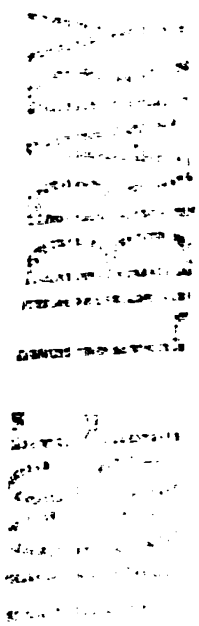
three identified transactivation domains at residues 97-109 (Xiao et al., 1997), 142-210 (Shepard et al., 1989) and 775-1298 (DeLuca et al., 1985; DeLuca and Schaffer, 1985; Shepard et al., 1989). In the absence of ICP4, only IE genes are expressed, underscoring the requirement for ICP4 in progression of the lytic cycle (Dixon and Schaffer, 1980; Watson and Clements, 1980).

EBV reactivation from latency

In EBV, epithelial cells are fully permissive for lytic infection (Niedobitek et al., 1991). Unfortunately,there are no good cell culture systems for the growth and lytic replication of EBV in epithelial cell lines (Sixbey et al., 1983). Reactivation from viral latency in B cells, however, is well characterized in EBV at the molecular level. Lytic induction can be chemically induced by phorbol esters or calcium ionophore but lytic replication can also be initated by cross-linking sIG (Takada, 1984), indicative of the probable participation of B cell activation in progress through the lytic cycle. EBV gene products Z (BZLF1, Zta, Zebra) and R (BRLF1, Rta) are expressed early and can be detected in the presence of cycloheximide (Takada and Ono, 1989), confirming their IE designation. Genetic dissection of the requirements for lytic replication demonstrate that Z is necessary for initiation of viral DNA synthesis (Fixman et al., 1995). Z most likely exerts its action through four AP-1 related Z responsive elements (ZREs) located in the core of EBV ori-Lyt (Lieberman and Berk, 1990; Lieberman et al., 1990; Schepers et al., 1996). The replication function of Z is located in the N-terminus (aa 11-25), separate from the domain required for transcriptional activation of its known downstream targets (Sarisky et al., 1996). Early studies placed Z as the sole

molecular switch to lytic reactivation in EBV (Takada et al., 1986). Recent work suggests that R may also play a major role in reactivation. In both epithelial cells and B cells, expression of R is able to disrupt latency and stimulate the expression of lytic cycle genes (Adamson and Kenney, 1998; Ragoczy et al., 1998; Zalani et al., 1992). Activation of lytic replication, however, is less robust with R alone, implying a cooperative model of Z and R interaction for full lytic activation.

In addition to the activation of lytic replication, both Z and R are important transactivators of the transcription of lytic cycle genes. Z mRNA encodes an N-terminal activation domain (Chi and Carey, 1993; Flemington et al., 1992), nuclear localization signal (NLS) (Mikaelian et al., 1993), and a coiled coil (Chang et al., 1990; Kouzarides et al., 1991). Phosphorylation of Z regulates its DNA binding activity (Daibata et al., 1992; Kolman et al., 1993). R contains also contains domains for transcriptional activation and DNA binding (Manet et al., 1991). In addition, the N-terminus of R is able to bind Rb (outside of the E2F binding pocket) and displace E2F (Zacny et al., 1998), freeing this limiting cellular transcription factor for activation of viral promoters bearing E2F binding sites (Liu et al., 1996).



KSHV reactivation from latency

Study of the KSHV genome is still in its infancy. Many of the KSHV ORFs have been assigned functions based on homology to known proteins in other herpesviruses. The first genes to be studied in molecular detail have been those candidates thought to be important to establishment and progression of KS disease, including what is now generally thought of as the molecular switch of

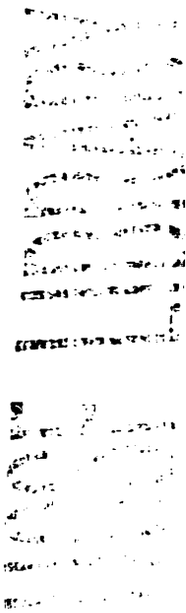
KSHV, ORF50. ORF50 is a nuclear protein which has homology to R, the IE transcriptional transactivator of EBV (Lukac et al., 1998). Overexpression of R in latently infected B cells results in lytic reactivation. Moreover, R is able to activate the promoters of several DE genes in transient transfection reporter assays (Lukac et al., 1998; Seaman et al., 1999; Sun et al., 1998). Further studies have shown that R is extensively phosphorylated *in vivo* (Lukac et al., 1999). Additionally, removal of the activation domain of R results in a dominant negative protein which is able to prevent reactivation of BCBL-1, even in the presence of strong chemical inducers such as TPA. Furthermore, R activates the promoters of K-bZIP and ORF57, homologs to EBV IE proteins Z and M, suggesting that in KSHV ORF50 may be the initiating protein in the cascade of lytic gene expression.

Late gene expression and viral replication

EBV late gene regulation - a requirement for replication *in trans*

EBV late gene expression is also dependent upon viral DNA synthesis. However, in contrast to the alpha herpesviruses, this dependence appears not to reflect a requirement *in cis*. In 1997, Serio et al showed that late gene promoters could be transferred to plasmid vectors and still exhibit proper late gene regulation when viral replication occurs *in trans* (Serio et al., 1997). Expression from these promoters was inducible by TPA or Z, the EBV IE transactivator, and repressed by viral polymerase inhibitors, but only in cell lines latently infected with replication competent EBV. Recent studies suggest that the minimal core promoter of late genes is sufficient to drive regulated late gene expression (Serio et al., 1998).

Computer analysis suggests that the composition of the TATA box may also help determine expression kinetics but further work needs to be done to establish the precise sequence requirements within the TATA box for late gene regulation. Interestingly, as in HSV, addition of an early gene upstream region to a late TATA converted the late promoter to one which exhibited early gene expression. It is not known whether the lack of a *cis*-requirement for DNA replication in EBV is specific to that virus, or is a general feature of gammaherpesvirus late gene regulation. I shall return to theis question in Chapter 3.



Overview of the thesis

Chapter 1 describes the screening of various cytokines for the induction of KSHV lytic growth using an immunofluoresence assay or flow cytometry. Our search resulted in the identification of interferon gamma as a positive signal for lytic reactivation and interferon alpha as a negative signal that represses the latent to lytic switch.

In **Chapter 2,** a genetic appraoch was used to identify motifs responsible for *cis*-activation by the KSHV immediate early protein, ORF50. Two distinct elements were identified, suggesting ORF50 may act through a variety of motifs.

Chapter 3 explores the mechanisms of KSHV late gene regulation. These studies show that proper late gene expression cannot be recapitulated when late promoters are taken out of the viral context.

References

Adamson, A. L., and Kenney, S. C. (1998). Rescue of the Epstein-Barr virus BZLF1 mutant, Z(S186A), early gene activation defect by the BRLF1 gene product. Virology *251*, 187-197.

Ambroziak, J., Blackbourn, D., Herndier, B., Glogan, R., Gullet, J., McDonald, A., Lennette, E., and Levy, J. (1995). Herpesvirus-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients (technical comment). Science *268*, 582-583.

Arvanitakis, L., Geras-Raaka, E., Varma, A., Gershengorn, M. C., and Cesarman, E. (1997). Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation [see comments]. Nature *385*, 347-50.

Bais, C., Santomasso, B., Coso, O., Arvanitakis, L., Raaka, E. G., Gutkind, J. S., Asch, A. S., Cesarman, E., Gershengorn, M. C., Mesri, E. A., and Gerhengorn, M. C. (1998). G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator [see comments] [published erratum appears in Nature 1998 Mar 12;392(6672):210]. Nature *391*, 86-9.

Barillari, G., Buonaguro, L., Fiorelli, V., Hoffman, J., Michaels, F., Gallo, R. C., and Ensoli, B. (1992). Effects of cytokines from activated immune cells on vascular cell growth and HIV-1 gene expression. J Immunol *149*, 3727-3734.

Beral, V., Peterman, T. A., Berkelman, R. L., and Jaffe, H. W. (1990). Kaposi's sarcoma among persons with AIDS: a sexually transmitted infection? [see comments]. Lancet *335*, 123-8.

Blasig, C., Zietz, C., Haar, B., Neipel, F., Esser, S., Brockmeyer, N. H., Tschacler, E., Colombini, S., Ensoli, B., and Sturzl, M. (1997). Monocytes in Kaposi's sarcoma lesions are productively infected by human herpesvirus 8. J. Virol. *71*, 7963-7968.

Blyth, W. A., Hill, T. J., Field, H. J., and Harbour, D. A. (1976). Reactivation of herpes simplex virus infection by ultraviolet light and possible involvement of prostaglandin. J Gen Virol *33*, 547-550.

Boshoff, C., Whitby, D., Hatziionnou, T., Fisher, C., Walt, J. v. d., Hatzakis, A., Weiss, R., and Schulz, T. (1995). Kaposi's sarcoma-associated herpesvirus in HIV-negative Kaposi's sarcoma. Lancet *345*, 1043-1044.

Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS- related body-cavity-based lymphomas [see comments]. N Engl J Med *332*, 1186-91.

Cesarman, E., Moore, P., Rao, P., Inghirami, G., Knowles, D., and Chang, Y. (1995). In vitro establishment and characterization of two acquired immunodefieiencey syndrome-related lymphoma cell lines (BC-1 and BC-2) containing Kaposi's sarcoma-associated herpesvirus-like (KSHV) DNA sequences. Blood *86*, 2708-2714.

Cesarman, E., Nador, R. G., Aozasa, K., Delsol, G., Said, J. W., and Knowles, D. M. (1996). Kaposi's sarcoma-associated herpesvirus in non-AIDS related lymphomas occurring in body cavities. Am J Pathol *149*, 53-7.

Cesarman, E., Nador, R. G., Bai, F., Bohenzky, R. A., Russo, J. J., Moore, P. S., Chang, Y., and Knowles, D. M. (1996). Kaposi's sarcoma-associated herpesvirus contains G protein-coupled receptor and cyclin D homologs which are expressed in Kaposi's sarcoma and malignant lymphoma. J Virol *70*, 8218-23.

Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma [see comments]. Science *266*, 1865-9.

Chang, Y., Moore, P. S., Talbot, S. J., Boshoff, C. H., Zarkowska, T., Godden, K., Paterson, H., Weiss, R. A., and Mittnacht, S. (1996). Cyclin encoded by KS herpesvirus [letter]. Nature *382*, 410.

Chang, Y. N., Dong, D. L., Hayward, G. S., and Hayward, S. D. (1990). The Epstein-Barr virus Zta transactivator: a member of the bZIP family with unique DNA-binding specificity and a dimerization domain that lacks the characteristic heptad leucine zipper motif. J virol *64*, 3358-3369.

Chi, T., and Carey, M. (1993). The ZEBRA activation domain: modular organization and mechanism of action. Mol Cell Biol *13*, 7045-7055.

S., Grant, R.
presence of
sarcoma. J.

M. L., Basone
herpes simple

S. D., Paveloff
Ocular he
associated tr

M., Humph
Barr virus B

N. A., McC
terization of d
immediate

N. A., and
promoters by te
type 1 protein

R. A., and Sc
ss of tempera
type 1 immed

W., and Stout

B., Barillari,
protein of HIV-
ts of AIDS pat

B., Gendelm
A., Chang,
broblast gro
ma. Nature 3

Chuck, S., Grant, R. M., Katongle-Mbidde, E., Conant, M., and Ganem, D. (1996). Frequent presence of herpesviral-like DNA sequences in lesions of HIV-negative Kaposi's sarcoma. J. Inf. Dis. *173*, 248-251.

Cook, M. L., Basone, V. B., and Stevens, J. G. (1974). Evidence that neurons harbor latent herpes simplex virus. Infect Immun *9*, 946-951.

Cook, S. D., Paveloff, M. J., Doucet, J. J., Cottingham, A. J., Sederati, F., and Hill, J. M. (1991). Ocular herpes simplex virus reactivation in mice latently infected with latency-associated transcript mutants. Invest Ophthalmol Visual Sci *32*, 1558-1561.

Daibata, M., Humphreys, R. E., and Sairenji, T. (1992). Phosphorylation of the Epstein-Barr virus BZLF1 immediate-early gene product ZEBRA. Virology *188*, 916-920.

DeLuca, N. A., McCarthy, A., and Schaffer, P. A. (1985). Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protien ICP4. J Virol *56*, 558-570.

DeLuca, N. A., and Schaffer, P. A. (1985). Activation of immediate-early, early and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. Mol Cell Biol *5*, 1997-2008.

Dixon, R. A., and Schaffer, P. A. (1980). Fine structure mapping and functional analysis of temperature sensitive mutant in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. J Virol *36*, 189-203.

Dutz, W., and Stout, A. P. (1960). Kaposi's sarcoma in infants and children. Cancer *13*, 684-694.

Ensoli, B., Barillari, G., Salahuddin, S. Z., Gallo, R. C., and Wong-Staal, F. (1990). Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Nature *345*, 84-86.

Ensoli, B., Gendelman, R., Markham, P., Fiorelli, V., Colombini, S., Raffeld, M., Cafaro, A., Chang, H. K., Brady, J. N., and Gallo, R. C. (1994). Synergy between basic fibroblast growth factor and HIV-1 tat protein in induction of Kaposi's sarcoma. Nature *371*, 674-680.

Ensoli, B., Nakamura, S., Salahuddin, S., Biberfeld, P., Larsson, L., Beaver, B., Wong-Staal, F., and Gallo, R. (1989). Aids-Kaposi's sarcoma derived cells express cytokines with autocrine and paracrine growth effect. Science *243*, 223-6.

Everett, R. D. (1984). Transactivation of transcription by herpes virus products: requirement for two HSV-1 immediate early polypeptides for maximum activity. EMBO J *3*, 3135-3141.

Everett, R. D., Paterson, T., and Elliott, M. (1990). The major transcriptional regulatory protein of herpes simplex virus type 1 includes a protease resistant DNA binding domain. Nucleic Acids Rec *18*, 4579-4585.

Fixman, E. D., Hayward, G. S., and Hayward, S. D. (1995). Replication of Epstin-Barr virus orilyt: Lack of a dedicated virally encoded origin-binding protein and dependence on Zta in cotransfection assays. J Virol *69*, 2998-3006.

Fixman, E. D., Hayward, G. S., and Hayward, S. D. (1992). trans-acting requirements for replication of Epstein-Barr virus ori-Lyt. J Virol *66*, 5030-9.

Flemington, E. K., Borras, A. M., Lytle, J. P., and Speck, S. H. (1992). Characterization of the Epstein-Barr virus BZLF1 protein transactivation domain. J Virol *66*, 922-929.

Flore, O., Rafii, S., Ely, S., O'Leary, J. J., Hyjek, E. M., and Cesarman, E. (1998). Transformation of primary human endothelial cells by Kaposi's sarcoma-associated herpesvirus. Nature *394*, 588-592.

Gallinari, P., Wiebauer, K., Nardi, M. C., and Jiricny, J. (1994). Localization of a 34-amino acid segment implicated in dimerization of the herpes simplex virus type 1 ICP4 polypeptide by a dimerization trap. J Virol *68*, 3809-3820.

Gao, S., Boshoff, C., Jayachandra, S., Weiss, R., Chang, Y., and Moore, P. (1997). KSHV ORF K9 (vIRF) is an oncogene which inhibits the interferon signaling pathway. oncogene *15*, 1979-85.

Gelman, I. H., and Silverstein, S. (1985). Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc Natl Acad Sci USA *82*, 5265-5269.

Gessain, A., Briere, J., Angelin-Duclos, C., Valensi, F., Beral, H. M., Davi, F., Nicola, M. A., Sudaka, A., Fouchard, N., Gabarre, J., Troussard, X., Dulmet, E., Audouin, J., Diebold, J., and de The, G. (1997). Human herpes virus 8 (Kaposi's sarcoma herpes virus) and malignant lymphoproliferations in France: a molecular study of 250 cases including two AIDS-associated body cavity based lymphomas. Leukemia *11*, 266-72.

Giraldo, G., Beth, E., and Haguenau, F. (1972). Herpes-type virus particles in tissue culture of Kaposi's sarcoma from different geographic regions. J Natl Cancer Inst *49*, 1509-1526.

Giraldo, G., Beth, E., and Henle, W. (1978). Antibody patterns to herpesviruses in Kaposi's sarcoma. II. Serological association of American Kaposi's sarcoma with cytomegalovirus. Int J Cancer *22*.

Giraldo, G., Beth, E., Kourilsky, F. M., Henle, W., Henle, G., Mike, V., Huraux, J. M., Andersen, H. K., Gharbi, M. R., Kyalwazi, S. K., and Puissant, A. (1975). Antibody patterns to herpesviruses in Kaposi's sarcoma: serological association of European Kaposi's sarcoma with cytomegalovirus. Int J Cancer *15*, 839-848.

Glesby, M. J., Hoover, D. R., Weng, S., Graham, N. M. H., Phair, J. P., Detels, R., Ho, M., and Saah, A. J. (1996). Use of Antiherpes Drugs and the Risk of Kaposi's Sarcoma: Data from the Multicenter AIDS Cohort Study. Journal of Infectious Diseases *173*, 1477-1480.

Haverkos, H. W., and Curran, J. W. (1982). The current outbreak of Kaposi's sarcoma and opportunistic infections. Ca-A Cancer Journal for Clinicians *32*, 330-339.

Honess, R. W., and Roizman, B. (1974). Regulation of herpesvirus macromolecular synthesis. J Virol *14*, 8-19.

Huang, Y.-Q., Li, J., Kaplan, M., Poiesz, B., Katabira, E., Zhang, W., Feiner, D., and Friedman-Kien, A. (1995). Human herpesvirus-like nucleic acid in various forms of Kaposi's sarcoma. Lancet *345*, 759-761.

Ioachim, H. L. (1995). Kaposi's sarcoma and KSHV [letter; comment] [published erratum appears in Lancet 1996 Jan 6;347(8993):68]. Lancet *346*, 1360.

Jones, J. L., Hanson, D. L., Chu, S. Y., Ward, J. W., and Jaffe, W. H. (1995). AIDS-associated Kaposi's sarcoma. Science *267*, 1078-1079.

Kaposi, M. (1872). Idiopathic multiple pigmented sarcoma of the skin. Archiv Fur Dermatologie Und Syphillis 4, 265-173. Translated from the German in Ca-A Cancer Journal for Clinicians. (1982). *32*, 342-347.

Kedes, D. H., and Ganem, D. (1997). Sensitivity of Kaposi's sarcoma-associated herpesvirus replication to antiviral drugs. Implications for potential therapy. J Clin Invest *99*, 2082-6.

Kirshner, J. R., Staskus, K., Haase, A., Lagunoff, M., and Ganem, D. (1999). Expression of the Open Reading Frame 74 (G-protein-coupled receptor) gene of Kaposi's sarcoma (KS)-associated herpesvirus: implications for KS pathogenesis. J. Virol. *in press*.

Knipe, D. M. (1989). The role of viral and cellular nuclear proteins in herpes simplex virus replication. Adv Virus Res *37*, 85-123.

Kolman, J. L., Taylor, N., Marshak, D. R., and Miller, G. (1993). Serine-173 of the Epstein-Barr virus ZEBRA protein is required for DNA binding and is a target for casein kinase II phosphorylation. Proc Natl Acad Sci USA *90*, 10115-10119.

Kouzarides, T., Packham, G., Cook, A., and Farrell, P. J. (1991). The BZLF1 protein of Epstein-Barr virus has a coiled coil dimerisation domain without a heptad leucine repeat but with homology to the C/EBP leucine zipper. Oncogene *6*, 195-204.

Laycock, K. A., Lee, S. F., Brady, R. H., and Pepose, J. S. (1991). Characterization of a murine model of recurrent herpes simplex virus keratitis induced by ultraviolet B radiation. Invest Ophthalmol Visual Sci *32*, 2741-2746.

Li, M., Lee, H., Guo, J., Neipel, F., Fleckenstein, B., Ozato, K., and Jung, J. U. (1998). Kaposi's sarcoma-associated herpesvirus viral interferon regulatory factor. J. Virol. *72*, 5433-5440.

Lieberman, P. M., and Berk, A. J. (1990). In vitro transcriptional activation, dimerization, and DNA-binding specificity of the Epstein-Barr virus Zta protein. J Virol *64*, 2560-2568.

Lieberman, P. M., Hardwick, J. M., Sample, J., Hayward, G. S., and Hayward, S. D. (1990). The Zta transactivator involved in induction of lytic cycle gene expression in Epstein-Barr virus-infected lymphocytes binds to both AP-1 and ZRE sites in target promoter and enhancer regions. J Virol *64*, 1143-1155.

Lisitsyn, N., Lisitsyn, N., and Wigler, M. (1993). Cloning the difference between two genomes. Science *259*, 946-959.

Liu, C., Sista, N. D., and Pagano, J. S. (1996). Activation of the Epstein-Barr Virus DNA Polymerase Promoter by the BRLF1 Immediate-Early Protein is Mediated through USF and E2F. J. Virol. *70*, 2545-2555.

Lukac, D. M., Kirshner, J. R., and Ganem, D. (1999). Transcriptional Activation by the Product of Open Reading Frame 50 of Kaposi's Sarcoma-Associated Herpesvirus Is Required for Lytic Viral Reactivation in B Cells. J. Virol. *73*, 9348-9361.

Lukac, D. M., Renne, R., Kirshner, J. R., and Ganem, D. (1998). Reactivation of Kaposi's sarcoma-associated herpesvirus infection from latency by expression of the ORF 50 transactivator, a homolog of the EBV R protein. Virology *252*, 304-312.

Manet, E., Rigolet, A., Gruffat, H., Giot, J.-F., and Sergeant, A. (1991). Domains of the Epstein-Barr virus (EBV) transcription factor R required for dimerization, DNA binding and activation. Nucleic Acids Res. *19*, 2661-2667.

Martin, D. F., Kuppermann, B. D., Wolitz, R. A., Palestine, A. G., Li, H., and Robinson, C. A. (1999). Oral ganciclovir for patients with cytomegalovirus retinitis treated with a ganciclovir implant. Roche Ganciclovir Study Group. N. Engl. J. Med. *340*, 1063-1070.

Mesri, E. A., Cesarman, E., Arvanitakis, L., Rafii, S., Moore, M. A., Posnett, D. N., Knowles, D. M., and Asch, A. S. (1996). Human herpesvirus-8/Kaposi's sarcoma-associated herpesvirus is a new transmissible virus that infects B cells. J Exp Med *183*, 2385-90.

Mikaelian, I., Drouet, E., Marechal, V., Denoyel, G., Nicolas, J. C., and Sergeant, A. (1993). The DNA-binding domain of two bZIP transcription factors, the Epstein-Barr virus switch gene product EB1 and Jun, is a bipartite nuclear targeting sequence. J Virol *67*, 734-742.

Mocroft, A., Youle, M., Gazzard, B., Morcinek, J., Halai, R., and Phillips, A. N. (1996). Anti-herpesvirus treatment and risk of Kaposi's sarcoma in HIV infection. AIDS *10*, 1101-1105.

Moore, P. S., Boshoff, C., Weiss, R. A., and Chang, Y. (1996). Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. Science *274*, 1739-44.

Moore, P. S., Gao, S. J., Dominguez, G., Cesarman, E., Lungu, O., Knowles, D. M., Garber, R., Pellett, P. E., McGeoch, D. J., and Chang, Y. (1996). Primary characterization of a herpesvirus agent associated with Kaposi's sarcomae [published erratum appears in J Virol 1996 Dec;70(12):9083]. J Virol *70*, 549-58.

Moses, A. V., Fish, K., Ruhl, R., Smith, P. P., Strussenberg, J. G., Zhu, L., Chandran, B., and Nelson, J. A. (1999). Long-term infection and transformation of dermal vicrovascular endothelial cells by human herpesvirus 8. J Virol *73*, 6892-6902.

Mullen, M. A., Ciufo, D. M., and Hayward, G. S. (1994). Mapping of intracellular localization domains and evidence for colocalization interactions between the IE110 and IE175 nuclear transactivator proteins of herpes simplex virus. J Virol *68*, 3250-3266.

Nakamura, S., Salahuddin, S. Z., Biberfeld, P., Ensoli, B., Markham, P. D., Wong-Staal, F., and Gallo, R. C. (1988). Kaposi's sarcoma cells: long-term culture with growth factor from retrovirus-infected CD4+ T cells. Science *242*, 426-430.

Neipel, F., Albrecht, J. C., Ensser, A., Huang, Y. Q., Li, J. J., Friedman-Kien, A. E., and Fleckenstein, B. (1997). Human herpesvirus 8 encodes a homolog of interleukin-6. J Virol *71*, 839-42.

Neipel, F., Albrecht, J. C., and Fleckenstein, B. (1997). Cell-homologous genes in the Kaposi's sarcoma-associated rhadinovirus human herpesvirus 8: determinants of its pathogenicity? J Virol *71*, 4187-92.

Nicholas, J., Ruvolo, V. R., Burns, W. H., Sandford, G., Wan, X., Ciufo, D., Hendrickson, S. B., Guo, H. G., Hayward, G. S., and Reitz, M. S. (1997). Kaposi's sarcoma-associated human herpesvirus-8 encodes homologues of macrophage inflammatory protein-1 and interleukin-6. Nat Med *3*, 287-92.

Nicholas, J., Zong, J., Alcendor, D. J., Ciufo, D. M., Poole, L. J., Sarisky, R. T., Chiou, C., Zhang, X., Wan, X., Guo, H., Reitz, M. S., and Hayward, G. S. (1998). Novel organizational features, captured cellular genes, and strain variability within the genome of KSHV/HHV8. J Natl Cancer Inst Monographs *23*, 79-88.

Niedobitek, G., Young, L. S., Lau, R., Brooks, L., Greenspan, D., Greenspan, J. S., and Rickinson, A. B. (1991). Epstein-Barr virus infection in oral hairy leukoplakia: virus replication in the absence of a detectable latent phase. J Gen Virol *72*, 3035-3046.

O'Hare, P., and Hayward, G. S. (1985). Evidence for a direct role for both the 175,000 and 110,000-molecular weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J Virol *53*, 751-760.

Orenstein, J. M., Alkan, S., Blauvelt, A., Jeang, K. T., Weinstein, M. D., Ganem, D., and Herndier, B. (1997). Visualization of human herpesvirus type 8 in Kaposi's sarcoma by light and transmission electron microscopy. Aids *11*, F35-45.

Ragoczy, T., Heston, L., and Miller, G. (1998). The Epstein-Barr virus Rta protein activates lytic cycle genes and can disrupt latency in B lymphocytes. J. Virol. *72*, 7978-7984.

Renne, R., Blackbourn, D., Whitby, D., Levy, J., and Ganem, D. (1998). Limited transmission of Kaposi's sarcoma-associated herpesvirus in cultured cells. J. Virol. *72*, 5182-5188.

Renne, R., Lagunoff, M., Zhong, W., and Ganem, D. (1996). The size and conformation of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) DNA in infected cells and virions. J Virol *70*, 8151-4.

Renne, R., Zhong, W., Herndier, B., McGrath, M., Abbey, N., Kedes, D., and Ganem, D. (1996). Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. Nat Med *2*, 342-6.

Russo, J. J., Bohenzky, R. A., Chien, M. C., Chen, J., Yan, M., Maddalena, D., Parry, J. P., Peruzzi, D., Edelman, I. S., Chang, Y., and Moore, P. S. (1996). Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). Proc Natl Acad Sci U S A *93*, 14862-7.

Safai, B., and Good, R. A. (1981). Kaposi's sarcoma: A review and recent developments. Ca-A Cancer Journal for Clinicians *31*, 2-12.

Said, J. W., Chien, L., Tasaka, T., and Koeffler, H. P. (1997). Ultrastructural characterization of human herpesvirus 4 (Kaposi's sarcoma-associated herpesvirus) in Kaposi's sarcoma lesions: electron microscopy permits distinction from cytomegalovirus (CMV). J Pathol *182*, 273-281.

Salahuddin, S. Z., Nakamura, S., Biberfeld, P., Kaplan, M. H., Markham, P. D., Larsson, L., and Gallo, R. C. (1988). Angiogenic properties of Kaposi's sarcoma-derived cells after long-term culture in vitro. Science *242*, 430-433.

Sarid, R., Sato, T., Bohenzky, R. A., Russo, J. J., and Chang, Y. (1997). Kaposi's sarcoma-associated herpesvirus encodes a functional bcl-2 homologue. Nat Med *3*, 293-8.

Sarisky, R., Gao, Z., Lieberman, R. M., Fixman, E. D., Hayward, G. S., and Hayward, S. D. (1996). A replication function associated with the activation domain of the Epstein-Barr virus Zta transactivator. J Virol *70*, 8340-8347.

Sawtell, N. M., and Thompson, R. L. (1992). Rapid in vivo reactivation of herpes simplex virus type 1 in latently infected murine ganglionic neurons after transient hyperthermia. J Virol *66*, 2150-2156.

Schalling, M., Ekman, M., Kaaya, E. E., Linde, A., and Biberfeld, P. (1995). A role for a new herpes virus (KSHV) in different forms of Kaposi's sarcoma. Nat Med *1*, 707-8.

Schepers, A., Pich, D., and Hammerschmidt, W. (1996). Activation of oriLyt, the lytic origin of DNA replication of Epstein-Barr Virus, by BZLF1. Virology *220*, 367-376.

Seaman, W. T., Ye, D., Wang, R. X., Hale, E. E., Weisse, M., and Quinlivan, E. B. (1999). Gene expression from the ORF50/K8 region of Kaposi's sarcoma-associated herpesvirus. Virology *263*, 436-449.

...T R, Cab
...Habou recu
...Virol 72 8850

...T R, Kol
...Stein-Barr Vi
...cus J Virol 7

...ngard A A,
...mmal comp
...ding propert
...Pl Virol 65

...ose J W, Ve
...ngo J S (19
...mad in vitro

...ller J, Grol
...ngo M F, C
...oma-associa
...ase, see com

...ous K A, Z
...ngey J, A
...oma-associa
...Virol 71,

...M, Blasi
...S, Brock
...ald P (19
...and endot
...oma Int J C

...R, Lin S.-
...Kinetics
...2 2332-

...R, Lin S.
...ular activ
...ogenous P

Serio, T. R., Cahill, N., Prout, M. E., and Miller, G. (1998). A functionally distinct TATA box required for late progression through the Epstein-Barr Virus life dycle. J. Virol. *72*, 8338-8343.

Serio, T. R., Kolman, J. L., and Miller, G. (1997). Late gene expression from the Epstein-Barr Virus BcLF1 and BFRF3 promoters does not require DNA replication in *cis*. J. Virol. *71*, 8726-8734.

Shepard, A. A., Imbalzano, A. N., and DeLuca, N. A. (1989). Separation of primary structural componenets conferring autoregulation, transactivation and DNA-binding properties to the herpes simplex virus transcriptional regulatory protein ICP4. J virol *63*, 3714-3728.

Sixbey, J. W., Vesterinen, E. H., Nedrud, J. G., Raab-Traub, N., Walton, L. A., and Pagano, J. S. (1983). Replication of Epstein-Barr virus in human epithelial cells infected in vitro. Nature *306*, 480-483.

Soulier, J., Grollet, L., Oksenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., d'Agay, M. F., Clauvel, J. P., Raphael, M., Degos, L., and et al. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease [see comments]. Blood *86*, 1276-80.

Staskus, K. A., Zhong, W., Gebhard, K., Herndier, B., Wang, H., Renne, R., Beneke, J., Pudney, J., Anderson, D. J., Ganem, D., and Haase, A. T. (1997). Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. J Virol *71*, 715-9.

Sturzl, M., Blasig, C., Schreier, A., Neipel, F., Hohenadl, C., Cornali, E., Ascherl, G., Esser, S., Brockmeyer, N. H., Ekman, M., Kaaya, E. E., Tschachler, E., and Biberfeld, P. (1997). Expression of HHV-8 latency-associated T0.7 RNA in spindle cells and endothelial cells of AIDS-associated, classical and African Kaposi's sarcoma. Int J Cancer *72*, 68-71.

Sun, R., Lin, S.-F., Staskus, K., Gradoville, L., Grogan, E., Haase, A., and Miller, G. (1999). Kinetics of Kaposi's sarcoma-associated herpesvirus gene expression. J. Virol. *73*, 2232-2242.

Sun, R., Lin, S. F., Gradoville, L., Yuan, Y., Zhu, F., and Miller, G. (1998). A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus. Proc. Natl. Acad. Sci. USA *95*, 10866-10871.

Takada, K. (1984). Cross-linking of cell surface immunoglobulin induces Epstein-Barr virus in Burkitt lymphoma lines. Int J Cancer *33*, 27-32.

Takada, K., and Ono, Y. (1989). Synchronous and sequential activation of latently infected Epstein-Barr virus genomes. J Virol *63*, 445-449.

Takada, K., Shimuzu, N., Sakuma, S., and Keating, A. (1986). Transactivation of the latent Epstein-Barr virus (EBV) genome after transfection of the EBV BamHI Z DNA fragment. J Virol *57*, 1016-1022.

Tenser, R. B., Edris, W. A., and Hay, K. A. (1988). Herpes simplex virus latent infection: reactivation and elimination of latency after neurectomy. Virology *167*, 302-305.

Watson, R. J., and Clements, J. B. (1980). A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. Nature *285*, 329-330.

Wu, C. A., Nelson, N. J., McGeoch, D. J., and Chalberg, M. D. (1988). Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. J Virol *62*, 435-443.

Wu, C. L., and Wilcox, K. W. (1990). Codons 262 to 490 from the herpes simplex virus ICP4 gene are sufficient to encode a sequence-specific DNA binding protein. Nucleic Acids Res *18*, 531-538.

Xiao, W., Pizer, L. I., and Wilcox, K. W. (1997). Identification of a promoter-specific transactivation domain in the herpes simplex virus regulatory protein ICP4. J Virol *71*, 1757-1765.

Zacny, V. L., Wilson, J., and Pagano, J. S. (1998). The Epstein-Barr virus immediate-early gene product, BRLF1, interacts with the retinoblastoma protein during the viral lytic cycle. J Virol *72*, 8043-8051.

Zalani, S., Holley-Guthrie, E. A., Gutsch, D. E., and Kenney, S. C. (1992). The Epstein-Barr virus immediate-early promoter BRLF1 can be activated by the cellular Sp1 transcription factor. J Virol *66*, 7282-7292.

Zhong, W., Wang, H., Herndier, B., and Ganem, D. (1996). Restricted expression of Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) genes in Kaposi sarcoma. Proc Natl Acad Sci U S A *93*, 6641-6.

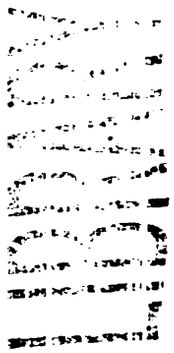
Zhu, F., Cusano, T., and Yuan, Y. (1999). Identification of the immediate-early transcripts of Kaposi's sarcoma-associated herpesvirus. J Virol *73*, 5556-5567.

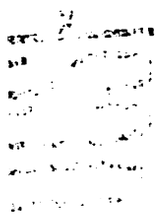


Chapter 1 - Inflammatory cytokines and the reactivation of Kaposi's sarcoma-associated herpesvirus (KSHV) lytic replication

Jean Chang, Rolf Renne, Dirk Dittmer and Don Ganem

This chapter is in press: Virology, Academic Press





Statement of contributions

In this paper, a panel of cytokines was screened for their ability to activate KSHV from latency. Interferon gamma and interferon alpha were identified as cytokines which modulate viral reactivation.

I carried out the time course for establishing the kinetics of viral induction in Figures 1 and 2. I executed a confirmatory second screen of the cytokines tested in Table 1. I performed the induction and flow cytometry for Figures 3a and 4. I generated the RNA samples used for quantitative Taqman PCR depicted in Figure 3b.

Chapter 1 - Inflammatory cytokines and the reactivation of Kaposi's sarcoma-associated herpesvirus (KSHV) lytic replication

Abstract

Kaposi's sarcoma (KS) is a complex proliferative lesion long suspected of being dependent upon exogenous paracrine signaling molecules to stimulate its proliferative, angiogenic and inflammatory components. In particular, both clinical and experimental observations have pointed to a potential role for inflammatory cytokines as permissive factors for KS development. But KS pathogenesis is also critically dependent on infection by an exogenous herpesvirus, the KS-associated herpesvirus (KSHV). To examine the possible links between inflammatory cytokines and KSHV replication, we tested for the ability of such cytokines to induce lytic viral reactivation in the latently infected BCBL-1 cell line. Interferon gamma (IFNγ) consistently activated KSHV replication, while TNF, IL-1, IL-2, IL-6, GM-CSF and bFGF did not. Glucocorticoids also failed to induce lytic KSHV growth in these cells, but ionomycin, a calcium ionophore, induced replication and strongly augmented the known inductive effects of phorbol esters. Interferon alpha (IFNα) had a dose-dependent inhibitory effect upon KSHV induction by ionomycin. The identification of IFNγ as an activator and IFNα as an inhibitor of KSHV induction *in vitro* correlates well with *in vivo* observations and demonstrates for the first time that inflammatory cytokines can directly modulate KSHV replication.

Introduction

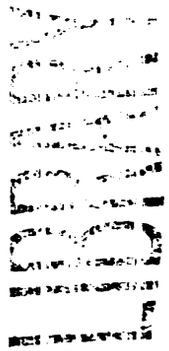
Kaposi's sarcoma-associated herpesvirus (KSHV), also called human herpesvirus 8 (HHV8), is a novel human gammaherpesvirus that is tightly linked to Kaposi's sarcoma (KS) and several other human diseases. KSHV sequences have been found in virtually all KS lesions tested, and several lines of epidemiologic evidence suggest that KSHV is necessary for KS pathogenesis (reviewed in Boshoff and Weiss, 1997; Ganem, 1997; Schulz et al., 1998). Infection with KSHV precedes clinical KS, and antecedent KSHV seropositivity strongly predicts increased KS risk (Martin et al., 1998; Renwick et al., 1998; Whitby et al., 1995). Globally, the seroprevalence of KSHV in different regions is tightly correlated with the prevalence of the tumor (Gao et al., 1996; Kedes et al., 1996; Mayama et al., 1998; Simpson et al., 1996; Whitby et al., 1998). At the cellular level, viral DNA and RNA sequences are present in the majority of the spindle-shaped endothelial cells thought to be the prime movers of KS histogenesis (Boshoff et al., 1995; Staskus et al., 1997). Most such cells are latently infected, but a small subpopulation also supports lytic viral growth (Staskus et al., 1997). KSHV infection is also found in infiltrating monocytes in KS tumors (Blasig et al., 1997), and in the B cell compartment of peripheral blood mononuclear cells (Ambroziak et al., 1995).

Since KSHV is a lymphotropic herpesvirus whose natural target is the B cell (Ambroziak et al., 1995; Mesri et al., 1996), lytic reactivation from its lymphoid reservoir would appear to be required for spread to the endothelium during the early stages of KS pathogenesis. In addition, since several lytic gene products of

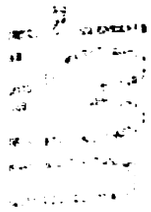
the virus can stimulate angiogenesis (Bais et al., 1998; Boshoff et al., 1997), it is possible that lytic replication in the target endothelium can also directly contribute to this aspect of KS lesions. Thus, factors associated with enhanced lytic KSHV growth can plausibly be envisioned to increase KS risk. The known linkage of KS with immune deficiency may operate, at least in part, on this basis - i.e. by immunodeficiency leading to a failure to control KSHV replication and spread.

Clinical and experimental observations suggest that many paracrine factors are also likely to be involved in KS pathogenesis. The dramatic acceleration of KS sometimes observed following severe bouts of sepsis (Krown, 1999), or following glucocorticoid administration (Perez et al., 1998; Trattner et al., 1993) has suggested that modulators of inflammation may play an important permissive role in KS development. In the mid 1980's, attempts to treat KS with certain immunomodulatory cytokines produced mixed results - while interferon alpha (IFNα) was modestly beneficial (Evans et al., 1991; reviewed in Krown, 1998; Krown et al., 1983), treatment trials with tumor necrosis factor (TNF) and interferon gamma (IFNγ) were abandoned because of several episodes of dramatic worsening of the tumor (Aboulafia et al., 1989; Ganser et al., 1986; Krigel et al., 1985) . Beyond these clinical observations, extensive laboratory experiments have also suggested a role for cytokines and growth factors in KS biology. KS lesions display elevated levels of mRNA for many cytokines, including basic fibroblast growth factor (bFGF), interleukin 1 (IL-1), platelet-derived growth factor (PDGF), IL-6, TNF, and IFNγ (reviewed in Ensoli and Sturzl, 1998; Fiorelli et al., 1998; Oxholm et al., 1989; Sturzl et al., 1995; Xerri et al., 1991) and cultured KS spindle

cells express a wide variety of cytokines and growth factors, including IL-1, IL-6, IL-8, vascular endothelial growth factor (VEGF) and bFGF (Ensoli et al., 1989; Miles et al., 1990; Samaniego et al., 1998; Sciacca et al., 1994). The growth of KS spindle cells *in vitro* generally requires conditioned media from activated lymphocytes or PBMCs (Nakamura et al., 1988); such media typically contain high concentrations of TNF, IFNγ , IL-1, IL-6, and oncostatin M (Barillari et al., 1992; Nair et al., 1992; Samaniego et al., 1995). Recently, Sirianni et al. reported increased cytokine (IFNγ) production in PBMCs and tumor infiltrating lymphocytes derived from KS patients (Sirianni et al., 1998). Based on these and other findings, it has been postulated that KS pathogenesis may be critically dependent upon the presence of appropriate inflammatory signals emanating from the tumor's microenvironment.



What has remained unclear is how the viral and cytokine components of the pathogenesis of KS are related. To shed light on this question, we have examined the ability of a large panel of cytokines and growth factors to modulate the lytic reactivation of KSHV from latency. Our results indicate that at least one inflammatory cytokine, IFNγ , can induce KSHV replication from its latent state in B cells, providing one plausible mechanism that directly links these two distinctive contributors to KS risk. Consistent with such a linkage, IFNα, a compound known to ameliorate KS lesions, has the ability to blunt KSHV growth.



Results

A quantita

The BCBL-

lymphoma

Reduced

Survival in

The Ren

activation

BCBL-1 cell

treatment

effect pov

reduction

release BC

activation

PEL be d

reduction in

NF- phys

Survival co

Accordi

Meaningfu

Resveratr

NF- protect

Results

A quantitative assay system for lytic reactivation.

The BCBL-1 cell line is a B cell line derived from a KSHV-related primary effusion lymphoma. In the ground state, the cells are latently infected with KSHV, but can be induced to undergo lytic viral replication upon treatment with a variety of chemical inducers such as the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Renne et al., 1996; Renne et al., 1996). Our previous studies of lytic reactivation involved the assay of lytic viral mRNAs or DNA in mass cultures of BCBL-1 cells (Renne et al., 1996). While definitive, these assays do not allow assessment of subtle alterations in reactivation levels; as such, while they work well for powerful artificial inducers like TPA they cannot detect the lower levels of induction likely to be produced by physiologically relevant compounds. This is because BCBL-1 cells have a measurable background level of spontaneous reactivation (Renne et al., 1996); two - to four-fold induction above this level would be difficult to credibly detect in a mass culture, as would higher levels of induction in small subpopulations of cells - but either of such scenarios could be highly physiologically relevant. In order to test a variety of different chemical and biological compounds for their ability to reactivate BCBL-1 cells we used an immunofluorescence-based assay (IFA) which allows examination of reactivation at the single cell level.

The assay employs a monoclonal antibody which specifically recognizes the viral K8.1 protein, an envelope glycoprotein expressed with late kinetics. This protein is

specific to lytically infected cells; as a late protein, it is expressed only in cells that have initiated viral genomic replication (Chandran et al., 1998; Raab et al., 1998). Thus, cells staining for this marker are irreversibly committed to lytic reactivation. To standardize the assay and optimize the time points chosen for examination, we determined the kinetics of lytic induction using TPA as our reference standard. About 2.5 x 10^5 BCBL-1 cells were plated into 24-well plates 24 hours prior to induction. At time zero, 20 ng of TPA was added and incubation continued 1, 3, 5, and 7 days; at those times post induction, induced and uninduced cells were harvested by mild centrifugation for IFA. All IFA assays were done in quadruplicate and the results expressed as the percentage of cells scoring positive for K8.1 expression. In selected instances, reactivity of induced BCBL-1 cells with K8.1 mAb was also examined by flow cytometry to allow facile screening of large numbers of cells.

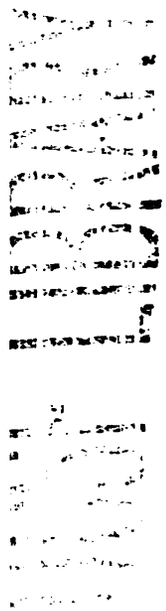


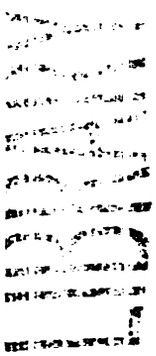
Figure 1 shows data from a typical immunofluoresence (IF) assay. Untreated BCBL-1 have a few cells which score for K8.1 expression, reflecting the level of spontaneous lytic reactivation in this cell line (Fig. 1A and B). Cells treated with ionomycin (Fig. 1C and D) and TPA (Fig 1E and F) show a significant increase in the number of positive cells while cells treated with both inducers results in even greater lytic induction (Fig. 1G and H). The kinetics of this induction is shown in Fig 2. As early as 3 days post induction, 4.1% of TPA-treated cells are positive for K8.1 expression, a 12.5-fold increase over the background spontaneous rate of activation (0.33%). Ionomycin, a calcium ionophore known to induce EBV from latency (Faggioni et al., 1986), was also found to be effective in KSHV induction of

BCBL, reaching an induction ratio of 9.2-fold over background. The combination of TPA and ionomycin was more potent than either alone, allowing an induction ratio of 33-fold at 3 days post-induction. Similar results are obtained when cells are scored by flow cytometry for reactivity with K8.1 mAb (data not shown). In both systems, there is considerable day-to-day variation in the percentage of cells induced by a given inducer. However, within each experiment the rank order of inducing stimuli was always constant: TPA + ionomycin > TPA > ionomycin. The ability of ionomycin to induce KSHV replication indicates an important stimulatory role for intracellular calcium in this process - a potentially important feature, since calcium mobilization is one of the hallmarks of the signal transduction cascade in B cells activated by antigen and other exogenous stimuli (DeFranco, 1999), including the viral K1 gene product (Lagunoff et al., 1999; Lee et al., 1998).

Screening of inflammatory cytokines for ability to induce KSHV.

Using the immunofluoresence assay, we screened a large number of inflammatory cytokines, singly and in combination, for effects on the induction of KSHV from latency. Table 1 lists the cytokines we tested and the results of the screen. IL-2, IL-6 and GM-CSF are cytokines regularly found in the conditioned medium of activated T cells often used to support KS spindle cell cultures (Barillari et al., 1992); IL-6 has previously been suggested to stimulate the proliferation of such cells (Miles et al., 1990). Although bFGF is not a mediator of inflammation, its effects were examined because of its posited role in the stimulation of KS-like neovascular lesions in mice. We placed particular emphasis on TNFα and IL-1β,

since Ensoli and coworkers have shown that these cytokines can stimulate spindle cell growth (Nakamura et al., 1988) and can act in concert to stimulate production and release of bFGF from both AIDS-KS derived spindle cells and normal endothelial cells (Samaniego et al., 1995; Samaniego et al., 1997). Dexamethasone was also tested as glucocorticoid treatment has been known to stimulate the development of KS lesions. All factors were screened at concentrations previously reported to have effects on KS cells, and scored at 3 days post addition of the compound. As shown in Table 1, none of these compounds were active inducers of KSHV replication. Several combinations of these molecules, chosen based on their known presence in T cell conditioned medium (Barillari et al., 1992), were similarly inactive.



Effects of interferons on KSHV.



Several considerations led us to explore the effects of interferons on KSHV induction. First, the type II interferon IFNγ is one of the predominant cytokines released from T cells recovered from KS lesions (Sirianni et al., 1998), making it a prime candidate for a paracrine regulator. Second, the viral genome encodes for homologs of the cellular interferon regulatory factor (IRF) family of transcription factors. Multiple reports have shown that the product of ORF K9, vIRF-1, can inhibit gene expression induced by type I interferons (Gao et al., 1997; Li et al., 1998; Zimring et al., 1998). This suggests that at least some type I interferons might be inhibitory to KSHV growth, providing a selective force for the evolution of viral strategies for the blunting of such inhibition. Accordingly we examined both type I and type II IFNs for their effects on KSHV.

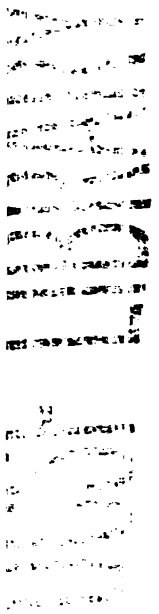
Figure 3a shows that, in contrast to the large panel of cytokines and immune modulators examined in Table 1, IFNγ is an effective inducer of KSHV lytic growth. IFNγ is less active than the known chemical inducers of reactivation; as shown in Fig 3a, it is only about half as active as ionomycin, when assayed at day 3 post induction. The induction of lytic gene expression by IFNγ could be confirmed by quantitative real-time RT-PCR for lytic mRNAs. Figure 3b shows relative transcript levels in treated BCBL-1 cells at day 5 post-infection. BCBL-1 cells exposed to IFNγ showed 10-fold increase in lytic K8.1 and orf50 mRNA levels, compared with untreated cells. As expected, TPA displayed much stronger induction, with over a 100-fold increase in lytic transcript levels. Neither treatment produced significant enhancement of the latent orf72 (v-cyclin) mRNA (Fig 3b).

By contrast, treatment of BCBL-1 cells with IFNα reduced the fraction of cells displaying K8.1 expression by flow cytometry, in a dose-dependent fashion (Fig 4). This suggests that IFNα can inhibit spontaneous lytic reactivation. Reactivation induced by ionomycin exposure was similarly inhibited by IFNα, again in a dose-dependent way (Fig 4). Partial inhibition of TPA induction by IFNα is also observed (data not shown). Thus, both type I and type II IFNs are able to exert effects upon viral reactivation.

Discussion

These data establish that products of host immune and inflammatory responses can directly modulate KSHV replication. These modulatory effects are notable for

their selectivity - of the large number of cytokines (and cytokine combinations) tested, only two were active, with IFNγ positively regulating lytic replication while IFNα inhibited induction. The failure of most other compounds tested to influence KSHV replication in our *in vitro* system does not, of course, exclude potential roles for these compounds in the modulation of viral replication *in vivo*. Our experiments employs a PEL (B-cell) line, but KSHV also targets endothelial (spindle) cells and monocytes *in vivo*. Such cells may respond to entirely different sets of stimuli. In addition, we think it is likely that other inflammatory mediators will also be found to influence KSHV dynamics in B-cells *in vivo*. For example, since IFNγ is a major cytokine involved in Th1-mediated responses, factors known to upregulate IFNγ release (e.g. IL-18 or IL-12 (Dinarello, 1999; Yoshimoto et al., 1998)) or augment Th1 responses more generally might also be expected to influence viral replication. However, the effects of Th1 upregulation on KSHV growth *in vivo* might be opposed by augmented CTL killing of infected cells - so the net effects of such Th1 modulation *in vivo* cannot be unambiguously predicted from our simple *in vitro* experiments.



As expected, the stimulatory effects of IFNγ were smaller than those induced by artificial chemical stimuli, but were highly reproducible and achieved using concentrations of cytokine comparable to levels seen in sera of patients at late stages of HIV disease (Rossol et al., 1989). Although our results reflect *in vitro* assays, there is good reason to believe that they may be relevant to KS pathogenesis *in vivo*. They provide a plausible mechanistic basis for long-standing clinical observations linking inflammatory processes to KS progression *in vivo*.

They are also consistent with the failure of IFNγ in the therapy of clinical KS and provide at least one potential explanation for the acceleration of disease progression observed in early trials of the drug (Ganser et al., 1986; Krigel et al., 1985). In addition, PBMCs from KSHV-positive patients grown in culture tend to lose the virus unless IFNγ is present to maintain and in some cases, increase viral load (Monini et al., 1999); though such experiments do not differentiate direct effects of IFNγ on virus producing cells from indirect effects of factors released from uninfected monocytes or other cells present in these complex populations. Together with these data, our results raise the question of whether downregulation of IFNγ would be of prophylactic or theraputic benefit in KS.

Our studies with IFNα also accord well with the known effects of this compound *in vitro* and *in vivo*. Culture of PBMCs from KSHV-infected subjects with high doses of IFNα results in a two-fold decrease in viral load (Monini et al., 1999) while this result could reflect direct or indirect effects of IFNα, our data suggests that direct inhibition is the likely mechanism. IFNα is also known to be modestly active in the therapy of KS (Evans et al., 1991; Krown, 1998; Krown et al., 1983). Our results show that it is a weak inhibitor of KSHV induction, with significant inhibition of viral growth is observed principally at high concentrations of the protein. This may be the result of KSHV ORF K9 expression, which acts to oppose IFNα mediated gene expression (Gao et al., 1997; Li et al., 1998; Zimring et al., 1998) and thus can blunt its antiviral actions. Formal proof of this, however, must await the development of genetic systems of the isolation and characterization of

K9 mutant viruses - which would be predicted to display enhanced sensitivity to type I IFNs.

Methods

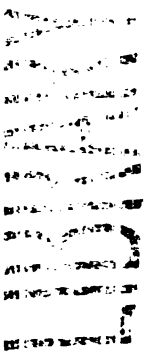
Cell culture and KSHV induction.

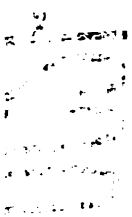
The BCBL-1 cell line was propagated and maintained as described previously (Renne et al., 1996). Cells were split to a density of 2.5 x 10^5/ml at 12-20 hrs prior to induction. On day 0, cells were washed once with PBS and then resuspended to the original volume with fresh media. For immunofluorescence analysis (IFA), cells were aliquotted in 0.8 ml volumes to 24-well plates and 200 ul of RPMI 1640 media with or without 5x inducing agent: TPA (Calbiochem-Nova-biochem Corp., La Jolla, CA), ionomycin (Sigma Chemical Co., St. Louis, MO), recombinant IL-2 (Genzyme Diagnostics, Cambridge, MA), IL-1α , IL-1β , IL-6, TNFα , GM-CSF, bFGF, dexamethasone, IFNα or IFNγ (Boehringer Mannheim GmbH, Mannheim, Germany). Each induction was performed in quadruplicate. Cells were harvested at the appropriate time post-induction for IFA. For FACS, cells were aliquotted in 40ml volumes to culture flasks and cultured with or without inducing agent.

Antibodies and IFA.

The monoclonal antibody 19B4 specific for K8.1 was provided by Dr. B. Forghani. For IFA, cells were washed once in PBS and resuspended in 150 ul of PBS. 10 ul of washed cells were applied to individual wells of a 32-well staining slide (Erie Scientific, Portsmouth, New Hampshire). Cells were allowed to adhere for 20

minutes and remaining liquid was removed by aspiration. Slides were immediately fixed in 50% methanol/50% acetone for 10 min at -20°C. The slides were then removed, air-dried and stored at -70°C or analyzed immediately. Cells in each well were rehydrated and blocked in 10 ul of 3% bovine serum albumin (BSA)/ 0.05% sodium azide (blocking buffer) for 30 min. Blocking buffer was removed by aspiration and 10 ul of 19B4 was applied to each well for one hour. The slides were then washed in 1x PBS/4% Tween-20 for 30 min, followed by one 30-min wash in 1x PBS. Excess PBS was removed by suction and 10 ul of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (ICN Biochemicals, Inc., Costa Mesa, CA) was added at a dilution of 1:200. Secondary antibody was removed after 30 min and slides were washed as above. Slides were treated with Vectashield (Vector Labs., Burlingame, CA), sealed and stored at 4°C.





Flow cytometric analysis.

1.25 x 10^6 cells were harvested, washed with PBS, fixed in 4% paraformaldehyde for 20 min, washed twice in PBS, resuspended in 200ul PBS/1%BSA and transferred to V-bottom 96-well plates. Cells were incubated with anti-K8.1 antibody (19B4) for 30 min at 4°C and washed twice with PBS/1%BSA. Then cells were incubated with FITC-conjugated goat anti-mouse secondary antibody (ICN Biochemicals, Inc., Costa Mesa, CA) for 30 min at 4°C, washed twice with PBS/1%BSA, and resuspended in PBS for analysis on a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). Living cells were gated based on forward and side scatter parameters.

RT-PCR.

Quantitative real-time RT-PCR was carried out in duplicate using Taqman™RT and Taqman™PCR with Amplitaq Gold™ reagents. Reverse transcription was carried out using 2.5 μM random hexamers at 25°C for 10 min, 48°C for 30 minutes and 95°C for 5 minutes. PCR was carried out using universal cycle conditions (2 min @ 50°C, 10 min @ 95°C then 40 cycles of 15 sec @ 95°C and 1 min at 60°C) on an ABI PRIZM 7000™ Sequence Detector. The primers used were: gapdhF; 5'-GAAGGTGAAGGTCGGAGTC, gapdhR 5'-GAAGATGGTGATGGGATTTC, gapdhP VIC-CAAGCTTCCCGTTCTCAGCC-TAMRA, orfK8.1s1 5'-AAAGCGTCCAGGCCACCACAGA, orfK8.1s45'-fam-ATGCCTTAATATCAGCCTTTTCA-tamra, orfK8.1a3 5'-CACCACTATTTCTGCCGTTTTC, orf50s1; 5'-CACAAAAATGGCGCAAGATGA, orf50p; 5'-6FAM-AGAAGCTTCGGCGGTCCTG-TAMRA, orf50a4; 5'-TGGTAGAGTTGGGCCTTCAGTT. All primers span splice junctions and hence only amplified RNA. To prevent contamination, PCR reactions were assembled in the segregated space in which neither KSHV virions nor cloned KSHV DNA were handled. Carryover of the amplification product was avoided using positive displacement pipettes and UNGglycosylase in the amplification reaction. To determine the sensitivity of the assay, RNA derived from BCBL-1 cells was used (data not shown). Quantitative analysis revealed that the signal for the lytic orf50 and orf K8.1 messages was on average 50-100 fold lower than the signal for spliced-latent messages in latently-infected BCBL-1 cells, consistent with prior observations that only 1-5% of BCBL-1 cells undergo spontaneous KSHV replication at any given time (Renne et al., 1996). The assay was linearly

dependent on input cell number in the range of 1 to 10^5 infected cells per reaction and we were consistently able to detect one infected cell in 10^5 uninfected cells.

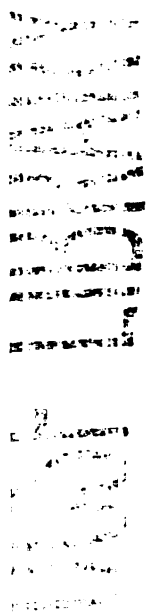


Table 1

Screening of inflammatory cytokines and growth factors for KSHV induction in BCBL-1 cells

Inducing agent:	Concentration:	Fold Induction
uninduced		1.0
TPA	20 ng/ml	11.7
IL-1a	0.1 ng/ml	1.0
	1 ng/ml	1.3
	10 ng/ml	1.0
IL-1b	0.35 ng/ml	1.3
	3.5 ng/ml	0.8
	35 ng/ml	1.0
IL-2	1 ng/ml	0.5
	10 ng/ml	0.5
IL-6	35 ng/ml	0.8
	175 ng/ml	1.0
TNFα	0.1 ng/ml	1.0
	1 ng/ml	1.3
	10 ng/ml	0.5
GM-CSF	0.5 ng/ml	0.8
	2.5 ng/ml	0.8
basic FGF	5 ng/ml	1.3
	25 ng/ml	0.8
Dexamethasone	1 nM	1.3
	10 nM	0.8
	100 nM	1.0
IL-1a, IL-1b and TNFα	1, 3.5, 1 ng/ml	0.8
	10, 35, 10 ng/ml	1.5
	10, 35, 1 ng/ml	0.5
	1, 3.5, 10 ng/ml	0.5
IL-1a, IL-1b, TNFα and GM-CSF	1, 3.5, 1, 0.5 ng/ml	1.0

BCBL-1 cells were treated with candidate inducing agents at the concentrations indicated above. Cells were harvested at 3 days post-treatment and quantitated as described for figure 1. Data is presented as fold induction relative to untreated cells.

References

Aboulafia, D., Miles, S. A., Saks, S. R., and Mitsuyasu, R. T. (1989). Intravenous recombinant tumor necrosis factor in the treatment of AIDS-related Kaposi's sarcoma. J Acquir Immune Defic Syndr *2*, 54-8.

Ambroziak, J. A., Blackbourn, D. J., Herndier, B. G., Glogau, R. G., Gullett, J. H., McDonald, A. R., Lennette, E. T., and Levy, J. A. (1995). Herpes-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients. Science *268*, 582-3.

Bais, C., Santomasso, B., Coso, O., Arvanitakis, L., Raaka, E. G., Gutkind, J. S., Asch, A. S., Cesarman, E., Gerhengorn, M. C., and Mesri, E. A. (1998). G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator. Nature *391*, 86-9.

Barillari, G., Buonaguro, L., Fiorelli, V., Hoffman, J., Michaels, F., Gallo, R. C., and Ensoli, B. (1992). Effects of cytokines from activated immune cells on vascular cell growth and HIV-1 gene expression. Implications for AIDS-Kaposi's sarcoma pathogenesis. J Immunol *149*, 3727-34.

Blasig, C., Zietz, C., Haar, B., Neipel, F., Esser, S., Brockmeyer, N. H., Tschachler, E., Colombini, S., Ensoli, B., and Sturzl, M. (1997). Monocytes in Kaposi's sarcoma lesions are productively infected by human herpesvirus 8. J Virol *71*, 7963-8.

Boshoff, C., Endo, Y., Collins, P. D., Takeuchi, Y., Reeves, J. D., Schweickart, V. L., Siani, M. A., Sasaki, T., Williams, T. J., Gray, P. W., Moore, P. S., Chang, Y., and Weiss, R. A. (1997). Angiogenic and HIV-inhibitory functions of KSHV-encoded chemokines. Science *278*, 290-4.

Boshoff, C., Schulz, T. F., Kennedy, M. M., Graham, A. K., Fisher, C., Thomas, A., McGee, J. O., Weiss, R. A., and O'Leary, J. J. (1995). Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. Nat Med *1*, 1274-8.

Boshoff, C., and Weiss, R. A. (1997). Aetiology of Kaposi's sarcoma: current understanding and implications for therapy. Mol Med Today *3*, 488-94.

Chandran, B., Smith, M. S., Koelle, D. M., Corey, L., Horvat, R., and Goldstein, E. (1998). Reactivities of human sera with human herpesvirus-8-infected BCBL-1 cells and identification of HHV-8-specific proteins and glycoproteins and the encoding cDNAs. Virology *243*, 208-17.

DeFranco, A. L. (1999). B-Lymphocyte Activation. In Fundamental immunology, W. E. Paul, ed. (Philadelphia: Lippincott-Raven), pp. 225-261.

Dinarello, C. A. (1999). IL-18: A TH1-inducing, proinflammatory cytokine and new member of the IL-1 family. J Allergy Clin Immunol *103*, 11-24.

Ensoli, B., Nakamura, S., Salahuddin, S. Z., Biberfeld, P., Larsson, L., Beaver, B., Wong-Staal, F., and Gallo, R. C. (1989). AIDS-Kaposi's sarcoma-derived cells express cytokines with autocrine and paracrine growth effects. Science *243*, 223-6.

Ensoli, B., and Sturzl, M. (1998). Kaposi's sarcoma: a result of the interplay among inflammatory cytokines, angiogenic factors and viral agents. Cytokine Growth Factor Rev *9*, 63-83.

Evans, L. M., Itri, L. M., Campion, M., Wyler-Plaut, R., Krown, S. E., Groopman, J. E., Goldsweig, H., Volberding, P. A., West, S. B., Mitsuyasu, R. T., and et al. (1991). Interferon-alpha 2a in the treatment of acquired immunodeficiency syndrome-related Kaposi's sarcoma. J Immunother *10*, 39-50.

Faggioni, A., Zompetta, C., Grimaldi, S., Barile, G., Frati, L., and Lazdins, J. (1986). Calcium modulation activates Epstein-Barr virus genome in latently infected cells. Science *232*, 1554-6.

Fiorelli, V., Gendelman, R., Sirianni, M. C., Chang, H. K., Colombini, S., Markham, P. D., Monini, P., Sonnabend, J., Pintus, A., Gallo, R. C., and Ensoli, B. (1998). gamma-Interferon produced by CD8+ T cells infiltrating Kaposi's sarcoma induces spindle cells with angiogenic phenotype and synergy with human immunodeficiency virus-1 Tat protein: an immune response to human herpesvirus-8 infection? Blood *91*, 956-67.

Ganem, D. (1997). KSHV and Kaposi's sarcoma: the end of the beginning? Cell *91*, 157-60.

Ganser, A., Brucher, W., Brodt, H. R., Busch, W., Brandhorst, I., Helm, E. B., and Hoelzer, D. (1986). Treatment of AIDS-related Kaposi's sarcoma with recombinant gamma-interferon. Onkologie *9*, 163-6.

Gao, S. J., Boshoff, C., Jayachandra, S., Weiss, R. A., Chang, Y., and Moore, P. S. (1997). KSHV ORF K9 (vIRF) is an oncogene which inhibits the interferon signaling pathway. Oncogene *15*, 1979-85.

Gao, S. J., Kingsley, L., Li, M., Zheng, W., Parravicini, C., Ziegler, J., Newton, R., Rinaldo, C. R., Saah, A., Phair, J., Detels, R., Chang, Y., and Moore, P. S. (1996). KSHV antibodies among Americans, Italians and Ugandans with and without Kaposi's sarcoma. Nat Med *2*, 925-8.

Kedes, D. H., Operskalski, E., Busch, M., Kohn, R., Flood, J., and Ganem, D. (1996). The seroepidemiology of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus): distribution of infection in KS risk groups and evidence for sexual transmission. Nat Med *2*, 918-24.

Krigel, R. L., Odajnyk, C. M., Laubenstein, L. J., Ostreicher, R., Wernz, J., Vilcek, J., Rubinstein, P., and Friedman-Kien, A. E. (1985). Therapeutic trial of interferon-gamma in patients with epidemic Kaposi's sarcoma. J Biol Response Mod *4*, 358-64.

Krown, S. E. (1999). Clinical Characteristics of Kaposi's Sarcoma. In The AIDS knowledge base : a textbook on HIV disease from the University of California, San Francisco School of Medicine, and San Francisco General Hospital, P. T. Cohen, M. A. Sande and P. Volberding, eds. (Philadelphia, PA: Lippincott, Williams and Wilkins), pp. 817-821.

Krown, S. E. (1998). Interferon-alpha: evolving therapy for AIDS-associated Kaposi's sarcoma. J Interferon Cytokine Res *18*, 209-14.

Krown, S. E., Real, F. X., Cunningham-Rundles, S., Myskowski, P. L., Koziner, B., Fein, S., Mittelman, A., Oettgen, H. F., and Safai, B. (1983). Preliminary observations on the effect of recombinant leukocyte A interferon in homosexual men with Kaposi's sarcoma. N Engl J Med *308*, 1071-6.

Lagunoff, M., Majeti, R., Weiss, A., and Ganem, D. (1999). Deregulated signal transduction by the K1 gene product of Kaposi's sarcoma-associated herpesvirus. Proc Natl Acad Sci U S A *96*, 5704-9.

Lee, H., Guo, J., Li, M., Choi, J. K., DeMaria, M., Rosenzweig, M., and Jung, J. U. (1998). Identification of an immunoreceptor tyrosine-based activation motif of K1 transforming protein of Kaposi's sarcoma-associated herpesvirus. Mol Cell Biol *18*, 5219-28.

Li, M., Lee, H., Guo, J., Neipel, F., Fleckenstein, B., Ozato, K., and Jung, J. U. (1998). Kaposi's sarcoma-associated herpesvirus viral interferon regulatory factor. J Virol *72*, 5433-40.

Martin, J. N., Ganem, D. E., Osmond, D. H., Page-Shafer, K. A., Macrae, D., and Kedes, D. H. (1998). Sexual transmission and the natural history of human herpesvirus 8 infection. N Engl J Med *338*, 948-54.

Mayama, S., Cuevas, L. E., Sheldon, J., Omar, O. H., Smith, D. H., Okong, P., Silvel, B., Hart, C. A., and Schulz, T. F. (1998). Prevalence and transmission of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in Ugandan children and adolescents. Int J Cancer *77*, 817-20.

Mesri, E. A., Cesarman, E., Arvanitakis, L., Rafii, S., Moore, M. A., Posnett, D. N., Knowles, D. M., and Asch, A. S. (1996). Human herpesvirus-8/Kaposi's sarcoma-associated herpesvirus is a new transmissible virus that infects B cells. J Exp Med *183*, 2385-90.

Miles, S. A., Rezai, A. R., Salazar-Gonzalez, J. F., Vander Meyden, M., Stevens, R. H., Logan, D. M., Mitsuyasu, R. T., Taga, T., Hirano, T., Kishimoto, T., and et al. (1990). AIDS Kaposi sarcoma-derived cells produce and respond to interleukin 6. Proc Natl Acad Sci U S A *87*, 4068-72.

Monini, P., Carlini, F., Sturzl, M., Rimessi, P., Superti, F., Franco, M., Melucci-Vigo, G., Cafaro, A., Goletti, D., Sgadari, C., Butto, S., Leone, P., Chiozzini, C., Barresi, C., Tinari, A., Bonaccorsi, A., Capobianchi, M. R., Giuliani, M., di Carlo, A., Andreoni, M., Rezza, G., and Ensoli, B. (1999). Alpha interferon inhibits human herpesvirus 8 (HHV-8) reactivation in primary effusion lymphoma cells and reduces HHV-8 load in cultured peripheral blood mononuclear cells. J Virol *73*, 4029-41.

Monini, P., Colombini, S., Sturzl, M., Goletti, D., Cafaro, A., Sgadari, C., Butto, S., Franco, M., Leone, P., Fais, S., Leone, P., Melucci-Vigo, G., Chiozzini, C., Carlini, F., Ascherl, G., Cornali, E., Zietz, C., Ramazzotti, E., Ensoli, F., Andreoni, M., Pezzotti, P., Rezza, G., Yarchoan, R., Gallo, R. C., and Ensoli, B. (1999).

Reactivation and persistence of human herpesvirus-8 infection in B cells and monocytes by Th-1 cytokines increased in Kaposi's sarcoma [see comments]. Blood *93*, 4044-58.

Nair, B. C., DeVico, A. L., Nakamura, S., Copeland, T. D., Chen, Y., Patel, A., O'Neil, T., Oroszlan, S., Gallo, R. C., and Sarngadharan, M. G. (1992). Identification of a major growth factor for AIDS-Kaposi's sarcoma cells as oncostatin M. Science *255*, 1430-2.

Nakamura, S., Salahuddin, S. Z., Biberfeld, P., Ensoli, B., Markham, P. D., Wong-Staal, F., and Gallo, R. C. (1988). Kaposi's sarcoma cells: long-term culture with growth factor from retrovirus-infected CD4+ T cells. Science *242*, 426-30.

Oxholm, A., Oxholm, P., Permin, H., and Bendtzen, K. (1989). Epidermal tumour necrosis factor alpha and interleukin 6-like activities in AIDS-related Kaposi's sarcoma. An immunohistological study. Apmis *97*, 533-8.

Perez, E., Barnadas, M. A., Garcia-Patos, V., Pedro, C., Curell, R., Sander, C. A., Kind, P., de Moragas, J. M., and Alomar, A. (1998). Kaposi's sarcoma in a patient with erythroblastopenia and thymoma: reactivation after topical corticosteroids. Dermatology *197*, 264-7.

Raab, M. S., Albrecht, J. C., Birkmann, A., Yaguboglu, S., Lang, D., Fleckenstein, B., and Neipel, F. (1998). The immunogenic glycoprotein gp35-37 of human herpesvirus 8 is encoded by open reading frame K8.1. J Virol *72*, 6725-31.

Renne, R., Lagunoff, M., Zhong, W., and Ganem, D. (1996). The size and conformation of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) DNA in infected cells and virions. J Virol *70*, 8151-4.

Renne, R., Zhong, W., Herndier, B., McGrath, M., Abbey, N., Kedes, D., and Ganem, D. (1996). Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. Nat Med *2*, 342-6.

Renwick, N., Halaby, T., Weverling, G. J., Dukers, N., Simpson, G. R., Coutinho, R. A., Lange, J. M. A., Schulz, T. F., and Goudsmit, J. (1998). Seroconversion for human herpesvirus 8 during HIV infection is highly predictive of Kaposi's sarcoma. Aids *12*, 2481-2488.

Rossol, S., Voth, R., Laubenstein, H. P., Muller, W. E., Schroder, H. C., Meyer zum Buschenfelde, K. H., and Hess, G. (1989). Interferon production in patients infected with HIV-1. J Infect Dis *159*, 815-21.

Samaniego, F., Markham, P. D., Gallo, R. C., and Ensoli, B. (1995). Inflammatory cytokines induce AIDS-Kaposi's sarcoma-derived spindle cells to produce and release basic fibroblast growth factor and enhance Kaposi's sarcoma-like lesion formation in nude mice. J Immunol *154*, 3582-92.

Samaniego, F., Markham, P. D., Gendelman, R., Gallo, R. C., and Ensoli, B. (1997). Inflammatory cytokines induce endothelial cells to produce and release basic fibroblast growth factor and to promote Kaposi's sarcoma-like lesions in nude mice. J Immunol *158*, 1887-94.

Samaniego, F., Markham, P. D., Gendelman, R., Watanabe, Y., Kao, V., Kowalski, K., Sonnabend, J. A., Pintus, A., Gallo, R. C., and Ensoli, B. (1998). Vascular endothelial growth factor and basic fibroblast growth factor present in Kaposi's sarcoma (KS) are induced by inflammatory cytokines and synergize to promote vascular permeability and KS lesion development. Am J Pathol *152*, 1433-43.

Schulz, T. F., Chang, Y., and Moore, P. S. (1998). Kaposi's Sarcoma-Associated Herpesvirus (Human Herpesvirus 8). In Human tumor viruses, D. J. McCance, ed. (Washington, D.C.: ASM Press), pp. 87-134.

Sciacca, F. L., Sturzl, M., Bussolino, F., Sironi, M., Brandstetter, H., Zietz, C., Zhou, D., Matteucci, C., Peri, G., Sozzani, S., and et al. (1994). Expression of adhesion molecules, platelet-activating factor, and chemokines by Kaposi's sarcoma cells. J Immunol *153*, 4816-25.

Simpson, G. R., Schulz, T. F., Whitby, D., Cook, P. M., Boshoff, C., Rainbow, L., Howard, M. R., Gao, S. J., Bohenzky, R. A., Simmonds, P., Lee, C., de Ruiter, A., Hatzakis, A., Tedder, R. S., Weller, I. V., Weiss, R. A., and Moore, P. S. (1996). Prevalence of Kaposi's sarcoma associated herpesvirus infection measured by antibodies to recombinant capsid protein and latent immunofluorescence antigen. Lancet *348*, 1133-8.

Sirianni, M. C., Vincenzi, L., Fiorelli, V., Topino, S., Scala, E., Uccini, S., Angeloni, A., Faggioni, A., Cerimele, D., Cottoni, F., Aiuti, F., and Ensoli, B. (1998). gamma-Interferon production in peripheral blood mononuclear cells and tumor infiltrating lymphocytes from Kaposi's sarcoma patients: correlation with the

presence of human herpesvirus-8 in peripheral blood mononuclear cells and lesional macrophages. Blood *91*, 968-76.

Staskus, K. A., Zhong, W., Gebhard, K., Herndier, B., Wang, H., Renne, R., Beneke, J., Pudney, J., Anderson, D. J., Ganem, D., and Haase, A. T. (1997). Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. J Virol *71*, 715-9.

Sturzl, M., Brandstetter, H., Zietz, C., Eisenburg, B., Raivich, G., Gearing, D. P., Brockmeyer, N. H., and Hofschneider, P. H. (1995). Identification of interleukin-1 and platelet-derived growth factor-B as major mitogens for the spindle cells of Kaposi's sarcoma: a combined in vitro and in vivo analysis. Oncogene *10*, 2007-16.

Trattner, A., Hodak, E., David, M., and Sandbank, M. (1993). The appearance of Kaposi sarcoma during corticosteroid therapy. Cancer *72*, 1779-83.

Whitby, D., Howard, M. R., Tenant-Flowers, M., Brink, N. S., Copas, A., Boshoff, C., Hatzioannou, T., Suggett, F. E., Aldam, D. M., Denton, A. S., and et al. (1995). Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. Lancet *346*, 799-802.

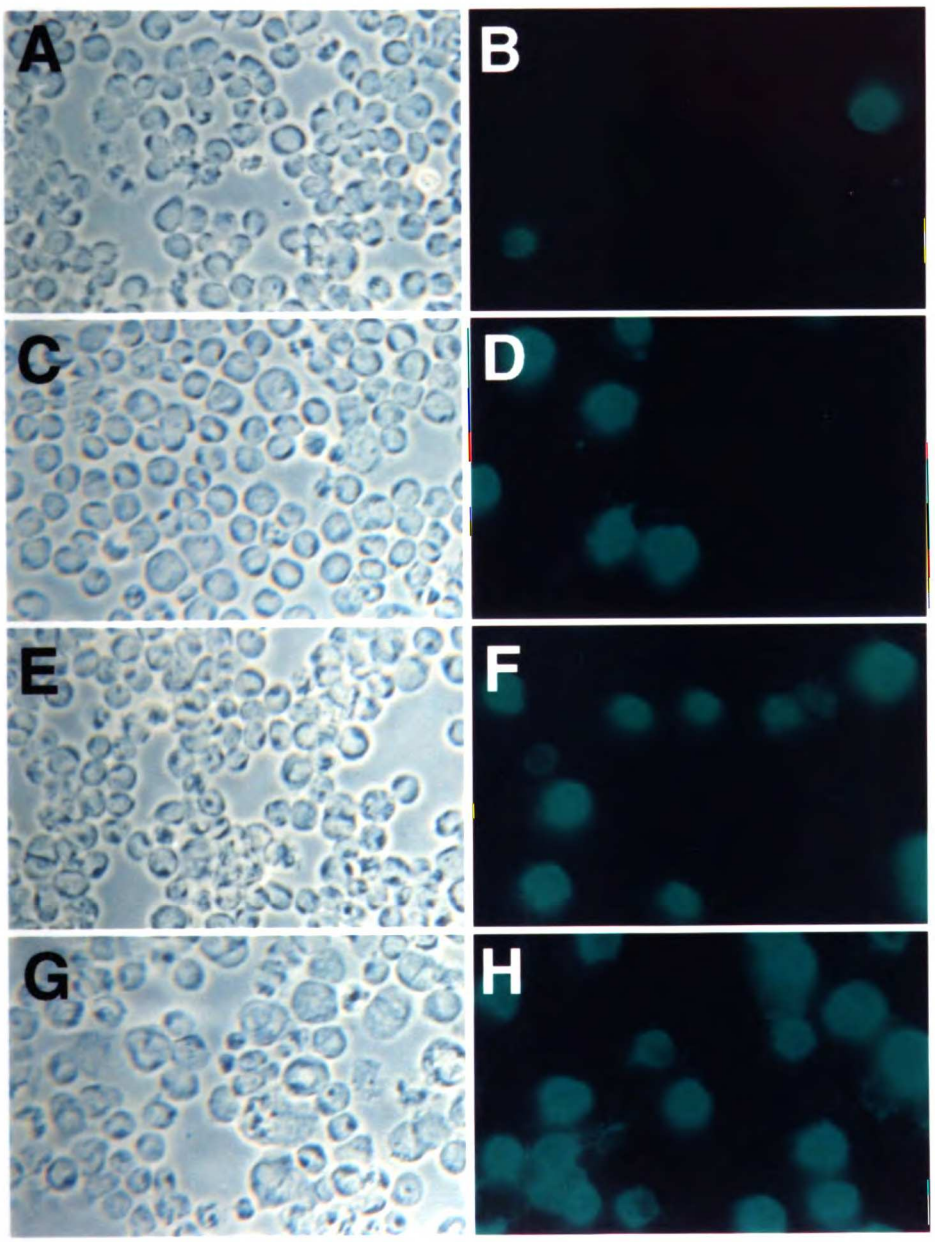
Whitby, D., Luppi, M., Barozzi, P., Boshoff, C., Weiss, R. A., and Torelli, G. (1998). Human herpesvirus 8 seroprevalence in blood donors and lymphoma patients from different regions of Italy [see comments]. J Natl Cancer Inst *90*, 395-7.

Xerri, L., Hassoun, J., Planche, J., Guigou, V., Grob, J. J., Parc, P., Birnbaum, D., and deLapeyriere, O. (1991). Fibroblast growth factor gene expression in AIDS-Kaposi's sarcoma detected by in situ hybridization. Am J Pathol *138*, 9-15.

Yoshimoto, T., Takeda, K., Tanaka, T., Ohkusu, K., Kashiwamura, S., Okamura, H., Akira, S., and Nakanishi, K. (1998). IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN-gamma production. J Immunol *161*, 3400-7.

Zimring, J. C., Goodbourn, S., and Offermann, M. K. (1998). Human herpesvirus 8 encodes an interferon regulatory factor (IRF) homolog that represses IRF-1-mediated transcription. J Virol *72*, 701-7.

Figure 1

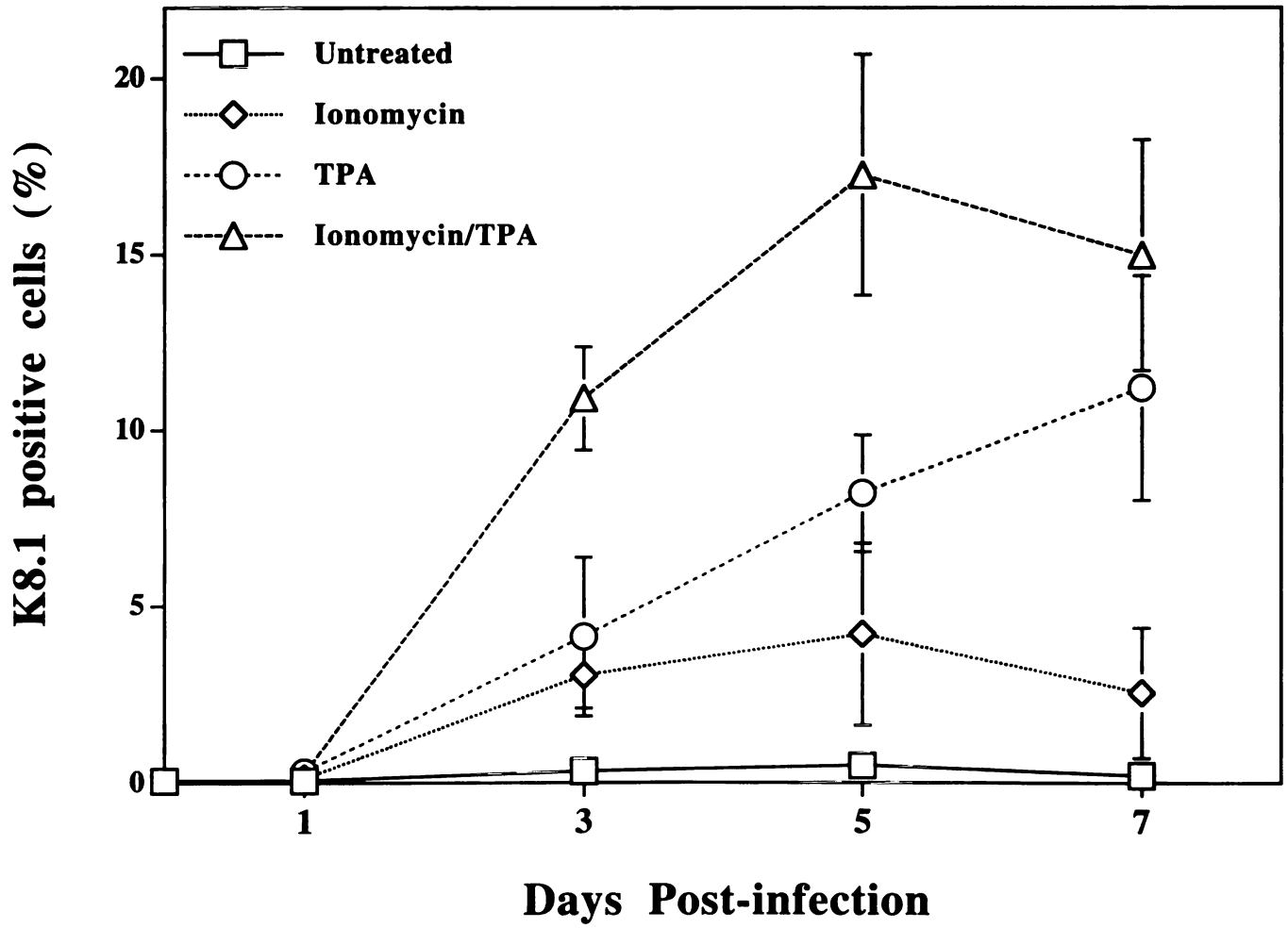


UCSF LIBRARY

Figure 1 Lytic reactivation of BCBL-1 by chemical induction.

Typical staining of BCBL-1 for K8.1 after chemical induction. BCBL-1 cells were left untreated (A, B) or induced with ionomycin (C, D), TPA (E, F) or both chemical inducers (G, H). At 3 days post-induction, cells were washed, resuspended in PBS, and fixed to 32-well slides for IFA analysis. Positive cells were detected using anti-K8.1 monoclonal antibody (19B4) followed by FITC-conjugated goat anti-mouse antibody and visualized by immunofluorescence microscopy. For each sample, a random field was chosen by light microscopy, then the corresponding immunofluoresence field was examined. Total cell count per field ranged from 330 to 1000 cells per field. Approx. 1/6 field is shown.

Figure 2

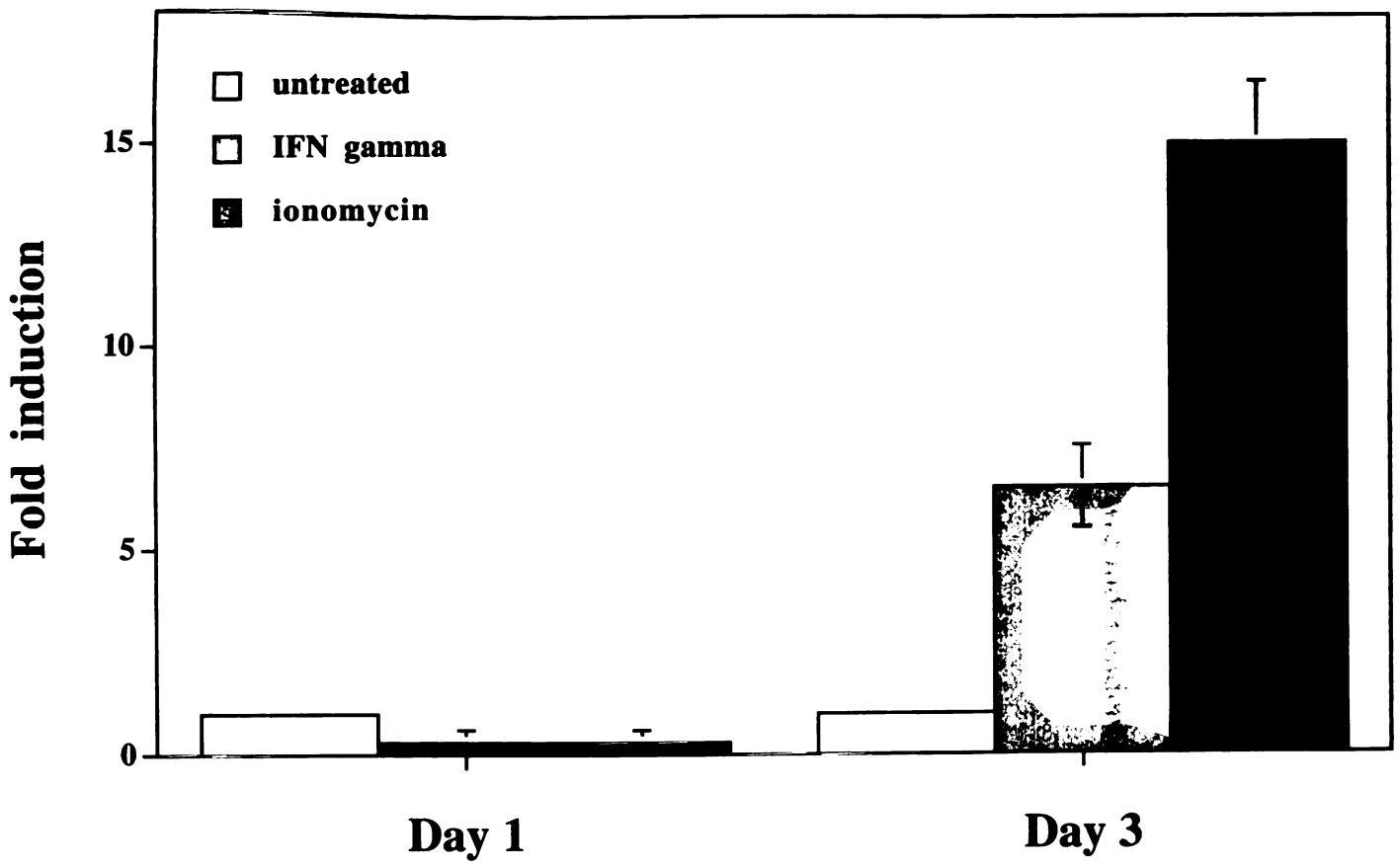


UNOT LIBRARY

Figure 2 Kinetics of induction. BCBL-1 cells were induced with TPA, Ionomycin or both chemical inducers.

Cells were harvested at 0, 1, 3, 5, and 7 days post-induction and analysed as above. Data points are the mean ± SD of quadruplicate inductions. Representative results from two independent experiments are shown.

Fig. 3a



PATENT LIBRARY

Figure 3a IFNγ and Ionomycin induction of KSHV.

BCBL-1 cells were induced with IFNγ (30U/ml) or ionomycin(500nM). At 1 and 3 days post-induction, cells were harvested and quantitated by FACS. Data was collected on 30,000 cells. Values are plotted as fold induction relative to untreated cells from the same time point. Error bars represent the standard deviation of triplicate inductions. Representative data from two independent experiments is shown.

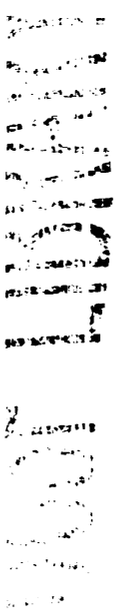


Fig. 3b

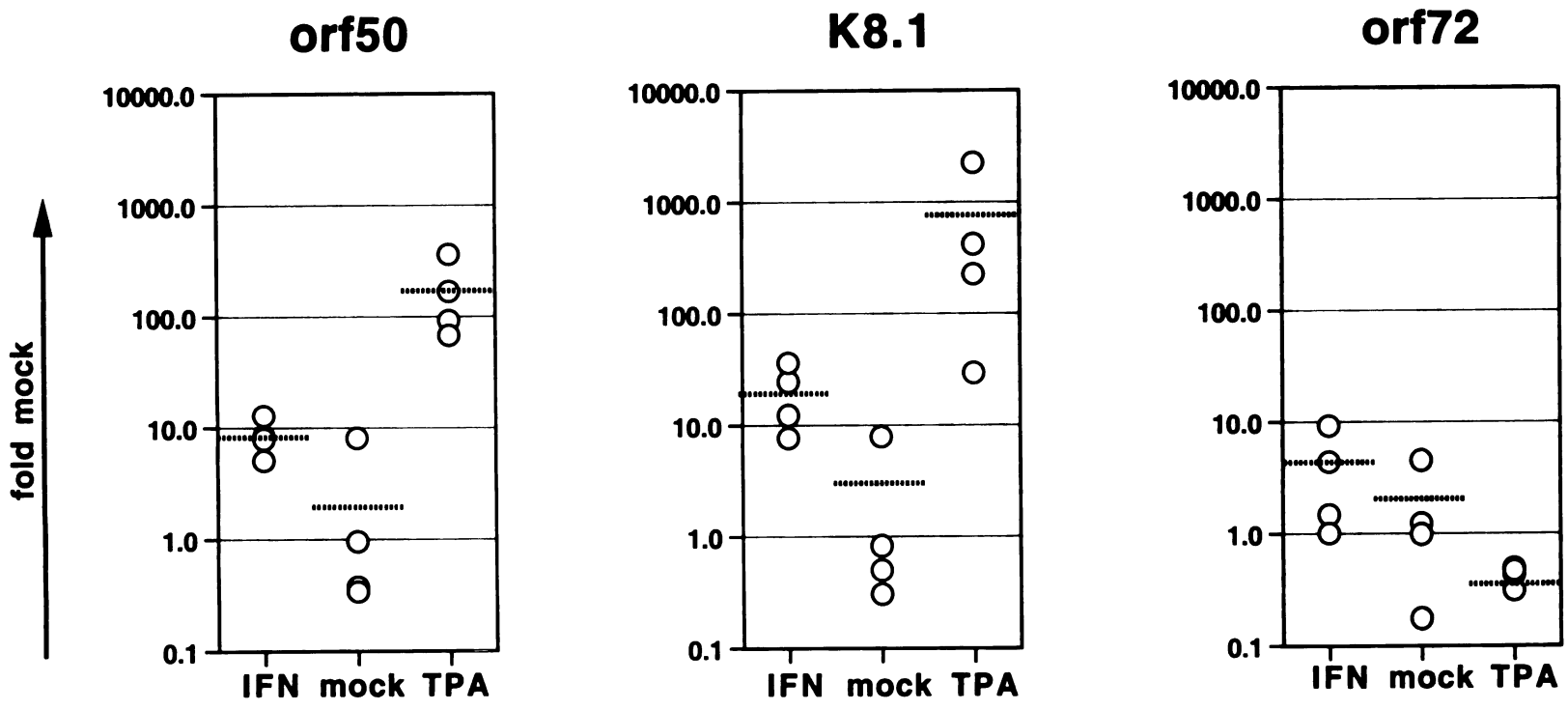
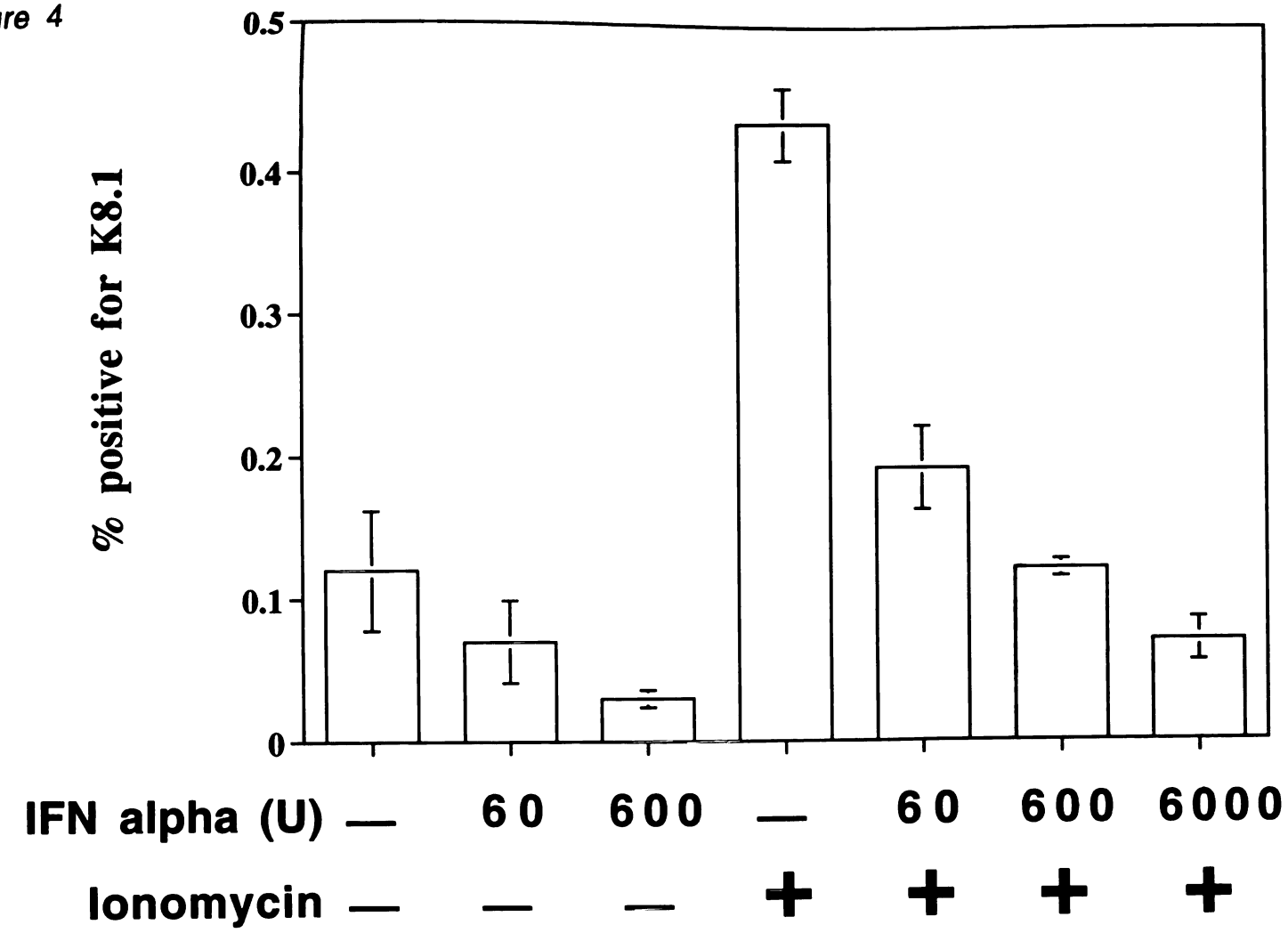

orf50
K8.1
orf72
fold mock
10000.0
1000.0
100.0
10.0
1.0
0.1
IFN mock TPA

Figure 3b Real-time RT-PCR quantitation of lytic induction by IFNγ and TPA.

Total RNA was isolated from 10^6 cells (mock treated, IFNγ-treated and TPA-treated). Cells were treated in duplicate to control for variability between inductions. RNA from each duplicate was analyzed in duplicate to account for variability in the RT and PCR reactions. To normalize for the amount of RNA in each reaction GAPDH was co-amplified in the same tube (multiplex) and quantified using a differently labeled probe. Ct-values for KSHV-specific transcripts (orf72, orf50, orfK8.1) were subtracted from corresponding Ct-values for the GAPDH and expressed as fold over mock treated cells for each treatment group. Mean values are represented by dotted lines.

Figure 4



BEST AVAILABLE COPY

Figure 4 IFNα inhibits induction of KSHV late gene (K8.1) expression by ionomycin.

BCBL-1 cells were induced with ionomycin(500nM) ± IFNα (60, 600 or 6000 U/ml). At five days post-induction, cells were harvested and examined by flow cytometry for K8.1 expressing cells, as described for figure 3a. The data is presented as the mean percentage of cells positive for K8.1 staining ± SD of triplicate inductions.

Chapter 2

Abstract

Expression of the KSHV ORF50 gene product triggers lytic reactivation and stimulates the expression of delayed early genes. In this study we identify an important cis-DNA element, 50RE-1, necessary for ORF50-responsive expression of the kaposin message. The 50RE-1, AAATGGGTGGCTAACC, is also present in the nut-1 promoter and is responsible for ORF50-regulated nut-1 expression. In addition we provide evidence for another ORF50 responsive element which is unrelated to 50RE-1. Transfer of this second element to a heterologous promoter confers ORF50 sensitivity to previously insensitive promoters. These findings suggest that ORF50 can exert its effects via multiple types of sites in the promoter regions of its target genes.

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) is a human gammaherpesvirus which has been linked to several different malignancies, including Kaposi's sarcoma (KS) (Chang and Moore, 1996; Schulz, 1998), primary effusion lymphoma (PEL) (Cesarman et al., 1995), and multicentric Castleman's disease (Soulier et al., 1995). Cells in KS and PEL are primarily latently infected, a form of infection in which only a small subset of viral genes are expressed (Dittmer et al., 1998; Kellam et al., 1997; Sarid et al., 1999; Zhong et al., 1996). These genes include: the latency-associated nuclear antigen (LANA) (Kedes et al., 1997; Kellam et al., 1997;

Rainbow et al., 1997) which is implicated in maintaining KSHV episomes (Ballestas et al., 1999), a homolog of FLICE inhibitor proteins (vFLIP) (Sturzl et al., 1999), a D-type cyclin homolog (ORF72) (Cesarman et al., 1996; Chang et al., 1996; Davis et al., 1997; Li et al., 1997), and a potential trio of related proteins, kaposin A, B, and C, for which no function has yet been determined (Sadler et al., 1999). Latency genes are undoubtedly important to the persistence of KSHV; however, it is unlikely that they are the only contributors to viral pathogenesis. Clinical studies have shown that the risk of KS can be reduced by 75 to 93 percent when at-risk patients are treated with ganciclovir, a drug which inhibits lytic replication (Martin et al., 1999). Within KS tumors and PEL cell lines lytic reactivation can be detected in a sub-population of tumor cells (Staskus et al., 1997; Sturzl et al., 1997). Lytic reactivation has been postulated to be an important step in the establishment of KSHV in endothelia and subsequent progression to disease (Lukac et al., 1998). Appealing models for the molecular pathogenesis of some features of KS can be built based upon the homology of various KSHV lytic cycle genes to cellular proteins which regulate proliferation and angiogenesis (Boshoff et al., 1997; Neipel et al., 1997; Russo et al., 1996). While it remains to be determined which particular lytic genes participate in tumor formation, the activation of lytic replication and the regulation of lytic gene expression is clearly an important issue in understanding KS pathogenesis.

Activation of the lytic cycle results in an ordered cascade of viral gene expression. First to be expressed are the immediate-early (IE) genes, typically viral activators which trigger the gene expression of the next temporal class, the delayed-early

(DE) genes. With DE gene expression, components of the viral replication machinery are available and lytic replication takes place. The last class of genes to be expressed, the late genes, are dependent upon viral replication and produce the structural components for packaging of the viral genome and virion assembly. In KSHV the molecular switch to the viral lytic cycle has been identified. The ORF50 gene product can trigger the lytic cycle in latently infected B cells ((Lukac et al., 1999; Lukac et al., 1998; Sun et al., 1998). Furthermore, deletion of the activation domain of ORF50 results in a dominant negative mutant of the protein which can inhibit lytic reactivation of BCBL-1 by known chemical inducers (Lukac et al., 1998), emphasizing ORF50's role as the key regulator of entry into the lytic cycle. KSHV ORF50 has distant homology to the EBV IE transcriptional regulator, R (or BRLF1 (Russo et al., 1996). Interestingly, two targets of ORF50, k-bZIP and ORF57 are also homologs of EBV IE genes, Z (also known as Zta, BZLF1, or Zebra) and M (Mta or BMLF1) ((Lukac et al., 1999). In EBV, both Z and R are able to trigger the lytic cycle (Grogan et al., 1987; Ragoczy et al., 1998; Rooney et al., 1989; Takada et al., 1986; Zalani et al., 1996). While the KSHV R homolog, ORF50, is able to disrupt latency (Lukac et al., 1999; Lukac et al., 1998; Sun et al., 1998), attempts to activate the lytic cycle via transfection of k-bZIP have not been successful (A. Polson, personal communication). Consistent with these observations, only the ORF50 transcript can be induced in absence of de novo protein synthesis (Sun et al., 1999; Zhu et al., 1999), the operational definition of herpesvirus IE genes.

Sequence analysis of KSHV ORF50 reveals a basic N-terminal domain, a potential leucine zipper, and a C-terminal domain which has homology to cellular and viral

transcription factor activation domains, including the EBV R protein (Lukac et al., 1999). When fused to the DNA-binding domain of the yeast GAL4 protein, this C-terminal segment of KSHV ORF50 strongly stimulated transcription from promoters bearing GAL4 binding sites (Lukac et al., 1999). The N-terminal portion (238 aas) of ORF50 has homology to the region responsible for DNA binding and dimerization in EBV R (Manet et al., 1991).

The molecular mechanism of ORF50 activation on downstream targets is unknown. In EBV, R has been shown to activate its downstream targets by both direct and indirect means. R-responsive elements (RRE) which support direct binding by R, have been characterized in both the EBV DR promoter (RRE-DR1 and RRE-DR2) and the EBV M promoter (RRE-M) (Chevallier-Greco et al., 1989; Gruffat et al., 1992). These three sites have limited similarity to one another, suggesting that EBV R recognizes relatively degenerate consensus sequences. In an attempt to characterize a consensus R binding site, Gruffat and Sergeant used iterative rounds of R binding to random 23 bp DNA sequences in an EMSA format to enrich for high affinity R binding sequences (Gruffat and Sergeant, 1994). The resulting consensus, GNCCN$_5$GGNG supports the observation that R binding sites are relatively variable in sequence. In addition to direct binding, R can also activate transcription of viral genes through upregulation of the expression of limiting cellular transcription factors, USF and E2F (Liu et al., 1996). Thus, EBV R is able to activate downstream targets by multiple mechanisms.

Kinetic studies of KSHV lytic cycle activation show that expression of ORF50 precedes that of both k-bZIP and ORF57 (Lukac et al., 1999; Sun et al., 1999). Such

an expression pattern is consistent with the observation that ORF50 expression can activate both k-bZIP and ORF57 promoters in transient transfection assays (Lukac et al., 1999). Additionally, ORF50 upregulates a number of classical DE genes, TK and DBP, as well as the promoters of several genes unique to KSHV, nut-1 and kaposin (Lukac et al., 1999; Lukac et al., 1998). In this study we examined selected downstream targets of ORF50. Deletion analysis and linker scanning mutagenesis identify two ORF50 responsive elements from KSHV DE promoters. We also show that these elements can impart ORF50 responsiveness upon heterologous promoters. These results show that ORF50 responsiveness can occurs through multiple types of sites.

Results

Linker scanning mutagenesis reveals an ORF50 responsive element (50RE-1) in the Kaposin B promoter

Previous work in our lab had demonstrated that a 1434 nt region of the Kaposin promoter showed moderate ORF50 activation (Lukac et al., 1998). 5' deletions of this region have shown that a small 98 bp region upstream of the Kaposin B start site is sufficient for ORF50 responsiveness (R. Sadler, personal communication; see also Fig 1b). To define functionally important *cis*-acting DNA elements within this 98 bp region, we constructed a series of linker scanning mutants, replacing 12 bp segments of the 98 bp promoter region with the linker sequence TATCATATGATA. Computer analysis of the Kaposin B promoter region proposed potential binding sites for several cellular transcription factors,

including AP-2, PEA3, and Sp-1 (Fig 1a). Linker scanning analysis should help determine the relevance of these proposed sites and identify new sites important in ORF50 activation.

We examined the ORF50 responsiveness of these mutant promoters in a luciferase reporter system. Assays were carried out in SLK cells, a diploid human cell line that was derived from a KS lesion (Herndier et al., 1994). These cells have an endothelial morphology, display von Willebrand's factor, an endothelial marker, and produce urokinase plasminogen activator (uPA) and plasminogen activator inhibitor 1 (PAI-1), indicative of its endothelial origin (Herndier et al., 1994). When tested in SLK, the full length (98 bp) Kaposin promoter, pGL3 B3X, shows a 24.7-fold increase in luciferase expression in response to the presence of ORF50 (Fig 1b). Within the linker series, basal levels of luciferase varied less than four-fold from the parental reporter construct, pGL3 B3X. When co-transfected with an ORF50 expression construct, only B3X (-93/-82) showed a significant loss of ORF50 responsiveness in SLK, approximately ~93% loss of the full activity of pGL3-B3X (Fig 1b). B3X (-69/-58) and B3X (-81/-70) showed consistent but less striking decrements in ORF50 activation (69% and 67%, respectively) when compared to pGL3-B3X (Fig 1b). Similar effects were seen in CV-1 and BJAB (Fig 1c and d). A three-fold decrease in ORF50 responsiveness was also observed for B3X (-12/-1) in SLK cells (Fig 1b), however, this effect was not seen in CV-1 or BJAB (Fig 1c and d). No significant loss of ORF50 responsiveness was observed in other constructs within the linker series.

The ORF50 responsive element of the kaposin promoter is also present and necessary for ORF50 activation of the nut-1 promoter

Previous work had shown that the 1.4kb pGL3 nut-1 construct was also ORF50 responsive (Lukac et al., 1998). Close examination of the nut-1 promoter revealed a 16 bp motif, AAATGGGTGGCTAACC, that is precisely conserved in the Kaposin promoter. Moreover, this 16 bp motif (50RE-1) includes the 12 bp region ablated in the kaposin promoter that was responsible for ORF50 activation. To determine whether this sequence was also necessary for ORF50 responsiveness in the expression of the kaposin gene, the 50RE-1 in the nut-1 promoter was ablated in the same manner as linker mutation (-93/-82) of the kaposin promoter. Mutation of the motif in the full length nut-1 promoter, pGL3 nut-1 (-67/-56), resulted in an 10-fold reduction of ORF50 responsiveness (Fig 2b). Deletion of 762 bp from the 5' end of the full length promoter, resulting in pGL3 nut-1 -706, did not drastically affect ORF50 responsiveness whereas linker mutation in the context of this shorter promoter region, pGL3 nut-1 -706 (-67/-56), resulted in complete abolition of ORF50 activation, comparable to truncation of the full length promoter to the TATA box, pGL3 nut-1 -44.

Additional 50RE-1 variants are present in the KSHV genome

We were interested in whether 50RE-1 might also be present at other sites within the viral genome. To determine the prevalence of this 16 bp element, we performed computer analysis using the GCG findpatterns program. Using the full 16bp element to search in the KSHV genome, only the two known sites in the

Kaposin and nut-1 promoters were found. Repeating the same search while allowing for two mismatches, we find a third recurrence with 13 contiguous bases (AcATGGGTGGCTAACg) of the 50RE, located at genomic position 24105 to 24118. Additional searches, allowing up to four mismatches, identified eight more potential sites (Table 1). Interestingly, several of these potential sites are clustered in areas proposed to contain the KSHV viral lytic origin (Nicholas et al., 1998).

The TK promoter also possesses an ORF50 responsive element

The TK promoter has also been shown to be ORF50 responsive (Lukac et al., 1998). Unlike the kaposin or nut-1 promoters, the TK promoter region does not have the 16 bp 50RE-1 motif. The plasmid pGL3 TK includes 1.4 kb upstream from the ATG of the TK ORF. Using this region to drive the expression of luciferase, up to 35-fold activation by ORF50 can be attained (Fig 3b). To determine the sequences within the 1.4 kb TK promoter region that are required for activation by ORF50, a 5' deletion series of the TK promoter was constructed (Fig 3a). Progressive deletion of 337, 656, 723, 800, 876, and 940 bp (constructs TK RI through TK4) from the 5' end of the full length TK promoter region results in constructs which behave similarly, yielding 15 to 40-fold activation in the presence of ORF50. Further deletion of 44 and 91 bp (TK Sma I and TK5) result in striking reductions of ORF50 activation, 78% and 88% loss of activity (relative to the full length TK region) (Fig 3b). Additional deletions (TK StuI and TK Pvu II) are similarly unresponsive to ORF50. These results suggest the presence of a potential ORF50 responsive element (or elements) between position 34867, the 5' end of the TK4 construct, and position 34911, the 5' end of the TK Sma I construct.

TK RRE confers ORF50 dependence upon heterologous promoters

The putative ORF50 responsive region was placed in front of the minimal E1B promoter to test its ability to confer ORF50 responsiveness to a heterologous promoter. The minimal E1B promoter was unresponsive to ORF50. In contrast, 117bp of the ORF50 responsive region (34836 - 34952) of the TK promoter resulted in 45-fold activation of TK117E1B in the presence of ORF50 (Fig 4b). Smaller regions of the TK ORF50 responsive region were also placed 5' to the minimal fos promoter. pfosluc was slightly ORF50 responsive (6.3 fold), however addition of 90bp (34867 - 34957) or 45bp (34867 - 34912) of the TK ORF50 responsive region resulted in 75.7-fold and 66.9 fold activation, respectively (Fig 4c). These results show that the ORF50 responsive element present in the TK promoter is able to transfer ORF50-directed activation to heterologous promoters.

Discussion

These results demonstrate that transcriptional activation by ORF50 can occur through at least two distinct *cis*-acting DNA elements. Analysis of two downstream targets of ORF50, kaposin and nut-1, showed that a 16 bp motif (50RE-1) is responsible for ORF50 responsiveness in both promoters. Deletion analysis of a third ORF50 responsive promoter, the TK promoter, identified an ORF50 responsive region which does not contain the 16 bp motif identified in the kaposin and nut-1 promoters. Further study of the region within the TK promoter shows that it can confer ORF50 responsiveness to heterologous promoters.

One obvious model for the mechanism of ORF50 activation is direct binding by ORF50 to these sites and subsequent stimulation of transcription via the activation domain of ORF50. Preliminary evidence suggests that this is not the case. Assay for ORF50 binding to the region identified in TK by electrophoretic mobility shift has been unsuccessful in detecting ORF50 bound to these sites (data not shown). Slower migrating complexes are seen when these sequences are incubated with nuclear extracts, regardless of the presence or absence of ORF50, suggesting that cellular proteins may bind these sequences. In assays using 50RE-1 sequences, similar results are seen (D. Lukac, personal communication). At least one of the 50RE-1 complexes may be specific as cold wild type 50RE-1 competitor is able to compete for complex formation while mutant 50RE-1 had no effect. This complex is also formed irrespective of the presence of ORF50 in the extracts. Taken together, these finding suggest that ORF50 may act through cellular factors to activate transcription of its downstream targets.

Upregulation of the expression of cellular factors would be one possible mechanism for indirect activation by ORF50. Several viral transcription factors, including E1A and EBV R, have been shown to activate transcription by binding to Rb (Dyson et al., 1992; Zany et al., 1998). The binding of these proteins to Rb displaces E2F which is then free to activate viral and cellular promoters containing E2F sites (Hiebert et al., 1991; Liu et al., 1996; Swenson et al., 1999; Zany et al., 1998). The Rb interacting region of EBV R has been localized to the first 200 aa of the R protein (Zany et al., 1998), a region of shared homology with ORF50 (Lukac et al., 1998). It would be interesting to see if ORF50 also encodes for an Rb binding

domain. Rb binding activity, resulting in displacement of E2F, would support the idea of ORF50 activation through the activity of cellular factors.

One argument against such a model is that the intensity of complex formation in the EMSA experiments is unaffected by the presence or absence of ORF50 (D. Lukac, personal communication). An increase in the concentration of a participating cellular factor should be reflected as an increase in bound probe that is specific to ORF50 containing extracts. An alternative explanation would require 50RE sites to be constantly bound by a cellular factor whose activity is altered by ORF50, while its DNA binding remains unchanged. Interestingly, the 3' half of 50RE-1 contains a potential CAAT box (Zhong et al., 1996). Certain isoforms of the CCAAT/enhancer binding protein (C/EBP)-related family have been shown to bind to their cognate sites with relatively constant affinity while their transcriptional activation function is modulated by phosphorylation (Trautwein et al., 1993; Wegner et al., 1992). If a C/EBP isoform binds to 50RE-1, addition of antibodies to C/EBP family members should result in a supershift of the complex of interest and allow rapid identification of the C/EBP isoform involved.

Of the ORF50 responsive regions identified thus far, many seem to be found relatively close to the transcriptional start of their respective messages. The reiterated motif of kaposin and nut-1 (50RE-1) occurs at -82 to -55 with respect to the start site of the kaposin and nut-1 transcripts, respectively. The close proximity of 50RE-1 to the start site of transcription in both promoters suggests that spacing between 50RE-1 and the transcriptional start may be important for ORF50-directed activation of promoters. Indeed, an ORF50 responsive region in the ORF57

promoter has been localized to a 50 bp region, 54 bp upstream of its cap site (L. Garibyan, personal communication). Additionally, a likely TATA box is 40 bp downstream of the ORF50-responsive region identified in the TK promoter. Although this TATA box is 425 bp upstream of the start of the TK ORF, studies of the TK promoter in related gammaherpesviruses suggest that our tentative designation of the KSHV TK TATA may be correct. In EBV, the EBV TK TATA is located 272 bp upstream of the translational start site (Liu et al., 1992). Additionally, S1 nuclease mapping and primer extension analysis of the TK promoter of bovine herpesvirus 4 (BHV-4) show that the transcriptional start of the BHV-4 TK lies 345 bp upstream of the TK ORF (Zhang and van Santen, 1995). Although initial computer analysis did not identify a TATA box for KSHV (Moore et al., 1996), mapping of the TK transcriptional start would determine whether the TK message begins near this potential TATA box and help determine whether the spacing between the ORF50 responsive element and the site of transcription initiation is preserved across ORF50 responsive promoters.

The significance of the variant 50RE-1 motifs detected within the KSHV genome is unknown. While some of these sites reside within open reading frames and most likely do not act as ORF50 responsive sites, a few of these sites are located in regions which bear resemblance to the lytic origin of other herpesviruses (Nicholas et al., 1998). In EBV, the immediate early gene Z plays a significant role in the activation of EBV oriLyt via several ZRE sites within the EBV lytic origin (Fixman et al., 1995; Sarisky et al., 1996; Schepers et al., 1996). Thus, it is of

particular interest to note that potential ORF50 binding sites are located in regions proposed to contain the lytic origin of KSHV.

The identification of multiple distinct ORF50 responsive sites in promoters of DE genes suggests that ORF50 may activate its downstream targets by more than one mechanism. Future progress in understanding these mechanism(s) will likely hinge on the identification of host and viral proteins that directly recognize the DNA sequences identified in this work.

Materials and Methods

Plasmids

pcDNA3-gORF50, pGL3 nut-1 and pGL3 TK have been described previously (Lukac et al., 1998).

pGL3-B3 (Lukac et al., 1998) was digested with XmaI and KpnI and recircularized, resulting in pGL3-B3X.

Linker scanning mutations of the kaposin promoter were generated by polymerase chain reaction (PCR). For each mutation, two PCR amplicons were generated: amplicon A (the 5' fragment) and amplicon B (the 3' fragment). Amplicon A was generated using primer pGL3rev (cttcatagccttatgcagttgctctc), which is specific to vector sequences downstream of the B3X fragment, and a forward primer, B3X(n)F, which contains the 12 bp linker sequence (tatcatatgata) at position n, flanked by sequence homologus to the kaposin promoter. Similarly,

amplicon B was generated using primer B5A (aggtacttggagcggccgcaataa) which is specific to vector sequences upstream of the B3X fragment, and an antisense primer, B3X(n)R , which contains the 12 bp linker sequence at position n, flanked by sequence homologus to the kaposin promoter. B3X forward (F) and reverse (R) primers:

B3X(-12/-1)F	taaagaagaggggagacctatcatatgatagcattacgcgcgcgg
B3X(-12/-1)R	ccgcgcgcgtaatgctatcatatgataggtctcccctcttcttta
B3X(-23/-12)F	caggaaacgctataaagaatatcatatgataacccccctcgcgc
B3X(-23/-12)R	gcgcgaggggggttatcatatgatattctttatagcgtttcctg
B3X(-33/-22)F	gccccttcgcaggaaacgtatcatatgataggggagaccgaccc
B3X(-33/-22)R	gggtcggtctcccctatcatatgatacgtttcctgcgaaggggc
B3X(-45/-34)F	acggttacgccccttatcatatgatactataaagaagagggga
B3X(-45/-34)R	tcccctcttctttatagtatcatatgataaggggcgtaaccgt
B3X(-57/-46)F	aagcagtttgtcctacgtatcatatgatatcgcaggaaacgctataaag
B3X(-57/-46)R	ctttatagcgtttcctgcgatatcatatgatacgtaggacaaactgctt
B3X(-69/-58)F	cccctacataagcatatcatatgatagttacgccccttcg
B3X(-69/-58)R	cgaaaggggcgtaactatcatatgatatgcttatgtagggg
B3X(-81/-70)F	gaaatgggtggctaacctatcatatgatagtttgtcctacggttacg
B3X(-81/-70)R	cgtaaccgtaggacaaactatcatatgataggttagccacccatttc
B3X(-93/-82)F	gcaggtgccgggaaattatcatatgatacctacataagcagtttgt

B3X(-93/-82)R acaaactgcttatgtaggtatcatatgataatttcccggcacctgc

Using primers pGL3rev and B5A, A and B amplicons were annealed and used as template to generate a PCR product containing the full B3X insert with linker scanning mutation. This PCR product was digested with Not I and Nar I and subcloned into pGL3-Basic which had been digested with the same enzymes.

pGL3 nut-1 deletion mutants:

pGL3 nut-1 -877was created by digestion of pGL3 nut-1 with Sma I followed by blunt end ligation.

pGL3 nut-1 -706 was created by digestion of SmaI -cut pGL3 nut-1 with Tth III followed by blunt end ligation.

pGL3 nut-1 -44 was created by digestion of MluI-cut pGL3 nut-1 with Pfl MI followed by blunt end ligation.

pGL3 nut-1 (-56/-67) and pGL3 nut-1 -706 (-56/-67) were generated in the same manner as the linker scanning mutations of the kaposin promoter (see above) except that pGL3 nut-1 or pGL3 nut-1 -706 were used as the initial template, and the following forward and reverse primers were used for amplicon generation:

nut1(-67/-49)F gcgtggcttccaaaaattatcatatgatatgtccaaaatatgggaaca

nut1(-67/-49)R tgttcccatattttggacatatcatatgataatttttggaagccacgc

TK deletions, TK RI, TK RV, TK Stu I and TK PvuII were created by double digest of pGL3-TK with the respective enzyme plus NheI and recircularizing by blunt end ligation. TK1-5 were generated by PCR amplification using TK RI as template and the following primers:

downstream primer:

pGL3rev cttcatagccttatgcagttgctctc

upstream primers:

TK1 cggaattcacgcgtgctagggtgtgcctttgtctttc

TK2 cggaattcacgcgtgctagcatggtgggcttgtgaat

TK3 cggaattcacgcgtgctagtttttgggtaccttagatag

TK4 cggaattcacgcgtgctagacaattttccttcggtggtg

TK5 cggaattcacgcgtgctagcagcaggggcgtgatagt

All PCR products were digested with Mlu I and Nar I and cloned into pGL3-Basic that had been digested with the same enzymes

TK Sma I was constructed by digesting pGL3-TK with Sma I and recircularizing by blunt end ligation.

TK117E1B was constructed by PCR amplification of 117bp of the TK promoter region from genomic positions 34836 to 34952 using primers TKX34836 ctagtctagagtcagcattttctct and TKN34952 agtcgctagcaagaatcagaaaaca. This product

was digested with Xho I and Nhe I and cloned into E1BCAT (Ueda et al., 1996) which had been digested with Xho I and Xba I.

plucfos was a gift from R. Grosschedl. It contains –56 to +109 of the minimal c-fos promoter as a SalI/BamHI. fragment cloned into pGL3 basic that had been digested with HinDIII.

TK90fos was constructed by PCR amplification of 90bp of the TK promoter region from genomic positions 34867 - 34957, using primers RvTKBgl tgcagatctttaagaatcagaaaac and BamTK4 aagggatccaattttccttcggtgg. This product was digested with Bam HI and Bgl II and cloned into pfosluc which had been digested with Bgl II.

TK45fos were constructed by ligation of two oligos containing 45bp of the TK promoter region from genomic positions 34867 - 34912 (primers TK45fwd gatctcaattttccttcggtggtgtgcaccggcgtgggaaacgccccggg and TK45rev gatccccggggcgtttcccacgccggtgcacaccaccgaaggaaaattga). The annealed product, which resulted in Bgl II compatible ends, was cloned into pfosluc which had been digested with Bgl II.

Cell lines and transfections

SLK cells were propagated and maintained in RPMI 1640 media supplemented with 10% fetal calf serum, 2mM glutamine and antibiotics (100U penicillin/ml, 100ug streptomycin/ml). For transfections, cells were plated at 1 x 10^5 cells per well of a 6-well plate and grown in 1.5 ml fresh media overnight. On the day of

transfection, 5µl of Fugene 6 transfection reagent (Boehringer Manneheim) was diluted in 95 µL of unsupplimented media, and added to 2 µg DNA. The mixture was mixed and complexes were allowed to form at room temperature for 15 min. Complexes were added to cells which were harvested 48 hrs post complex addition .

CV-1 cells were propagated and maintained in DME H-21 media supplemented with 10% FCS and antibiotics (100U penicillin/ml, 100ug streptomycin/ml). For transfections, cells were plated at 1 x 10^5 cells per well of a 6-well plate and grown overnight. On the day of transfection, 5µg of total DNA was diluted in 95 µL of unsupplimented media, 10 µL of Superfect transfection reagent (Qiagen) was added. The mixture was vortexed and complexes were allowed to form at room temperature for 10 min. Cells were washed once with 1x PBS and the complexes, diluted with 600 µL of complete media, were left on the cells for 3 h. Complexes were then removed, cells were washed once with 1x PBS, and fresh media was added. Cells were harvested 48 hrs post complex addition.

BJAB cells were propagated and maintained in RPMI 1640 media as in Renne *et al.* (Renne et al., 1996). On the day of electroporation, cells were washed once with 1x PBS, and resuspended in unsupplimented media. 1 x 10^6 cells were aliquotted in 0.4 µL volume to electroporation cuvettes (0.42cm) containing 20 µg of DNA. Cells were electroporated with 960 MF and 150 mV and subsequently transferred to 10 ml fresh media for 48 hours.

Luciferase, CAT and β-galactosidase Assay.

Cells were washed twice with 1mL 1x PBS. After the addition of 0.2 ml reporter lysis buffer (Promega) cells were harvested by scraping, vortexed and spun briefly to pellet cellular debris. 20- and 50 μL aliquots were analyzed by luciferase, CAT or β-galactosidase assay, respectively, according to the manufacturer's instructions (Promega).

Table 1 Additional 50RE-1 variants in the KSHV genome

Forward	mis=	AAATGGGTGGCTAACC	
22559	4	AAAgGGGTGcaTtACC	proposed left ori
24105	2	AcATGGGTGGCTAACg	proposed left ori
28596		AAATGGGTGGCTAACC	nut-1 promoter
31132	4	AAATGGGTGGgTgtaC	within ORF17
129327	3	AAAgGcGTGGCTAAaC	upstream of ORF74
129563	4	AAATGGaTtGgTcACC	within ORF74
Reverse	mis=	GGTTAGCCACCCATTT	
57841	4	tGcTgGCCACCCtTTT	within ORF37
118842		GGTTAGCtACCCATTT	kaposin promoter
120191	4	GGaTAGCCACCCAcca	proposed right ori
120228	4	GGTTAGCCACCCAcaa	proposed right ori
128958	4	GcTTAcCCACCCccTT	upstream of ORF74

References

Ballestas, M. E., Chatis, P. A., and Kaye, K. M. (1999). Efficient persistence of extrachromosomal KSHV DNA mediated by Latency-Associated Nuclear Antigen. Science *284*, 641-644.

Boshoff, C., Endo, Y., Collins, P. D., Takeuchi, Y., Reeves, J. D., Schweickart, V. L., Siani, M. A., Sasaki, T., Williams, T. J., Gray, P. W., Moore, P. S., Chang, Y., and Weiss, R. A. (1997). Angiogenic and HIV-inhibitory functions of KSHV-encoded chemokines [see comments]. Science *278*, 290-4.

Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS- related body-cavity-based lymphomas [see comments]. N Engl J Med *332*, 1186-91.

Cesarman, E., Nador, R. G., Bai, F., Bohenzky, R. A., Russo, J. J., Moore, P. S., Chang, Y., and Knowles, D. M. (1996). Kaposi's sarcoma-associated herpesvirus contains G protein-coupled receptor and cyclin D homologs which are expressed in Kaposi's sarcoma and malignant lymphoma. J Virol *70*, 8218-23.

Chang, Y., and Moore, P. S. (1996). Kaposi's Sarcoma (KS)-associated herpesvirus and its role in KS. Infect Agents Dis *5*, 215-22.

Chang, Y., Moore, P. S., Talbot, S. J., Boshoff, C. H., Zarkowska, T., Godden, K., Paterson, H., Weiss, R. A., and Mittnacht, S. (1996). Cyclin encoded by KS herpesvirus [letter]. Nature *382*, 410.

Chevallier-Greco, A., Gruffat, H., Manet, E., Calender, A., and Sergeant, A. (1989). The Epstein-Barr virus (EBV) DR enhancer contains two functionally different domains: Domain A is consititutive and cell specific, domain B is transactivated by the EBV early protein R. J Virol *63*, 615-623.

Davis, M. A., Sturzl, M. A., Blasig, C., Schreier, A., Guo, H. G., Reitz, M., Opalenik, S. R., and Browning, P. J. (1997). Expression of human herpesvirus 8-encoded cyclin D in Kaposi's sarcoma spindle cells [see comments]. J Natl Cancer Inst *89*, 1868-74.

Dittmer, D., Lagunoff, M., Renne, R., Staskus, K., Haase, A., and Ganem, D. (1998). A cluster of latently expressed genes in Kaposi's Sarcoma-associated herpesvirus. J. Virol. *72*, 8309-15.

Dyson, N., Guida, P., McCall, C., and Harlow, E. (1992). Adenovirus E1A makes two distinct contacts with the retinoblastoma protein. J Virol *66*, 4606-4611.

Fixman, E. D., Hayward, G. S., and Hayward, S. D. (1995). Replication of Epstin-Barr virus orilyt: Lack of a dedicated virally encoded origin-binding protein and dependence on Zta in cotransfection assays. J Virol *69*, 2998-3006.

Grogan, E. J., Jenson, J., Countryman, J., Heston, L., Gradoville, L., and Miller, G. (1987). Transfections of a rearranged viral DNA fragment, WZhet, stably converts latent Epstein-Barr virus infection to productive infection in lymphoid cells. Proc. Natl. Acad. Sci. USA *84*, 1332-1336.

Gruffat, H., Duran, N., Buisson, M., Wild, F., Buckland, R., and Sergeant, A. (1992). Characterization of an R-binding Site Mediating the R-Induced Activation of the Epstein-Barr Virus BMLF1 Promoter. J. Virol. *66*, 46-52.

Gruffat, H., and Sergeant, A. (1994). Characterization of the DNA-binding site repertoire for the Epstein-Barr virus transcription factor R. Nucleic Acids Res. *22*, 1172-1178.

Herndier, B. G., Werner, A., Arnstein, P., Abbey, N. W., Demartis, F., Cohen, R. L., Shuman, M. A., and Levy, J. A. (1994). Characterization of a human Kaposi's sarcoma cell line that induces angiogenic tumors in animals. AIDS *8*, 575-581.

Hiebert, S. W., Blake, M., Azizkhan, J., and Nevins, J. R. (1991). Role of E2F transcription factor in E1A-mediated trans activation of cellular genes. J Virol *65*, 3547-3552.

Kedes, D. H., Lagunoff, M. L., Renne, R., and Ganem, D. (1997). Identification of the gene encoding the major latency-associated nuclear antigen of the Kaposi's sarcoma-associated herpesvirus. Journal of Clinical Investigation *100*, 2606-2610.

Kellam, P., Boshoff, C., Whitby, D., Matthews, S., Weiss, R. A., and Talbot, S. J. (1997). Identification of a major latent nuclear antigen LNA-1 in the human herpesvirus 8 genome. J. Hum. Virol. *1*, 19-29.

Li, M., Lee, H., Yoon, D. W., Albrecht, J. C., Fleckenstein, B., Neipel, F., and Jung, J. U. (1997). Kaposi's sarcoma-associated herpesvirus encodes a functional cyclin. J Virol *71*, 1984-91.

Liu, C., Sista, N. D., and Pagano, J. S. (1996). Activation of the Epstein-Barr Virus DNA Polymerase Promoter by the BRLF1 Immediate-Early Protein is Mediated through USF and E2F. J. Virol. *70*, 2545-2555.

Liu, M. Y., Pai, C. Y., Shieh, S. M., Hsu, T. Y., Chen, J. Y., and Yang, C. S. (1992). Cloning and expression of a cDNA encoding the Epstein-Barr virus thymidine kinase gene. J. Virol. Meth. *40*, 107-118.

Lukac, D. M., Kirshner, J. R., and Ganem, D. (1999). Transcriptional Activation by the Product of Open Reading Frame 50 of Kaposi's Sarcoma-Associated Herpesvirus Is Required for Lytic Viral Reactivation in B Cells. J. Virol. *73*, 9348-9361.

Lukac, D. M., Renne, R., Kirshner, J. R., and Ganem, D. (1998). Reactivation of Kaposi's sarcoma-associated herpesvirus infection from latency by expression of the ORF 50 transactivator, a homolog of the EBV R protein. Virology *252*, 304-312.

Manet, E., Rigolet, A., Gruffat, H., Giot, J.-F., and Sergeant, A. (1991). Domains of the Epstein-Barr virus (EBV) transcription factor R required for dimerization, DNA binding and activation. Nucleic Acids Res. *19*, 2661-2667.

Martin, D. F., Kuppermann, B. D., Wolitz, R. A., Palestine, A. G., Li, H., and Robinson, C. A. (1999). Oral ganciclovir for patients with cytomegalovirus retinitis treated with a ganciclovir implant. Roche Ganciclovir Study Group. N. Engl. J. Med. *340*, 1063-1070.

Moore, P. S., Gao, S. J., Dominguez, G., Cesarman, E., Lungu, O., Knowles, D. M., Garber, R., Pellett, P. E., McGeoch, D. J., and Chang, Y. (1996). Primary characterization of a herpesvirus agent associated with Kaposi's sarcomae [published erratum appears in J Virol 1996 Dec;70(12):9083]. J Virol *70*, 549-58.

Neipel, F., Albrecht, J. C., and Fleckenstein, B. (1997). Cell-homologous genes in the Kaposi's sarcoma-associated rhadinovirus human herpesvirus 8: determinants of its pathogenicity? J Virol *71*, 4187-92.

Nicholas, J., Zong, J., Alcendor, D. J., Ciufo, D. M., Poole, L. J., Sarisky, R. T., Chiou, C., Zhang, X., Wan, X., Guo, H., Reitz, M. S., and Hayward, G. S. (1998). Novel organizational features, captured cellular genes, and strain variability within the genome of KSHV/HHV8. J Natl Cancer Inst Monographs *23*, 79-88.

Ragoczy, T., Heston, L., and Miller, G. (1998). The Epstein-Barr virus Rta protein activates lytic cycle genes and can disrupt latency in B lymphocytes. J. Virol. *72*, 7978-7984.

Rainbow, L., Platt, G. M., Simpson, G. R., Sarid, R., Gao, S. J., Stoiber, H., Herrington, C. S., Moore, P. S., and Schulz, T. F. (1997). The 222- to 234-kilodalton latent nuclear protein (LNA) of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) is encoded by orf73 and is a component of the latency-associated nuclear antigen. J Virol *71*, 5915-21.

Renne, R., Zhong, W., Herndier, B., McGrath, M., Abbey, N., Kedes, D., and Ganem, D. (1996). Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. Nat Med *2*, 342-6.

Rooney, C. M., Rowe, D. T., Ragot, T., and Farrell, P. J. (1989). The spliced BZLF1 gene of Epstein-Barr virus (EBV) transactivates an early EBV promoter and induces the virus productive cycle. J Virol *63*, 3109-3116.

Russo, J. J., Bohenzky, R. A., Chien, M. C., Chen, J., Yan, M., Maddalena, D., Parry, J. P., Peruzzi, D., Edelman, I. S., Chang, Y., and Moore, P. S. (1996). Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). Proc Natl Acad Sci U S A *93*, 14862-7.

Sadler, R., Wu, L., Forghani, B., Renne, R., Zhong, W., Herndier, B., and Ganem, D. (1999). A complex translational program generates multiple novel proteins from the latently expressed Kaposin (K12) locus of Kaposi's sarcoma-associated herpesvirus. J. Virol. *in press*.

Sarid, R., Wiezorek, J. S., Moore, P. S., and Chang, Y. (1999). Characterization and cell cycle regulation of the major Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) latent genes and their promoter. J. Virol. *73*, 1438-1446.

Sarisky, R., Gao, Z., Lieberman, R. M., Fixman, E. D., Hayward, G. S., and Hayward, S. D. (1996). A replication function associated with the activation domain of the Epstein-Barr virus Zta transactivator. J Virol *70*, 8340-8347.

Schepers, A., Pich, D., and Hammerschmidt, W. (1996). Activation of orilyt, the lytic origin of DNA replication of Epstein-Barr virus, by BZLF1. Virology *220*, 367-376.

Schulz, T. (1998). Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8). J Gen Virol *79*, 1573-1591.

Soulier, J., Grollet, L., Oksenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., d'Agay, M. F., Clauvel, J. P., Raphael, M., Degos, L., and et al. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease [see comments]. Blood *86*, 1276-80.

Staskus, K. A., Zhong, W., Gebhard, K., Herndier, B., Wang, H., Renne, R., Beneke, J., Pudney, J., Anderson, D. J., Ganem, D., and Haase, A. T. (1997). Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. J Virol *71*, 715-9.

Sturzl, M., Blasig, C., Schreier, A., Neipel, F., Hohenadl, C., Cornali, E., Ascherl, G., Esser, S., Brockmeyer, N. H., Ekman, M., Kaaya, E. E., Tschachler, E., and Biberfeld, P. (1997). Expression of HHV-8 latency-associated T0.7 RNA in spindle cells and endothelial cells of AIDS-associated, classical and African Kaposi's sarcoma. Int J Cancer *72*, 68-71.

Sturzl, M., Hohenadl, C., Zietz, C., Castanos-Velez, E., Wunderlich, A., Ascherl, G., Biberfeld, P., Monini, P., Browning, P. J., and Ensoli, B. (1999). Expression of K13/v-FLIP gene of human herpesvirus 8 and apoptosis in Kaposi's sarcoma spindle cells. J Natl Cancer Inst *91*, 1725-1733.

Sun, R., Lin, S.-F., Staskus, K., Gradoville, L., Grogan, E., Haase, A., and Miller, G. (1999). Kinetics of Kaposi's sarcoma-associated herpesvirus gene expression. J. Virol. *73*, 2232-2242.

Sun, R., Lin, S. F., Gradoville, L., Yuan, Y., Zhu, F., and Miller, G. (1998). A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus. Proc. Natl. Acad. Sci. USA *95*, 10866-10871.

Swenson, J. J., Mauser, A. E., Kaufmann, W. K., and Kenney, S. C. (1999). The Epstein-Barr virus protein BRLF1 activates S phase entry through E2F1 induction. J Virol *73*, 6540-6550.

Takada, K., Shimuzu, N., Sakuma, S., and Keating, A. (1986). Transactivation of the latent Epstein-Barr virus (EBV) genome after transfection of the EBV BamHI Z DNA fragment. J Virol *57*, 1016-1022.

Trautwein, C., Caelles, C., van der Geer, P., Hunter, T., Karin, M., and Chojkier, M. (1993). Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. Nature *364*, 544-547.

Ueda, K., Wei, Y., and Ganem, D. (1996). Cellular Factors controlling the activity of woodckuck hepatitis virus enhancer II. J Virol *70*, 4714-4723.

Wegner, M., Cao, Z., and Rosenfeld, M. G. (1992). Calcium-regulated phosporylation within the leucine zipper of C/EBPβ. Science *256*, 370-373.

Zany, V. L., Wilson, J., and Pagano, J. S. (1998). The Epstein-Barr virus immediate-early gene product, BRLF1, interacts with the retinoblastoma protein during the viral lytic cycle. J Virol *72*, 8043-8051.

Zalani, S., Holley-Guthrie, E., and Kenney, S. (1996). Epstein-Barr viral latency is disrupted by the immediate-early BRLF1 protein through a cell-specific mechanism. Proc Nat Acad Sci USA *93*, 9194-9199.

Zhang, L., and van Santen, V. L. (1995). Interaction of bovine herpesvirus 4 (BHV-4) immediate early 2 gene product with BHV-4 thymidine kinase promoter-regulatory region. J Gen Virol *76*, 2433-2445.

Zhong, W., Wang, H., Herndier, B., and Ganem, D. (1996). Restricted expression of Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) genes in Kaposi sarcoma. Proc Natl Acad Sci U S A *93*, 6641-6.

Zhu, F., Cusano, T., and Yuan, Y. (1999). Identification of the immediate-early transcripts of Kaposi's sarcoma-associated herpesvirus. J Virol *73*, 5556-5567.

Figure 1a

Linker-scanning mutants of the minimal kaposin promoter

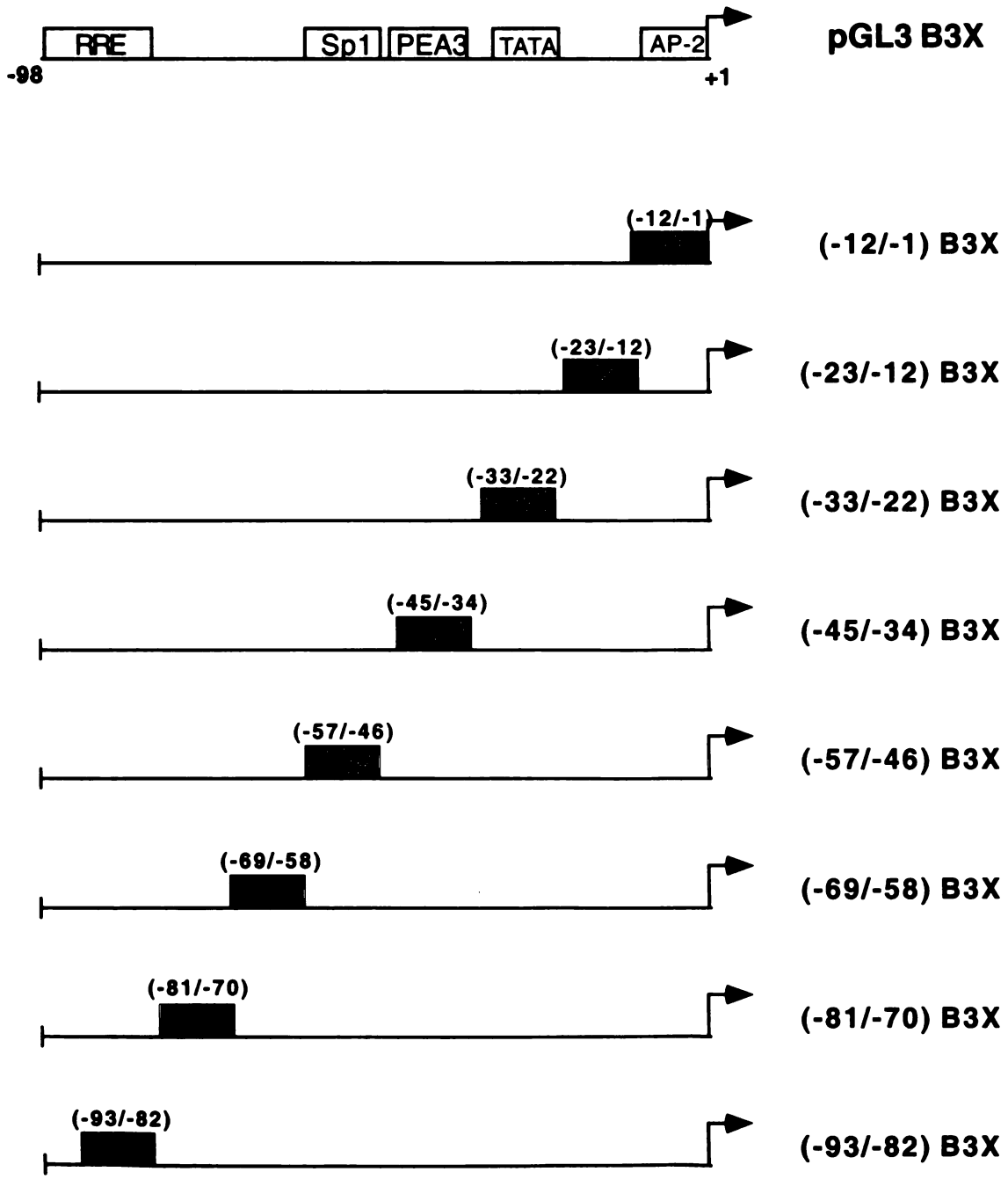


Figure 1a Analysis of the kaposin promoter using linker scanning mutants.

All mutants have kaposin sequences starting from position -98 and extending to position +33, with a 12bp linker TATCATATGATA replacement at the indicated positions. Each mutant is named by the beginning and ending positions of the linker replacement.

Fig 1b

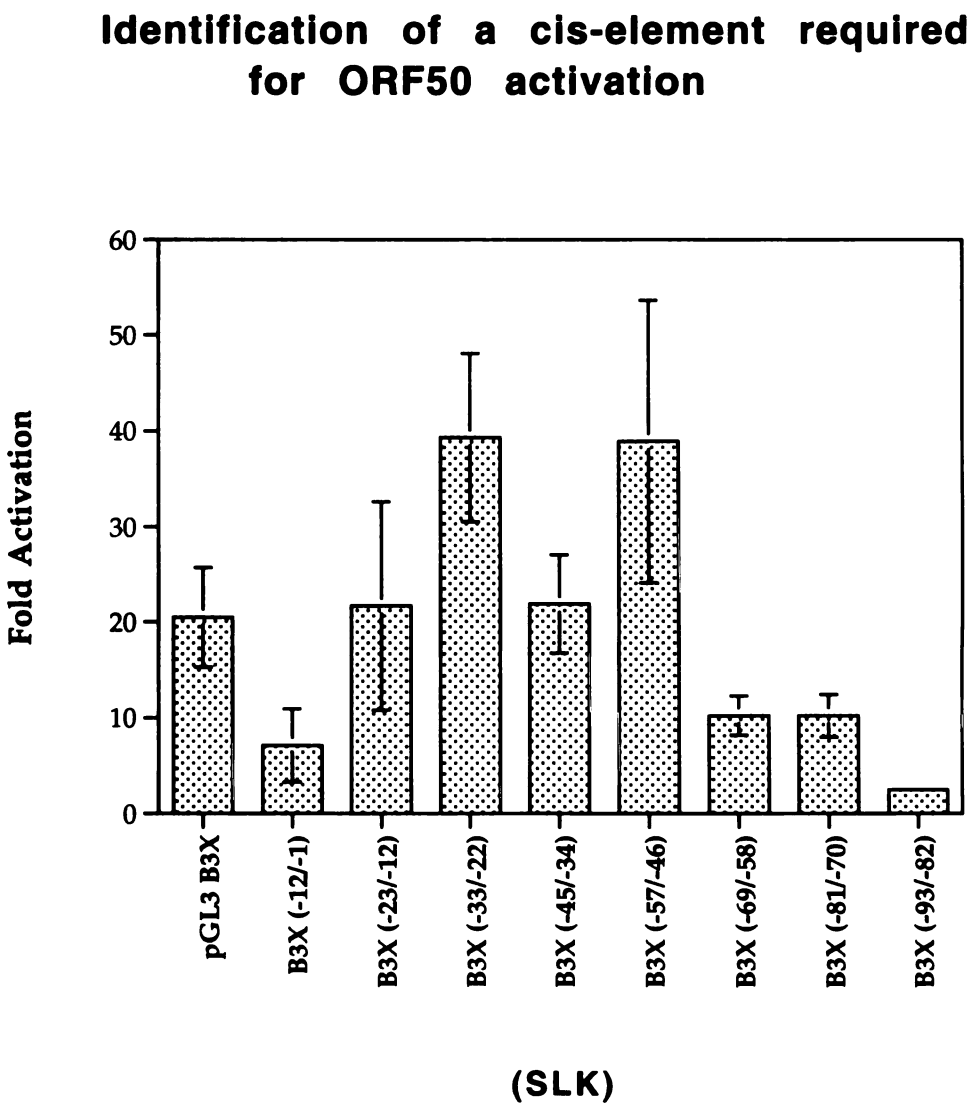
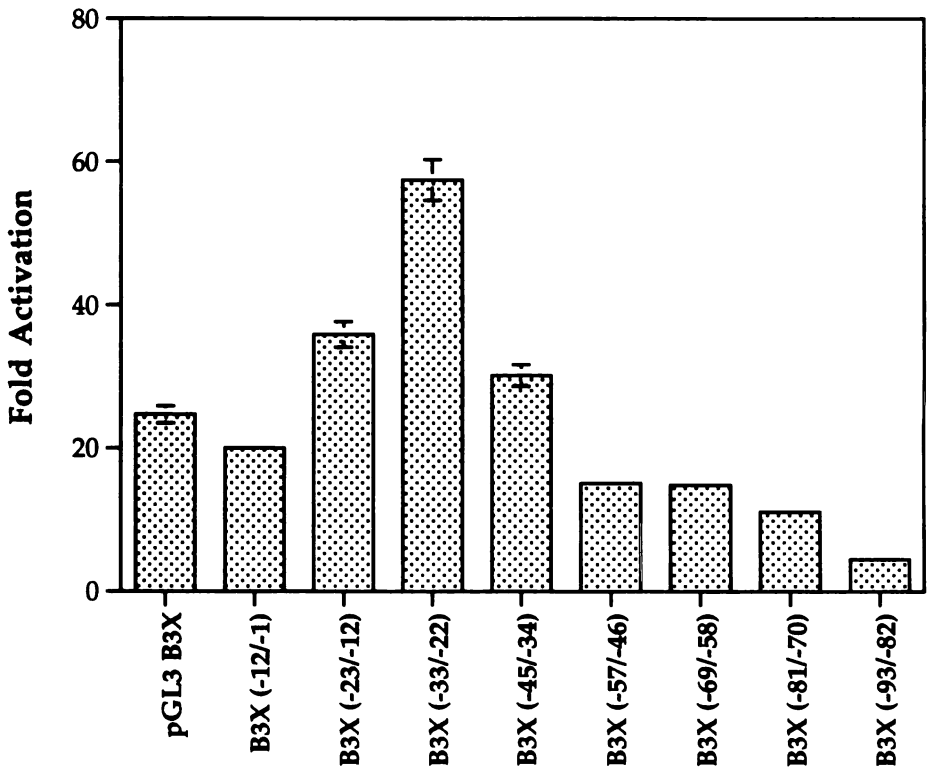

Identification of a cis-element required for ORF50 activation
Fold Activation
60
50
40
30
20
10
0
pGL3 B3X
B3X (-12/-1)
B3X (-23/-12)
B3X (-33/-22)
B3X (-45/-34)
B3X (-57/-46)
B3X (-69/-58)
B3X (-81/-70)
B3X (-93/-82)
(SLK)

Figure 1b Effect of linker scanning mutants on ORF50 activation of the Kaposin promoter in SLK cells.

SLK cells were transfected with 0.2ug pcDNA3.1 LacZ, 0.8 ug of reporter plasmid and 0.8 ug of pGL3-gORF50 or pcDNA3 (- ORF50). Cells were harvested 48 hrs after transfection for Bgal and luciferase assays. Values plotted represent fold activation relative to transfection in the absence of ORF50 (set to "1") for each reporter plasmid.

Fig 1c

Identification of a cis-element required for ORF50 activation



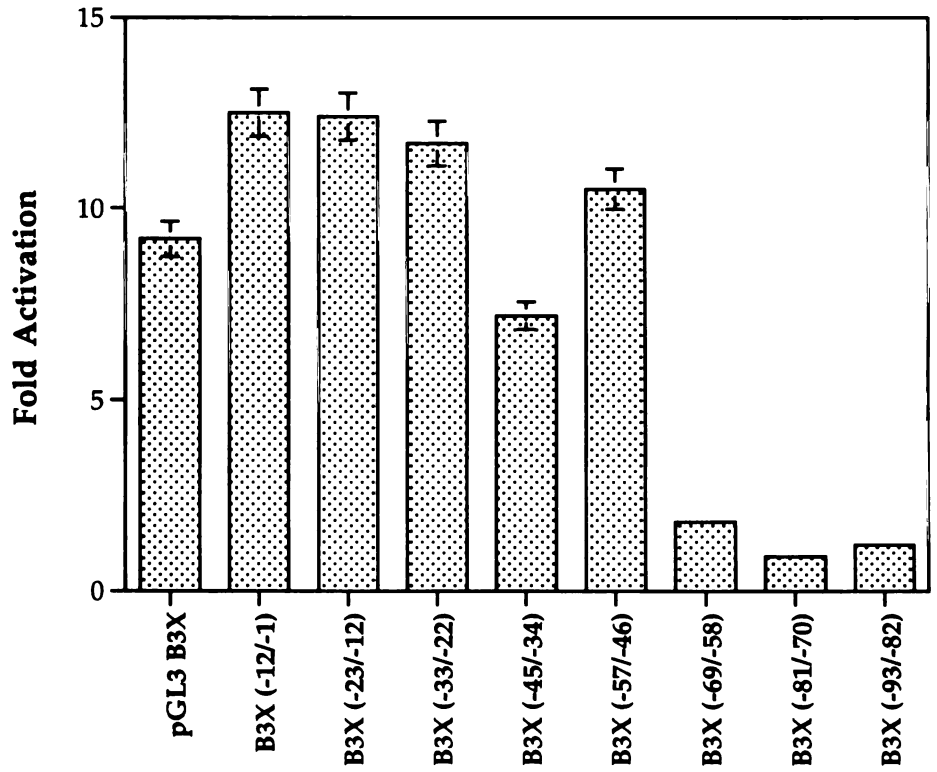
(CV1)

Figure 1c Effect of linker scanning mutants on ORF50 activation of the Kaposin promoter in CV-1.

CV-1 cells were transfected with 1 ug pcDNA3.1 LacZ, 2 ug of reporter plasmid, 2 ug of pGL3-gORF50 or pcDNA3 (- ORF50) and 5ug pBSIISK- (filler DNA). Cells were harvested 48 hrs after transfection for luciferase assays. Values plotted represent fold activation relative to transfection in the absence of ORF50 (set to "1") for each reporter plasmid.

Fig 1d

Identification of a cis-element required for ORF50 activation



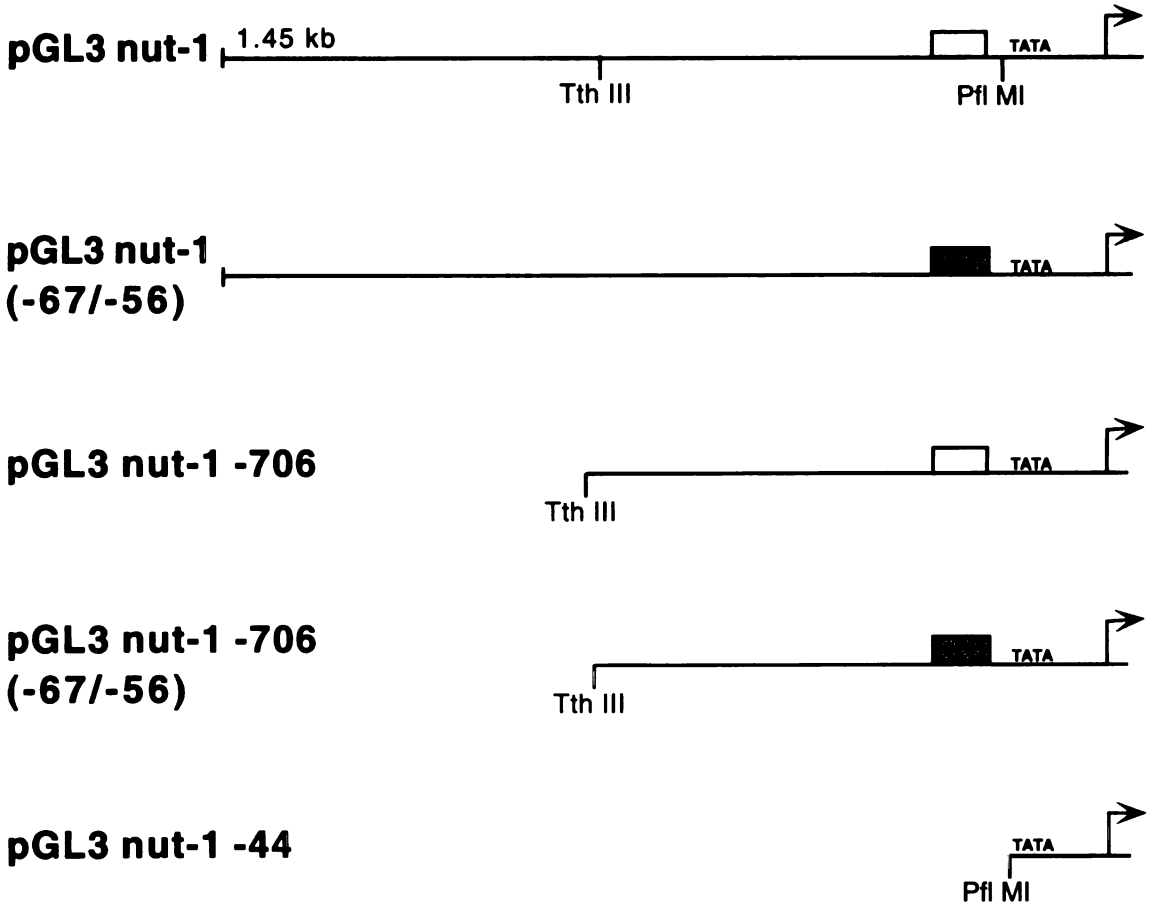
(BJAB)

Figure 1d Effect of linker scanning mutants on ORF50 activation of the Kaposin promoter in BJAB cells.

BJAB cells were transfected with 1 ug pcDNA3.1 LacZ, 2 ug of reporter plasmid, 5 ug of pGL3-gORF50 or pcDNA3 (- ORF50) and 6ug pBSIISK- (filler DNA). Cells were harvested 48 hrs after transfection for luciferase assays. Values plotted represent fold activation relative to transfection in the absence of ORF50 (set to "1") for each reporter plasmid. Transfection efficiencies were normalized using Bgal data.

Figure 2a

Deletion and linker mutants of the nut-1 promoter



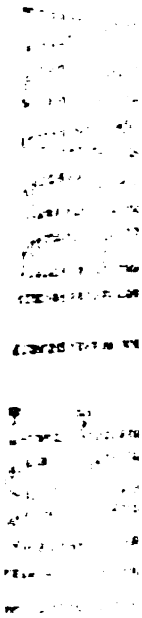


Fig 2b

50RE-1 is responsible for ORF50 responsiveness of the nut-1 promoter

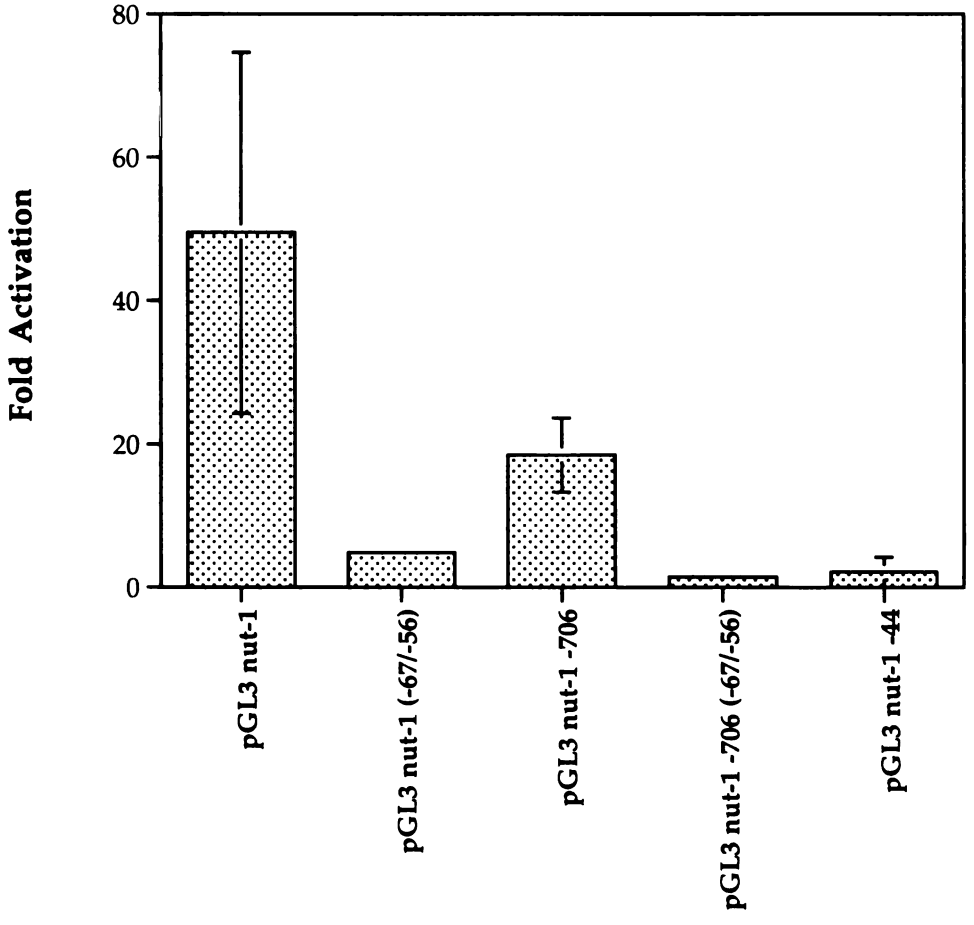
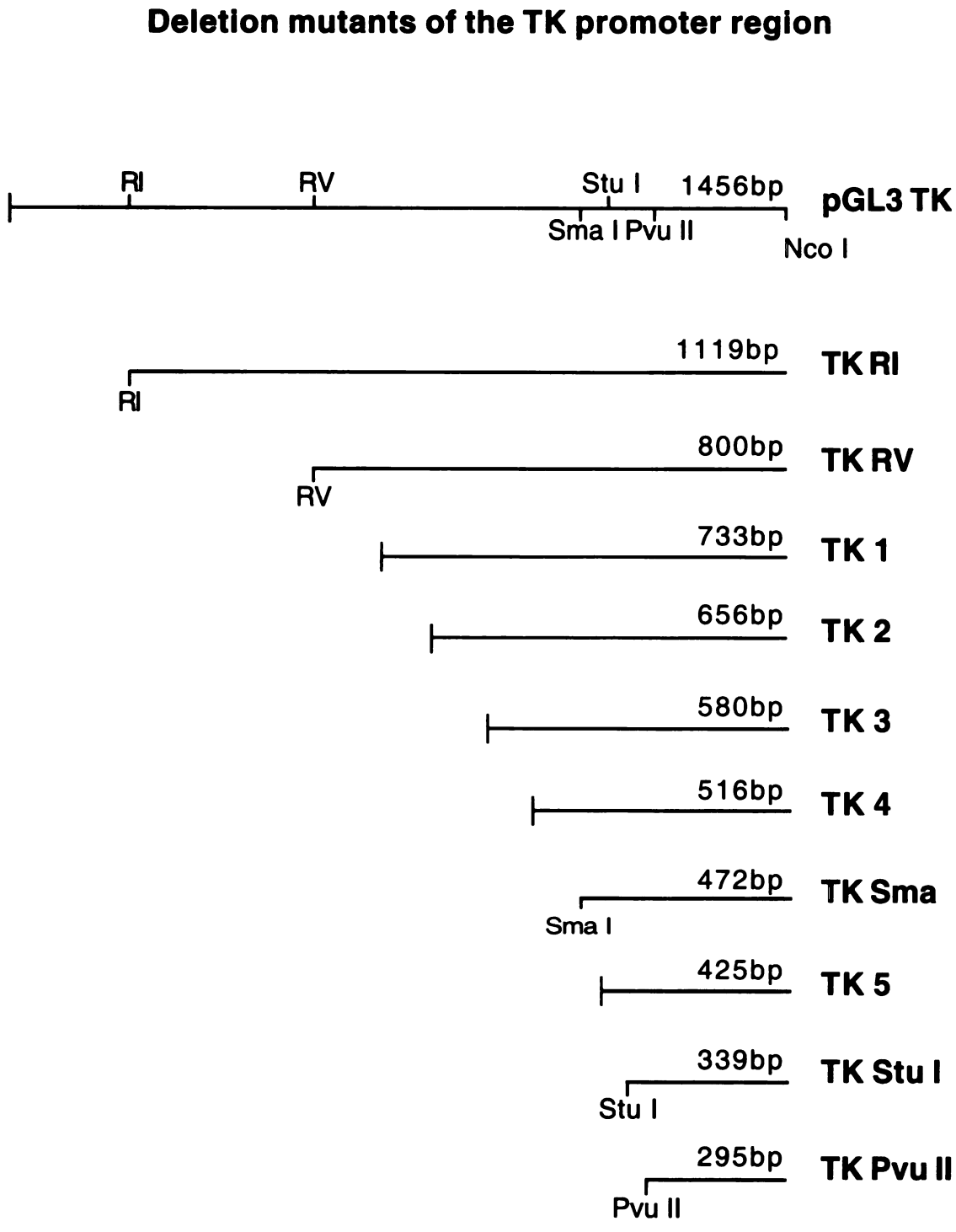


Figure 2b Effect of deletion and linker mutants on ORF50 activation of the nut-1 promoter in SLK cells.

SLK cells were transfected with 0.2ug pcDNA3.1 LacZ, 0.8 ug of reporter plasmid and 0.8 ug of pGL3-gORF50 or pcDNA3 (- ORF50). Cells were harvested 48 hrs after transfection for Bgal and luciferase assays. Values plotted represent fold activation relative to transfection in the absence of ORF50 (set to "1") for each reporter plasmid.

Figure 3a


Deletion mutants of the TK promoter region
RI
RV
Stu I
1456bp
pGL3 TK
Sma I Pvu II
Nco I
1119bp
TK RI
RI
800bp
TK RV
RV
733bp
TK 1
656bp
TK 2
580bp
TK 3
516bp
TK 4
472bp
TK Sma
Sma I
425bp
TK 5
339bp
TK Stu I
Stu I
295bp
TK Pvu II
Pvu II

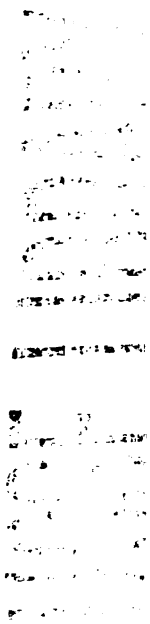


Figure 3a Schematic of the TK promoter deletion series.

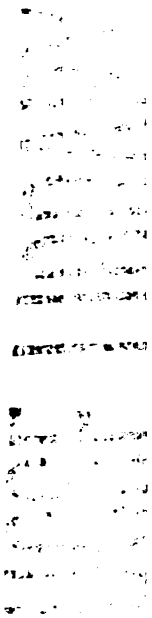
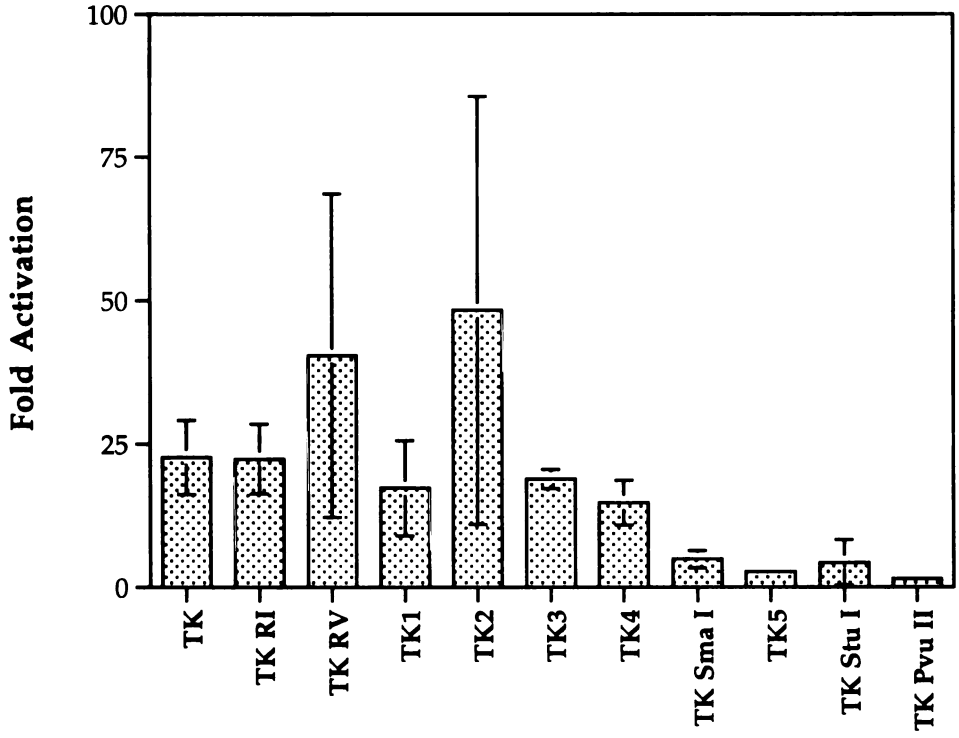


Fig 3b

An ORF50 responsive element resides within the TK promoter region



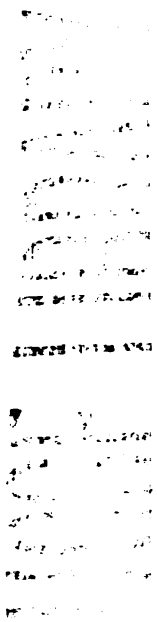
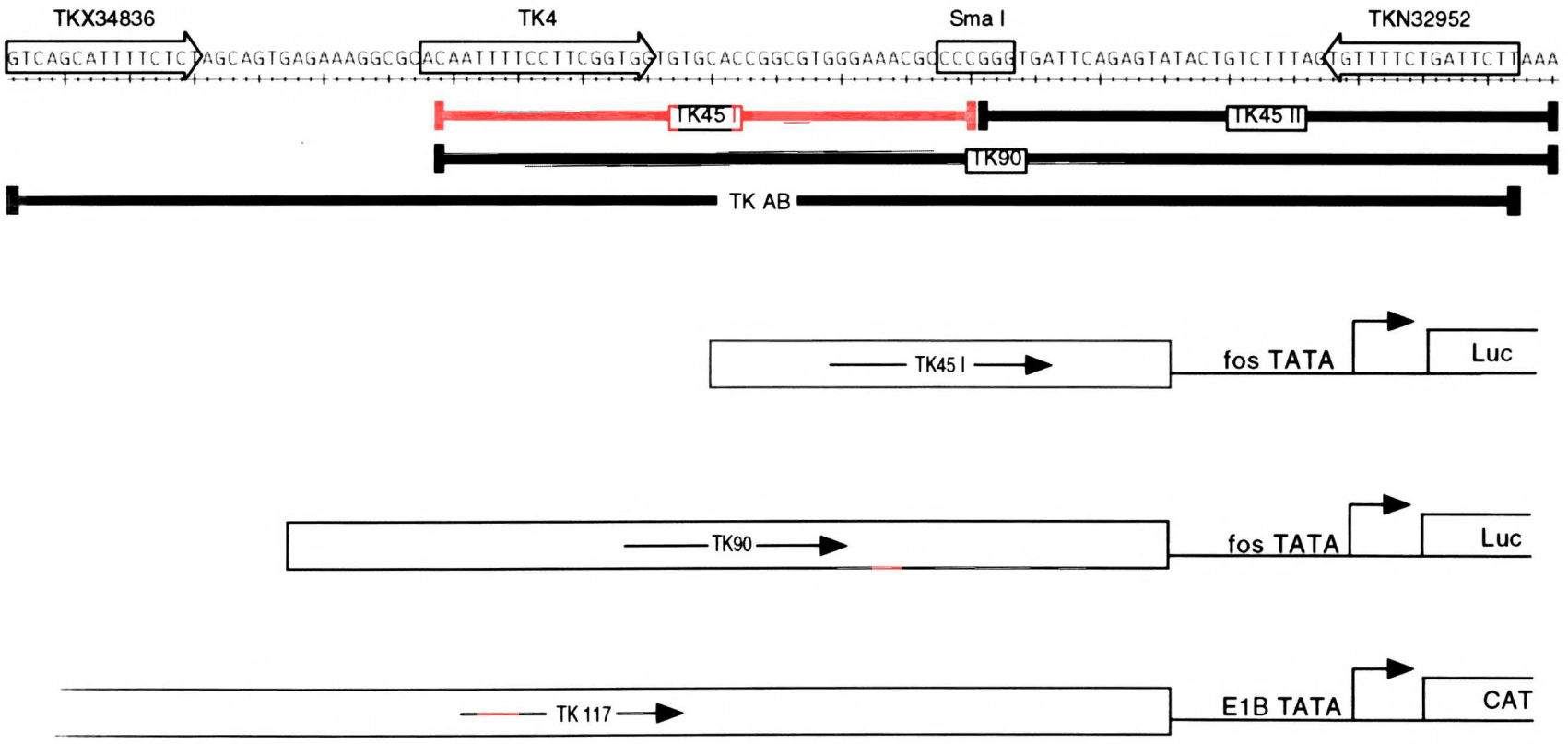


Figure 3b Effect of deletion and linker mutants on ORF50 activation of the TK promoter in SLK cells.

SLK cells were transfected with 0.2ug pcDNA3.1 LacZ, 0.8 ug of reporter plasmid and 0.8 ug of pGL3-gORF50 or pcDNA3 (- ORF50). Cells were harvested 48 hrs after transfection for Bgal and luciferase assays. Values plotted represent fold activation relative to transfection in the absence of ORF50 (set to "1") for each reporter plasmid.

Figure 4a

The ORF50 responsive region of the TK promoter linked to heterologous promoters



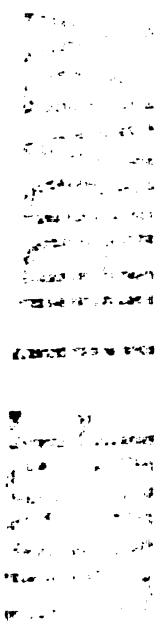


Figure 4a Schematic of TK ORF50-responsive regions placed in front of the minimal E1B or fos promoters.

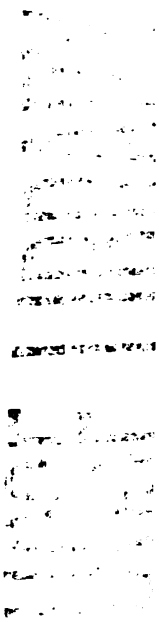
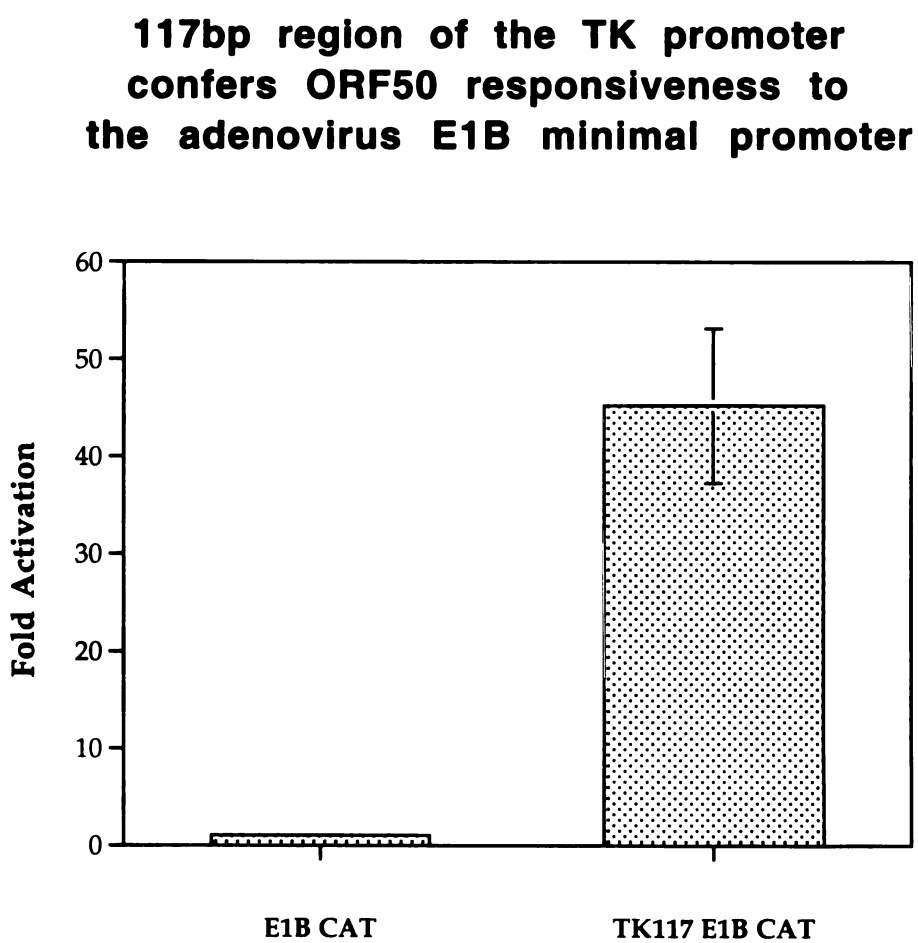


Fig 4b


117bp region of the TK promoter confers ORF50 responsiveness to the adenovirus E1B minimal promoter
Fold Activation
60
50
40
30
20
10
0
E1B CAT
TK117 E1B CAT

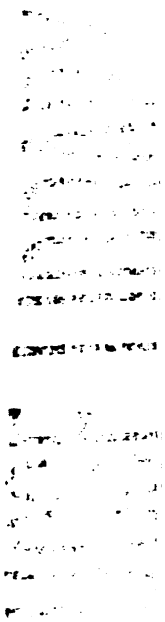


Figure 4b 117 bp region of the TK promoter confers ORF50 sensitivity to the minimal E1B promoter in SLK cells.

SLK cells were transfected with 0.2ug pcDNA3.1 LacZ, 0.8 ug of reporter plasmid and 0.8 ug of pGL3-gORF50 or pcDNA3 (- ORF50). Cells were harvested 48 hrs after transfection for Bgal and luciferase assays. Values plotted represent fold activation relative to transfection in the absence of ORF50 (set to "1") for each reporter plasmid.

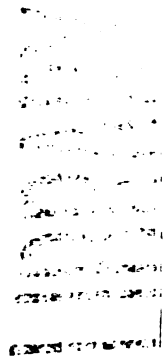
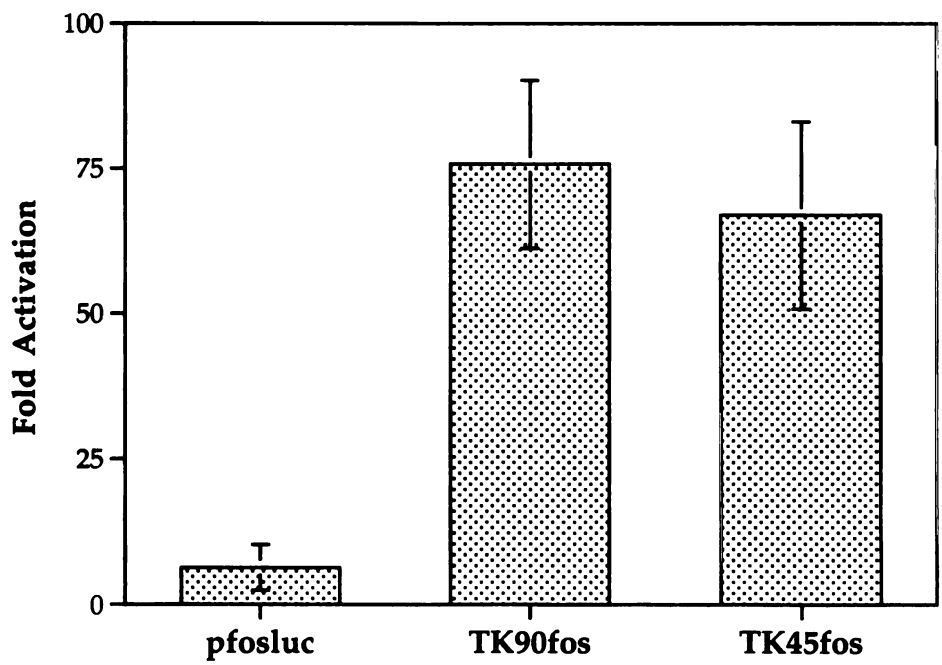
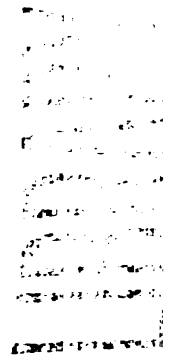




Fig 4c

A cis-DNA element in the TK promoter can confer ORF50 responsiveness to the minimal fos promoter





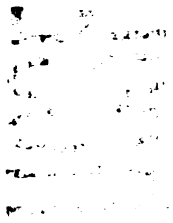
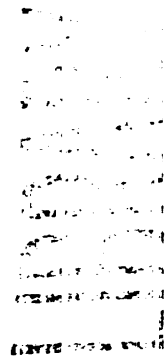
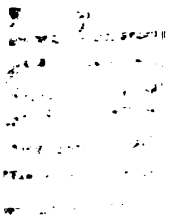


Figure 4c 90 and 45 bp regions of the TK promoter increases ORF50-dependent activation of the minimal fos promoter in SLK cells.

SLK cells were transfected with 0.2ug pcDNA3.1 LacZ, 0.8 ug of reporter plasmid and 0.8 ug of pGL3-gORF50 or pcDNA3 (- ORF50). Cells were harvested 48 hrs after transfection for Bgal and luciferase assays. Values plotted represent fold activation relative to transfection in the absence of ORF50 (set to "1") for each reporter plasmid.





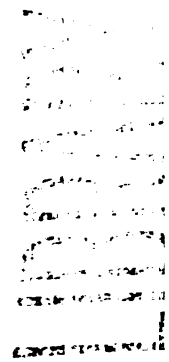
Chapter 3 - Regulation of late gene expression

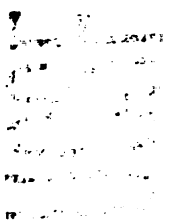
Abstract

To investigate the *cis*-acting sequence elements that are required for the regulation of KSHV late gene expression, we characterized the transcript of a classic late gene, assembly protein (ORF17.5), and constructed reporter plasmids bearing the assembly protein promoter region. We determined that late promoters taken out of the viral context are no longer dependent upon viral replication for expression. Minimal core late promoters are likewise unable to recapitulate late gene regulation. A limited search for the KSHV lytic origin demonstrated that our candidate regions were incapable of supporting lytic replication in transient or stable assays. Our results suggest that proper KSHV late gene expression is likely to be dependent upon viral lytic replication *in cis*.

Introduction

Kaposi's sarcoma (KS) -associated herpesvirus (KSHV) is the proposed etiologic agent of Kaposi's sarcoma (Beral et al., 1990). Epidemiologic evidence shows that infection is not ubiquitous, is strongly correlated with increased risk of disease, and precedes disease development (Gao et al., 1996; Gao et al., 1996; Kedes et al., 1996; Martin et al., 1998; Whitby et al., 1995). Viral DNA is present in KS lesions of all epidemiologic types: classical, HIV-associated, African endemic and transplant associated KS (Boshoff et al., 1995; Staskus et al., 1997). KSHV is also implicated in at least two lymphoproliferative disorders, primary effusion lymphoma (PEL)

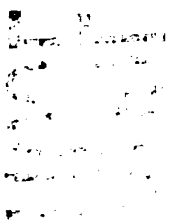




(Cesarman et al., 1995) and multicentric Castleman's disease (Soulier et al., 1995), consistent with its classification as a lymphotropic gammaherpesvirus (Moore et al., 1996; Russo et al., 1996). Viral sequences can also be detected in CD19+ peripheral B cells of infected individuals (Ambroziak et al., 1995; Mesri et al., 1996; Moore et al., 1996; Whitby et al., 1995), the proposed site of primary infection. The majority of cells in B cell lines derived from PEL are latently infected with KSHV (Cesarman et al., 1995; Renne et al., 1996). These latently infected cells can be induced to lytic reactivation by chemical inducers such as phorbol ester, sodium butyrate or calcium ionophore (Miller et al., 1997, see also chapter 1, this thesis; Moore et al., 1996; Renne et al., 1996).

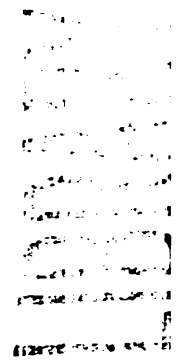
In herpesviruses, activation (or reactivation) of the viral lytic cycle initiates a temporally regulated cascade of gene expression, ultimately resulting in the release of infectious virons (Honess and Roizman, 1974). Lytic cycle genes can be roughly categorized into three classes: immediate-early (IE), delayed early (DE) and late genes. IE gene products direct the expression of DE genes, including components of the viral replication machinery. After replication of the viral genome, late genes, generally structural proteins, are expressed, virons are packaged and released. KSHV gene expression is no exception to this paradigm (Sun et al., 1999). In KSHV, the ORF50 protein (a homolog of the EBV IE R protein) has been shown to trigger reactivation from latency in B cell lines derived from PEL and direct expression of KSHV DE genes (Lukac et al., 1999; Lukac et al., 1998; Sun et al., 1998). KSHV assembly protein (ORF17.5) and small viral capsid antigen (ORF65), whose homologs in HSV and EBV are classified as late genes, have been

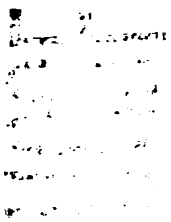




shown to behave with late kinetics in that they are induced by activators such as TPA but their induction is abrogated by inhibitors of the viral polymerase (Lin et al., 1997; Unal et al., 1997). Thus, viral DNA replication seems to constitute an important temporal boundary between early and late gene expression in KSHV.

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





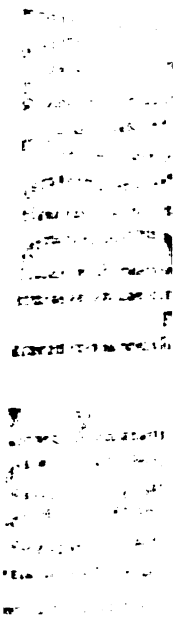
herpesvirus more closely related to KSHV, showed that EBV late promoter regions driving a CAT reporter gene exhibited late kinetics in infected cells even when the plasmid lacked an oriLyt *in cis* (Serio et al., 1997). More recently, it was shown that the region responsible for conferring late gene kinetics in this context was the minimal core promoter, bearing a unique TATA box (TATTAAA) (Serio et al., 1998). These results suggest DNA replication *in cis* might not be a strict requirement for late gene expression in all herpesviruses.

Sparked by the above EBV results, we set out to define the sequence requirements for properly regulated expression of KSHV late genes. In this work I show that KSHV late promoters, taken out of the viral context, do not recapitulate late gene regulation. Experiments designed to identify the KSHV lytic origin (for the generation of reporter constructs bearing the KSHV lytic origin *in cis*) are also described.

Results

Northern analysis of assembly protein (AP) transcript.

We chose a classic late gene, assembly protein, for our initial study of late gene regulation in KSHV. Figure 1a is a schematic of the genomic region around the AP ORF. The AP ORF is contained within the coding region of a larger, related ORF for the viral protease (Pr). Two transcripts are derived from this region (Fig 1b): a short transcript that initiates within the Pr ORF and encodes AP alone, and a

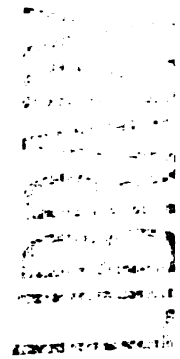


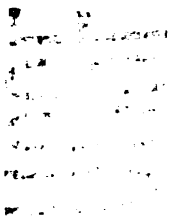
longer transcript encoding for protease and assembly protein (Pr/AP), expressed as a contiguous polyprotein (Fig 1c).

To study the expression of AP, we used a KSHV-infected B cell line, BCBL-1, which maintains the virus in a latent state. Treatment of BCBL-1 with phorbol esters induces lytic reactivation, viral DNA replication and subsequent late gene expression. Figure 2 shows Northern analysis of induced and uninduced BCBL-1 RNA using a double stranded DNA probe for the Pr/AP ORF. The band at ca. 950 bp corresponds to the predicted AP message. This mRNA is clearly upregulated by TPA induction. It can also seen in the uninduced lane, presumably due to the small subpopulation of cells undergoing spontaneous lytic reactivation. A less intense signal at 1.8 kb is also detected, representing the protease message, indicating that the viral protease is expressed at much lower levels than that of AP.

The 5′ end of the AP mRNA

Visual inspection of the region upstream of the AP ORF reveals a likely TATA box at genomic position 31747, 60nt upstream of the first potential start codon (Fig 3). A cDNA library derived from poly(dT)-primed, induced BCBL-1 mRNA was screened using a 774bp Cla I/Sac I fragment as probe, corresponding to a region within the AP ORF (genomic positions 30840- 31614). Four positive clones were isolated, representing three independent clones, as determined by sequence analysis. All three cDNA clones shared a likely poly(A) addition signal, AAUAA, at nt 30761. Although two of three cloned cDNAs start 12 bases upstream of the



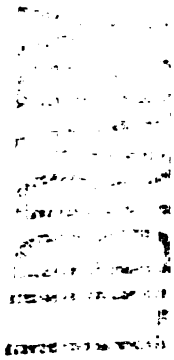


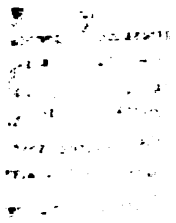
first AUG, the presence of a candidate TATA box suggested that the transcriptional start site might lie further upstream. To identify the start site of the AP transcript, we used RNase protection (RPA) and primer extension analysis. For RPA, a 266 nt riboprobe was hybridized to BCBL-1 RNA and subjected to T2 ribonuclease digestion of unannealed, single stranded regions. Two protected products, 166 and 171 bp - corresponding to genomic positions 31705 and 31710, were seen (Fig 4).

The start site was independently confirmed by primer extension. We used a labeled oligo (PE1) located 12nt downstream of the first potential initiating ATG for AP (Fig 3) and annealed it to induced and uninduced BCBL-1 RNA. Extension from the primer produced two products of 65 and 70 bp, confirming our RNase protection results (Fig 5). Significantly, the major start site occurs 28bp downstream of a possible TATA box.

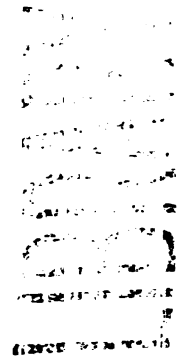
KSHV late promoters taken out of the viral context are not dependent upon viral replication

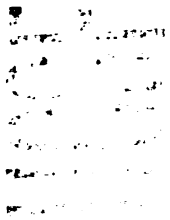
By definition, late genes are expressed only after viral replication has occurred. Viral induction by chemical inducers such as TPA or ionomycin would trigger the lytic cycle, including the eventual expression of late genes. This expression could be blocked if inhibitors of the viral polymerase prevented viral replication. To study the transcriptional regulation of KSHV late genes, we set out to identify regions of the AP promoter that could recapitulate late gene regulation in a heterologous context.





A 1.3kb H3/RV fragment and a 700bp SacI/RV fragment were cloned into a luciferase reporter plasmid in both orientations and tested for their ability to drive luciferase expression. When tested in 293 cells, both pGL3 H3/RV and pGL3 SacI/RV were able to direct the expression of luciferase up to 29.2 and 89.9 -fold over the promoterless, parent reporter vector, pGL3 basic (Figure 7a). This activity was dependent upon orientation as the same fragments cloned in the reverse direction, pGL3 RV/H3 and pGL3 RV/SacI, were much less active (1.8 and 15.4 fold, respectively) in the same assay. To test for proper late gene expression, these reporter constructs were transfected into latently infected cells that could be chemically stimulated to enter the lytic cycle. We were interested in whether these reporter constructs might also be responsive to effects of these chemical stimuli that were not directly linked to viral induction. Figure 7b shows that none of the reporter constructs were responsive to TPA in 293 cells. In contrast, when the same constructs are transfected into BCBL-1 cells, a context wherein viral activators and other cofactors necessary for lytic reactivation and replication are present, these reporters are modestly TPA responsive (Fig 7c). The relatively low level of TPA-induced activity in BCBL-1 was surprising. It concerned us that the initiating ATG from the AP ORF present in both the SacI/RV and H3/RV fragments might be causing initiation of translation upstream and out-of-frame with the luciferase ORF. To test this possibility, we used PCR mutagenesis to change the ATG to TTG in pGL3 SacI/RV, creating pGL3 APTTG. When tested in BCBL-1, the ablation of the upstream ATG resulted in approximately 3-fold greater luciferase expression than the parental construct (Fig 7d). All subsequent experiments were carried out using pGL3 APTTG as this construct would provide

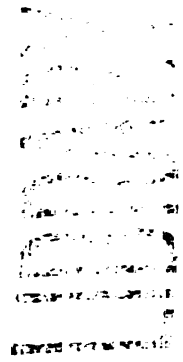


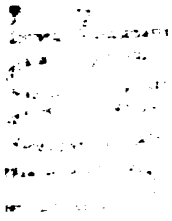


us with greater dynamic range to detect inhibition of activation of this late promoter.

In the presence of viral polymerase inhibitors, faithful recapitulation of late gene expression would result in the inability of chemical induction to give rise to late gene products. To test if this was the case, we transfected pGL3 APTTG into BCBL-1 under four different condition: chemical inducer alone (ionomycin or TPA), viral polymerase inhibitor ganciclovir (GCV) alone, inducer and inhibitor combined (ionomycin/GCV or TPA/GCV) or no treatment. Luciferase activity of untreated cells was set to "1". Fig 8a shows that pGL3 APTTG activation was not inhibited by GCV. Moreover, activation of this reporter construct was not dependent upon viral cofactors as pGL3 APTTG was readily activated in BJAB cells, an uninfected B cell line (Fig 8b).

To confirm our result we also tested the promoter regions of two other classic late genes: glycoprotein B (gB) and major capsid protein (MCP). Figure 8c show that these reporters were likewise insensitive to viral polymerase inhibitors (GCV and PFA). pGL3 H3/RV which contains a larger region upstream of AP than pGL3 APTTG was similarly unaffected by GCV, or cidofovir (data not shown). Fig 8d shows that both gB and MCP promoters could be activated by both TPA and ionomycin in BJAB cells, confirming our finding that late gene promoters can be activated through the stimulation of signaling pathways in B cells in the absence of viral cofactors. These results suggest that late gene regulation in KSHV cannot be faithfully recapitulated when late promoters are taken out of the viral context.



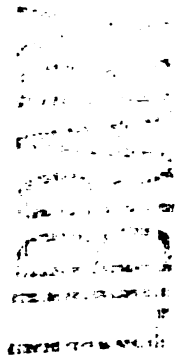


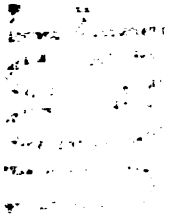
Minimal promoter constructs are also insufficient to direct gene expression with late gene kinetics

Serio et al showed that for late gene regulation, the minimal core of an EBV late promoter was able to confer late gene kinetics to a reporter construct (Serio et al., 1998). Their observation that upstream enhancers could override the temporal control provided by a core EBV late promoter led us to question whether the KSHV promoter regions we tested may have been too large, encompassing enhancer regions that would mask the ability of a KSHV core late promoter to confer regulated late gene expression. Moreover, many of the EBV late promoters have an unusual TATA element (TATTAAA) (Serio et al., 1998). Inspection of the promoter regions of various KSHV late promoters showed that a subset of the late promoters, including AP, also had unusual TATA elements (TATTTAAA) reminiscent of the EBV late promoter TATA identified by the Miller group. To test our hypothesis, we created a deletion mutant of pGL3 APTTG, truncating the upstream region to -30 from the start site. Figure 9 shows that, unlike the EBV case, this promoter region was unresponsive to viral polymerase inhibitors.

A directed search for the KSHV lytic origin by transient and stable replication assay

Our results are consistent with a model that requires DNA replication *in cis* as an integral component of late gene expression. To create a system for the study of late gene regulation, we sought to identify the KSHV lytic origin. Cloning of the lytic origin to reporter constructs bearing late gene promoters would allow us to test

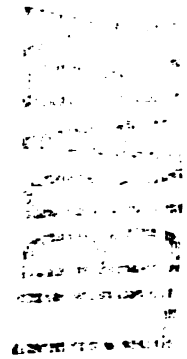


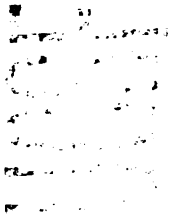


the hypothesis that replication *in cis* is required for recapitulation of late gene kinetics.

We focused our search towards two specific regions of the genome, the region between K4.2 and K5, and the region between K12 and ORF71 (Fig 10). These regions have landmarks characteristics of herpesvirus replication origins including areas of clustered transcription site motifs, regions of short repetitive GC-rich elements, and AT-rich palindromes (Nicholas et al., 1998). The two regions are highly homologous to one another and exist in inverted orientation relative to each other. Furthermore, their positioning is analogous to the positions of the EBV lytic origins, which are also duplicated regions that share extensive similarity (Kieff, 1996).

The candidate regions were tested using plasmid clones B4, which contains ~6 kb from genomic positions 22897 - 28888, and KS31 or KS50 (the same approximate region derived from KS lung tumor or BCBL-1 virion DNA, respectively), which contain ~6.8 or 5.5 kb from genomic positions 115,714 - 122,592 and 116,203 - 121,684. Using a transient transfection assay, we asked if plasmids harboring these regions would be replicated in BCBL-1 under conditions of lytic induction. DNAs for transfection were prepared from *E. coli* that naturally yields Dam methylated DNA. Upon replication in mammalian cells, progeny plasmids would not be methylated in this manner. This difference in methylation can be detected by digestion with DpnI, which only cleaves at methylated recognition sites. Accordingly, we transfected our candidate regions, KS31, KS50, B4, as well as a control plasmids, KS25 (which contains a region of KSHV that is a lesser candidate

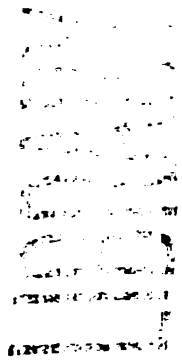




for carrying a replication origin) and pCEP4, into BCBL-1 with or without chemical inducer. After harvesting episomes by the Hirt method, DNAs were linearized with BamHI in the presence or absence of DpnI. Figure 11a shows that at 3 days post-transfection, all harvested episomes were DpnI sensitive, indicating that none of the transfected plasmids had undergone replication.

As a control for the sensitivity of our assay, we tested our ability to detect replicated plasmid bearing a known origin of replication in BCBL-1. pSVE1 bears the SV40 early region, including the SV40 origin and the ORF for its origin binding protein, SV40 T antigen (T-Ag), thus it contains all the components necessary for replication and maintenance in mammalian cells. Although the SV40 origin is not very robust in lymphoid cells, Figure 11b shows that we were able to detect DpnI resistant plasmid when episomes were harvested from BCBL-1 cells at 3 days post-transfection. pBSIISK-, a plasmid which does not have an origin for maintenance in mammalian cells, is completely digested by DpnI.

To exclude the possibility that our negative findings were due to technical problems with regard to transfection efficiencies in transient transfection, we also used a stable replication assay to detect replication. Our candidate regions were cloned to pCEP4, a plasmid with the EBV latent origin and EBNA-1, its origin binding protein. This plasmid would allow the for selection of stable transfectants such that every cell would harbor the candidate region of interest. Under these conditions, we would expect amplification of plasmid copy number under inducing conditions if the region of interest contained a lytic origin. This accumulation of plasmid would be abolished by inhibitors of the viral polymerase.



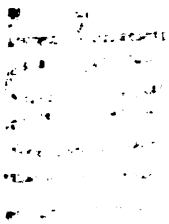
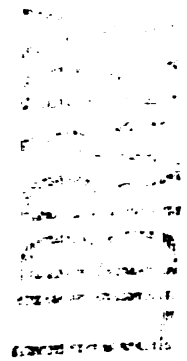
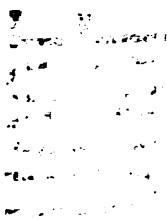


Figure 12 shows that TPA treatment causes a slight increase in signal but not in the amounts that one would expect from robust herpesviral lytic replication. Moreover, ganciclovir treatment did not inhibit this increase. These results confirmed the findings from our transient assay that our candidate regions are not sufficient to support lytic replication, despite the presence of all *trans*-acting factors, contributed by the induced virus in BCBL-1.

Discussion

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

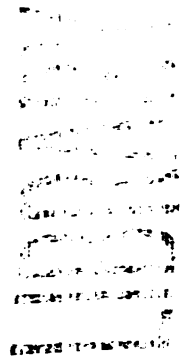


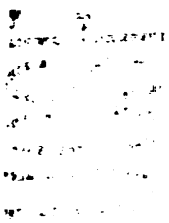


The presence of a candidate variant TATA motif, restricted to a subset of late promoters, suggested a possible explanation for our inability to recapitulate late gene regulation- that our promoter regions incorporated upstream elements which do not normally play a role in the regulation of late genes but in our assay, when the region is taken out of its native context, would override genuine regulation that is directed by minimal late gene promoters during the natural viral lytic cycle. Our results, however, show that a core late gene promoter, while responsive to TPA, is not responsive to viral polymerase inhibitors, confirming our experience that late gene expression in KSHV differs from that in EBV. These findings suggest a distinct mechanistic difference in late gene regulation between KSHV and EBV despite evolutionary relatedness of these two herpesviruses.

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

One possible model for the requirement of DNA synthesis *in cis* is that replication alters an inhibitory state such as methylation in promoter regions, restricted access of promoters packaged in nucleosomes, or repression of transcription initiation by negative regulatory proteins bound to the promoter. Replication of the viral genome would result in promoter regions free of inhibitory elements either due to the generation of DNA which has not been post-transcriptionally modified (ie. methylated) or the displacement of inhibitors (histones or negative regulatory





promoters) by the viral replication machinery. For any of these scenarios, the act of replication is central to the ability to express late genes. In which case, the study of late gene regulation will be dependent upon the identification of the KSHV lytic origin.

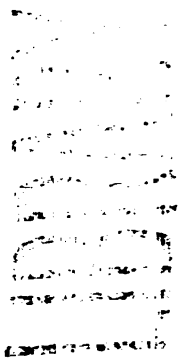
Our results are also consistent with that do not invoke a requirement for DNA replication *in cis*. Rather, a requirement for some form of chromatin structure could be the missing element in our assay. Transient transfection introduces a naked DNA template for activation. Such templates would be excluded from proper regulation if nucleosomal DNA were required. To test this hypothesis, late promoters driving reporter expression, in conjunction with a latent origin that would allow stable replication and proper nucleosome formation, could be transfected into BCBL-1 and tested for proper late gene expression.

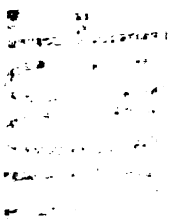
It is clear that sequences surrounding the transcriptional start of late genes are not sufficient to recapitulate authentic late gene expression. Further understanding of late gene regulation will likely require the identification of the KSHV lytic origin.

Materials and Methods

Plasmids

pGL3 SacI/RV and pGL3 RV/SacI were constructed by cloning the 696 nt *Sac*I/*Eco*RV fragment from genomic positions 31663 to 32359 in forward or reverse orientations, respectively, into pGL3 Basic that had been digested with *Sma*I. The *Sac*I site was blunted with Klenow polymerase.





pGL3 H3/RV and pGL3 RV/H3 were constructed by cloning the 1313 nt *HindIII*/*Eco*RV fragment from genomic positions 31663 to 32976 in forward or reverse orientations, respectively, into pGL3 Basic that had been digested with *Sma*I. The *HindIII* site was blunted with Klenow polymerase.

pGL3-AP was constructed by changing the initiating ATG of the AP ORF (nt position 31687) to TTG using PCR mutagenesis.

pGL3-gB has been described previously (Lukac et al., 1998)

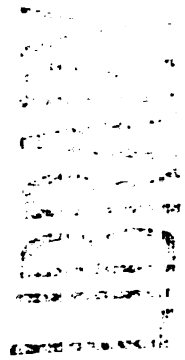
pGL3-MCP was constructed by cloning the 1935 nt *Xma*I/*Nco*I fragment from genomic positions 32362 to 31666 into pGL3 Basic that had been digested with the same enzymes.

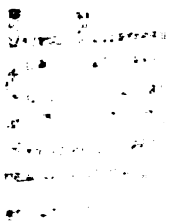
pSVE1 was a gift of T. Shenk. It contains the HpaII-BamHI fragment of SV40 (nt 346-2533, the entire early region) cloned into BamHI-ClaI of pML2 (a pBR322 derivative).

KS25 contains an 8kb XbaI fragment from genomic positions 43457 to ~51450 cloned into pBS SK+ cut with the same enzyme.

KS31 contains a 6.8 kb SacI fragment from genomic positions 115714 - 122592 cloned into pBS SK+ cut with the same enzyme.

KS50 contains a 7.0 kb SacII fragment from genomic positions 116203 - 121684 cloned into pBS SK+ cut with the same enzyme.





B4 contains a 6.0 kb SacI fragment from genomic positions 22897 - 28888 cloned into pBS KS+ cut with the same enzyme.

pCEP4 was digested with Bgl II to release the 844 bp fragment containing the CMV promoter and recircularized to produce pCEPΔP.

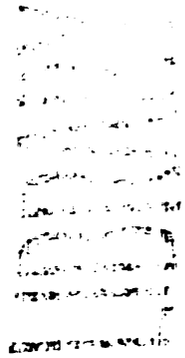
pcDNA3.1 B4 was created by subcloning the 6.0 kb SacI fragment from B4 to pcDNA3.1which had been digested with the same enzyme.

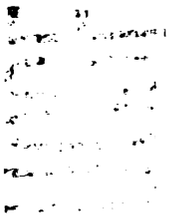
KS31, KS50, pcDNA3.1 B4 were digested with Hind III and Not I to release 6.8, 7.0 and 6.0 kb fragments, respectively. The fragments were cloned into pCEPΔP which was digested with the same enzymes.

pGL3 -30 AP was constructed by PCR amplification of the pGL3 AP plasmid using primers AP -30 (5'- gagccacgcgtatttaaaggccagc) and pGL3rev (5'- cttcatagccttatgcagttgctctc). The PCR product was digested with Mlu I and Nar I and cloned into the corresponding sites in pGL3 Basic.

Cell lines and transfections

BCBL-1 were propagated and maintained in RPMI 1640 media as in Renne *et al.* (Renne 1996 NM). Cells were split to a density of 2.5 x 10^6 cells/ml 18 - 24 hours prior to transfection. For electroporation, cells were washed once with 1x PBS, and resuspended in unsupplimented media with 10mM dextrose, 0.1 mM DTT. 4 x 10^6 cells were aliquotted in 0.4 μL volume to electroporation cuvettes (0.42cm) containing 10 μg of DNA. Cells were electroporated with 960 MF and 150 mV and

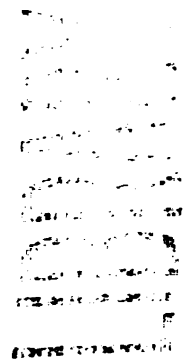


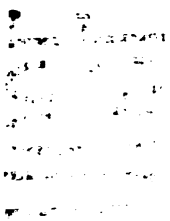


subsequently transferred to 10 ml fresh media for 72 hours. For Superfect transfection, cells were washed once with PBS and 1 x 10^7 cells were resuspended in 6 ml growth media for each transfection. 10ug of DNA was diluted in 150ul unsupplimented RPMI1640 media and 20 ul of Superfect (Qiagen) was added. Complexes were allowed to form for 10 min at room temp. 1 ml of growth media was mixed with the DNA/Superfect complexes added to the cells. Cells were harvested 48 hours post-transfection.

BJAB cells were propagated and maintained in RPMI 1640 media as in Renne *et al.* (Renne 1996 NM). On the day of electroporation, cells were washed once with 1x PBS, and resuspended in unsupplimented media. 1 x 10^6 cells were aliquotted in 0.4 μL volume to electroporation cuvettes (0.42cm) containing 20 μg of DNA. Cells were electroporated with 960 MF and 150 mV and subsequently transferred to 10 ml fresh media for 48 hours.

NIH293 were maintained and propagated in Dulbecco modified Eagle medium (DMEM) H16 supplemented with 10% FCS, 2mM glutamine and antibiotics. For transfections, cells were plated at 2 x 10^5 cells per 60-mm dish and grown overnight. On the day of transfection, cells were fed 3h before DNA addition. DNA was precipitated using the calcium phosphate procedure. 24h after DNA addition, cells were washed with PBS and fresh media ± TPA was added. Cells were harvested at 48h post DNA addition.





RNA preparation and Northern analysis

BCBL-1 were split to a density of 3 x 10^5 cells / ml and induced with 12-O - tetradecanoyl phorbol-13-acetate (TPA) 20ng/ml or mock treated. 24 hours post-induction, cells were harvested and RNA was isolated using RNazol B (Tel-Test, Inc., Friendswood, TX) following manufacturer's directions.

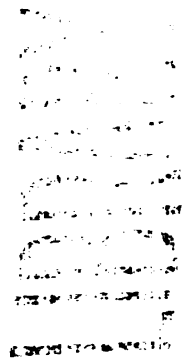
10ug of total RNA was separated on 1% agarose/1% formaldehyde gel, transferred to Hybond-N by alkaline transfer for 40 min. The blot was UV crosslinked and hybridized overnight in Church buffer. Probes were ^{32}P labeled by using a Random DNA Prime kit from Amersham.

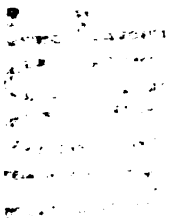
cDNA cloning

mRNA from induced BCBL-1 were poly (dT)-primed and cloned into Lambda Zap (Stratagene) by R. Renne. The library was screened using a ClaI/SacI fragment from the assembly protein ORF by using a Random DNA Prime kit from Amersham.

RNase protection assay

Riboprobe plasmid was constructed by PCR amplification of a 256nt region spanning genomic positions 31511 to 31736 using primers 5'-TGGAATTCAACTGACAGAGGTGTGG and 5'-TCGGGTACCGGGGAACATCTGGCC. The PCR product was digested with Kpn I and Eco RI and cloned into the corresponding sites in pBSIISK$^-$. Riboprobe plasmid was linearized with EcoRI and transcribed with T7 RNA polymerase in





the presence of radiolabeled ^{32}P UTP. Gel purified riboprobes (4×10^5 or 8×10^4 cpm) were hybridized to 10ug of total RNA overnight at 42°. RNase digestion was performed using 1:100 dilution of the RNase A / RNase T1 solution from the RPA II kit (Ambion). Digested RNAs were precipitated and separated on an 8% denaturing acrylamide gel. The gel was exposed to Kodak BioMax film for 18 hrs.

Primer extension

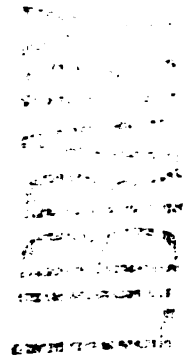
Primer extension was performed as described in Zhong et al. 10 ug of total RNA from BCBL-1 cells, induced or uninduced by TPA for 48 hours, were hybridized with primers PE1 (5′ gggatggatatgatatcctcttgac) or PE2 (5′ ggatgccgaccgggaattggctggc). Samples were separated on an 8% denaturing acrylamide gel. The gel was exposed to Kodak BioMax film for 4 days.

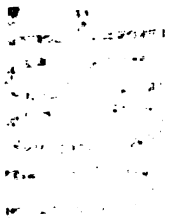
Luciferase assay.

Cells were washed twice with 1mL 1x PBS. After the addition of 0.2 ml reporter lysis buffer (Promega) cells were harvested by scraping, vortexed and spun briefly to pellet cellular debris. 20- and 50 μL aliquots were analyzed by luciferase or β-galactosidase assay, respectively, according to the manufacturer's instructions (Promega).

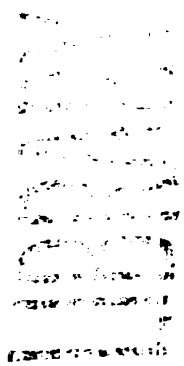
Hirt extracts and Southern Blot

At 72 hours post-transfection, DNA was prepared as in Hirt, 1967. Briefly, cells were spun down and resuspended in 400ul of 10mM Tris-HCl (pH 7.5), 0.6% SDS,





10 mM EDTA for 20 min. 100ul of 5M NaCl was added and allowed to precipitate overnight at 4°C. Samples were spun for 30 min and episomes were recovered from the supernatant by ethanol precipitation. Recovered DNA was digested overnight with 20U of the indicated plasmid ± 20U of DpnI. The digested DNAs were electrophoretically separated on 0.8% agarose gels and transferred to nylon membrane (Hybond N). The blot was UV crosslinked and hybridized overnight in Church buffer. Probes were ^{32}P labeled by using a Random DNA Prime kit from Amersham.





References

Ambroziak, J., Blackbourn, D., Herndier, B., Glogan, R., Gullet, J., McDonald, A., Lennette, E., and Levy, J. (1995). Herpesvirus-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients (technical comment). Science *268*, 582-583.

Beral, V., Peterman, T. A., Berkelman, R. L., and Jaffe, H. W. (1990). Kaposi's sarcoma among persons with AIDS: a sexually transmitted infection? [see comments]. Lancet *335*, 123-8.

Boshoff, C., Schulz, T. F., Kennedy, M. M., Graham, A. K., Fisher, C., Thomas, A., McGee, J. O., Weiss, R. A., and O'Leary, J. J. (1995). Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. Nat Med *1*, 1274-8.

Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS- related body-cavity-based lymphomas [see comments]. N Engl J Med *332*, 1186-91.

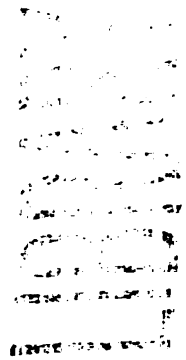
Cesarman, E., Moore, P., Rao, P., Inghirami, G., Knowles, D., and Chang, Y. (1995). In vitro establishment and characterization of two acquired immunodefieiencey syndrome-related lymphoma cell lines (BC-1 and BC-2) containing Kaposi's sarcoma-associated herpesvirus-like (KSHV) DNA sequences. Blood *86*, 2708-2714.

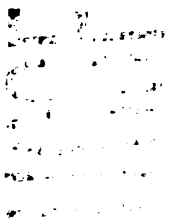
Dennis, D., and Smiley, J. R. (1984). Transactivation of a late herpes simplex virus promoter. Mol. Cell. Biol. *4*, 544-551.

Flanagan, W. M., Papavassiliou, A. G., Rice, M., Hecht, L. B., Silverstein, S., and Wagner, E. K. (1991). Analysis of the herpessimplex virus type 1 promoter controlling the expression of UL38, a true late gene involved in capsid assembly. J. Virol. *65*, 769-786.

Gao, S. J., Kingsley, L., Hoover, D. R., Spira, T. J., Rinaldo, C. R., Saah, A., Phair, J., Detels, R., Parry, P., Chang, Y., and Moore, P. S. (1996). Seroconversion to antibodies against Kaposi's sarcoma-associated herpesvirus-related latent nuclear antigens before the development of Kaposi's sarcoma. N Engl J Med *335*, 233-41.

Gao, S. J., Kingsley, L., Li, M., Zheng, W., Parravicini, C., Ziegler, J., Newton, R., Rinaldo, C. R., Saah, A., Phair, J., Detels, R., Chang, Y., and Moore, P. S. (1996).





KSHV antibodies among Americans, Italians and Ugandans with and without Kaposi's sarcoma [see comments]. Nat Med *2*, 925-8.

Homa, F. L., Glorioso, J. C., and Levine, M. (1988). A specific 15-bp TATA box promoter element is required for expression of a herpes simplex type 1 late gene. Genes Dev. *2*, 40-53.

Homa, F. L., Otal, T. M., Glorioso, J. C., and Levine, M. (1986). Transcriptional control signals of a herpes simplex virus type 1 late (gamma 2) gene lie within bases -34 to +124 relative to the 5' terminus of the mRNA. Mol. Cell. Biol. *6*, 3652-3666.

Honess, R. W., and Roizman, B. (1974). Regulation of herpesvirus macromolecular synthesis. J Virol *14*, 8-19.

Johnson, P. A., and Everett, R. D. (1986). The control of herpes simplex virus type-1 late gene transcription: a 'TATA-box'/cap site region is sufficient for fully efficient regulated activity. Nucleic Acids Res. *14*, 8247-8264.

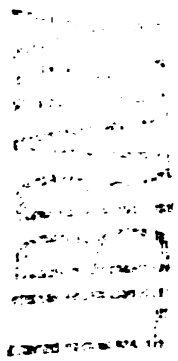
Johnson, P. A., and Everett, R. D. (1986). DNA replication is required for abundant expression of a plasmid-borne late US11 gene of herpes simplex virus type 1. Nucleic Acids Res. *14*, 8247-8256.

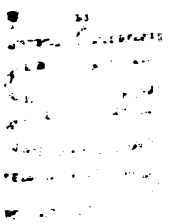
Kedes, D., Operskalski, E., Busch, M., Kohn, R., Flood, J., and Ganem, D. (1996). The seroepidemiology of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus): distribution of infection in KS risk groups and evidence for sexual transmission. Nature Med. *2*.

Kibler, P. K., Duncan, J., Keith, D., Hupel, T., and Smiley, J. R. (1991). The regulation of herpes simplex virus true late gene expression: sequences downstream from the US11 TATA box inhibit expression from an unreplicated template. J. Virol. *65*, 6749-6760.

Kieff, E. (1996). Epstein-Barr Virus and Its Replication. In Fields Virology, B. N. Fields, D. M. Knipe, P. M. Howley and e. al, eds. (Philadelphia: Lippincott-Raven Publishers), pp. 2343-2396.

Lin, S. F., Sun, R., Heston, L., Gradoville, L., Shedd, D., Haglund, K., Rigsby, M., and Miller, G. (1997). Identification, expression, and immunogenicity of Kaposi's





sarcoma- associated herpesvirus-encoded small viral capsid antigen. J Virol *71*, 3069-76.

Lukac, D. M., Kirshner, J. R., and Ganem, D. (1999). Transcriptional Activation by the Product of Open Reading Frame 50 of Kaposi's Sarcoma-Associated Herpesvirus Is Required for Lytic Viral Reactivation in B Cells. J. Virol. *73*, 9348-9361.

Lukac, D. M., Renne, R., Kirshner, J. R., and Ganem, D. (1998). Reactivation of Kaposi's sarcoma-associated herpesvirus infection from latency by expression of the ORF 50 transactivator, a homolog of the EBV R protein. Virology *252*, 304-312.

Martin, J. N., Ganem, D. E., Osmond, D. H., Page-Shafer, K. A., Macrae, D., and Kedes, D. H. (1998). Sexual transmission and the natural history of human herpesvirus 8 infection. N Engl J Med *338*, 948-54.

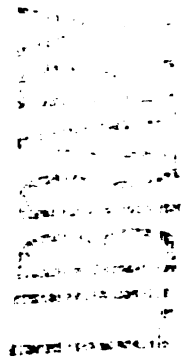
Mavromara-Nazos, P., and Roizman, B. (1987). Activation of herpes simplex virus 1 γ2 genes by viral DNA replication. Virology *161*, 593-598.

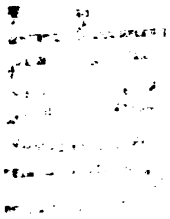
Mavromara-Nazos, P., and Roizman, B. (1989). Delineation of regulatory domains of early (β) and late (γ2) genes by construction of chimeric genes expressed in herpes simplex virus type 1 genomes. Proc. Natl. Acad. Sci USA *86*, 4071-4075.

Mesri, E. A., Cesarman, E., Arvanitakis, L., Rafii, S., Moore, M. A., Posnett, D. N., Knowles, D. M., and Asch, A. S. (1996). Human herpesvirus-8/Kaposi's sarcoma-associated herpesvirus is a new transmissible virus that infects B cells. J Exp Med *183*, 2385-90.

Miller, G., Heston, L., Grogan, E., Gradoville, L., Rigsby, M., Sun, R., Shedd, D., Kushnaryov, V. M., Grossberg, S., and Chang, Y. (1997). Selective switch between latency and lytic replication of Kaposi's sarcoma herpesvirus and Epstein-Barr virus in dually infected body cavity lymphoma cells. J Virol *71*, 314-24.

Moore, P. S., Gao, S. J., Dominguez, G., Cesarman, E., Lungu, O., Knowles, D. M., Garber, R., Pellett, P. E., McGeoch, D. J., and Chang, Y. (1996). Primary characterization of a herpesvirus agent associated with Kaposi's sarcomae [published erratum appears in J Virol 1996 Dec;70(12):9083]. J Virol *70*, 549-58.





Moore, P. S., Kingsley, L. A., Holmberg, S. D., Spira, T., Gupta, P., Hoover, D. R., Parry, J. P., Conley, L. J., Jaffe, H. W., and Chang, Y. (1996). Kaposi's sarcoma-associated herpesvirus infection prior to onset of Kaposi's sarcoma. AIDS *10*, 175-180.

Nicholas, J., Zong, J., Alcendor, D. J., Ciufo, D. M., Poole, L. J., Sarisky, R. T., Chiou, C., Zhang, X., Wan, X., Guo, H., Reitz, M. S., and Hayward, G. S. (1998). Novel organizational features, captured cellular genes, and strain variability within the genome of KSHV/HHV8. J Natl Cancer Inst Monographs *23*, 79-88.

Renne, R., Zhong, W., Herndier, B., McGrath, M., Abbey, N., Kedes, D., and Ganem, D. (1996). Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. Nat Med *2*, 342-6.

Russo, J. J., Bohenzky, R. A., Chien, M. C., Chen, J., Yan, M., Maddalena, D., Parry, J. P., Peruzzi, D., Edelman, I. S., Chang, Y., and Moore, P. S. (1996). Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). Proc Natl Acad Sci U S A *93*, 14862-7.

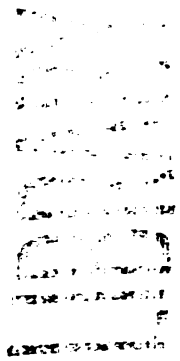
Serio, T. R., Cahill, N., Prout, M. E., and Miller, G. (1998). A functionally distinct TATA box required for late progression through the Epstein-Barr Virus life dycle. J. Virol. *72*, 8338-8343.

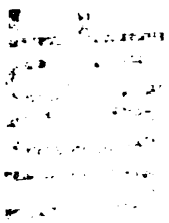
Serio, T. R., Kolman, J. L., and Miller, G. (1997). Late gene expression from the Epstein-Barr Virus BcLF1 and BFRF3 promoters does not require DNA replication in *cis*. J. Virol. *71*, 8726-8734.

Silver, S., and Roizman, B. (1985). γ2- Thymidine kinase chimeras are identically transcribed but regulated as γ2 genes in herpes simplex virus genomes and as β genes in cell genomes. Mol. Cell. Biol. *5*, 518-528.

Soulier, J., Grollet, L., Oksenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., d'Agay, M. F., Clauvel, J. P., Raphael, M., Degos, L., and et al. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease [see comments]. Blood *86*, 1276-80.

Staskus, K. A., Zhong, W., Gebhard, K., Herndier, B., Wang, H., Renne, R., Beneke, J., Pudney, J., Anderson, D. J., Ganem, D., and Haase, A. T. (1997). Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. J Virol *71*, 715-9.





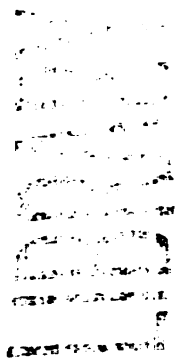
Steffy, K. R., and Weir, J. P. (1991). Upstream promoter elements of the herpes simplex virus type 1 glycoprotein H gene. J. Virol. *65*, 972-975.

Sun, R., Lin, S.-F., Staskus, K., Gradoville, L., Grogan, E., Haase, A., and Miller, G. (1999). Kinetics of Kaposi's sarcoma-associated herpesvirus gene expression. J. Virol. *73*, 2232-2242.

Sun, R., Lin, S. F., Gradoville, L., Yuan, Y., Zhu, F., and Miller, G. (1998). A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus. Proc. Natl. Acad. Sci. USA *95*, 10866-10871.

Unal, A., Pray, T. R., Lagunoff, M., Pennington, M. W., Ganem, D., and Craik, C. S. (1997). The protease and the assembly protein of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) [In Process Citation]. J Virol *71*, 7030-8.

Whitby, D., Howard, M. R., Tenant-Flowers, M., Brink, N. S., Copas, A., Boshoff, C., Hatzioannou, T., Suggett, F. E., Aldam, D. M., Denton, A. S., and et al. (1995). Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma [see comments]. Lancet *346*, 799-802.



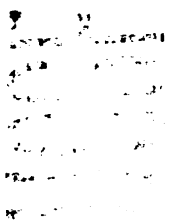
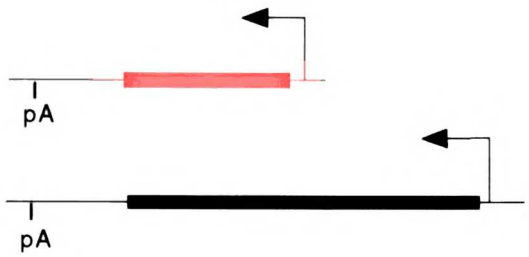


Figure 1

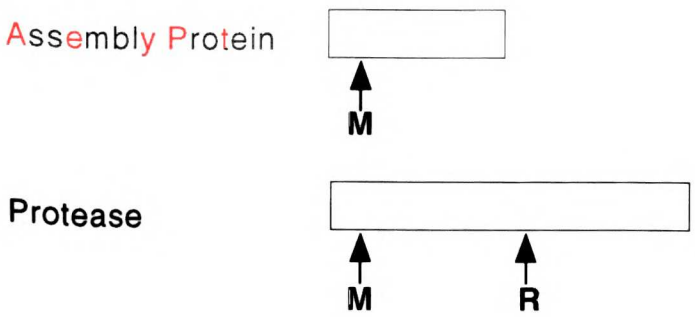
A. Genomic locus

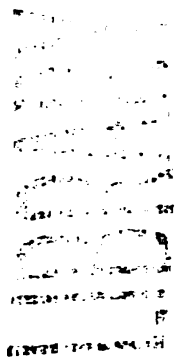


B. mRNA



C. Protein





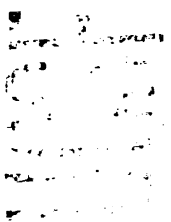
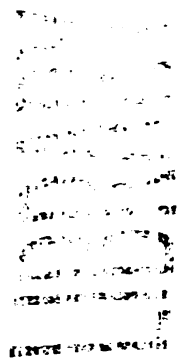


Figure 1 Schematic of ORF17.5 locus, mRNA and gene product, Assembly Protein

a. Genomic organization of ORF 17.5 locus.

b. ORF 17 and 17.5 mRNA structure.

c. Gene products of ORF 17 (Protease) and 17.5 (Assembly Protein).



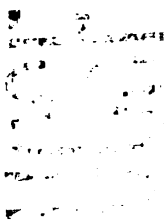
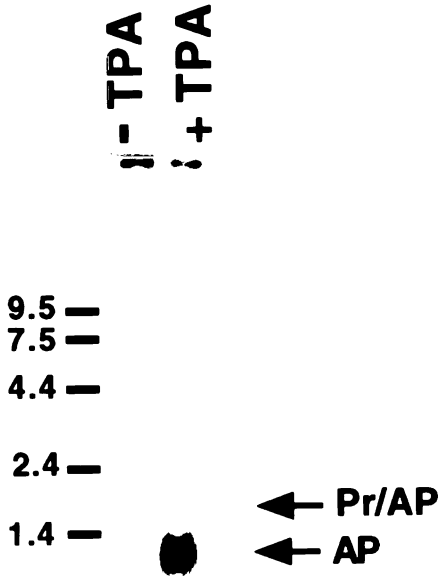
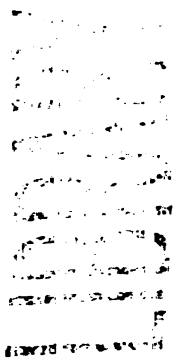


Figure 2





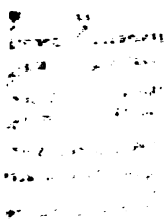
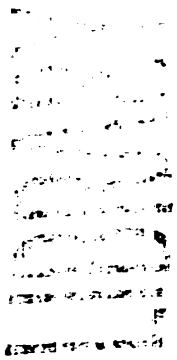


Figure 2 Northern analysis of AP expression

Total RNA from untreated and TPA-treated (48 hrs) BCBL-1 cells were Northern blotted and probed with a double stranded probe for the assembly protein ORF. Sizes of RNA molecular weight markers are shown at left.



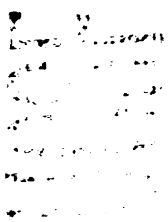
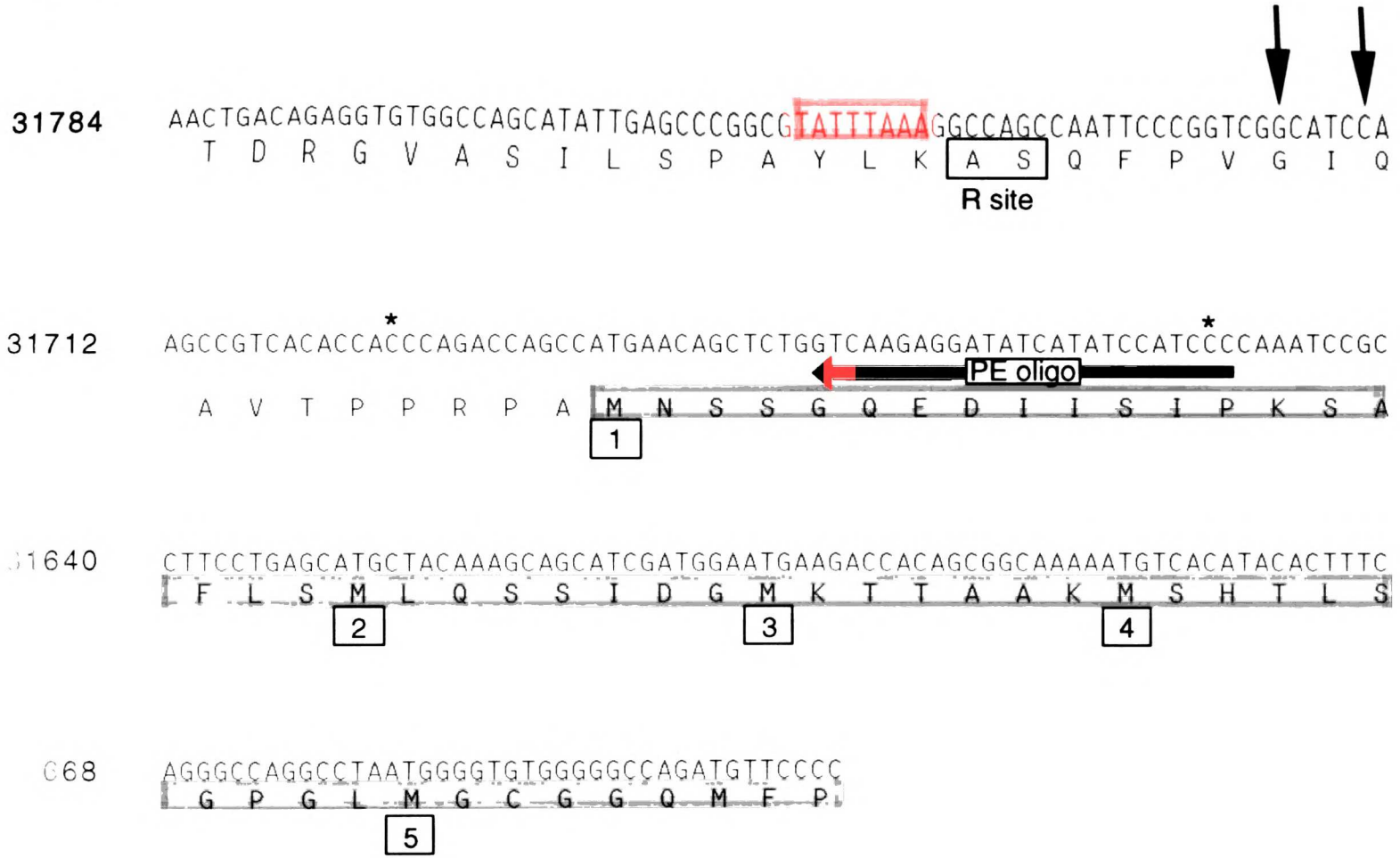
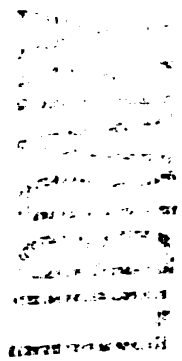


Figure 3


31784
AACTGACAGAGGTGTGGCCAGCATATTGAGCCCGGCGTATTTAAAGGCCAGCCAATTCCCGGTCGGCATCCA
T D R G V A S I L S P A Y L K A S Q F P V G I Q
R site
31712
AGCCGTCACACCACCCAGACCAGCCATGAACAGCTCTGGTCAAGAGGATATCATATCCATCCCCAAATCCGC
PE oligo
A V T P P R P A M N S S G Q E D I I S I P K S A
1
1640
CTTCCTGAGCATGCTACAAAGCAGCATCGATGGAATGAAGACCACAGCGGCAAAAATGTCACATACACTTTC
F L S M L Q S S I D G M K T T A A K M S H T L S
2
3
4
668
AGGGCCAGGCCTAATGGGGTGTGGGGGCCAGATGTTCCCC
G P G L M G C G G Q M F P
5



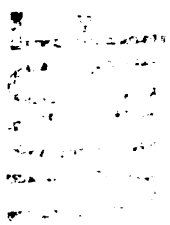
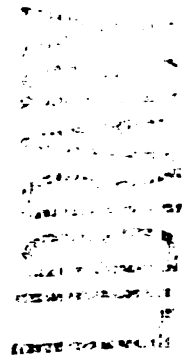


Figure 3 AP cDNA sequences

Nucleotide sequence of the riboprobe for RNase protection assay is depicted. The asterisk shows the locations of the 5' end of cloned cDNAs. The largest possible AP ORF is highlighted in blue with potential initiating AUG codons numbered. The long arrow beneath the DNA sequence represents the oligo used for primer extension analysis. The vertical arrows denote the mapped transcription start site.



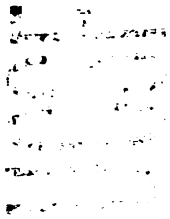
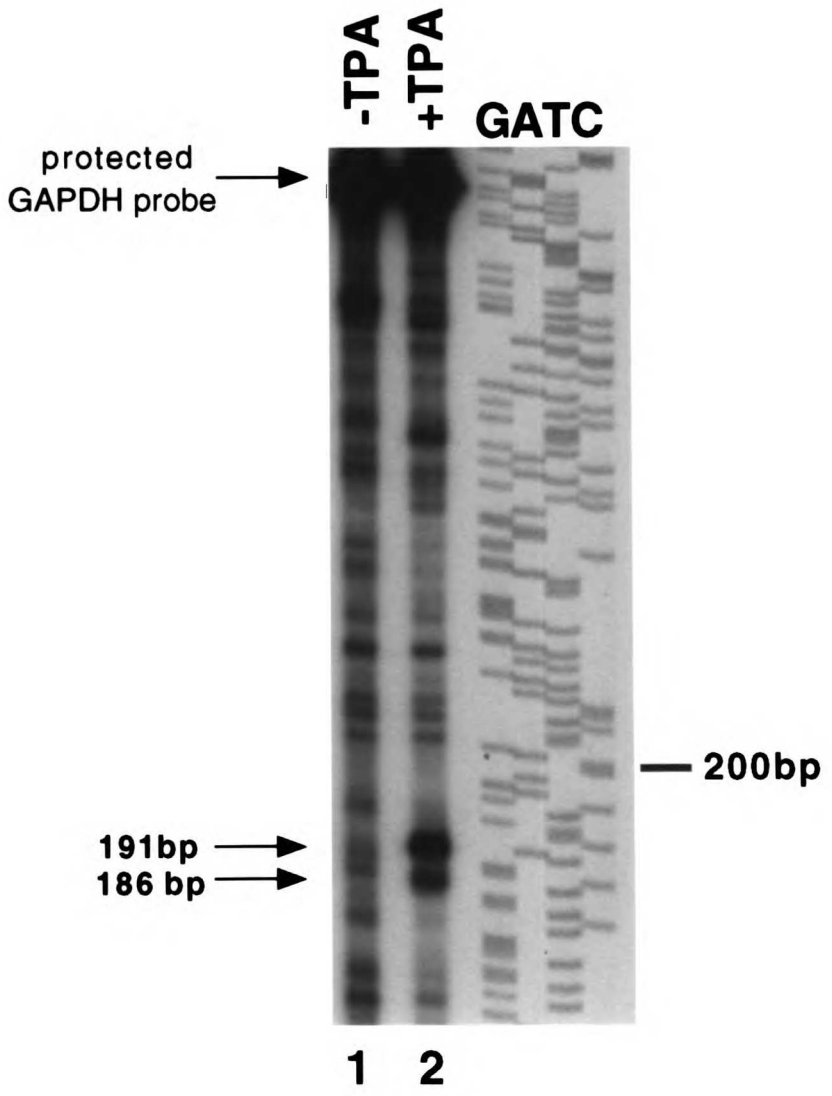
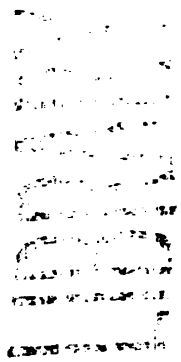


Figure 4





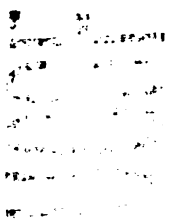
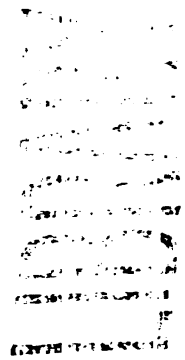


Figure 4 RNase protection analysis of the AP transcript

Total BCBL-1 RNA was isolated as in Fig 1, and hybridized to the riboprobe shown in Fig 3. RNA hybrids were digested with RNase, denatured and electrophoresed. A sequencing ladder (right) was used for sizing.



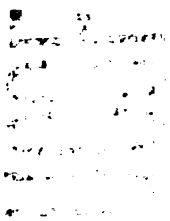
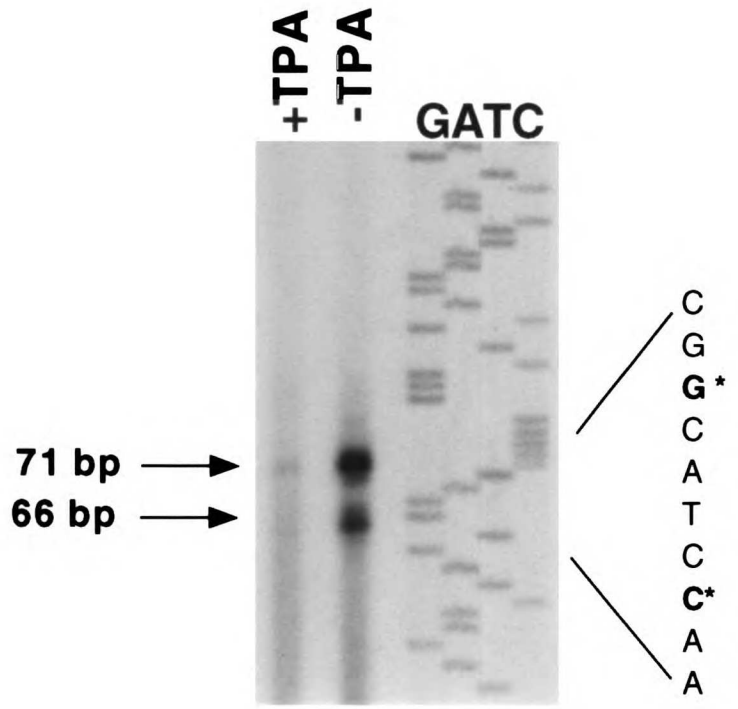
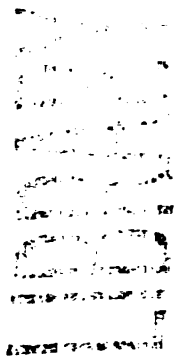


Figure 5





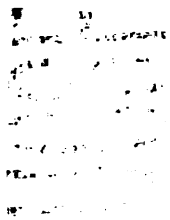
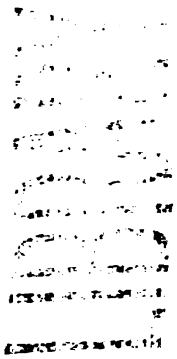


Figure 5 Primer extension analysis of the start site of the AP transcript

Total BCBL-1 RNA was isolated as in Fig 1, and primer extension was performed using the primer indicated in Fig 3. Products were visualized as in Fig 4. A sequencing ladder at right provides a size reference.



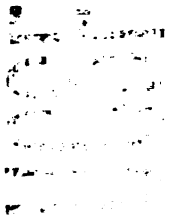
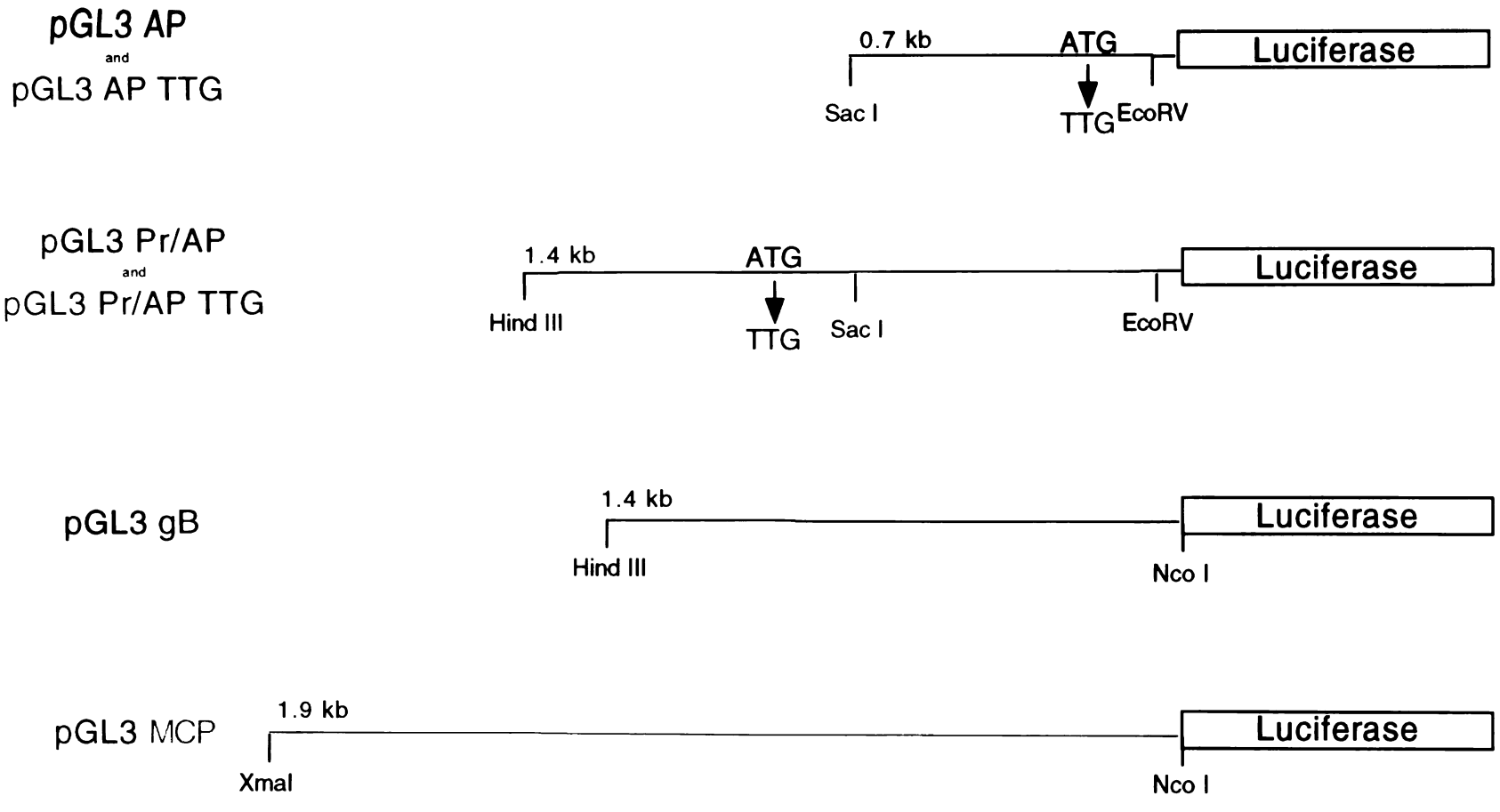
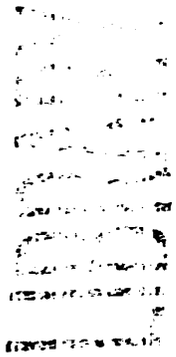


Figure 6


pGL3 AP
and
pGL3 AP TTG
0.7 kb
ATG
Luciferase
Sac I
TTG
EcoRV
pGL3 Pr/AP
and
pGL3 Pr/AP TTG
1.4 kb
ATG
Luciferase
Hind III
TTG
Sac I
EcoRV
pGL3 gB
1.4 kb
Luciferase
Hind III
Nco I
pGL3 MCP
1.9 kb
Luciferase
XmaI
Nco I



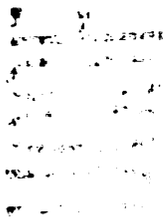
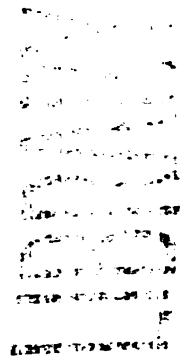


Figure 6 Late gene promoter constructs

Structure of reporter plasmids containing promoter regions from several KSHV late genes.



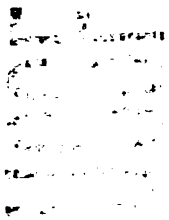
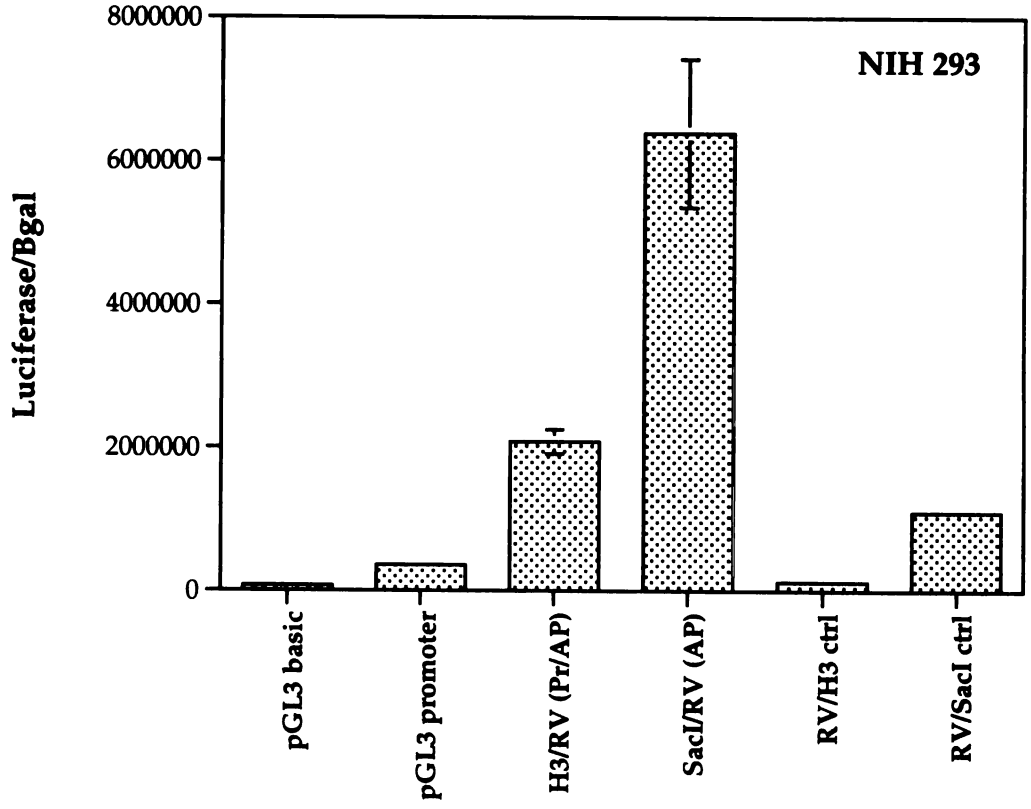
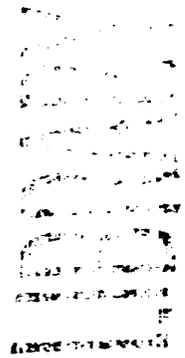


Fig 7a



Fold Activation:	1	5.1	29.2	89.9	1.8	15.4



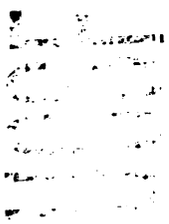
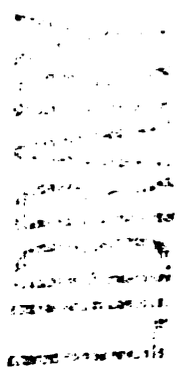


Figure 7a The 1.3kb and 700bp promoter regions of AP are both able to drive expression of a reporter gene.

293 cells were transfected with 4 ug of the indicated reporter plasmid and 2 ug of with β-galactosidase expressing plasmid. Cells were harvested 48 hrs post-transfection, β-galactosidase and luciferase activities were measured. Luciferase activity, normalized to β-gal activity for transfection efficiency is plotted with fold activation relative to pGL3-basic noted below.



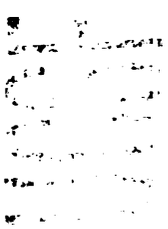
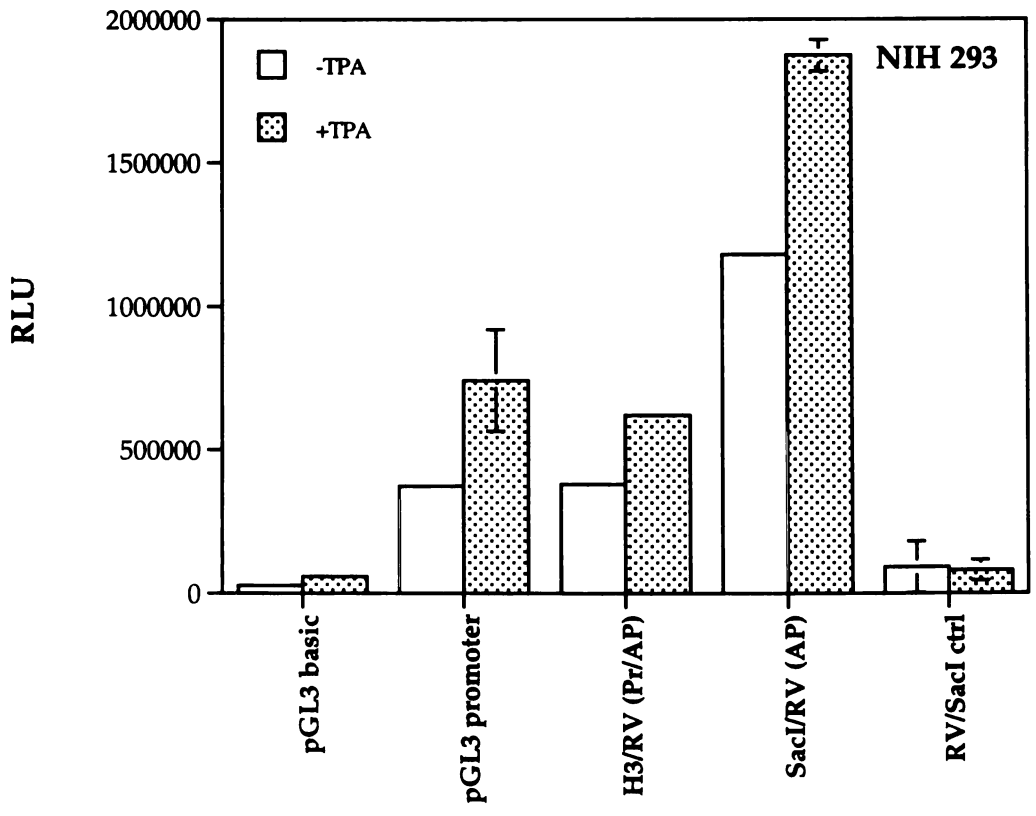
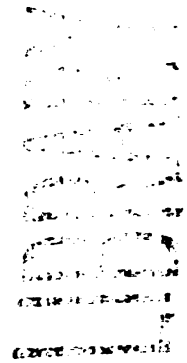


Fig 7b



Fold Activation:	2	2	2	2	1



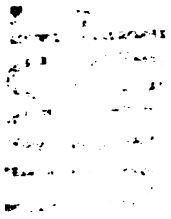
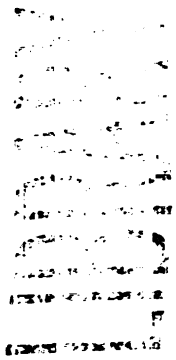


Figure 7b AP promoter regions are not responsive to TPA in 293 cells.

Cells were transfected as above and divided to two sets: treated with TPA (1ul 5000x to 5ml media) and untreated. Cells were harvested and assayed as above. Luciferase activity is plotted with fold activation calculated relative to untreated cells transfected with the same reporter plasmid.



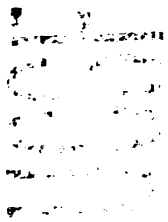
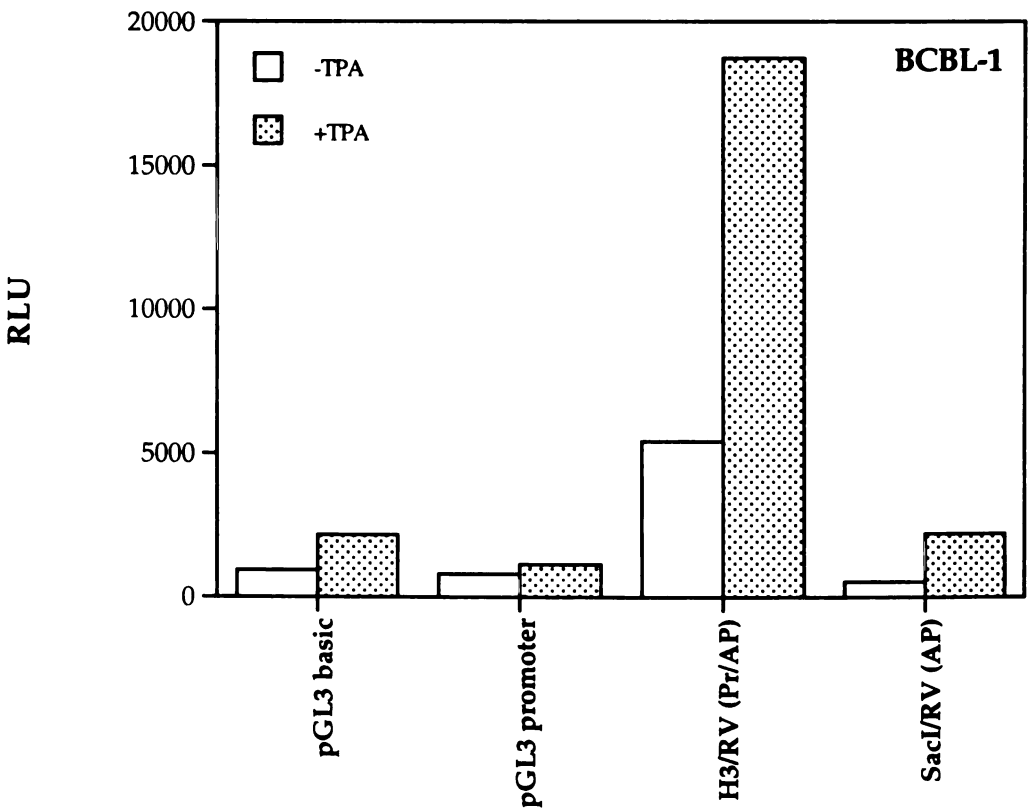
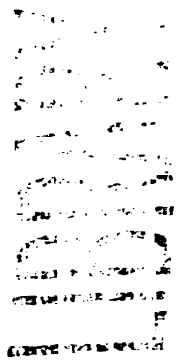


Fig 7c



Fold Act:	**2.3**	**1.4**	**3.4**	**4.2**



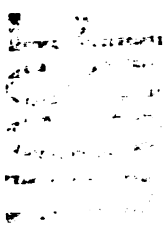
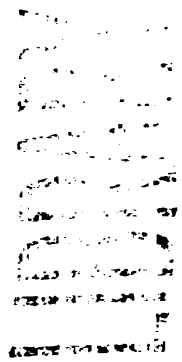


Figure 7c AP promoter regions are mildly responsive to TPA in BCBL-1 cells.

BCBL-1 were transfected with 5ug reporter plasmid and 1 ug β-galactosidase expressing plasmid and treated as in B. Cells were harvested and assayed with luciferase activity plotted and fold activation calculated relative to untreated cells transfected with the same reporter plasmid.



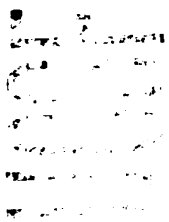
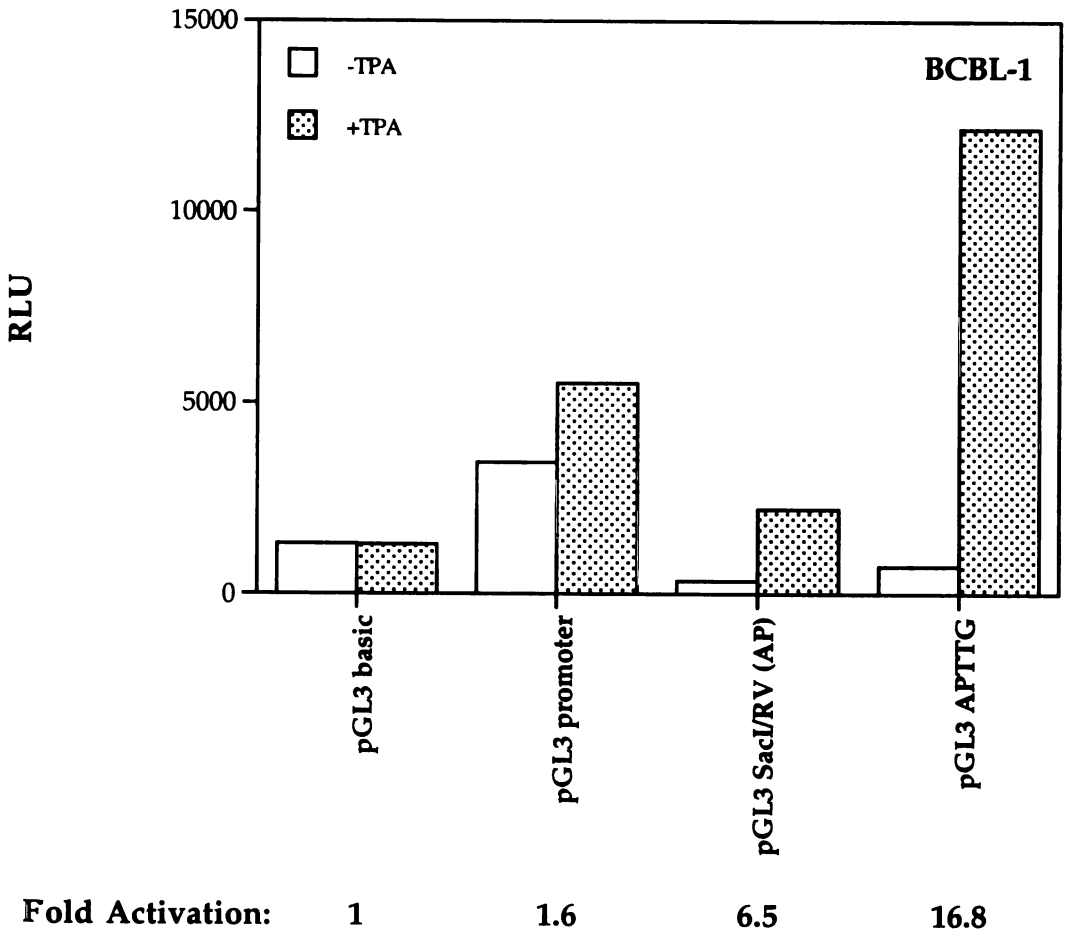
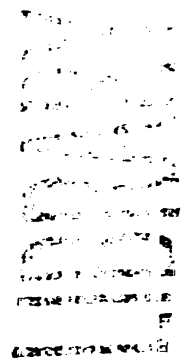


Fig 7d



Fold Activation:	1	1.6	6.5	16.8



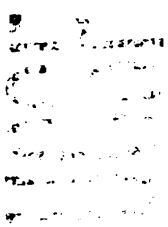
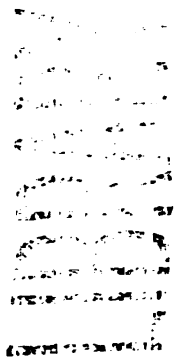


Figure 7d Ablation of ATG in AP promoter region increases reporter activity in response to TPA.

Cells were transfected, treated and assayed as in Fig 7c.



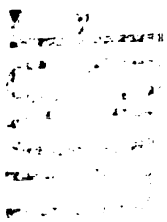
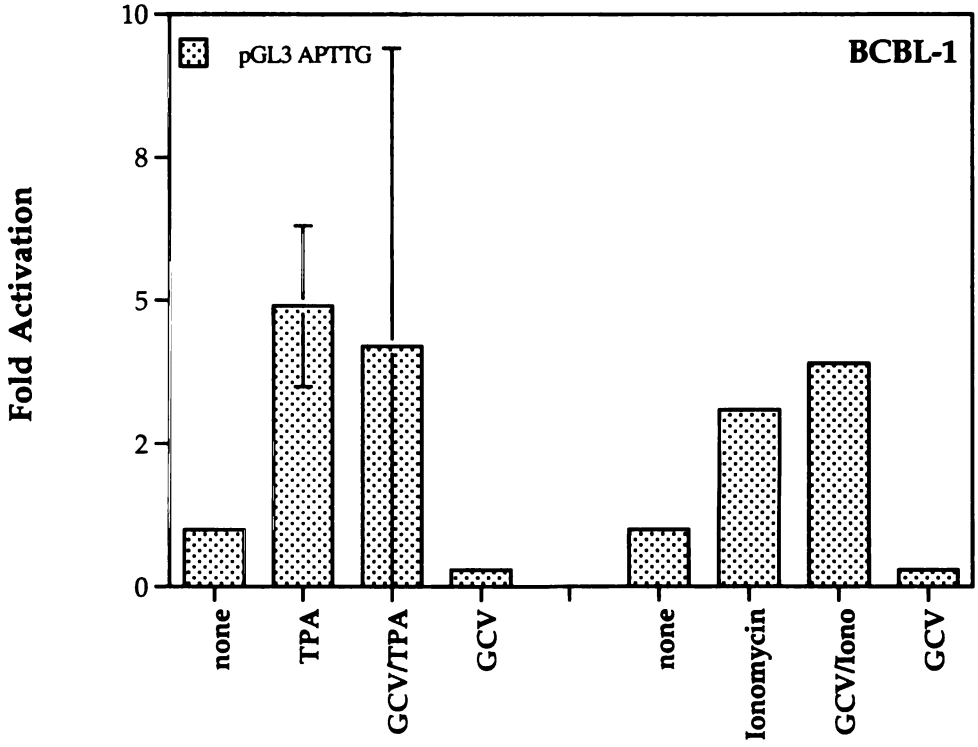
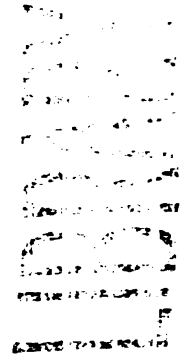


Fig 8a


Fold Activation
10
8
5
2
0
pGL3 APTTG
BCBL-1
none
TPA
GCV/TPA
GCV
none
Ionomycin
GCV/Iono
GCV



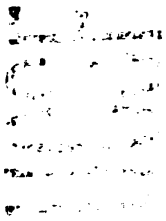
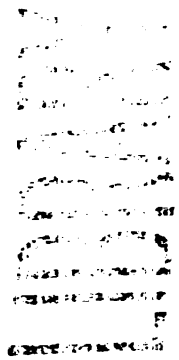


Figure 8a pGL3 AP TTG is responsive to both TPA and ionomycin but insensitive to ganciclovir.

BCBL-1 cells were transfected with 5 ug of reporter plasmid and 1 ug β-galactosidase expressing plasmid then treated with the indicated chemical inducers and viral inhibitors as indicated. Fold activation is plotted with all values calculated relative to untreated cells.



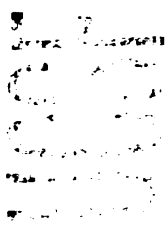
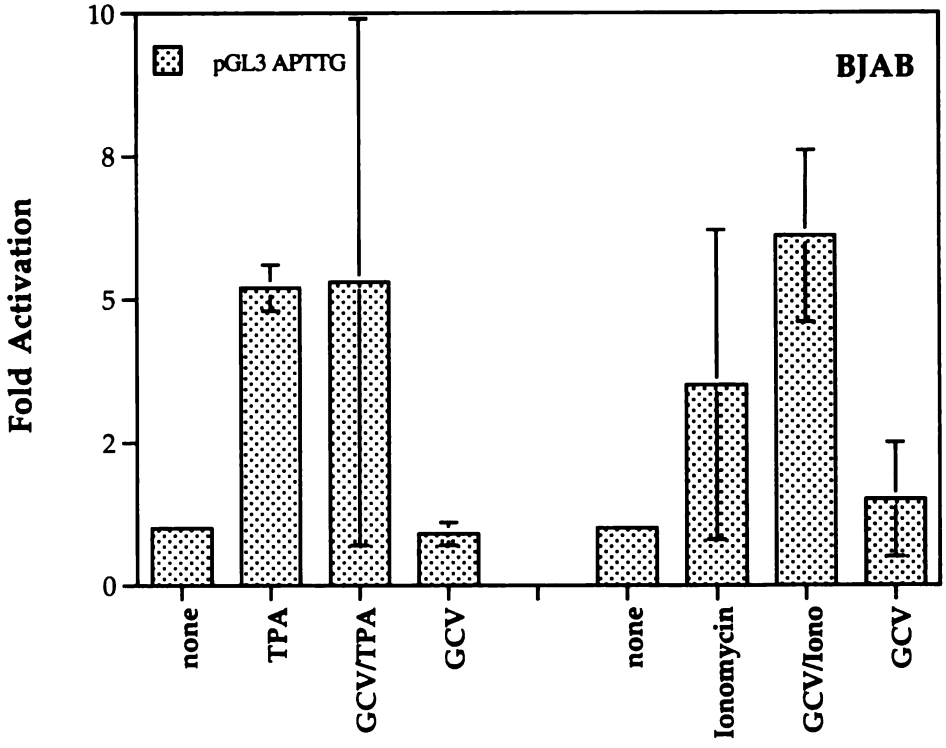
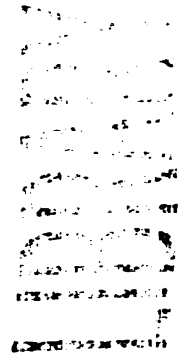


Fig 8b


pGL3 APTTG
BJAB
Fold Activation
10
8
5
2
0
none
TPA
GCV/TPA
GCV
none
Ionomycin
GCV/Iono
GCV



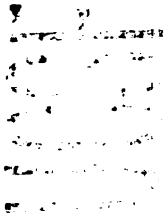
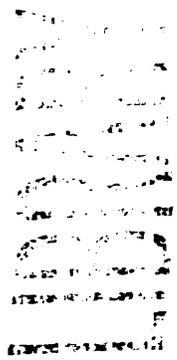


Figure 8b pGL3 AP TTG is responsive to both TPA and ionomycin in BJAB cells.

Cells were transfected 5 ug of reporter plasmid and 1 ug β-galactosidase expressing plasmid. Cells were treated and analyzed as in Fig 8a.



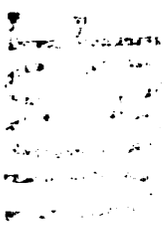
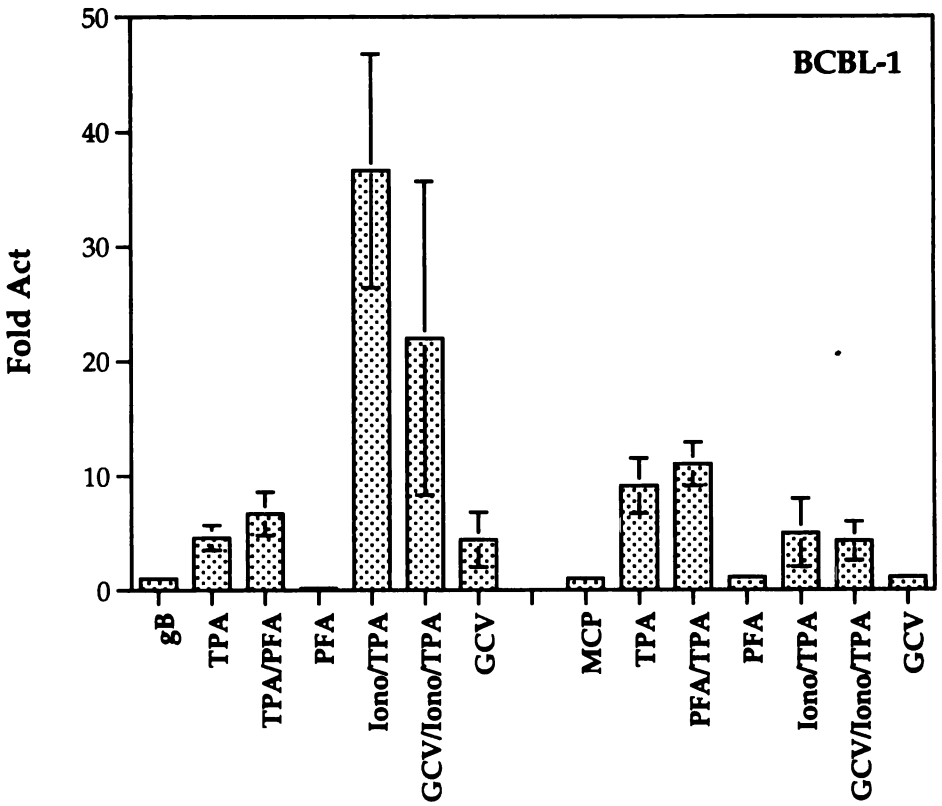
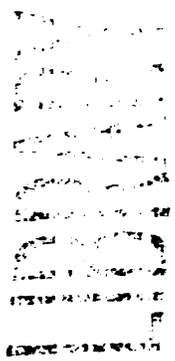


Fig 8c


BCBL-1
Fold Act
50
40
30
20
10
0
gB
TPA
TPA/PFA
PFA
Iono/TPA
GCV/Iono/TPA
GCV
MCP
TPA
PFA/TPA
PFA
Iono/TPA
GCV/Iono/TPA
GCV



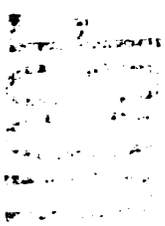
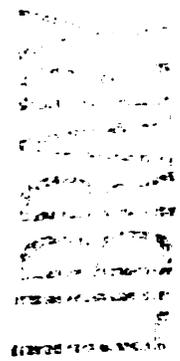


Figure 8c gB and MCP promoter regions are unresponsive to ganciclovir in BCBL-1.

BCBL-1 cells were transfected with 5 ug of reporter plasmid and 1 ug β-galactosidase expressing plasmid then treated with the indicated chemical inducers and viral inhibitors as indicated. Fold activation is plotted with all values calculated relative to untreated cells.



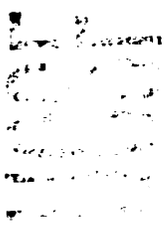
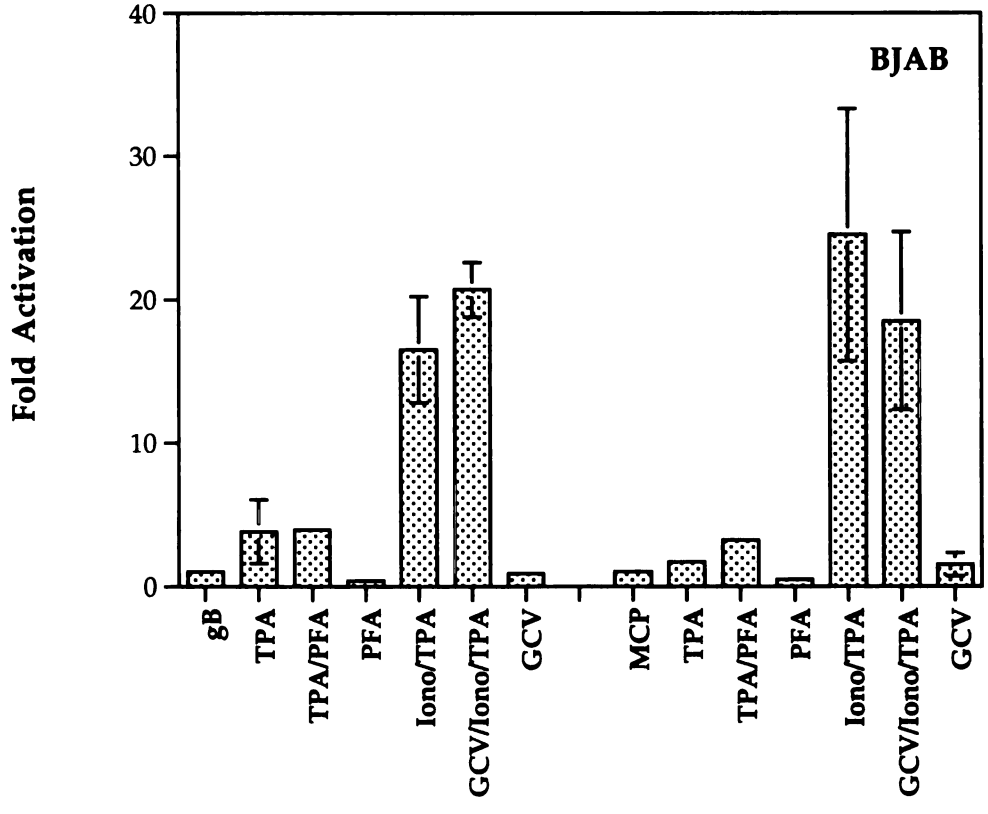
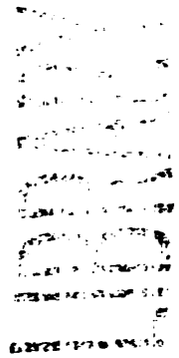


Fig 8d


BJAB
Fold Activation
40
30
20
10
0
gB
TPA
TPA/PFA
PFA
Iono/TPA
GCV/Iono/TPA
GCV
MCP
TPA
TPA/PFA
PFA
Iono/TPA
GCV/Iono/TPA
GCV



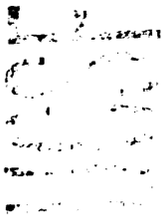
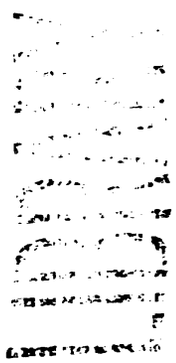


Figure 8d gB and MCP promoter regions are also inducible in BJAB cells.

BJAB cells were transfected, treated and analyzed as in Fig 8b.



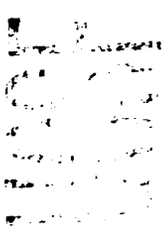
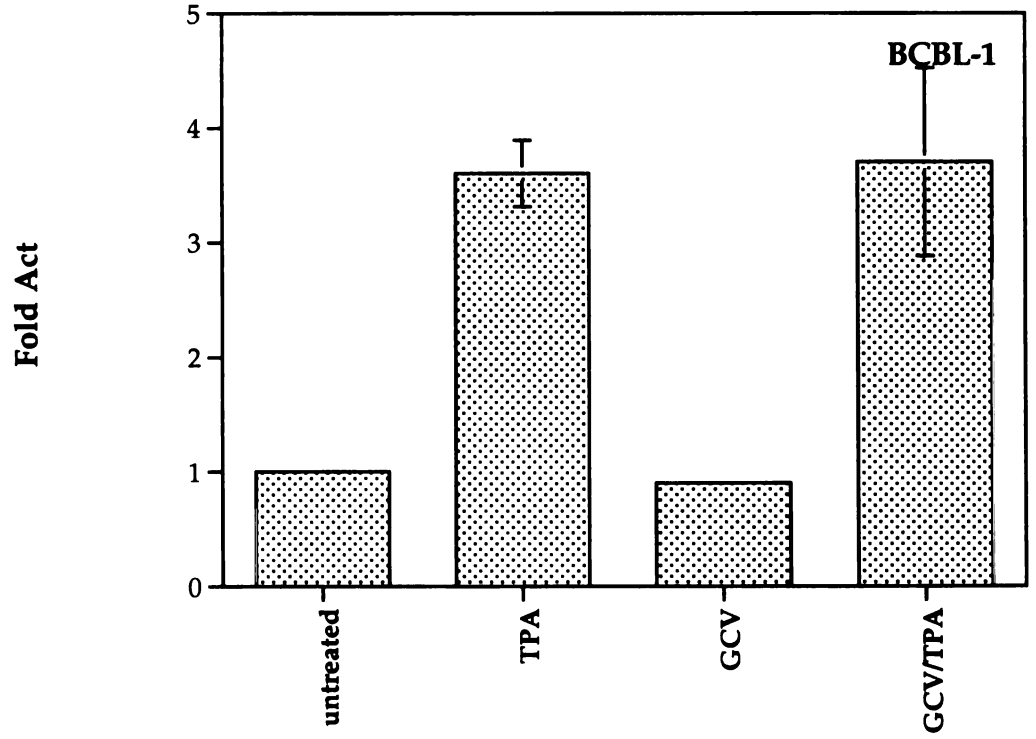
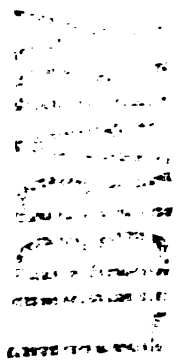


Fig 9





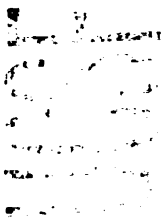
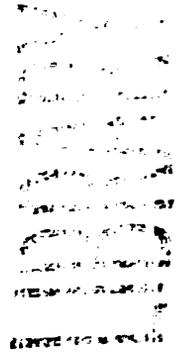


Figure 9 Minimal late gene promoter in BCBL-1

pGL3 AP -30 is responsive to TPA but insensitive to ganciclovir. BCBL-1 cells were transfected with 5 ug of reporter plasmid and 1 ug β-galactosidase expressing plasmid then treated with TPA and/or ganciclovir as indicated. Fold activation is plotted with all values calculated relative to untreated cells.



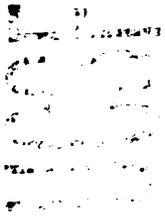
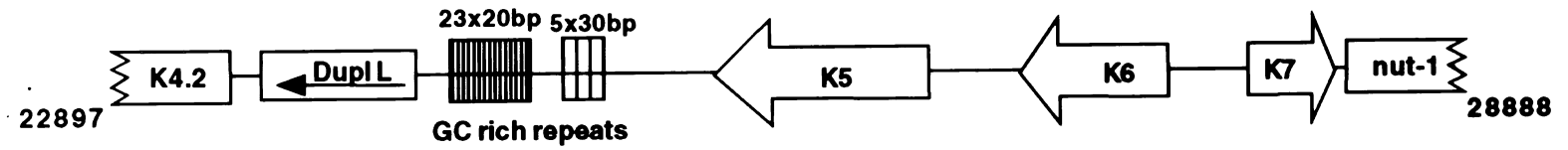
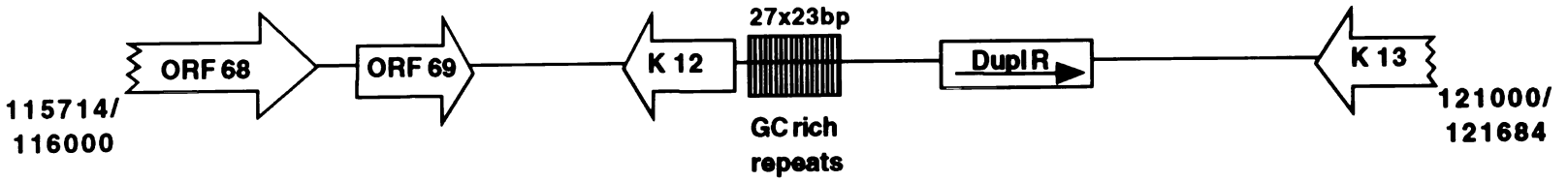


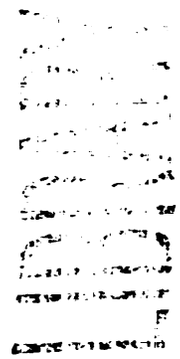
Figure 10

B4



KS31/KS50





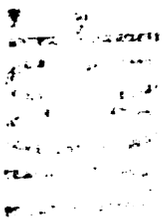


Figure 10 Genomic locus of candidate sites for the viral lytic origin.

Schematic of candidate regions from clones B4 and KS31/KS50.

Figure 11a

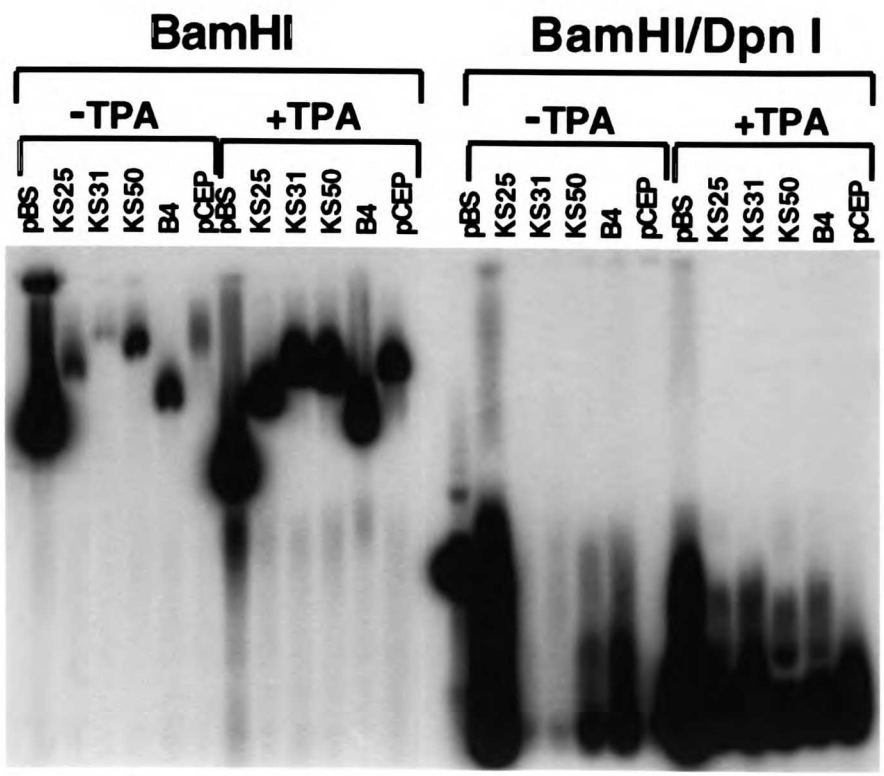

BamHI
BamHI/Dpn I
-TPA
+TPA
-TPA
+TPA
pBS
KS25
KS31
KS50
B4
pCEP
pBS
KS25
KS31
KS50
B4
pCEP
pBS
KS25
KS31
KS50
B4
pCEP
pBS
KS25
KS31
KS50
B4
pCEP

Figure 11a Assay for the KSHV lytic origin by transient transfection assay.

Candidate oriLyt loci do not undergo replication and become Dpn I resistant after BCBL-1 induction in transient assay. BCBL-1 cells were transfected with 10 ug of plasmid bearing the candidate locus indicated. At 72 hrs post-transfection DNA was extracted by the method of Hirt. DNAs were purified and digested with Bam HI ± Dpn I and electrophoresed in a 0.8% agarose gel, transferred to nylon membrane and detected by hybridization to a ^{32}P-labeled Amp probe and autoradiography.

Figure 11b

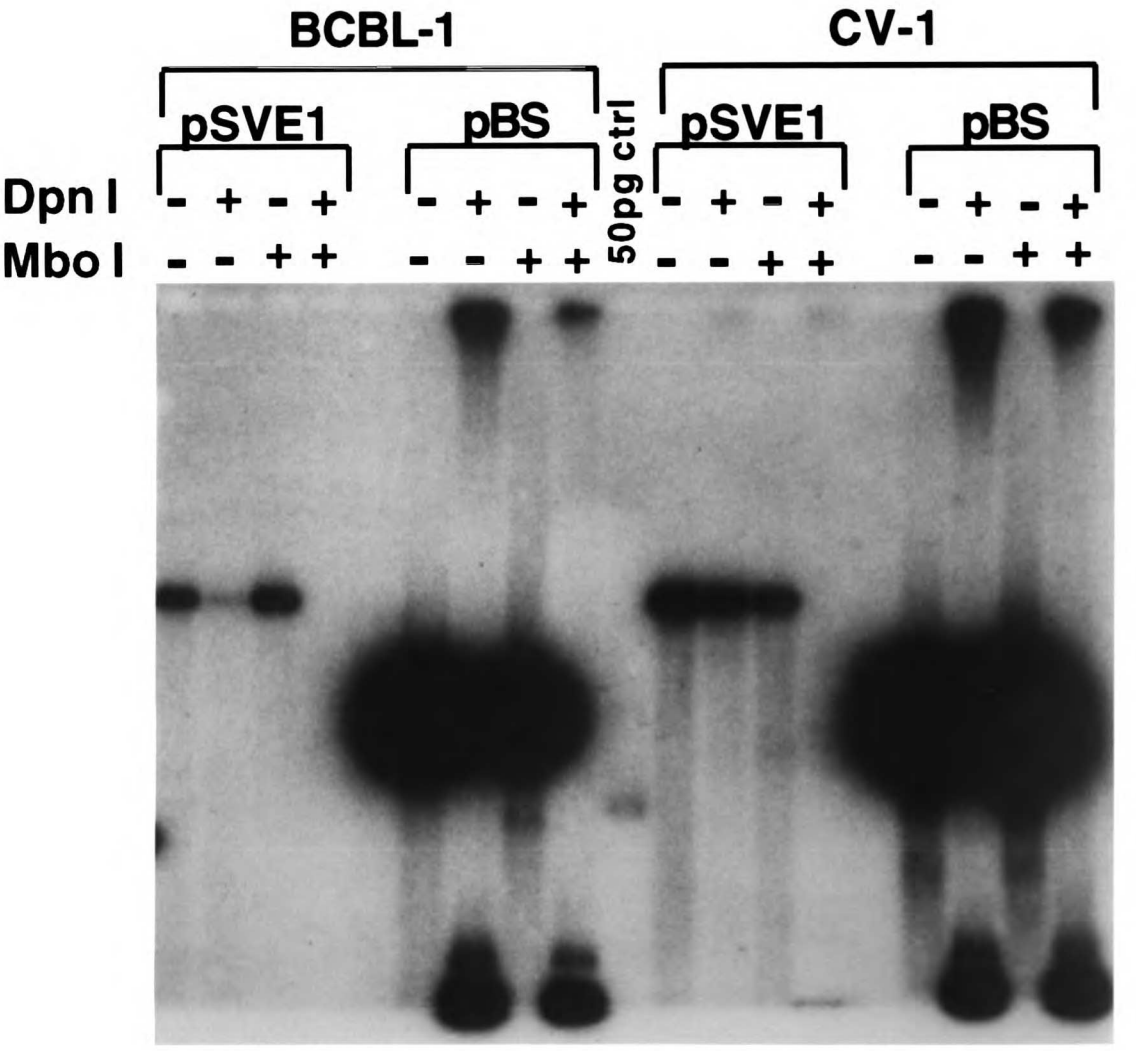


Figure 11b Replicated DNA can be detected by transient assay in BCBL-1.

BCBL-1 cells and CV-1 were transfected with 10 ug of pSVE1 or pBSIISK-. At 72 hrs post-transfection DNA was extracted by the method of Hirt. DNAs were purified and digested with Eco RI ± Dpn I ± Mbo I and electrophoresed in a 0.8% agarose gel, transferred to nylon membrane and detected by hybridization to a ^{32}P-labeled Amp probe and autoradiography.

Figure 12

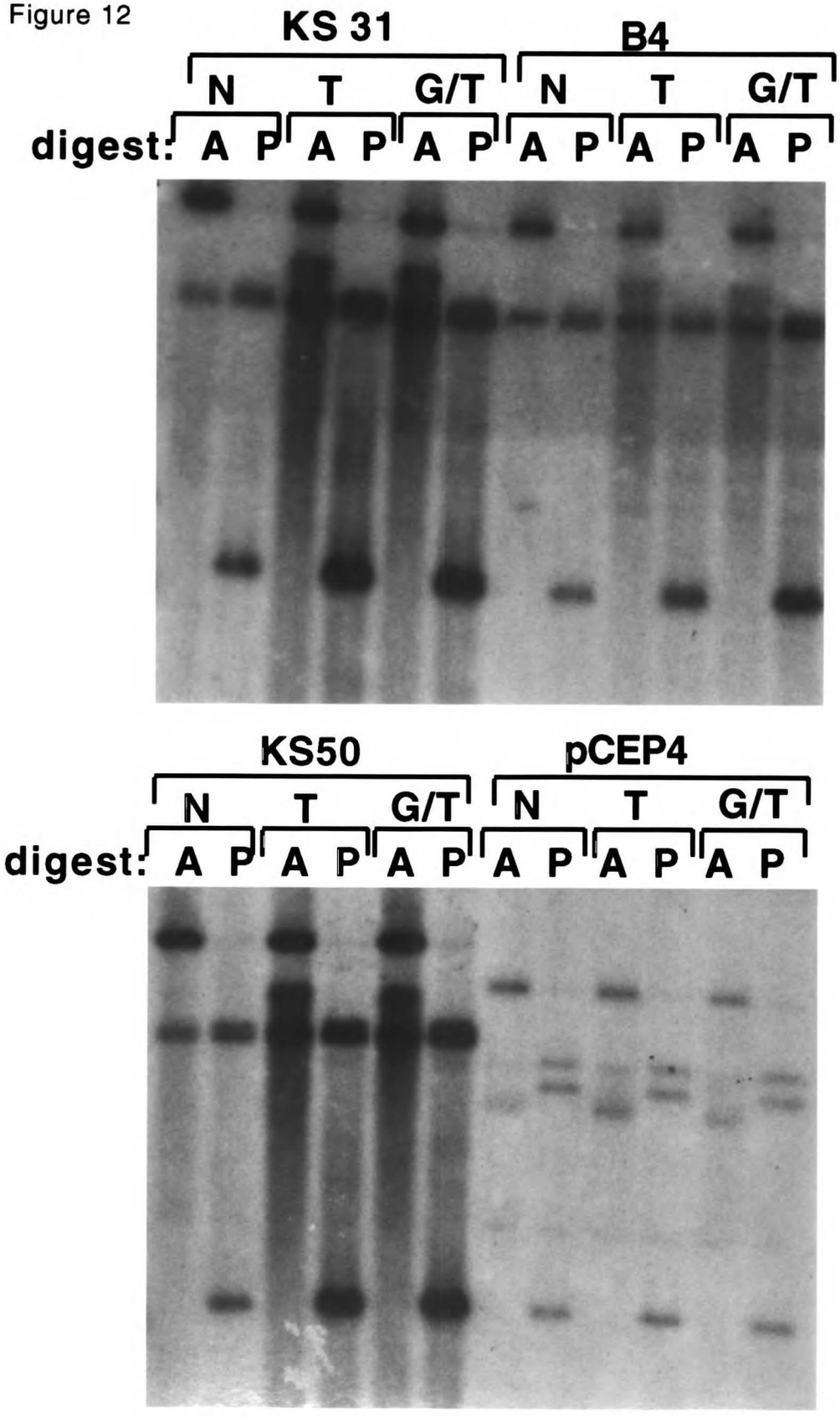

KS 31
B4
N T G/T N T G/T
digest: A P A P A P A P A P A P
KS50
pCEP4
N T G/T N T G/T
digest: A P A P A P A P A P A P

Figure 12 Assay for the viral lytic origin by stable transfection

Candidate oriLyt loci do not undergo significant amplification after BCBL-1 induction. BCBL-1 cells were transfected with 10 ug of plasmid bearing the candidate locus indicated. Cells were selected for stable transformants by G418 selection for ca. 2 weeks. Stable transfectants were treated with the indicated chemical inducer and/or viral inhibitor. At 72 hrs post-treatment, DNA was extracted by the method of Hirt. DNAs were purified and digested with Afl II or Pvu I and electrophoresed in a 0.8% agarose gel, transferred to nylon membrane and detected by hybridization to a ^{32}P-labeled Amp probe and autoradiography.

Conclusion

Progression through the viral lytic cycle is a carefully ordered process involving multiple steps serially reuqired for virus production. Exogenous agents can alternately stimulate or repress lytic reactivation. The viral response to these agents may have evolved to interpret specific molecules as signals representing the external milieu of its host cell. If so, such a sensing mechanism could help assure that reactivation would occur only when conditions are favorable for continued viral spread.

The studies described in chapter 1 of this thesis show that B cells latently infected with KSHV are insensitive to a number of cytokines but respond specifically to interferons gamma and alpha. Although chemical agents such as phorbol ester or calcium ionophore may effectively induce latently infected B cell lines such as BCBl-1, stimulation by these methods may have multiple downstream effects which are not biologically related to KSHV induction. The identification of two cytokines which can specifically modulate KSHV reactivation provide tools for elucidating the nature of the intracellular signal which is responsible for the expression of ORF50, the proposed molecular switch of KSHV.

Once lytic reactivation is initiated, presumably through the expression of the immediate early transactivator, ORF50, progression through the lytic cycle requires the activation of various delayed early promoters, including those driving the expression of products responsible for viral DNA synthesis. Our work in chapter 2 identifies two types of *cis*-elements that are necessary for ORF50-

dependent activation of transcription. Further delineation of the ORF50-responsive element in the TK promoter would enable sequence analysis efforts to determine whether ORF50 acts through a collection of loosely related sites. Characterization of additional ORF50-dependent promoters would also prove useful in deriving a consensus ORF50-responsive site. Such characterization may identify motifs that ORF50 might utilize for direct binding and promoter activation. Alternatively, the sites described in this thesis is may not involve direct binding by ORF50. Indirect mechanisms of activation presuppose the involvement of cellular factors. The presence or absence of such cellular factors within the host cell may prove to be another checkpoint within the lytic cycle.

A third checkpoint in the lytic cycle is viral replication. In the absence of viral DNA synthesis, only low levels of late gene expression can be detected in herpesviruses. Our data in chapter 3 suggests that late gene expression in KSHV requires viral replication *in cis*, suggesting that the passage of a replication fork through late promoter regions provides a necessary *cis*-modification of the DNA, resulting in an accessible template for transcription initiation. Identification of the KSHV lytic origin will most likely be necessary for the understanding of late gene regulation. Future work will determine whether the *cis*-modification required is the displacement of nucleosomes or negative regulatory factors or a positive action contributed by viral replication.

The passing of successive checkpoints culminates in the production of infection progeny virons. Broadening our understanding of lytic gene regulation will allow

future research to focus on methods to block lytic cycle progression, inhibit virion production, and prevent disease progression.

UC San Francisco LIBRARY
UNIVERSITY OF CALIFORNIA

For reference

Not to be taken from the room.

7064937

3 1378 00706 4937

UC San Francisco LIBRARY

UNIVERSITY OF CALIFORNIA · UNIVERSITY OF CALIFORNIA