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LYTIC GENE REGULATION IN
KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS

by

JESSICA R. KIRSHNER

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of
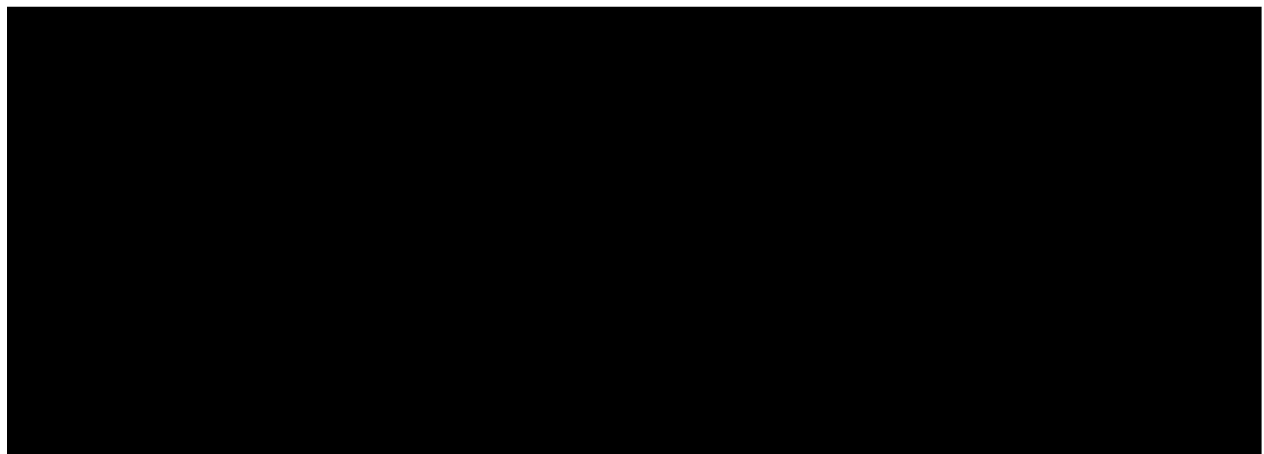
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in

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To my parents
For their constant love and encouragement

ACKNOWLEDGEMENTS

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LYTIC GENE REGULATION IN

KAPOSI'S SARCOMA –ASSOCIATED HERPESVIRUS

ABSTRACT

Jessica R. Kirshner

Infection with Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) is essential for development of KS and several B-cell lymphomas. KSHV DNA is found in virtually all spindle cells of KS tumors, and latent infection of KSHV is established long before the onset of KS. The virus exists in two replicative phases: latency, where few genes are expressed, and a productive lytic phase. Although the virus is predominantly latent in tumor cells, several lines of epidemiological and clinical evidence suggest that lytic replication contributes to tumor development. Furthermore, many genes with angiogenic, pro-inflammatory and growth-promoting properties are expressed solely during the lytic phase. Therefore, lytic KSHV replication likely plays a significant role in disease progression.

Control of lytic cycle gene expression in KHSV is regulated by several virally encoded proteins, including ORF 57, a protein with homology to post-transcriptional regulators in

other herpesviruses. Expression of ORF 57 results in the accumulation of the early viral transcripts: nut-1, and DNA polymerase processivity factor (PF/ ORF 59). This accumulation occurs by post-transcriptional mechanisms. Furthermore, ORF 57 can augment the activity of the transcriptional activator and switch protein, ORF 50, on the nut-1 promoter. ORF 57 contains at least two nuclear localization signals at the N-terminus, and a conserved C/H (putative zinc–finger) region at the C-terminus. Both the zinc-finger region and a arginine rich region at the N-terminus are required for synergy between ORF 57 and ORF 50.

One of the KSHV lytic genes thought to be involved in KS pathogenesis is the G-protein coupled receptor (GCR) which influences proliferation and angiogenesis when ectopically expressed in fibroblasts in vitro and in transgenic mice. We examined the expression of the KSHV GCR gene in virus-infected lymphoid cells and in KS tumors. In both situations the gene is expressed primarily during the early phases of lytic replication. The major transcript bearing GCR sequences is bicistronic, harboring coding sequences for another viral gene, K14, at its 5' end. These results suggest that the KSHV GCR protein is translated by unconventional mechanisms and may play a paracrine role in pathogenesis of KS.

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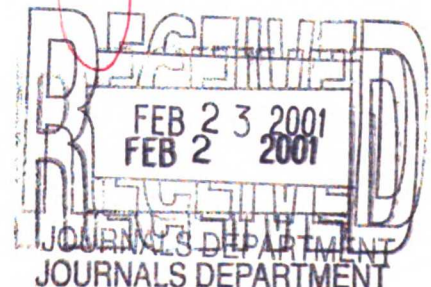
Chapter 1

Introduction

INTRODUCTION

History of Kaposi's Sarcoma

Kaposi's sarcoma (KS) was first described in 1872 by a Hungarian doctor named Moritz

Kaposi, a prominent figure in the legendary Viennese school of dermatology. He

published a paper in '*Archiv fur Dermatologie und Syphillis*' describing five cases of a

condition he called "idiopathic multiple pigmented sarcoma of the skin", eponymously

designated Kaposi's Sarcoma in 1891. All patients were middle aged or elderly men.

Common characteristics of this disease were red to bluish nodules that typically formed

on the feet or hands, but could involve the legs, trunk and even the mucous linings of the

respiratory and gastointestinal tracts. Importantly, he noted the 'striking pigmentation'

caused by the hemorrhaging and red blood in the nodules, a hallmark of the disease,

which would become a harbinger of the AIDS epidemic 90 years later.

Epidemiological types of KS

Scientists now recognize four clinical variants of Kaposi's Sarcoma. Interestingly, adult

males are predominantly affected in all forms. Classical KS, the type described

originally by Kaposi, is a rare disease that affects elderly men of Mediterranean and Eastern European descent. This form is the least aggressive of all the KS variants (7).

The endemic form of KS was described by Dutz and Stout (20) and Oettle (59) in 1960, who noted a high incidence of KS in several equatorial African countries. This form is more common and affects somewhat younger adults than classical KS. A variant of endemic KS has been described in children where it can present with widespread lymph node involvement; this form is considerably more aggressive than the adult endemic variety.

Iatrogenic KS can develop in post-organ transplant patients who are immunosuppressed (70). In many cases, the KS lesions regress or disappear if the immunosuppressive therapy is ceased (7).

In 1981, the fourth form of KS, AIDS associated-KS was identified. The Centers for Disease Control and Prevention (CDC) in Atlanta reported an increased incidence of two rare diseases, KS and *Pneumocystis carinii* in young homosexual men from California and New York (81). The simultaneous occurrence of these rare diseases, KS with *Pneumocystis carinii*, in patients who were otherwise healthy, alerted doctors and

scientists to an underlying immunosuppressive state. Scientists soon discovered a previously unknown infectious agent, now known as the retrovirus Human Immunodeficiency Virus (HIV), as the cause this severe immunosuppression, or Acquired Immune Deficiency Syndrome (AIDS). Currently, AIDS-associated KS is the most common form of the disease, and the most aggressive. As in classical KS, skin is the most frequently involved site, but the involvement is more widespread and severe, often producing disfiguring local edema and induration. In addition, visceral involvement is much more common, with pulmonary KS often leading to respiratory failure and death.

Histology of KS

KS is a histologically complex lesion, and unlike typical cancers, does not appear to be monoclonal in origin (28). The bulk of the lesions, or nodules, are characterized by spindle cells of endothelial origin, as well as infiltrating lymphocytes, macrophages, mast cells, and neutrophils. The spindle cells are thought to be the proliferating cell of the lesion. Slit-like vascular spaces lined with endothelium, along with extravasated erythrocytes, contribute to the red-brown color of this unique tumor.

Early investigations of KS etiology

KS lesions, like all tumors, produce cytokines, specifically high levels of Il-6, bFGF,

TNF α and γ interferon (7). Because KS spindle cells produce Il-6, and exogenous IL-6

induces spindle cell proliferation, it was thought that these lesions were cytokine driven.

AIDS-associated KS was the most aggressive form of the disease, and this data led

researchers to investigate the role of HIV encoded proteins in KS development.

However, HIV DNA and RNA was absent in most spindle cells, so direct cell-

autonomous proliferation driven by HIV was ruled out. Gallo has speculated that HIV-

infected cells may produce substances that drive proliferation in a paracrine fashion. The

viral transactivator Tat was a good candidate, as it can be released by infected cells, and

can be taken up by cells resulting in transactivation of the HIV promoter (25).

Furthermore, Tat can induce KS spindle cells to proliferate in vitro and is angiogenic in

vivo (21, 22). However, epidemiological evidence indicates that there exist HIV-

independent forms of KS; moreover, the risk of KS in all HIV-infected population groups

is not uniform. For example, the risk is much lower for HIV infected hemophiliacs,

transfusion recipients and children exposed through vertical transmission than for

homosexual men (5). Therefore, HIV infection alone cannot be sufficient for KS

development.

Search for an infectious cause of KS

Interestingly, an infectious cause for KS was postulated even prior to the AIDS epidemic.

Herpesvirus like particles were discovered using electron microscopy in KS cells that

were thought to be cytomegalovirus (CMV)(29, 30). DNA from many viruses, including

CMV, HHV-6, HPV and BK have been found in some, but not all lesions tested (7). The

lack of 100% concordance between viral DNA detection and KS suggested a novel

infectious agent.

Discovery of KSHV

The unique epidemiology of KS among various HIV-transmission groups suggested a

sexually transmitted co-factor other than HIV. In 1994, Patrick Moore and Yuan Chang

used representational difference analysis (RDA)(46) to search for DNA sequences

present in KS lesions, but absent in healthy tissues. They identified two fragments that

were homologous to herpesvirus structural genes (15). Using these fragments, several

labs were able to clone the165 kb genome of a novel human gamma, or lymphotropic,

herpesvirus (53, 57, 67). This novel virus called Kaposi's Sarcoma-associated

herpesvirus (KSHV) or Human herpesvirus 8 (HHV8) has a 140.5 kb unique coding

region flanked on either side by large G-C rich terminal repeats (64). It is highly

homologous to Rhesus Rhadinovirus (RRV) and Herpesvirus Saimiri (HVS), which

causes lymphomas in non-host monkeys, and other members of the gammaherpesvirus

family including Epstein-Barr virus, which causes infectious mononucleosis and is linked

to Burkitts lymphoma. Initial sequence identified 81 potential ORFs, 15 of which were

unique to KSHV (67). In addition to the genes common to all herpesviruses, KSHV

encodes genes that are homologs of cellular genes involved in cellular proliferation,

including vGCR (ORF 74)(2, 4, 14), v-bcl-2(ORF 16) (77), v-IL6 (ORF K2)(54, 55) , v-

cyclin (ORF 72)(31, 56) and v-IRF (ORF K9), a homolog of interferon regulatory factors

(26, 44, 54). The exact contribution of these genes to pathogenesis is currently under

investigation, and the role of vGCR will be explored in Chapter 2.

Epidemiology and disease association of KSHV

Discovery of KSHV led scientists to investigate whether it was indeed the agent

responsible for KS, or simply a passenger virus whose replication is favored in spindle

cells. Several pieces of epidemiological evidence suggest that KSHV infection is a

necessary factor for development of disease. First, KSHV DNA is detected in nearly 100

percent of the spindle cells (the cells that are likely transformed in lesions) in all clinical

forms of KS (8, 85, 86) reviewed in (78). KSHV is also found in tumor infiltrating

monocytes (6) and circulating B cells (1), (89). Second, infection with KSHV precedes

onset of disease and confers increased risk for KS development: in the context of

untreated HIV infection, infection with KSHV results in a 50% incidence of KS in ten

years (27, 40, 50, 54, 89). The KSHV genome is also present in two other diseases:

body-cavity lymphoma (13), or primary effusion lymphoma (PEL), and multicentric

Castleman's disease (84) where it also appears to be involved in pathogenesis.

BCBL-1 cells as model system

To date, no cell line can be infected de novo with KSHV to a reliable degree, making

studies of viral biology dependent upon clinically isolated infected cell lines such as the

BCBL-1 (body cavity based lymphoma) cells, derived from a PEL which is latently

infected with KSHV (65). Lytic replication is activated and a program of gene expression

is initiated upon stimulation with the phorbol ester, 12-O-tetradecanoyl phorbol-13-

acetate (TPA). 48 hours post-stimulation, mature, enveloped virions are detected in the

cytoplasm by TEM. Viral DNA increases 15 fold and transcription across the genome is

observed. In addition, a small percentage (3-5%) of cells in a BCBL-1 population

spontaneously undergo lytic reactivation, similar to what is observed in the spindle cells

of a KS lesion (85).

Biology of KSHV lytic replication

As in all herpesviruses, KSHV replication involves a temporally regulated pattern of gene expression. The three classes of herpesviral gene expression are experimentally defined by their ability to be expressed in the presence of various chemical inhibitors (36). The first class are the immediate early (IE) genes, whose transcripts are expressed following infection or reactivation of the virus in the presence of inhibitors of *de novo* protein synthesis. IE genes most often encode regulators of gene expression, for example the transcriptional activators ICP 4, and ICP 0 of Herpes Simplex virus, and Z and R of Epstein-Barr virus. Examination of KSHV lytic gene expression kinetics in BCBL-1 cells revealed several transcripts that are expressed within 6 hours of TPA induction; ORF 50, ORF 57, K-bZIP, K3 and K5 (47, 87, 96). More recently, a study using DNA array technology to examine expression of KSHV ORFs following TPA-induced lytic replication confirmed the earlier findings that ORF 57, K-bZIP, K5, v-GPCR as well as nut-1 RNA are the earliest genes to be transcribed, between 0 and 10 hours post-induction (39). Use of the chemical inhibitors of protein synthesis, such as cycloheximide, is difficult in most PEL cell lines due to high levels of toxicity, and therefore, our lab has been unable to define KSHV IE genes in that manner. Their early expression kinetics, together with their homology to essential herpesviral regulators of

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gene expression, suggest that ORF 50 and ORF 57 may play crucial roles in the regulation of KSHV gene expression.

Delayed early (DE) genes are transcribed following IE gene transcription, and their expression is regulated by IE gene products. This class of genes is experimentally defined as those whose transcripts are cycloheximide sensitive, while unaffected by inhibitors of viral DNA polymerase. Many KSHV genes are homologs of HVS and EBV DE genes involved in DNA replication (67). These include ORF 59/58, encoding DNA polymerase processivity factor, vGCR, and nut-1 RNA .

The third classes, late genes, are only expressed after DNA synthesis occurs, and these genes mostly encode virion structural proteins, such as MCP. These messages are sensitive to both cycloheximide and to inhibitors of the viral DNA polymerase.

Role of KSHV lytic replication in pathogenesis

The majority of KSHV in spindle cells of KS and B-cells of PEL persists in a latent form, similar to the behavior of EBV and other tumor viruses. This latent state is persistent with limited gene expression, and maintenance of the viral episome. The latent state can be switched to lytic replication following treatment with chemical inducers including

phorbol esters, or Na-butyrate(65). Once productive lytic replication ensues, virions are released and the cells lyse. However, there is growing evidence for a prominent role for lytic KSHV replication in KS pathogenesis. First, a recent study demonstrated that drugs such as ganciclovir, which inhibit viral DNA replication, can reduce risk of KS development(49). Second, lytic reactivation of the virus would be required for the spread of KSHV from its presumed lymphoid reservoir to target endothelial cells. This correlates well with evidence that latent KSHV infection peripheral blood is established long before the onset of KS (50, 54). Third, there is a small but consistent population of cells both in KS lesions and PEL cultures that support lytic growth (65, 85). Finally, many of the genes that have proinflammatory or pro-angiogenic properties are expressed only in the lytic phase; including v-GCR, vMIPS, K1, the anti-apoptotic BCL-2 homolog, and v-IL6. Since lytically infected cells are not expected to survive, it is not fully understood how these lytic genes contribute to transformation. One possibility is that KSHV gene products involved in signaling might affect the proliferation of other cells in the tumor by indirect mechanisms. Because of the evidence cited above, it is important to understand how the lytic cycle is regulated, in order to more fully determine the mechanisms by which KSHV contributes to pathogenesis

Regulation of the lytic cycle in other herpesviruses

The switch from latency to lytic replication, and the control of the ensuing gene

expression is best understood in the alphaherpesvirus HSV. Therefore, this system has

served as the paradigm for our study of KSHV replication. HSV encodes ICP0, and ICP

4, transcriptional activators which regulate expression of IE genes as well as DE genes.

ICP 4 is an essential gene that activates both early and late genes (66). The essential post-

transcriptional regulator ICP 27, which will be discussed in detail below, is also required

for late gene expression, as well as host-cell shut-off, and is the only HSV regulator that

has homologs in alpha, beta and gamma herpesviruses (66).

In EBV, the transition from latency to lytic replication in B cells infected with EBV is

dependent upon the BZLF-1 (Z) gene product, a viral bZip family transactivator (17, 24).

R, a transcriptional activator, and M, a post-transcriptional regulator are also important

regulators of the lytic cycle (43). Furthermore, Z and R activate transcription from the

M promoter (12, 41). The EBV protein R can upregulate target DE genes with Z and can

serve as a switch from latency to lytic replication in non-lymphoid cells and in B-cells

(63). (43, 94). The M protein is a post-transcriptional activator (42) and will be

discussed in detail below.

Regulation of KSHV Lytic cycle

Our analysis of KSHV lytic gene regulation focused on ORF 50 and ORF 57, genes

whose homologs in other herpesviruses are essential for replication. ORF 50 is a homolog

of the R protein of EBV, and ORF 57 is a homolog of ICP 27 of HSV and M of EBV.

Our lab has shown that ORF 50 is a potent transactivator of viral transcription; it can

robustly activate the promoters for ORF 57, and nut-1 among others (47). Importantly,

ORF 50 is also the crucial 'switch' protein, like Z and R of EBV, that can reactivate

KSHV from latency in BCBL-1 cells (47, 88). At its N-terminus is a basic domain ,

followed by a leucine zipper, and an acidic C-terminal domain with homology to

transcriptional activation domains (48). Recently, it was demonstrated that the amino

terminal portion of ORF 50 can bind directly to the ORF 57 promoter through a specific

element and this is required for activation (D.Lukac, personal communication). ORF 57

can enhance ORF 50's activation of specific promoters as well as upregulating the

accumulation of viral RNAs; its characterization is the major focus of this thesis.

Post-transcriptional regulation by viruses

Control of gene expression after transcription occurs at many levels including: mRNA

splicing, mRNA export , stability, translation and post-translational events such as protein

stability. Post-transcriptional regulation of gene expression by viral genes is a common

feature of both DNA and RNA viruses. The most well studied of these is the Rev

protein, a viral post-transcriptional regulator, of the retrovirus HIV. Studies of Rev have

provided a paradigm of viral post-transcriptional gene regulation. HIV is a small virus of

about 9 kb, which encodes nine genes transcribed by many different splicing events. Rev

is a shuttling protein that selectively binds and exports *unspliced* RNAs from the nucleus

to the cytoplasm, thus ensuring their translation (37). An arginine–rich domain at Rev's

N-terminus functions in trans to bind a sequence called the Rev response element (RRE)

in target viral RNAs (38). Another RNA virus, Influenza, encodes two proteins that affect

viral RNA; NS1 and NS2. NS1 contributes to host-cell shutoff of gene expression by

inhibiting nuclear export of spliced polyadenylated mRNA (16). Influenza RNA

however, is exported through a complex processing event that allows it to be

distinguished from cellular mRNAs (16). The NS 2 protein mediates the nuclear export of

viral RNA by acting as an adapter between viral RNPs and host nuclear export machinery

(58).

Adenovirus also encodes two proteins, E1B and E4, that work together to promote the

export of viral mRNA and inhibit the export of cellular mRNA (19). The usage of post-

transcriptional regulation by virally encoded genes is found in Herpesviruses from each

subclass. Alpha (HSV), beta (Human cytomegalovirus -HCMV) and gamma (KSHV,

EBV, HVS) all encode post-transcriptional regulators that are positionally conserved in a

gene block that codes for proteins involved in DNA replication. The similarities and

differences between each are discussed below.

Herpesviral homologs of ORF 57

HSV ICP 27

Functions of ICP 27

ICP 27 is a 63kD nuclear phospho-protein that is essential for replication (69).

Temperature sensitive mutants of ICP 27 have revealed that it is required for efficient

expression of many DE and Late genes. In addition, ICP 27 contributes to host cell shut-

off and down-regulation of viral IE gene expression (35, 82).

This pleiotropic protein functions by several different mechanisms. ICP 27 contributes to

host-cell shut-off by downregulating the expression of intron-containing genes. Because

most HSV transcripts are intronless, and since most cellular mRNA is spliced, this

provides a selective advantage for viral gene expression (34, 35, 62, 75). Interestingly,

the few spliced transcripts in HSV are predominantly from IE genes; therefore HSV has

evolved a mechanism by which it can downregulate expression of IE and DE genes at

late times after infection in order to tightly control the kinetic cascade of viral lytic gene

expression. Although ICP 27 colocalizes with and redistributes small nuclear

ribonucleoproteins in HSV infected cell nuclei, that may not account for the host-cell

splicing shutoff (61, 73, 74). ICP 27 is a shuttling protein that can bind and export

intronless late viral mRNA from the nucleus to the cytoplasm. ICP 27 binds RNA

through an N-terminal RGG domain and uses a leucine-rich nuclear export signal (71, 72, 76, 83).

ICP 27 employs a different mechanism to control expression of late genes; it induces a cellular factor, CstF(cleavage stimulation factor) that stimulates use of a subclass of inefficient viral polyadenylation signals, ensuring that many late genes are well expressed (51, 52). ICP 27 interacts with a cellular splicing factor, p32 as well as hnRNP K and casein kinase (10). Furthermore, ICP 27 expression reduces virus-induced apoptosis, although the exact mechanism for this function is unknown (3)

ICP 27 interactions with viral proteins

Overexpression of ICP 27 can alter the localization of both ICP 4 and ICP 0 (97) as well as possibly affecting the ability of ICP 4 to bind DNA by altering its post-translational modifications (60). Although alone ICP 27 does not activate transcription from promoters, it can further enhance or repress the transactivation abilities of ICP4 and ICP 0 (79). ICP 27 also interacts with itself in vivo through a C-terminal zinc-finger like region (95).

EBV BMLF-1

The EBV homolog of ORF 57, variously called BMLF-1, M and EB2, is a phosphoprotein of about 60kDa. Similar to ICP27, M can regulate gene expression in a post-transcriptional manner, however, the exact mechanism(s) by which this occurs are still under investigation. Early studies reported that M activates intronless genes, but inhibits expression of intron containing genes (18, 42, 45, 68). Like HSV, the majority of EBV genes are intronless and the spliced genes in EBV are mainly immediate early genes. Recently, it was demonstrated that M can increase cytoplasmic accumulation of EBV replication gene mRNA regardless of the presence of an intron in the target vector (80). The arginine rich N-terminal half of M can bind to RNA, and can shuttle from the nucleus to the cytoplasm (11, 80). In addition, the C-terminal region, but not the arginine-proline rich region at the N-terminus, seems to be required for M's effects on both splicing and transport (11). Another recent study focused on the general effects of M on mRNA processing; Buisson et al (11) showed that M inhibits the accumulation of polyadenylated RNA that is generated by weak (cryptic) splice sites and has no effect on 3' processing of RNAs. Furthermore, M can also induce the accumulation of unspliced RNA. Finally, M has been reported to mediate RNA export through both Crm-1 dependent and independent pathways (9, 23).

HVS ORF 57

Herpesvirus Saimiri shares more overall homology to KSHV than either EBV or HSV,

and contains homologs of ORF 57 and ORF 50, also called ORF 57 and ORF 50. HVS

ORF 50 can activate transcription of promoters, including the HVS ORF 57 promoter

(90). HVS ORF 57 is a 52 kDa protein and similar to ICP 27 and EBV BMLF-1, is a

multifunctional post-transcriptional regulator. HVS ORF 57 can modestly decrease

expression from reporter constructs containing introns as well as repress the

transactivating capabilities of the spliced version of HVS ORF 50 (91). Recently, it was

demonstrated that HVS ORF 57 can shuttle from the nucleus to the cytoplasm, bind viral

gB RNA and increase cytoplasmic levels of gB and capsid mRNAs (33). The C-terminus

of HVS ORF 57, which contains a conserved putative zinc finger motif, is required for

both its transactivation and repression properties (32).

CMV UL 69: In addition to the IE1 and IE2 gene products of HCMV, the key regulatory

proteins that control viral gene expression, HMCV encodes the early/late protein called

pUL69 which has regulatory capabilities (93). Although it is a homolog of ICP27, and is

positionally conserved, it cannot complement ICP 27 HSV mutants (93). Instead, it

appears to activate some viral promoters and may antagonize chromatin remodeling by

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binding to a chromatin remodeling protein (92). Unlike ICP27, pUL69 does not

negatively regulate intron-containing genes (93).

Overview of the Thesis

The work in this thesis was conducted to understand mechanisms regulating lytic gene expression in KSHV; we first investigated the expression characteristics of a gene, v-GCR, whose product likely plays an important role in pathogenesis. We then investigated in detail the characteristics and functions of ORF 57, a multi-functional post-transcriptional regulator whose product controls the expression of other lytic genes.

Chapter 2 describes our analysis of the structure and expression of the mRNA encoding the KSHV G-protein coupled receptor (ORF 74), an important signaling molecule that contributes to angiogenesis, a hallmark of KS tumors. We found that GCR is expressed as a bicistronic transcript with K14, an OX-2 homolog, and is a lytic gene, which impacts investigation of its role in pathogenesis. This was originally published in the Journal of Virology as follows: *Expression of the Open Reading Frame 74 (G-protein-coupled receptor) gene of KSHV: implications for pathogenesis.* Jessica R. Kirshner, Katherine Staskus, Ashley Haase, Michael Lagunoff, and Don Ganem.

J. Virol. Vol. 73. July 1999, p.6006-6014.

Chapter 3 is an examination of ORF 57 including basic characterization of its mRNA and cellular localization and a study of its functions. We find that ORF 57 increases the accumulation of two viral RNAs: DNA polymerase processivity factor (PF) and nut-1, as well as augmenting the activity of ORF 50 on the nut-1 promoter. This was originally published in the Journal of Virology as follows: *Kaposi's Sarcoma-Associated Herpesvirus Open Reading Frame 57 encodes a posttranscriptional regulator with multiple distinct activities.* Jessica R. Kirshner, David M. Lukac, Jean Chang, and Don Ganem. J. Virol. Vol.74. April 2000, p. 3586-3597.

Chapter 4 documents our further studies into the mechanism and domain structure of ORF 57. The putative zinc-finger region at the C-terminus is shown to be required for ORF 57's augmentation of ORF 50's activation of the nut-1 promoter. An arginine rich region at the N-terminus also plays an essential role in synergy between ORF 50 and ORF 57. Finally, we identify two distinct arginine rich nuclear localization signals in the N-terminal portion of ORF 57.

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Chapter 2

The expression of the ORF 74 (G protein coupled receptor) gene of Kaposi's sarcoma (KS) -associated herpesvirus: implications for KS pathogenesis.

INTRODUCTION

Kaposi's Sarcoma (KS), an endothelial tumor with neoangiogenic and inflammatory components, is the most common neoplasm of AIDS patients. The epidemiology of KS strongly implicates a sexually -transmitted cofactor other than HIV in its pathogenesis (3). In recent years, a novel human herpesvirus, Kaposi's sarcoma-associated herpesvirus (KSHV; also called human herpesvirus 8, HHV-8) has emerged as the leading candidate for this cofactor (8) (reviewed in (13, 40)). KSHV DNA is found in all KS tumors, localized to the endothelial (spindle) cells of the lesion (5, 42). Infection is found at high rates in groups at high KS risk, and much lower rates in the general population; in individual subjects, infection precedes KS development and is strongly correlated with increased KS risk (15, 16, 22, 27, 46). In the majority of KS spindle cells and PEL cells, the KSHV genome exists in a latent state, with only a small subpopulation of cells in either tumor displaying lytic viral replication (29, 36, 37, 42, 45). Genes known to be expressed in latency (9, 10) include those encoding (i) LANA, the latency-associated nuclear antigen (22, 23, 35) (ii) v-cyclin, a homolog of cellular D-type cyclins (18, 25, 43) (iii) v-FLIP, a protein whose homologs impair caspase activation and programmed cell(31, 32, 44); and (iv) products of the K12 locus (48), (30), (39) whose exact biochemical activities are unknown but which may influence cell proliferation in vitro.

In addition, KSHV harbors numerous additional genes that bear homology to cellular functions involved in growth control, signal transduction and other regulatory processes (4, 14, 32-34, 38). One of these, ORF 74, encodes a protein with homology to known G-protein coupled receptors (GCRs), in particular several human chemokine receptors and an IL8-receptor homolog (7). The KSHV GCR has been demonstrated to be a constitutively active receptor that signals through the phosphoinositide-inositoltrisphosphate-protein kinase C pathway and binds several C-C and CXC chemokines (1, 17). Mouse fibroblasts stably transfected with the KSHV GCR gene form foci, and these foci are tumorigenic in nude mice (2). Furthermore, expression of the KSHV GCR in such fibroblasts has been shown to induce secretion of vascular endothelial growth factor (VEGF), a known mediator of angiogenesis (2). Based on these findings, it has been proposed that the KSHV GCR may be a key mediator of both the proliferative and angiogenic components of KS. In fact, recent studies (after publication of this work) revealed that a mouse transgenic for KSHV GCR in hematopoietic cells developed angioprolferative lesions that resemble KS (47).

Here we examine the structure and expression of the mRNAs encoding ORF 74, which is located immediately rightward of a previously described cluster of latently expressed genes (Fig 1A)(10). We show that in a PEL cell line, BCBL-1, ORF 74 is expressed as an early lytic gene. In keeping with this, in situ hybridization of BCBL-1 cells and KS

tumors reveals that ORF 74 is transcribed predominantly in the subpopulation of cells

that express lytic cycle genes. A detailed analysis of the fine structure of this transcript

reveals that ORF 74 is the downstream gene of a bicistronic message whose 5' gene

encodes ORF K14, a homolog of the cellular OX-2 protein (38). The implications of

these findings for the potential role(s) of GCR in KS are discussed.

MATERIALS AND METHODS

Cell lines, plasmids and probes.

BCBL-1 cells, a B-cell lymphoma line latently infected with KSHV (24, 37) were

cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2mM penicillin,

streptomycin and L-glutamine, 1mM sodium bicarbonate and 0.05 mM 2-

mercaptoethanol at 37° 5% CO_2. BJAB cells are an EBV negative (26) and KSHV

negative B-cell lymphoma line and are cultured in the same media and conditions as

BCBL-1 cells.

The KSHV GCR gene was subcloned from phage lambda 4, a genomic KSHV clone

derived from a pulmonary KS tumor (48). A 1kb Bam HI fragment from clone lambda 4

(nt 129211-130212) nucleotide positions are based on the sequence of Russo et al. (38)

was subcloned into the BamHI site of pBS SKII + (Stratagene) to create the plasmid

pML14. pML14 was linearized with Eco RI within the polylinker and transcribed with

T3 RNA polymerase and ^{32}P-UTP to produce an antisense riboprobe for ORF 74. For a

double-stranded DNA probe for ORF 74, the 1 Kb BamHI fragment from pML14 was gel

purified and labeled with DNA polymerase using a random priming procedure

(Rediprime: Amersham). ORF K14-specific probe was made by cutting clone lambda 4

with Sma 1 (at nt. 127909) and Nde 1 (nt. 128320) to release a ~ 400 bp fragment;

following gel purification, this fragment was labeled by random priming as above.

Probe for the K14/ORF 74 intergenic region was created by cutting a cDNA clone that contained the 5' untranslated region of ORF 74 with AvrII (within KSHV sequences) and EcoRI (within the plasmid backbone) to release a fragment containing KSHV nt. 128948 -129214; following gel purification, the fragment was labeled by random priming as above. To create a GCR expression vector for the 3-(4,5-dimethylthiazol-2-yl)2,5- diphenyltetrazolium bromide (MTT) assay, the AvrII (nt 129125)-SacII (nt 130405) fragment from lambda 4 containing the entire GCR ORF and 156 bp upstream of the ATG was cloned into the XbaI-SacII sites of pBluescript (Stratagene) to create pML 16. The insert of pML16 was subsequently cloned into an expression plasmid under the control of the SRα promoter (composed of the simian virus 40 [SV40] early promoter and part of the human T-lymphotropic virus type 1 [HTLV-1] long terminal repeat) to create plasmid pML17.

RNA preparation and Northern blotting.

BCBL-1 cells were diluted to 2.5×10^5 cells/ml 12 hours prior to treatment with PFA (phosphonoformic acid) (0.7mM) (21, 28) or mock treatment; 12 hours later, half were treated with TPA (12-*O* -tetradecanoyl phorbol-13-acetate) (20 ng/ml) and half were mock treated for 12 hours. At that point, cells were washed and then maintained at a

constant PFA level for another 48 hours, at which time RNA was isolated using RNAzol

B (Tel-Test, Inc., Friendswood, TX) as recommended by the manufacturer. 50 ug of

each RNA sample was used for poly (A) enrichment using the Oligotex mRNA

purification system (Qiagen) as recommended by the manufacturer. 1.5 ug of

polyadenylated RNA that resulted from this purification was separated on a 1% agarose,

17% formaldehyde gel and transferred to Hybond N membrane for twelve hours in 10x

SSC. The blot was then UV cross-linked and hybridized with the ORF 74 riboprobe in

hybridization buffer (0.5 M NaCl, 5% SDS, 6% PEG 8000, 250 ug/ml salmon sperm

DNA, 50 % Formamide) at 72. After overnight hybridization, the blot was rinsed then

washed with 2X SSC / 0.1 % SDS for 15 min, with 0.5X SSC/ 0.1 % SDS for 15 min,

and finally with 0.1X SCC/ 0.1 % SDS for 15 minutes. Subsequently, the blot was

exposed to Kodak XAR5 film for 72 hrs.

cDNA cloning.

The cDNA clones containing the ORF 74 open reading frame were isolated from a poly

(dT) primed library made by R. Renne (unpublished) from induced BCBL-1 cells in

Lambda Zap (Stratagene). Screening was performed with the genomic ORF 74 Bam

fragment described above.

Ribonuclease Protection Assay.

For ORF 74, plasmids to make riboprobes were constructed using PCR to amplify

fragments of genomic DNA from the lambda 4 subclone. For probe G1, the following

primers were created: primer KG1: 5'gcggtgcatcacctacttcag,

primer GK2: 5' ctcacacacgctcscttctaggc. For probe G2, the primers used were GCR2: 5'

ccccttctgattctgacagacaac and primer GK2 above. The PCR products were cloned into

pCR2.1 (Invitrogen). The plasmids were linearized with the HindIII site from the

polylinker, and transcribed with T7 RNA polymerase in the presence of radiolabeled ^{32}P

UTP. The riboprobes were purified on a 4 % denaturing acrylamide gel, and eluted from

the gel for 12 hours at 37° in elution buffer (2.5M NaOAc, 0.5 mM EDTA, 0.5 % SDS).

The probes were precipitated with ethanol, and resuspended in 10 ul of TE and specific

activity was determined. The RNase protection was performed essentially as described in

Current Protocols in Molecular Biology (eds. Ausbel et al.) Section 4.7.1). In brief,

$5x10^5$ cpm of the probes were added to 10 ug of total RNA that had been precipitated and

resuspended in hybridization buffer. Hybridization was carried out overnight at 37° then

300 ul of RNase solution (10mM Tris pH 7.6, 5mM EDTA, 300 mM NaCl, 40 ug/ml

RNase A, 0.4 Units/ul RNase T1) was added for 1 hour at 60°. The reactions were

stopped with SDS and Proteinase K addition, and purified by phenol/chloroform

extraction and ethanol precipitation. The samples were separated on an 5% denaturing

acrylamide gel and exposed to film for 72 hours.

5' RACE.

5' rapid amplification of cDNA ends (5' RACE) was performed on 2 ug of 24 hour TPA-

treated BCBL-1 total RNA. Primer KR5 (5' ctgcaaagcagacacgccttctt) was used for

cDNA synthesis. Primer K5-2 (5' gatataactccgccctccactacg) and primer 7306 (5'

cgcggcgcccgggacaatc) were used for subsequent PCR steps according to the

manufacturers instructions (Boehringer Mannheim Inc.). RACE products generated after

two rounds of PCR were cloned into pCR2.1 (Invitrogen) and sequenced.

Primer Extension.

Primer extension was performed as in described in

Zhong et al (48). 10 ug of total RNA from BCBL-1 cells that were either uninduced or

induced for 24 hours with TPA were used with the primer K5-1 (5'

ggcaccaatcagaaagtagcttg). 10 ug of yeast RNA was also used as a control. The reactions

were loaded on a 6% denaturing acrylamide gel and then exposed to Kodak Biomax MS

film for 5 days.

S1 nuclease assay.

S1 nuclease mapping was performed using the S1 mapping kit from Ambion (Austin,TX) according to the manufacturers instructions. 10 ug of total RNA from BCBL-1 cells or BJAB cells treated with TPA for 24 hours was used with the end-labeled primer SK3 (5' ggaggcagctgcgccacgaagcagtcacgtccccaagagcagcagcttggtccatt). Hybridization (12 hr) and S1 nuclease digestion (30 min) was performed at 37°. After the reactions were complete, the samples were separated on a 6% denaturing acrylamide gel, and exposed to Kodak Biomax MS film for 5 days.

RT-PCR.

Total RNA (0.5 ug) from TPA treated BCBL-1 cells was first treated with RQ1 DNase (Promega) as recommended by the manufacturer. The RNA was then reverse transcribed with 10 pmol of primer GCRcR: (5' gagtttcattccaggattcatcatc) and 200 U of MMLV reverse transcriptase (Gibco BRL) for 35 minutes at 42° as in Dittmer et al. (10). The cDNA was then purified and resuspended in 100 ul using the High Pure PCR purification kit (Boehringer Mannheim) according to the manufacturers instructions. 10 ul of this cDNA was used in a PCR reaction with the following primers K9 (5' gcagctgcctccaaatgatacacac) and GCR1 (5' gaagatggttaggaaatcctcggc) and 1 U of Taq polymerase and buffer (Perkin Elmer). The amplification conditions were 30 cycles of 1

min at 94°, 1 min at 65° and 1.5 min at 72° followed by 10 min at 72°. The amplification

products were cloned into pCR2.1 (Invitrogen) and sequenced.

In Situ Hybridization.

Riboprobes were generated by run-off transcription of linearized plasmid DNA

containing ORF 74 (pML14), K12 (T0.7), or nut-1 (48) inserts. Radiolabeled (to a

specific activity of ~1x10^9 dpm/ug) and non-isotopic probes were synthesized with ^{35}S-

and digoxigenin-labeled UTP, respectively. In situ hybridization protocols described in

detail by Haase (20)were used for both tissue specimens and cultured cells. Briefly, 6_m

sections of a formalin-fixed paraffin-embedded dermal KS lesion from an HIV-infected

individual were deparaffinized and pretreated in a series of solutions containing 0.2N

HCl, 0.15M triethanolamine, pH 7.4, 0.005% digitonin, and 5 _g/ml proteinase K.

Slides with sections of BCBL-1 cells were pretreated with 0.2 N HCl, 2X SSC at 70° C,

and 1 _g/ml proteinase K. The slides were then acetylated and hybridized for 16-18 h at

45° C with a solution containing 10% dextran sulfate, 50% deionized formamide, 20mM

Hepes, pH 7.4, 1mM EDTA, 1X Denhardt's medium, 1mg/ml polyA, 0.6 M NaCl,

100mM dithiothreitol, 250 ug/ml yeast RNA and 1x10^5 cpm/ul ^{35}S-labeled riboprobe or

0.15 ng/ul digoxigenin-labeled riboprobe. The slides were then treated with RNase and

washed under conditions of increasing stringency, dehydrated, coated with photographic

emulsion and exposed at 4° C (42). For double-label, slides were hybridized with a mixture of radiolabeled and non-isotopic probes. Following the post-hybridization wash, digoxigenin was detected with alkaline phosphatase-conjugated antibody and NBT/BCIP substrate (Boehringer Mannheim). The slides were then coated with photographic emulsion and exposed at 4° C to develop the radiolabel signal. The slides were stained with standard hematoxylin and eosin protocols or, in the case of the double-label, briefly with hematoxylin alone.

MTT assay.

Subconfluent Cos7 cells were transfected with a GCR expression plasmid (pML17) or the empty vector, using Lipofectamine (Gibco BRL)according to the manufacturers instructions. At 48 h posttransfection, cells were trypsinised and resuspended in medium. MTT(0.3 mg/ml) was added with occassional mixing for 2.5 h. Cells were pelleted and lysed in 10% Triton-X-100-0.1 N HCl in isopropanol. The A_{570} and A_{690} were determined spectrophotometrically. The assay was performed on two separate transfections and the results are displayed as the means and standard deviations of these measurements.

RESULTS

Size and expression of ORF 74 mRNA.

Because of the proposed role(s) of the ORF 74 gene product in the pathogenesis of KS (1,

2) , we sought to determine the structure and temporal class of its mRNA. To

characterize the expression of this mRNA, we examined polyadenylated RNA from

BCBL-1 cells by Northern blotting with a probe specific for ORF 74. BCBL-1 is a B cell

line derived from an AIDS-related body-cavity based lymphoma that is latently infected

with KSHV (24, 37) it can be induced to undergo lytic replication upon addition of

phorbol esters, such as 12-*o*-tetradecanoylphorbol-13-acetate (TPA). The drug

phosphonoformic acid (PFA) is known to selectively inhibit viral DNA synthesis, leading

to suppression of late gene transcription while immediate early and early genes remain

unaffected (21, 28). BCBL-1 cells were treated with TPA alone, PFA alone, TPA plus

PFA, or mock treated, and RNA was isolated from these cells 48 hours later. 1.5 ug of

RNA poly A purified from each of the samples above were separated on a 1.2% agarose,

17% formaldehyde gel, and transferred to a Hybond-N membrane. An antisense

riboprobe for the coding region of ORF 74 was used to detect the ORF 74-containing

mRNA. Fig 2 A shows a major mRNA species of approximately 2.8 kb hybridizing to

the ORF 74 probe, as well as a much less abundant larger species of about 9 kb. The 2.8

kb mRNA is strongly upregulated by TPA (lane 3), suggesting that it is a lytic gene, and

is unaffected by treatment with PFA (lane 4), indicating that it is not a late gene, but

rather an immediate-early or early gene. The small amount of mRNA that hybridizes to

ORF 74 in the absence of TPA (lane 1) comes from the 1-3 % of cells in the BCBL-1

culture in which KSHV is spontaneously reactivated to lytic replication. The larger 9 kb

mRNA is temporally co-regulated with the major 2.8 kb species. We speculate that it

may be derived by readthrough of the 2.8 kb RNA past its polyA site into 3' sequences,

since the polyA signal of the 2.8 kb species is a variant (AUUAAA) from the consensus

AAUAAA (see below) that is known to result in a modestly reduced efficiency of

polyadenylation (41). However, we have not characterized this RNA in detail, and other

models for its structure are not excluded.

Since the ORF 74 coding region is only 1.0 kb, the 2.8 kb transcript was

presumed to contain sequences for an adjacent gene. The best candidate for this gene is

ORF K14, which encodes an OX-2 homolog, as it is the only adjacent ORF whose coding

region has the same polarity as ORF 74 (see Fig. 1A). To determine whether K14 is in

fact part of the 2.8 kb mRNA, 5 ug of total RNA from TPA and PFA-treated BCBL-1

cells was examined by Northern blotting using probes for K14 and for the K14/ORF 74

intergenic region. Figure 2B shows that the same 2.8 kb transcript anneals with probes

for the K14 gene (lane 1), the intergenic region (lane 2), and the ORF 74 coding region

(lane 3). The low abundance 9 kb band is not seen here, presumably owing to the lower

amount of RNA used in this experiment and the lower specific activity of the probes

employed. These results suggest that K14 and ORF 74 mRNAs are contained in a single

2.8 kb lytic transcript that is expressed early in viral replication.

In situ hybridization confirms that ORF 74 is a lytic cycle gene

The above population analyses were performed using RNA extracted from the PEL cell

line BCBL-1. To determine the profile of ORF 74 transcription at the single cell level we

performed in situ hybridization with antisense riboprobes. We found that in uninduced

BCBL-1 cultures only a few percent of the cells contained detectable levels of ORF 74

RNAs (Fig. 3E). These cells, which co-label with probes for lytic genes (not shown), are

the source of the low levels of ORF 74 mRNAs seen in the Northern analysis of

comparable uninduced cultures (see Fig. 2). As expected, TPA treatment induces ORF

74 transcription consistent with lytic expression in these cells (Fig. 3F). To explore the

relevance of these findings for KS, we examined primary KS tumors. Figure 3A shows

that few cells in the tumor express ORF 74 mRNA, and these cell numbers are

comparable to those expressing the known lytic gene, nut-1 (Fig. 3B). In contrast,

hybridization to a subjacent section with probe for the viral K12 (T0.7) gene that is

transcribed in both lytically and latently infected cells reveals the large number of

infected cells within the lesion (Fig. 3C). Double labeling with radiolabeled ORF 74

probe and digoxigenin-labeled nut-1 (whose expression is confined to lytic infection)

probe confirmed that the ORF 74 transcripts are produced predominantly in those cells

which are in the lytic cycle (Fig. 3D). This result accords well with the BCBL-1 finding

and suggests that most of the spindle cells in KS tumors do not express ORF 74.

Fine structure of the 3' portion of K14/GCR RNA

To begin the study of the fine structure of the GCR transcript, we screened an oligo dT-

primed cDNA library prepared from TPA induced BCBL-1 cells, using a DNA probe

specific for ORF 74. Six clones that contained ORF 74 sequences were isolated and

sequenced; none was full length. The 2 longest clones (1.6 kb) were identical and

included the entirety of the GCR coding region, together with additional 5' and 3'

sequences. At the 3' end of the clones was a run of T residues corresponding to the

polyA tail; 29 nt 5' to this polyA tail was a likely polyA addition signal, AUUAAA, at nt

130517. At the 5' end of the two clones, just upstream of the initiator AUG codon for

GCR, comparison of the cDNA sequence with the genomic sequence revealed the

presence of a small intron of 149 nt, with a splice donor at 129219 and splice acceptor at

129367, (see Fig 1B). The splice donor and acceptor sites conform to consensus splice

sites found in higher eukaryotes. (Of the 4 remaining cDNA clones, one was similarly

polyadenylated but shorter, while the others bore evidence of 3' deletions most likely due to rearrangements during cloning).

To confirm the presence of this intron and to see if any unspliced RNAs were also generated from this region, an RT-PCR analysis was employed. Primers spanning the putative intron were used to amplify randomly primed cDNAs from both total and oligo dT-selected RNA from TPA treated BCBL-1 cells. Sequencing of the major RT-PCR product revealed that it was indeed spliced and confirmed the splice donor and acceptor sites determined by cDNA cloning (not shown). No products corresponding to unspliced RNA were detected.

Searching for monocistronic GCR mRNAs

Although the major RNA containing GCR sequences appears to be a K14/GCR bicistronic transcript, we were concerned about the possibility that a minor monocistronic GCR mRNA might initiate within the K14/ORF 74 intergenic region. To search for such an RNA, we performed RNase protection across this region using the antisense riboprobes (G1 and G2) depicted in Fig. 4B. Both were transcribed from *genomic* KSHV DNA and shared a common 5' end within GCR sequences, but terminated at different sites within ORF K14 or the intergenic region. Each probe was hybridized to total RNA from BCBL-1 cells that were either untreated, or treated with PFA and TPA.

After hybridization, the samples were treated with RNAse A/T1 for 1 hour at 37° and separated on a 6% denaturing acrylamide gel. As shown in Figure 4A (lanes 1 and 4), hybridization with both probes G1 and G2 resulted in protection of a common 120 nt fragment representing the sequence 3' to the intron. Each probe also generated a larger protected fragment (Fig 4A); as depicted in Fig. 4B, these correspond exactly to the sizes predicted from protection by RNA segments arising from the spliced RNA in the region 5' to the splice donor. No other protected species were detected in this analysis. Additional RNase mapping studies of this region, including studies using cDNA probes bearing the 149 nt splice (not shown) similarly yielded no evidence for any transcripts other than the spliced bicistronic RNA discussed above. In particular, we detected no start sites corresponding to the 5' end of the 1.6 kb ORF 74-containing cDNAs that emerged from our cDNA cloning analyses; these are therefore incomplete cDNAs derived by premature termination during cDNA synthesis.

The 5' end of K14/GCR mRNA

Given the location of the polyA addition site at nt. 130546 and the approximate size of the transcript (2.8 kb), we estimated that the 5' end of the RNA would lie just upstream of the K14 open reading frame. To search for the start site of the 2.8 kb transcript, we employed primer extension, 5' RACE, and S1 nuclease analysis. For the primer

extension analysis, we used a labeled oligo (K5-1) located 81 nt downstream of the ATG

of K14 (see Fig. 5B). Following annealing of this end-labeled primer to RNA from TPA-

induced BCBL-1 cells and extension with reverse transcriptase, a single product of 116

bp was detected (Fig. 5A, lane 6); as expected, the amount of this extension product was

greater with RNA from TPA-induced vs. uninduced cells (Fig. 5A, lanes 5 and 6). There

was no extension product in the control reaction with yeast RNA (Fig. 5A, lane 7). The

size of this product, determined by comparison to the sequencing ladder generated using

the same primer, indicates a potential start site at nt 127848. A confirmatory 5' RACE

analysis (see Materials and Methods for details) yielded 4 clones, two of which

terminated precisely at nt 127848, in excellent agreement with the primer extension (the

sequence of the remaining two clones indicated that they resulted from mispriming).

Since both primer extension and 5' RACE rely on the use of reverse transcriptase,

which can prematurely terminate at RNA secondary structures, we employed S1 nuclease

analysis to further confirm the mapping. This was done by using an oligonucleotide of

53 nt (probe SK3, Fig. 5B) that spanned the putative start site at nt. 127848. RNA from

TPA-induced BCBL-1 and BJAB (a KSHV-negative Burkitt's lymphoma cell line) was

hybridized to the 53 bp 5' end-labeled probe, and then treated with S1 nuclease. The

resulting 32+/- 1 nt protected fragment, seen only in the BCBL-1 sample (Fig. 5C),

confirms nt 127848 as the presumed start site and implies that the 5' noncoding region

upstream of ORF K14 is only ca. 35 nt. Transcripts initiating from this start site and

polyadenylated at nt. 130546 would result in a mRNA of 2698 bp, which accords well

with the estimated size of the transcript by Northern blotting. Importantly, this start site

is 30 bp downstream of a recognizable TATA box. By scanning the region 5' to this start

site we found several potential transcription factor binding sites, including a SP1 site at

127755, and an AP-1 site overlapping the TATA box (Fig. 5B). Interestingly, the latency

promoter directing ORF 73 (LANA) expression (10)lies in the body of the K14 open

reading frame on the opposite stand. This raises the possibility that lytic cycle K14/GCR

mRNAs could have the potential to serve as antisense RNA regulators of the latency

transcripts from the ORF 71-73 cluster, and tests of this hypothesis are in progress.

Searching for alternatively spliced GCR RNAs from the K14 promoter

 Another potential way to generate a monocistronic mRNA for ORF 74 would be by

alternative splicing from a putative splice donor within the K14 5' noncoding region to a

splice acceptor site 5' to ORF 74 (for instance, the known splice site at nt 129368, just 5'

to the GCR AUG). To search for such spliced RNAs, we employed a sensitive RT-PCR

assay. cDNA was prepared from TPA-induced BCBL-1 RNA using a primer in the body

of the GCR coding region. PCR amplification from this template was carried out using

an upstream primer from the K14 5'NC region (K9) and a downstream primer just 3' to

the GCR AUG (GCR1). As shown in Fig. 6, the only PCR products detected were of a

size expected from amplification of the full-length, bicistronic RNA species. No smaller

species reflecting the splicing out of K14 coding sequences was observed. Sequencing of

these products confirmed that they corresponded to the intact sequence between the

primers. The longer species corresponded to the genomic sequence and may have

resulted from the presence of contaminating DNA or nuclear pre-mRNA; the shorter

species is identical in sequence, except for the excision of the 149 nt intron previously

described.

Consequences of GCR overexpression

The positioning of GCR coding sequences at the 3' end of a bicistronic mRNA would

seem designed to ensure their inefficient expression. To examine what would occur if the

GCR gene was strongly expressed, we constructed an expression vector in which the

strong SRα promoter (composed of the simian virus 40 [SV40] early promoter and part

of the human T-lymphotropic virus type 1 [HTLV-1] long terminal repeat) was used to

drive transcription of a monocistronic GCR message. This plasmid was transfected into

Cos7 cells, which allow for additional amplification of expression based on SV40 DNA

replication. Cell viability was assayed by the MTT assay, which measures the ability of

viable cells to metabolize the compound MTT to formazan, which is detected

spectrophotometrically (see Materials and Methods). As shown in Fig.7, substantial cell

death was observed when GCR was overexpressed.

DISCUSSION

These studies demonstrate that the principal KSHV mRNA bearing GCR coding

sequences is a lytic transcript composed of the coding regions of both ORF K14 and ORF

74, with the K14 gene located in the 5' position. Extensive searches (by sensitive RNAse

mapping and RT-PCR approaches) for alternatively initiated or alternatively spliced

RNAs in which ORF 74 would be the sole (or at least the 5') coding region revealed no

evidence for such transcripts, though of course the existence of such RNAs at levels

below the detection thresholds of our methods cannot be formally excluded. The

evidence points instead to the conclusion that the 2.8 kb RNA is likely to be functionally

bicistronic, with the K14 gene translated by conventional initiation mechanisms and the

GCR gene translated by nonclassical strategies, e.g. modified ribosomal scanning,

translational re-initiation or internal ribosome entry. Although unusual, this arrangement

may not be without logic. Published data indicates that the KSHV GCR is a powerful

and constitutively active signalling molecule (1). Presumably even modest levels of

KSHV GCR would suffice to carry out its signalling functions, and overexpression of

such a potent activity might even be deleterious. Indeed, in our hands, overexpression of

the GCR in transiently transfected COS cells triggers cell death (Fig.7). Thus, relegation

of the GCR to the 3' position in the transcript, where its translation would be expected to

be inefficient, may be a strategy for ensuring levels of expression commensurate with cell survival.

The finding that KSHV GCR is a lytic cycle gene is consistent with observations in other herpesvirus infections. For example, CMV encodes several GCR homologs as late lytic genes (45). The limitation of KSHV GCR expression to the lytic cycle has implications for its potential role(s) in KS tumorigenesis. Since most lytically infected cells eventually die, it is unlikely that cells expressing detectable GCR transcripts in KS will go on to proliferate - consistent with the observation that most spindle cells in KS tumors are not expressing GCR mRNA. Of course, it is formally possible that abortively infected cell types might express some lytic cycle genes yet escape destruction; but even assuming that *every* spindle cell expressing GCR in a KS lesion is abortively infected, this would still represent only a tiny fraction of proliferating spindle cells in the tumor. Still another model for how GCR expression could be linked to proliferation would involve the induction and secretion of growth-inducing factors from GCR-expressing cells. However, proliferative effects from this mechanism would be expected to operate on uninfected as well as infected cells. As such, this model would fail to explain one of the central features of KS - that most proliferating spindle cells are latently infected with KSHV (5, 42). We therefore find it improbable that the viral GCR will be principally

responsible for spindle cell proliferation in KS tumors, despite its demonstrated ability to deregulate growth in 3T3 cells and its effects when expressed in transgenic mice (1, 47).

On the other hand, expression of GCR in lytically infected cells could have numerous and profound pathologic consequences for the two other components of a KS lesion: neoangiogenesis and inflammatory cell infiltration. Bais et al (2) have clearly shown that GCR signalling initiates a program of cellular gene expression that culminates in VEGF synthesis and release. Clearly, paracrine release of such potent angiogenic factors from even a small subpopulation of cells could trigger dramatic neovascularization, a hallmark of KS histology. It is also possible to imagine that paracrine mediators released under the influence of GCR signalling might have effects on inflammatory cell infiltration. It is more likely that GCR plays its role in KS pathogenesis by these paracrine mechanisms than by cell-autonomous effects of GCR expression, and the consensus among GCR researchers is similar (6).

Finally, regardless of any potential role in KS pathogenesis, lytic cycle GCR expression likely plays additional roles in the KSHV life cycle - a cycle which in normal immunocompetent hosts is largely played out in lymphoid rather than endothelial cells. The cellular chemokine receptor, BLR1 (Burkitt's lymphoma receptor 1) is known to regulate the migration of B cells both systemically and within lymphoid organ microenvironments (11, 12, 19). If the product of ORF 74 shares this function, then

perhaps its expression during lytic reactivation would provide a mechanism to home

virus-laden cells to lymphoid organs, where the released viral progeny could more readily

infect other susceptible B cells. Alternatively, GCR-activated signalling pathways may

result in the activation or induction of host and/or viral proteins that upregulate lytic

growth. Decisive tests of the GCR in viral growth and KS pathogenesis will likely

require the development of genetic systems for constructing and propagating mutations in

individual viral genes.

ACKNOWLEDGMENTS

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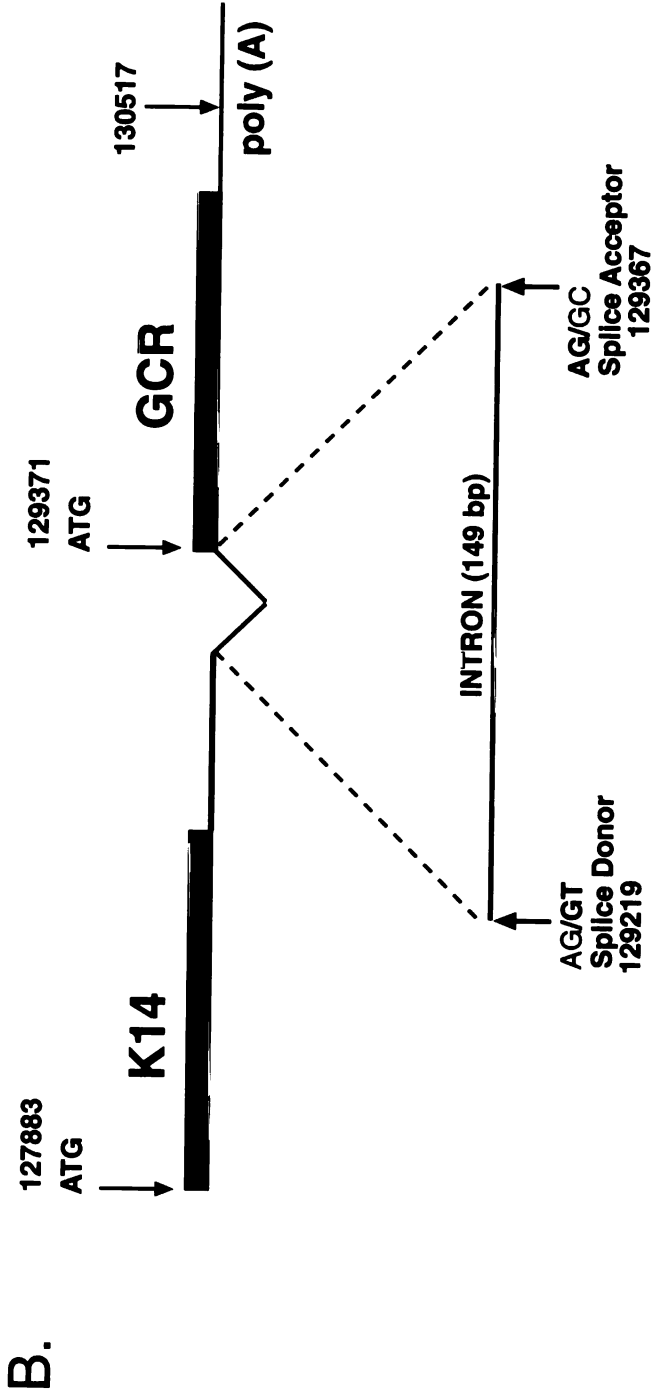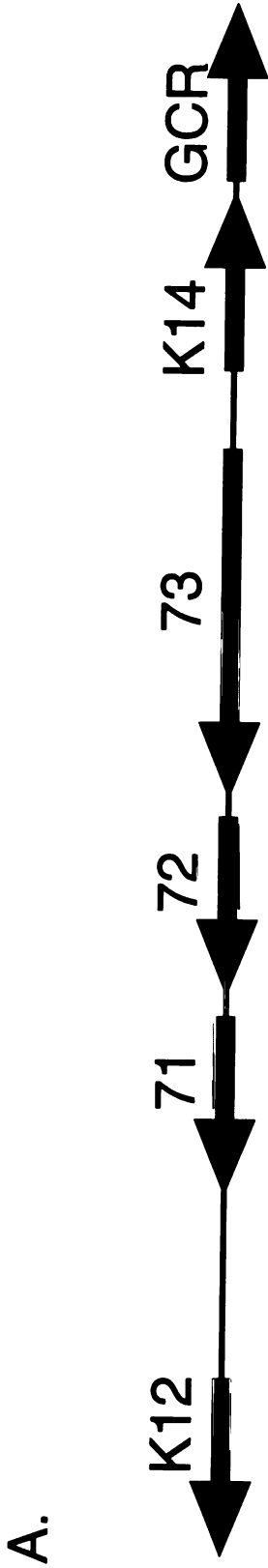
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Figure 1

A.

K12 71 72 73 K14 GCR

B.

127883
ATG

K14

AG/GT
Splice Donor
129219

INTRON (149 bp)

AG/GC
Splice Acceptor
129367

129371
ATG

GCR

130517

poly (A)

78

FIG. 1. Diagram of KSHV ORF 74 genomic location and mRNA structure. The open reading frames for GCR (ORF 74) and K14 are adjacent and located immediately to the right of the cluster of latency genes (ORFs 73,72,71 and K12); arrows indicate direction of transcription (A). Shown in (B) are the locations of the intron in the intergenic region of K14 and GCR, and the poly (A) signal. Also shown are the start codon positions for both open reading frames, as well as the positions for the consensus splice donor and acceptor sites. The nt numbers below the splice sites refer to the first (129219) and last (129367) nucleotide of the intron indicated by the two bold G's. Nucleotide positions are according to the sequence of Russo et al. (38).

Figure 2

80
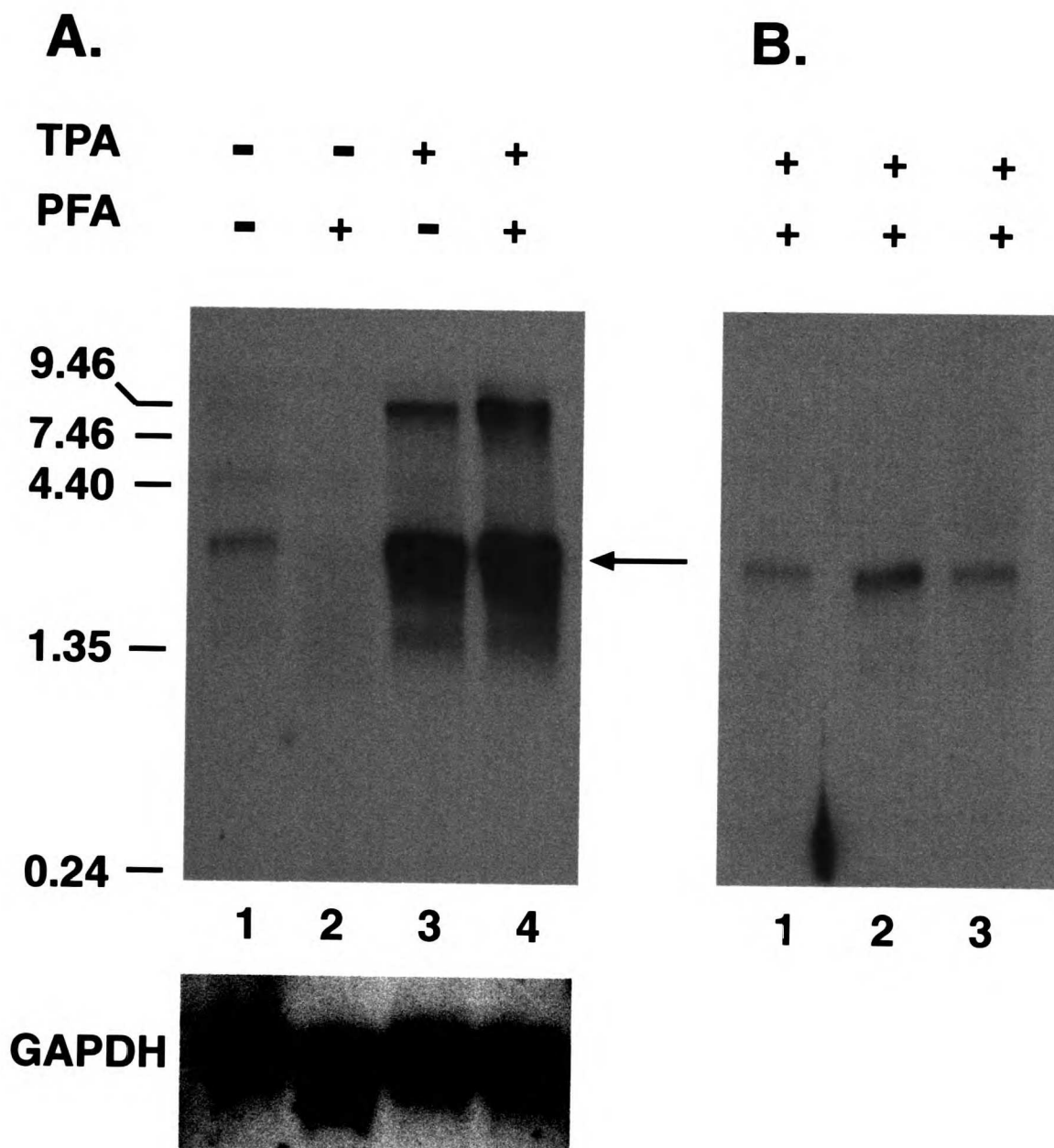
FIG. 2. Analysis of GCR mRNA expression. (A) (top panel) Autoradiogram of a Northern blot containing poly A purified mRNA from BCBL-1 cells either mock-treated (lane 1), PFA treated (lane 2), TPA treated (lane 3) or TPA and PFA treated (lane 4). The probe used was an antisense ORF 74 riboprobe, and kb size markers are shown on the left. The blot was re-probed with GAPDH specific probe as a loading control (bottom panel). The arrow indicates the major mRNA at 2.8 kb. (B) Autoradiogram of a Northern blot of total RNA from BCBL-1 cells treated with TPA and PFA and hybridized with probes for K14 (lane 1), the intergenic region (lane 2) and ORF 74 (lane 3). The arrow indicates the 2.8 kb mRNA species.

Figure 3
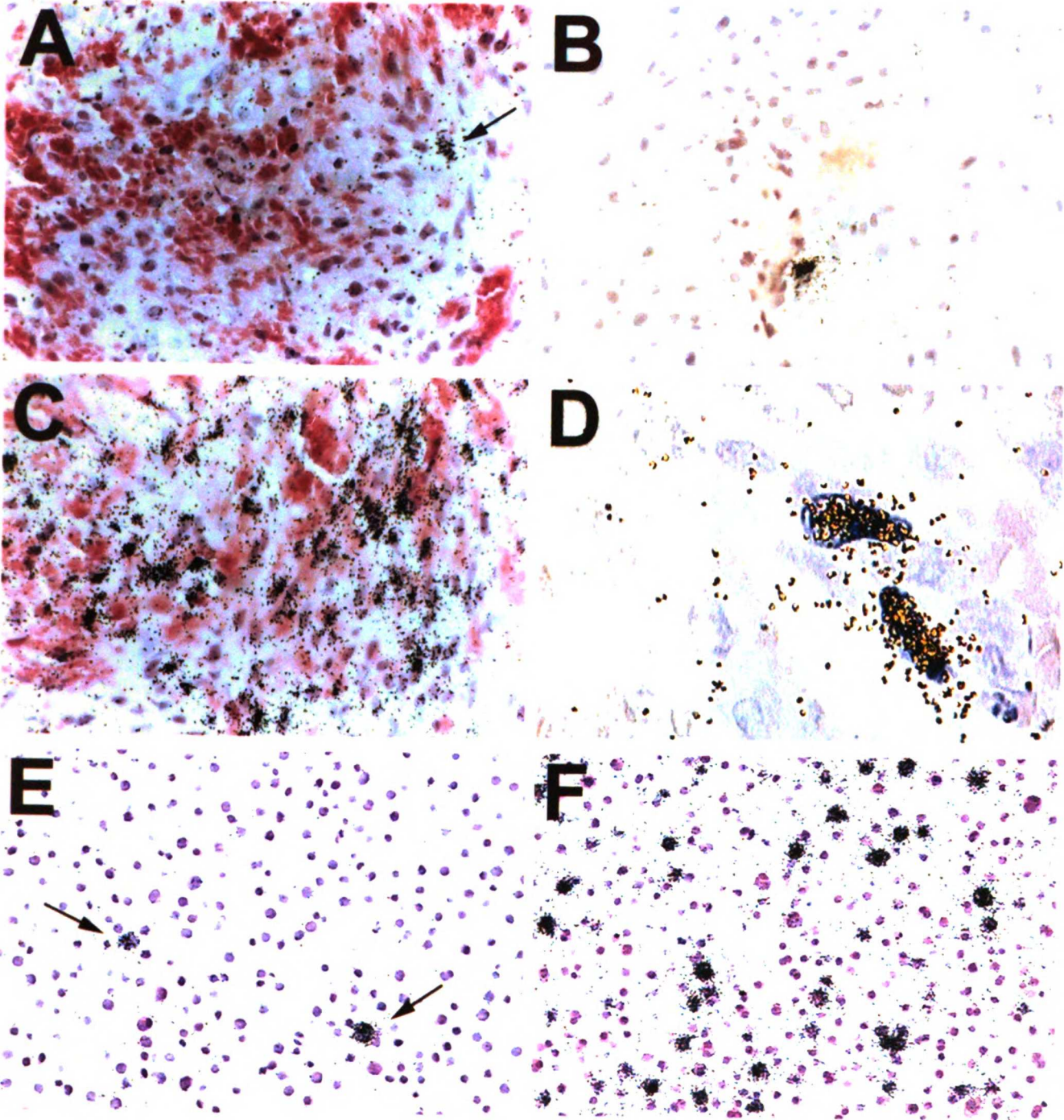
FIG. 3. In situ localization of KSHV GCR gene expression to lytically infected cells in KS and BCBL-1 cell culture. In situ hybridization of ^{35}S-labeled riboprobes specific for KSHV RNAs to thin sections of a dermal KS lesion reveals that few cells are transcribing ORF 74 (visualized as silver grains that have developed in a photographic emulsion coating the specimen) (A, arrow; 7 d exposure). The frequency of ORF 74-positive cells is similar to that which is transcribing the lytic gene, nut-1. (B; 4 h exposure) and represents only a fraction of the total population of infected cells that are revealed by hybridization with probe to detect the viral K12 (T0.7) gene, which transcribed in latent as well as lytically infected cells (C; 3 d exposure). Double-label in situ hybridization with ^{35}S-labeled ORF 74 riboprobe (yellow-green silver grains as viewed with epipolarized illumination) and digoxigenin-labeled nut-1 riboprobe (dark purple nuclei) shows co-localization to the same subpopulation of spindle tumor cells (D; 7 d exposure). Hybridization of ORF 74 probe to thin sections of paraffin -embedded BCBL-1 cells before TPA treatment (E, arrows; 3 d exposure) and two days after treatment with TPA (F; 3 d exposure) reveals that uninduced cultures contain a minor subset (1-3%) of cells expressing ORF 74 and that GCR transcription is turned on in cells induced to undergo lytic replication. A-C, E, F - counterstained with hematoxylin and eosin; D - lightly counterstained with hematoxylin.
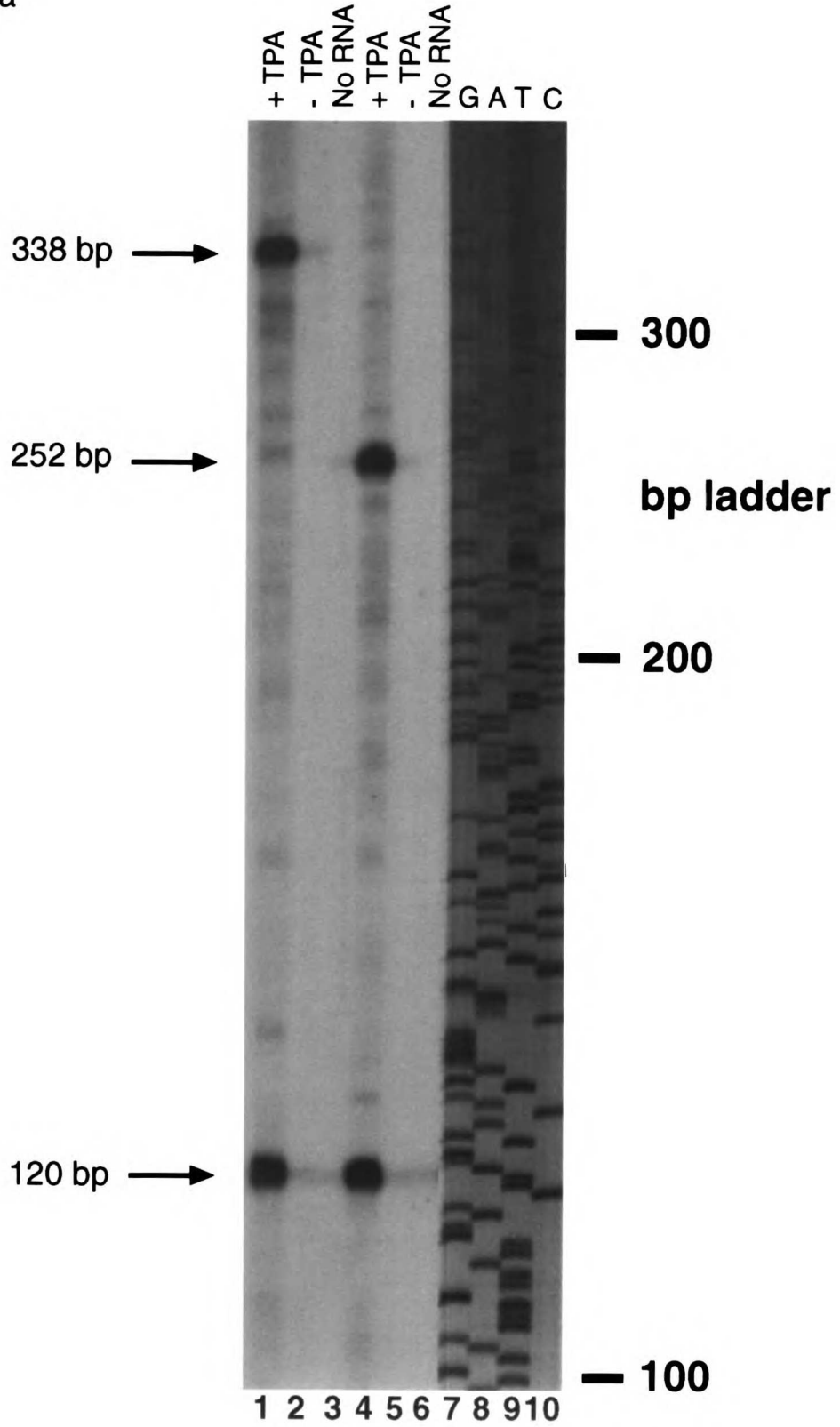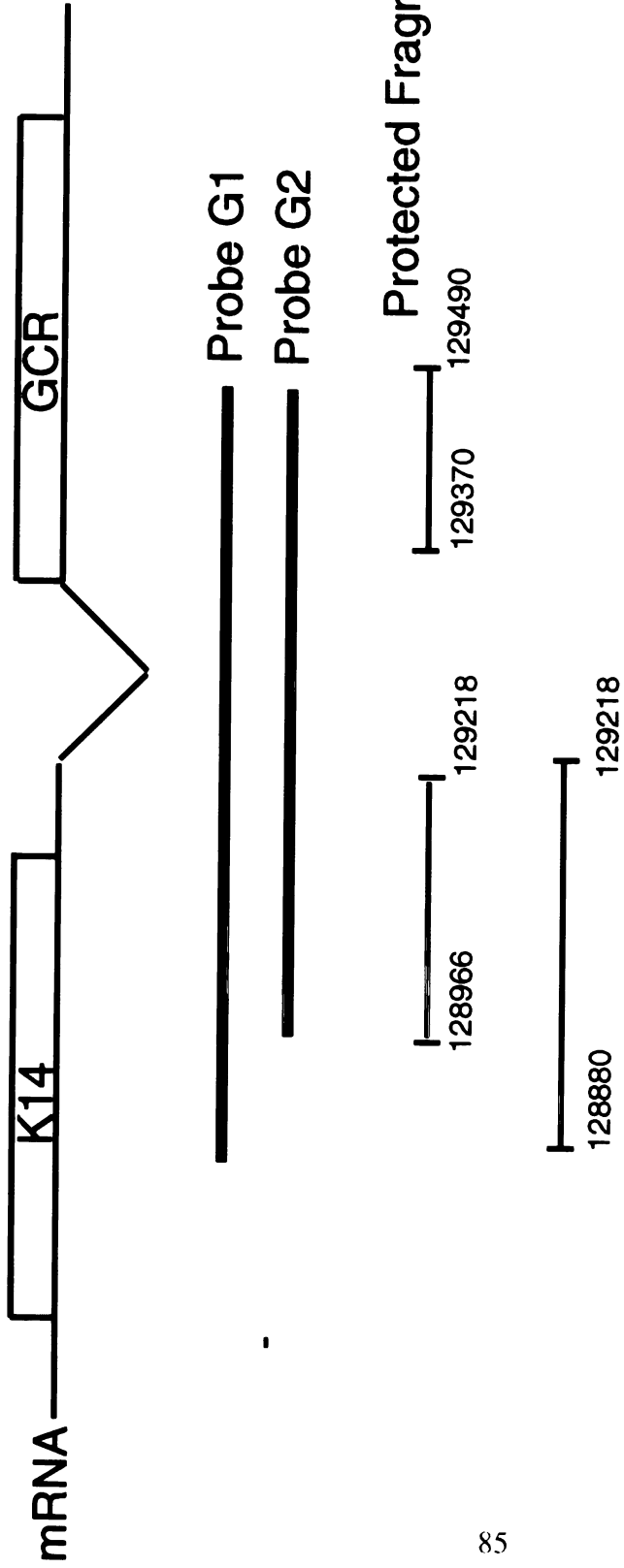
Figure 4a

338 bp →

252 bp →

120 bp →

300

bp ladder

200

100

+ TPA
- TPA
No RNA
+ TPA
- TPA
No RNA
G A T C

1 2 3 4 5 6 7 8 9 10

84

Figure 4b

GCR

K14

mRNA

Probe G1

Probe G2

Protected Fragments

129490

129370

129218

129218

128966

128880

85

FIG. 4. Ribonuclease protection analysis to identify a potential monocistronic GCR mRNA. The location of the antisense riboprobes used and sizes of the resultant protected fragments are diagrammed in (B). (A) shows an autoradiogram of a 6% denaturing acrylamide gel containing the protected species. Total RNA from either TPA treated BCBL-1 cells, (lanes 1 and 4), untreated BCBL-1 cells (lanes 2 and 5) or no RNA (lanes 3 and 6) was hybridized to probe G1 (lanes 1-3) or probe G2 (lanes 4-6), digested with RNase, denatured and electrophoresed. A sequencing ladder was run on the same gel and used for sizing (lanes 7-10).
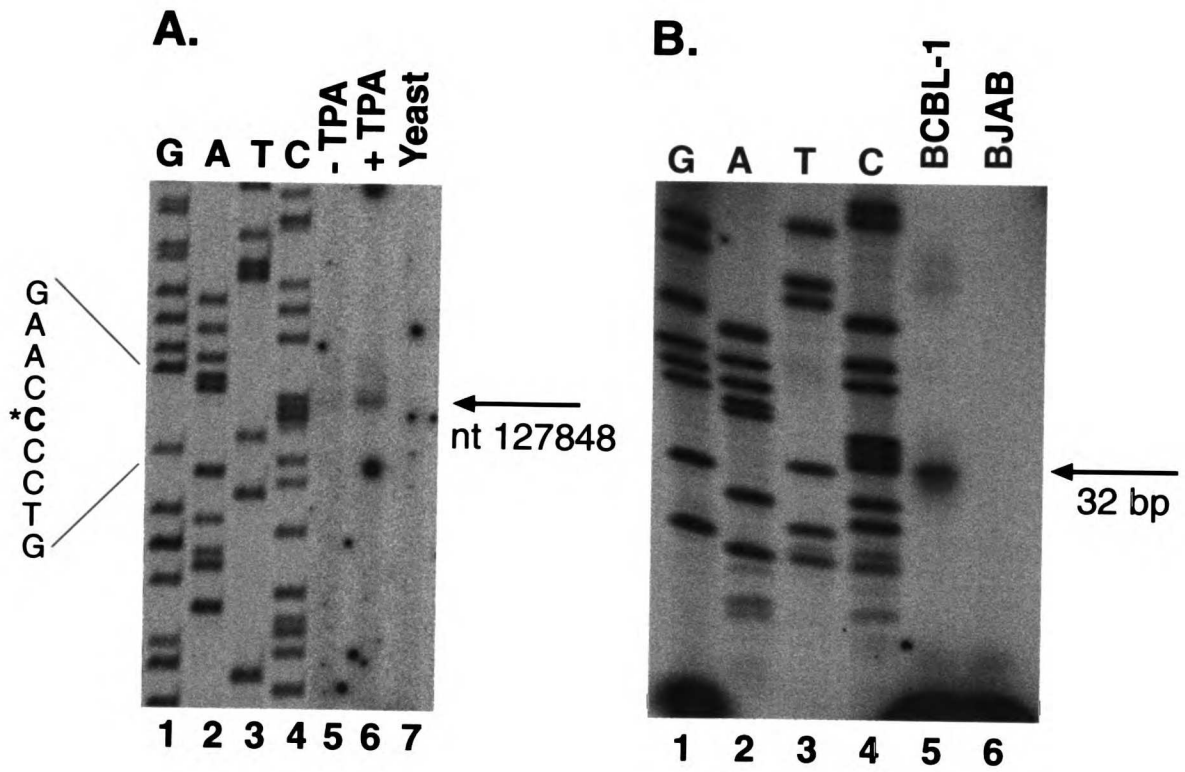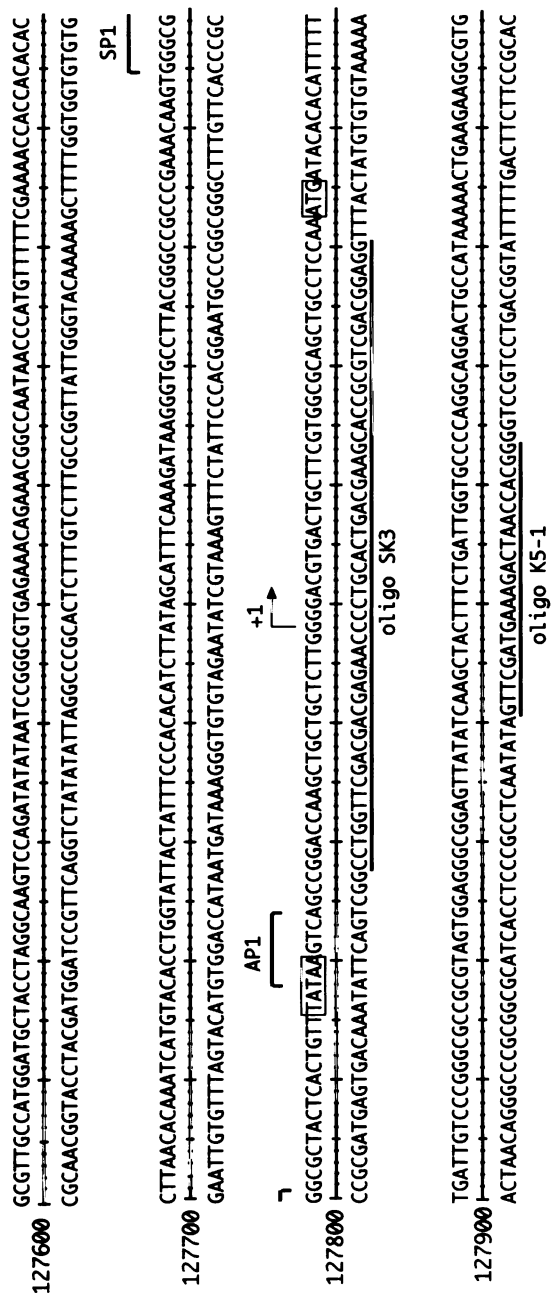
Figure 5 a,b

Figure 5c

FIG. 5 Mapping the 5' end of the 2.8 kb transcript using primer extension and S1 nuclease protection. (A) Primer extension was performed with a labeled oligo K5-1 (diagrammed in C) annealed to RNA from either untreated BCBL-1 cells, (lane 5), TPA treated BCBL-1 cells (lane 6) or yeast RNA (lane 7). A sequencing ladder generated using the same primer on genomic DNA was run on this gel (lanes 1-4) for size comparison. The arrow points to the extension product and its corresponding nt position, also indicated by the asterix on the left. (B) S1 nuclease protection was performed on RNA from TPA treated BCBL-1 cells, (lane 5), or TPA treated BJAB cells (lane 6). A sequencing ladder was run on the same gel to provide size standards (lanes 1-4). The primer used for the sequencing ladder was positioned 2 bp 5' to the oligo SK3. The arrow points to the protected fragment of 32 bp. (C) Sequence of the region surrounding the start site for the 2.8 kb mRNA. Shown are the start site, binding sites for transcription factors and positions of oligos used for S1 analysis and primer extension. The start site is indicated by +1. The arrows below the sequence represent the oligo directionality, and the transcription factor binding sites are indicated by brackets above the sequence. The TATA box and ATG start codon are indicated by boxes.
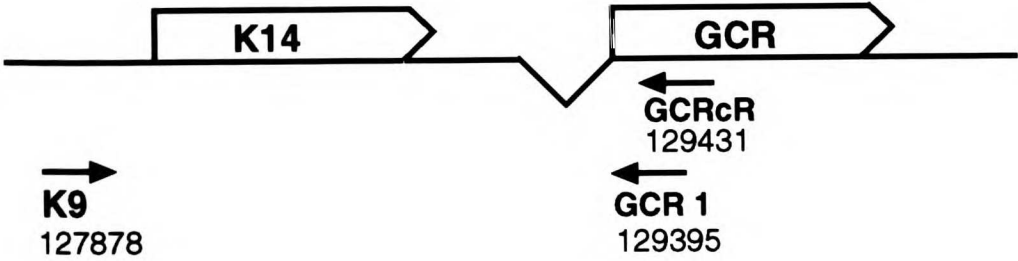
Figure 6 a,b

A.
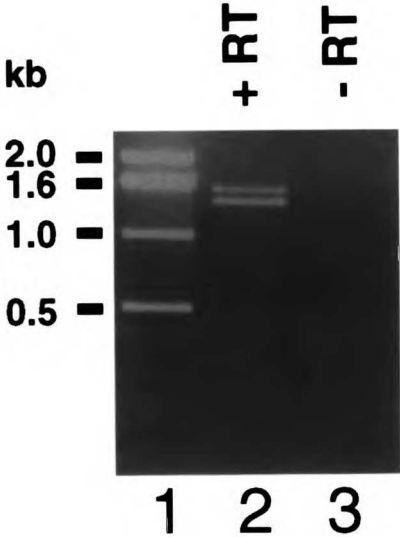
B.

FIG. 6 RT-PCR analysis of K14 and GCR to search for alternatively spliced GCR RNAs. (A) PCR products resulting from reverse transcription and subsequent amplification of BCBL-1 TPA treated RNA (lanes 2,3). RNA was annealed to primer GCRcR (see diagram in B), and cDNA synthesis undertaken in the presence (lane 2), or absence (lane 3) of MMLV reverse transcriptase. PCR amplification of this cDNA with the primers K9 and GCR1 (see diagram in B) was performed and products electrophoresed on a 1% agarose gel and stained with Ethidium bromide. Molecular weight standards are shown in lane 1. (B) Diagram of the K14-GCR region and the primers used in the experiment of Fig. 6A.

Figure 7
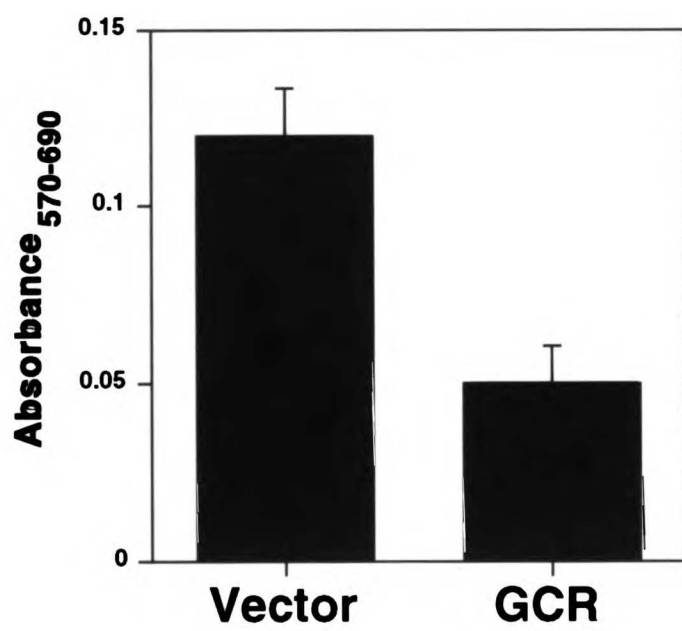
FIG. 7 MTT assay of GCR-transfected Cos7 cells. Cos 7 cells were transfected with

GCR expression plasmid or the empty vector, exposed to MTT, and lysed 48 hr

posttransfection. The A_{570} and A_{690} were measured, and A_{570}-A_{690} was plotted.

Chapter 3

Kaposi's sarcoma-associated herpesvirus ORF 57 encodes a post-transcriptional regulator with multiple distinct activities.

INTRODUCTION

Kaposi's Sarcoma (KS), an endothelial tumor with neoangiogenic and inflammatory

components, is a common neoplasm of AIDS patients. Recent evidence strongly

implicates a novel lymphotropic herpesvirus, KS-associated herpesvirus (KSHV; also

called human herpesvirus 8) in the pathogenesis of KS (5, 12); see (14, 41) for reviews.

KSHV DNA is found in virtually all of the spindle (endothelial) cells of clinically

apparent KS lesions (8, 46), as well as in tumor-infiltrating monocytes (6) and circulating

B cells (1, 27, 50). Consistent with its classification as a lymphotropic (γ) herpesvirus,

KSHV is also tightly linked to certain B lymphomas, termed primary effusion

lymphomas (PEL)(9, 45). Although KSHV infection of KS spindle cells is predominantly

latent, lytic replication is also evident in the tumor (28, 35, 36, 46), and growing evidence

suggests that the lytic cycle contributes importantly to tumorigenesis. For example, the

incidence of KS is greatly decreased when AIDS patients at risk for KS are treated with

ganciclovir, a drug that specifically blocks lytic viral replication (25). Moreover, several

lytic cycle gene products can stimulate inflammatory and angiogenic responses in

surrounding cells and tissues (2, 3, 7). Lytic replication has also been posited to be

required for KSHV spread from its presumed lymphoreticular reservoir to its endothelial

targets (24). Thus, the study of the KSHV lytic cycle (and the switch from latency to lytic

growth) is important not only to fully characterize the molecular basis of viral replication

but also to further inform our evolving notions of KS pathogenesis.

Lytic herpesviral replication is characterized by a temporally regulated cascade of

viral gene expression. Immediate-early (IE) genes, many of which encode activators of

gene expression, are expressed first. Their expression leads to upregulation of delayed-

early (DE) genes, whose products include proteins involved in viral DNA replication;

following replication, the so-called late (L) genes, primarily encoding virion structural

proteins, are expressed. Two of the earliest genes to be transcribed in KSHV infected B-

cells are ORF 50 and ORF 57. We (23, 24) and others (47) have recently shown that

ORF 50 expression can trigger lytic reactivation of KSHV in infected B cells. ORF 50 is

an IE gene (53) whose product is a transcriptional transactivator (23, 24, 47), and this

activity is required for viral reactivation by all known chemical inducers (e.g. TPA and

sodium butyrate) (24). In addition to its classical delayed-early targets (24), the ORF 50

gene product can also upregulate the promoter for ORF 57 (23).

KSHV ORF 57 is homologous to known post-transcriptional regulators in other

herpesviruses. One of these, ICP 27 of Herpes Simplex Virus (HSV), is a pleiotropic

regulator whose functions include downregulation of intron-containing transcripts and

upregulation of certain late messages (40, 43). Temperature sensitive mutations have

shown that ICP 27 is essential for lytic viral replication and is required for inhibition of

host cell splicing, an activity that contributes to host shutoff and to the down-regulation of intron-containing genes in transient assays (17, 18). ICP 27 has also been shown to shuttle from the nucleus to the cytoplasm and to promote the export of intronless viral RNAs (26, 32, 39, 44). The other gamma herpesviruses, EBV and HVS, also encode ICP 27 homologs (13, 21, 30) which, while less extensively studied, also appear to modulate gene expression in a post-transcriptional fashion (38, 42, 51).

Here we have examined the fine structure and expression of KSHV ORF 57 mRNA in BCBL-1 cells, a PEL cell line harboring latent but inducible KSHV genomes, and present an initial characterization of the activities of its product. Our results show that ORF 57 is a complex pleiotropic effector that can act on several levels to augment viral gene expression.

MATERIALS AND METHODS

Cell lines , plasmids and probes.

CV-1 cells were propagated and maintained in DME – H21 media supplemented with

10% fetal calf serum and penicillin/steptomycin at 37 ° in 5% CO_2.

To create intron containing versions (used in Figs. 3, 8) of the KSHV reporter vectors

described previously (24), we used PCR to amplify the SV40 t antigen intron from the

pGL2 promoter vector (Promega) using the following primers (which introduced terminal

Xba I sites): IN 1 : (5' gcgctctagaagagatttaaagctc) IN 2: (5' gcgctctagacagttccataggttg).

The resulting PCR fragment was cut with Xba I and cloned into the Xba I site of each

KSHV-promoter pGL3 vector, at the 3' end of the luciferase coding region.   The nut-1

reporter (pGL3 nut-1 A) used in Fig. 4 was created by PCR amplification of pGL3 nut-

1(24) as template using an internal pGL3 basic primer containing a Bgl II site, and the

following primer: 5' gcgagatctagccaaggtgact. The PCR amplified fragment was digested

with Bgl II and ligated into the Bgl II site of pGL3 basic (Promega). To create the C-

terminal epitope-tagged version of ORF 57, we performed two cloning steps. First, we

digested pcDNA 3.1 ORF 57 (24) with BamHI and Xho I to release the ORF 57 coding

region, and cloned that fragment into the Bam HI and Xho I sites of pcDNA 3.1/V5-His

A (Invitrogen) . Second, we used PCR to amplify the 3' end of ORF 57 without its stop

98

codon, so that the C-terminal tag could be expressed in frame with ORF 57. The following primers were used to PCR amplify the ORF 57 3' end from a genomic clone; primer 57 V1: (5' gcagagcgagctagcactgatcaaac), and primer 57V1: (5' gcgggttcgaaagaaagtggataaaag). The PCR fragment was digested with Nhe I and Sfu I, and cloned into the Nhe I and Sfu I sites of the vector created in step 1 to make pcDNA ORF 57-V5.

The ORF 59/58 expression plasmid (pcDNA 3.1 ORF 59/58) expresses the bicistronic 59/58 message encompassing the AUG to 152 bases downstream of the poly A signal. The following primers introduced restriction enzyme sites by PCR amplification using a lambda genomic clone from a KS lung tumor as template. Primer 59 (added Xba I site) : 5' gcgctctagaatgcctgtggattttcac, primer 58 (added Eco RV site) : 5' gcgcgatatcccaatgcagttgaactac. The 2.4 kb PCR product was cloned into pCR2.1 (Invitrogen). The PCR product was released from pCR2.1 with Hind III, treated with Klenow, then digested with Xba I . This product was cloned into pcDNA 3.1 (Invitrogen) which had been digested with Bbs I (to remove the vector-provided poly A signal), Klenow treated, and then cut with Xba I, to create pcDNA 3.1 ORF 59/58. To create a probe for the ORF59/ 58 northern, we digested pcDNA 3.1 ORF 59/58 with Eco RV to release a 500 bp fragment spanning nt 94330 to nt 94852.

The nut-1 expression plasmid was created by PCR amplification of a genomic lambda

clone using the following primers: nut1 L: 5' actgggactgcccagtc,

Nut 1R: 5' atggattaaacattgccatttat. The resulting product was cloned into pCR 2.1

(Invitrogen), this was digested with Hind III and Eco RV. The resulting fragment was

cloned into the Hind III and Eco RV sites of pcDNA 3 (Invitrogen) to create pcDNA 3

nut 1.   A ds DNA probe for nut-1 was created by digesting pcDNA 3 nut-1 with Eco RI.

The GCR and K5 expression plasmids were made by PCR amplification of genomic

lamda clones with the following primers, and subsequent cloning of the PCR products

into pCR 3.1 (Invitrogen). For GCR, GCR 1: 5'cgatcgcggccgcacctatactacttgtt, GCR 2: 5'

cagcttgatcaccgcgggctacgtggtggc. For K5, K5 1: 5' ccaagtggttgttcaaccgt, K5 2: 5'

agctgcaaagatggcgtcta.   Double stranded DNA probes for GCR and K5 contained only

coding sequences.

To create the truncated version of the nut1 reporter, pGL3 nut1 –706, pGL3 nut1(24) was

digested with Sma1 and TthIII followed by blunt end ligation. The mutant version of this

reporter, pGL3nut1 –706 mut, was created by linker scanning and resulted in substitution

of the ORF 50 response element with a 12 bp linker containing a Nde1 site and three

nucleotides on either side. (J. Chang, D. Lukac and D. Ganem, unpublished)

cDNA cloning.

The cDNA clones containing ORF 57 were isolated from a poly (dT)-primed library

made by R. Renne from induced BCBL-1 cells in Lambda Zap (Stratagene). Screening

was performed with a 436 bp ds DNA fragment spanning nt 82839 to nt 83278.

RNase protection and Primer Extension.

RNase protection was performed with the RPA II kit from Ambion (Austin, Tex.)

according to the manufacturers instructions with the following modifications. The

following plasmid was constructed to make the single stranded RNA probe for RNase

protection: a 400 bp Kpn genomic fragment containing the genomic sequence from nt

81948 to nt 82346 was cloned into the Kpn site of pSP72 (Promega). The plasmid was

linearized with Xba and transcribed with T7 RNA polymerase in the presence of ^{32} P

UTP to create an antisense probe. Approximately 1×10^6 cpm of probe was hybridized

to 7.5 ug of RNA from either uninduced or TPA -induced BCBL-1 cells overnight at 42

° . The samples were then digested with 1:100 dilution of the kit provided RNase A/

RNase T1 solution, plus 1:100 dilution of the kit provided RNase T1 solution for one

hour at 37°. Following precipitation of the digested RNA, the samples were separated on

an 8% denaturing acrylamide gel. The gel was exposed to Kodak XAR-5 film for 3 days.

Primer extension was performed essentially as described in Zhong et al. (52). Briefly, 10

ug of RNA from TPA -induced BCBL-1 cells was hybridized with the primer 57PE : (

5' ctctaggatgcccttcataatgtc) . The samples were separated on a 8% denaturing gel and

exposed to Kodak XAR 5 film for 3 days. A sequencing ladder created by sequencing a

genomic clone with the primer 57 PE was loaded on the gel adjacent to the Primer

Extension products for size comparison.

Immunoflourescence.

CV-1 cells were plated to 60% confluency on glass cover slips, and transfected with

either pcDNA 3.1His V5 or pcDNA 3.1His V5 ORF 57 (Invitrogen). 48 hrs after

transfection with Lipofectamine (Gibco BRL) according to the manufacturers

instructions, the cells were washed, then fixed for 10 minutes in fresh 4%

paraformaldehyde in PBS. After washing, the cells were permeabilized in 1xPBS/0.1 %

Triton-X/ 0.1 % Sodium Citrate for 10 minutes at 4° C. Subsequently, the cells were

blocked in 1xPBS/ 1.0% Triton-X/ 0.5 % Tween/ 3.0 % BSA for 30 minutes at room

temperature. The cells were then incubated with a 1:300 dilution of the mouse -anti-V5 antibody (Invitrogen) diluted into the blocking solution at room temperature for one hour and washed 3 times with 1xPBS. Finally, the cells were incubated with TRITC conjugated goat anti-mouse F(ab)2 fragments at a dilution of 1:100 for one hour at room temperature, then washed as above.

Transfections.

CV-1 cells were plated at $1x10^5$ cells per well of a 6-well tissue culture dish the day prior to transfection. In all transfections, pcDNA 3 was used as a filler plasmid to normalize total DNA per transfection. For the Luciferase assays, 4-5 ug total of DNA was diluted into 100 ul of serum-free DME H21 medium and 10 ul of Superfect transfection reagent (Qiagen) was added. For amounts of effector plasmids, refer to Figure Legends. After a 10 minute incubation during which time the cells were washed once in 1 x PBS, 700 ul of complete medium was mixed with the DNA –superfect –media mixture, and added to each well. After 3 hours of incubation, the cells were washed once with 1 x PBS, and 2 mls of fresh complete medium was added. Following a 48 hour incubation, the cells were washed twice with 1x PBS and were scraped into 150 ul of 1X Reporter Lysis Buffer (Promega). The cell extracts were vortexed for 30 seconds, and the debris was removed by centrifugation 15 seconds at 16,000 rpm in a microcentrifuge. The

supernatant was transferred to a new tube, and 20 ul aliquots were analyzed by luciferase

assays according to the manufacturers instructions (Promega). All graphs of luciferase

activity represent the results of experiments performed at least three times, in duplicate,

unless otherwise noted. Error bars represent standard deviations from these experiments.

Because all of the reporters we attemped to use as internal standards were affected by

ORF 57 expression, we chose to perform mutiple replicates of each experiment and

indicate standard deviations of the replicates as error bars. In some experiments, standard

deviation is so small that the error bars are not visible.

Northern Blotting.

For Northern blotting, CV -1 cells were plated at $5x 10^5$ cells per 100 mm dish

the day prior to transfection. For the ORF 50 northern, cells were transfected with 10 ug

total plasmid DNA consisting of 2.5 ug of pcDNA ORF 50, and either 0, 0.75, 2.5 or 7.5

ug of pcDNA ORF 57. For the ORF 59/58, nut-1, GCR and K5 Northerns, cells were

transfected as above with 10 ug total plasmid DNA consisting of 5 ug of the expression

vector for the target gene and either 0 ug or 1 ug of pcDNA 3 ORF 57. Superfect

transfection reagent (Qiagen) was used according to the manufacturers instructions. 48

hours after transfection, total RNA was harvested using RNAzol B (Tel-Test, Inc.,

Friendswood, Tex.) as recommended by the manufacturer. 10 ug of total RNA was

separated on a 1% agarose-17% formaldehyde gel and transferred to Hybond-N

membrane for 12 hr in 10X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

The blots were UV cross-linked and hybridized with either the ORF 50 antisense

riboprobe (described in Lukac et al (23)), as in Kirshner et al.(19), or with ds DNA

probes for ORF 59/58, GCR, K5 and nut-1 as in (Lagunoff et al (20)) . The blots were

exposed to Kodak XAR5 film for 2 days.

Western Blotting.

For Western blotting, cells were transfected as described above. 48 hours after

transfection, the cells were scraped into 10-S buffer (23), incubated on ice for 10 minutes,

then centrifuged for 5 minutes at 16, 000 rpm in a microcentrifuge, whereupon the

supernatant was removed. The protein concentration of the extracts was determined by

Bradford assay, and equal amounts of protein (10 ug) were separated on either a 8%

SDS-polyacrylamide gel (ORF 50), or a 12.5 % polyacrylamide gel (ORF 59/58).

Following electrophoresis, proteins were transferred and detected as in Lukac et al (23).

The primary antibody to ORF 59/58 (24)was used at a 1:500 dilution.

RESULTS

Expression of KSHV ORF 57

We have previously shown that KSHV ORF 57 is a lytic gene expressed between 2 and 4

hrs after TPA induction of BCBL-1 cells, immediately following the appearance of ORF

50 transcripts but prior to most DE mRNAs (23). To determine the fine structure of ORF

57 mRNA, we first screened an oligo-dT-primed cDNA library from TPA-induced

BCBL-1 cells with a probe for ORF 57. Six clones were obtained, none of which were

full length. The longest (schematically depicted in Fig 1A) contained a 3' end at nt.

83637, 29 nt. downstream of a canonical poly A signal, and a 5' end at nt 82100 . An

intron of 108 bp was located at the 5' end of this clone, with consensus spice donor and

acceptor sites (Fig.1 A) . To locate the transcriptional start site, we performed RNase

protection and primer extension analyses. The antisense riboprobe used for RNase

protection extended from the beginning of the second exon to 400 bp upstream (Fig 1 A)

. After hybridization to RNA from either TPA induced or uninduced BCBL-1 cells, the

samples were treated with RNases A and T1 for 1 hr at 37°C and separated on a 6 %

denaturing gel. Two protected fragments were generated (Fig 1B). These protected

fragments are more abundant in the induced lane but are also present in the uninduced

lane due to the 1-4% of BCBL-1 cells that undergo spontaneous reactivation. These results indicate a start site of transcription at nt 82003. To confirm these results, we performed primer extension with an end-labeled oligonucleotide that hybridized to the start of exon 1 (Fig 1 A). After annealing of this primer to RNA from TPA-induced BCBL-1 cells and extension with reverse transcriptase, a single product of 116 nt. was detected (Fig. 1C). By comparing the size of this product to a sequencing ladder generated by using the same primer, we were able to confirm the start site at nt 82003. Analysis of the region 5' to the start site revealed a TATA box 24 base pairs upstream of the transcriptional start site as well as several consensus transcription factor binding sites (Fig. 1D). Scanning the region downstream of the start site revealed the existence of four methionine (ATG) codons in frame with exon 1. As we do not yet know which of the potential start codons is/are utilized, in constructing an ORF 57 expression vector, a genomic fragment containing all potential start codons was employed. Although the genomic sequence originally predicted a size of 275 aa for ORF 57, our cDNA cloning and transcript mapping reveals that ORF 57 actually has a larger coding region (potentially 456 aa) in vivo.

Subcellular localization of ORF 57 protein

Analysis of the predicted amino acid sequence of ORF 57 reveals several arginine-rich

potential nuclear localization signals. To determine the cellular localization of ORF 57,

we first constructed an expression vector for ORF 57 with an epitope tag (pcDNA3.1

V5, Invitrogen) fused to the C-terminus. After verification that this expression vector

generated a functional ORF 57 product (as defined by its ability to synergize with ORF

50; see below), we examined CV-1 cells transiently transfected with this construct by

immunoflourescence with anti-V5 mAbs. Fig. 2 (left panel) shows the control vector-

transfected cells 48 hrs post transfection; no staining is observed. When transfected with

the vector expressing ORF 57 expression vector, intense staining is observed only in the

nuclei of CV-1 cells. (Fig 2, right panel).

Effect of ORF 57 on KSHV promoter-driven reporter genes

To begin the analysis of ORF 57 function, we examined the ability of the protein to

regulate expression of luciferase reporter genes driven by a variety of KSHV DE and

latent promoters. The tested promoters included those from the following KSHV genes:

nut-1 (also called PAN), tk (ORF 21), DBP (ORF 6), DNA polymerase (ORF 9) and

Kaposin. CV-1 cells were cotransfected with pcDNA 3.1 ORF 57 and the reporter

constructs, and luciferase activity was measured. Each target was examined over a 10-

fold range of concentrations of the ORF 57 expression vector; most were unaffected, and

in no case did we observe more than a twofold effect over the basal level of expression in

the absence of ORF 57. Fig. 3 shows representative results for four promoters: DNA pol,

Kaposin, nut-1 and tk (white bars) . Several heterologous promoters were also examined

in this fashion (CMV IE, SV40 E), with similar results (JK, unpublished data; see also

Fig. 7). These data indicate that ORF 57 is not a broad-spectrum transcriptional activator

such as adenovirus E1A or HSV ICP 0.

Because HSV-1 ICP 27 regulation of reporter expression is influenced by the presence or

absence of introns in the body of the transcript, we constructed isogenic, intron-

containing versions these luciferase reporters (driven by the promoters for: DNA pol,

Kaposin, nut-1 and tk) and tested them alongside their intronless counterparts. We used

the SV40 t antigen intron, which has been shown to be a target of regulation by ICP27

(40). Fig 3 (black bars) shows the results obtained for each of the 4 constructs, again

tested over a 10-fold range of co-transfected ORF 57 plasmid. We saw no consistent

effect of the presence of intronic sequences for each KSHV reporter tested. Some

constructs (e.g. that driven by the DNA pol promoter) were completely unaffected, while

others showed small (2-fold) effects at selected concentrations of ORF 57 (see Fig. 3 for

representative examples). Interestingly, this finding was not limited to the SV40 t intron:

an SV40 early promoter-driven luciferase construct bearing a synthetic intron derived

from ß-globin likewise failed to display regulation by ORF 57, as did its intronless

counterpart (data not shown). Moreover, an authentic KSHV gene (ORF 50) bearing its

native intron was not downregulated by ORF 57 coexpression (see below and Fig. 7).

These results argue against the existence of an ORF 57-encoded activity that globally

impairs splicing or actively represses expression from intron-containing genes. However,

they do not exclude the possibility that some viral genes might display intron-dependant

responses to ORF 57, and examples of this phenomenon will be presented below (see Fig.

8).

ORF 57 alone can regulate KSHV gene expression

Thus far, in the context of artificial reporter constructs, ORF 57 expression was observed

to have little effect on gene expression on its own. However, such chimeric reporters

bear little resemblance to the natural targets of ORF 57 regulation in vivo. We therefore

turned to the examination of transcription units containing authentic viral sequences, to

more closely mimic the targets found in a viral infection. In scanning for KSHV genes

that might be subject to ORF 57 regulation, we focused on the subclass of DE genes

involved in DNA replication, as these genes have been implicated in such regulation in

both HSV (49) and EBV (42). ORF 59 is a KSHV homolog of EBV BMRF1, a DNA

polymerase accessory factor whose expression has been shown to be enhanced by EBV

M protein expression(42). This gene was a particularly attractive target for our work

because of the availability of monoclonal antibodies to the ORF 59 protein (24). ORF 59

is expressed as the 5' gene in an unspliced bicistronic transcript with ORF 58;

polyadenylation occurs 3' to ORF 58 (10) (and R. Renne and DG, unpubl.data).

Accordingly, we cloned the genomic region spanning ORFs 58 and 59, including the

relevant KSHV poly A signal, downstream of the CMV IE promoter, which we have

shown is not substantially regulated by ORF 57 (see Fig. 7). This construct was

transfected into CV-1 cells in the presence or absence of pcDNA3.1ORF 57, and the

levels of RNA and protein measured by Northern blotting and immunoblotting,

respectively. Fig. 4A shows that in total RNA from whole cell extracts of such

transfectants, ORF 59 mRNA levels in the presence of ORF 57 were nearly 20 fold

higher than those produced in its absence. This stimulation was also reflected in the

levels of ORF59 protein (Fig. 4B). Since the CMV promoter is not significantly

upregulated by ORF 57, the bulk of this upregulation is post-transcriptional. Similar

induction was observed when the KSHV polyadenylation signal was replaced by the

polyadenylation signal from a heterologous gene (bovine growth hormone, BGH)

Similar screens of other early KSHV genes revealed that this effect is not limited

to ORF 59 (Fig. 5). A particularly interesting case is that of nut-1 RNA (Fig. 5A). In this

experiment, full-length nut-1 sequences bearing their native polyA signals were cloned

downstream of the CMV promoter; this allows examination of ORF 57 effects on the nut-

1 transcript independent of any possible effects on the nut-1 promoter. A nearly 20-fold

enhancement in the accumulation of nut-1 RNA is observed in total RNA from whole-

cell lysates (Fig. 5A). However, other KSHV-containing transcripts (GCR, K5) driven

by the CMV promoter (and employing the BGH poly A site) were unaffected (Fig. 5B),

indicating that post-transcriptional upregulation by ORF 57 is transcript specific, and

confirming that ORF 57 does not upregulate the CMV promoter.

Augmentation of ORF 50 activity by ORF 57 protein

In an authentic KSHV infection, ORF 57 is not expressed in isolation; rather, it is

produced with other powerful regulatory proteins, of which the best-characterized is the

IE transactivator encoded by ORF 50. Accordingly, we examined the effects of ORF 57

expression on the (intronless) nut-1-driven luciferase reporter in the presence or absence

of the ORF 50 protein, which we have previously established can strongly upregulate this

promoter (24). As shown in Fig 6A, while ORF 57 alone had little effect on nut-1

promoted luciferase expression, ORF 50 alone, as expected, activated reporter expression

80 fold. We then selected a level of ORF 50 expression vector that produced maximal

activation (1 ug/transfection), and to this, added increasing amounts of ORF 57

expression vector and assayed luciferase expression. Addition of even low levels of ORF

57 to ORF 50 resulted in a striking (80-90 fold) further up-regulation of expression over that generated by ORF 50 alone.

To further investigate the nature of this synergistic effect, we used a dominant negative mutant of ORF 50 termed 50 ΔSTAD. This mutant, whose transcriptional activation domain is deleted, retains dimerization capacity and has been shown to specifically inhibit the ability of wt ORF 50 to transactivate reporter genes and to induce lytic replication of KSHV in BCBL1 cells(23). Increasing amounts of an ORF 50 ΔSTAD expression vector were added to our co-transfection assay containing ORF 50, ORF 57 and the nut 1 reporter. Fig. 6B shows that addition of 50 ΔSTAD inhibits the stimulation of gene expression by the ORF50-57 combination in a dose-dependent fashion. Similarly, we found that syngergistic upregulation of the nut-1 promoter by the 50/57 combination is abolished by mutations in the promoter that ablate ORF 50 responsiveness. In studies to be reported elsewhere, mutagenesis of the nut-1 promoter identified a small region required for ORF 50 reponsiveness-mutations in this element do not effect basal transcription but completely abrogate upregulation by ORF 50 (J. Chang, D. Lukac and D. Ganem, unpublished). Fig. 6C shows that such mutations also abrogate synergy between ORF 50 and ORF 57 proteins. These results indicate that synergy between ORF 50 and 57 on this target requires the specific transactivation activity of ORF 50.

Two independent lines of evidence support the idea that ORF 57 is not simply

increasing the amount of the ORF 50 transactivator and therefore further activating the

nut 1 promoter. First, the observed effect of ORF 57 operates at saturating levels of the

ORF 50 protein; under these conditions, increasing ORF 50 has no effect on

transactivation (see Fig 6A). Second, direct examination of ORF 50 mRNA and protein

levels shows no significant augmentation by ORF 57 expression (Fig. 7). CV-1 cells were

co-transfected with pcDNA-ORF 50 and increasing amounts of pcDNA-ORF 57; 48

hours after transfection, total RNA was isolated. Equal amounts of this RNA was

separated on a 1.2 % agarose –17% formaldehyde gel, transferred to a Hybond –N

membrane and hybridized to an antisense riboprobe for ORF 50. Fig. 5A shows that with

increasing amounts of ORF 57, the ORF 50 mRNA levels do not significantly increase.

Phosphorimager (Molecular Dynamics) quantitation of the bands in the Northern blot

reveals that there is at most a 3 fold increase in the amount of mRNA at the highest

concentration of ORF 57 – but this ORF 57 level is well above the concentration needed

for the superinduction. Extracts of the same transfected cells were also examined by

immunoblotting with a rabbit antiserum to ORF 50 (Fig. 7B); no increase was detected in

ORF 50 protein levels at concentrations of ORF 57 at which marked synergy was

observed by luciferase assay. (At high levels of ORF 57, no more than a three fold

increase in ORF 50 protein levels was observed). The possibility of ORF 57s toxicity to

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host protein synthesis can be excluded for two reasons; (i) basal luciferase levels are not decreased by ORF 57 expression in reporters whose transcripts are not upregulated (Fig. 3); and (ii) levels of ORF 59 protein rise in concert with with RNA levels in response to ORF 57 (Fig. 4).

Re-examination of intron effects in the presence of ORFs 50 and 57

In the HSV system, the repressive effect of ICP 27 on intron-containing reporters was best seen when expression of the reporters was activated by coexpression of the ICP4 and ICP0 IE transactivators (40). This fact, together with the fact that in natural infection ORF 57 expression is always accompanied by ORF 50 production, led us to re-examine the effects of introns in this context. We tested three pairs of isogenic luciferase reporters containing or lacking the SV40 t intron, driven by the promoters of the nut-1, kaposin and tk genes, respectively. Each pair of reporters was examined for luciferase expression in the presence of a fixed saturating dose of ORF 50 and increasing amounts of the ORF 57 expression vector (Fig 8). Several interesting facts emerged from this analysis. First, the synergistic 50/57 interaction is promoter-specific. While all three promoters are ORF 50-responsive (23), the tk promoter is not synergistically upregulated by coexpression of ORF 57, while the kaposin promoter is superinduced by at most 25-fold (versus 80-100 fold for the nut-1 promoter) (compare Fig. 8A and B). Second, significant effects of

introns were observed in some but not all of these contexts. Specifically, the presence of

an intron in the nut-1 construct still allowed upregulation by ORF 57, but this synergy

was reduced to 10-fold (versus nearly 100-fold for the intronless reporter (Fig. 8 A)). By

contrast, little effect of the intron was observed on the kaposin reporter (Fig 8B).

Notably, we did not observe actual *repression* of gene expression – i.e. a decrease in

luciferase activity below the level observed in the absence of ORF 57 expression. Since

the "basal" level of luciferase was typically quite high in these assays (due to ORF 50

activation), this represents a sensitive assay for detecting repression.

DISCUSSION

These results demonstrate that ORF 57 is a nuclear protein expressed from a spliced lytic

mRNA. In transient reporter gene assays, ORF 57 expression has little effect on a wide

variety of promoters, suggesting that it is not a broad-spectrum transcriptional activator.

Despite extensive searches, we also observed little consistent effect of the presence or

absence of introns in such chimeric constructs. Nonetheless, the accumulation of some

viral RNAs (e.g. those for ORF 59 and nut-1) can be strongly augmented in the presence

of ORF 57, in a manner that suggests post-transcriptional regulation. Moreover,

expression from some promoters (especially nut-1) that are upregulated by ORF 50 can

be synergistically enhanced by coexpression with ORF 57. This synergy results from a

post-translational enhancement of ORF 50's transcriptional activity. These data reveal

ORF 57 to be a complex pleiotropic effector that can act on several levels to augment

viral gene expression.

Although homologous to ICP27, the KSHV ORF 57 protein differs in some

important respects from its HSV-1 counterpart. In HSV, ICP27 expression leads to a

strong inhibition of RNA splicing, an effect that is thought to be one important

contributor to the shut-off of host macromolecular synthesis. (Accordingly, most delayed-

early and late mRNAs of HSV are unspliced). Coexpression of ORF 57 with ORF 50 did

not impair the accumulation of ORF 50 mRNA or full-length protein (Fig. 7). Since ORF

50 bears an intron in its coding region, this result clearly indicates that ORF 57 does not

impair the splicing of this intron. (And notably, functional ORF 57 is itself the product of

RNA splicing). Moreover, even though research on KSHV gene expression is at an early

stage, numerous examples of spliced viral mRNAs have already been documented,

including the DE genes for K14/74 (19, 48) and KbZIP (16, 22), and the late genes

encoding K8.1 (11, 33) and ORF 29 (34). Recent studies (15) also show that multiply

spliced transcripts for K15/LAMP, while expressed at low levels in latency, are strongly

upregulated during lytic growth. These findings are inconsistent with a substantial block

to RNA splicing or to expression of intron-containing genes throughout the KSHV lytic

cycle.

However, although the above considerations argue strongly against a general

downregulation of intron-bearing sequences, they do not exclude that *in selected genes

activated by ORF 57* such activation might operate preferentially or exclusively on the

unspliced version of the RNA. That is, in such genes ORF 57 might be relatively

indifferent to intron-containing sequences but actively upregulate those lacking introns.

In other herpesviruses, evidence is growing that ORF 57's homologs can shuttle from

nucleus to cytoplasm and can mediate the preferential cytoplasmic accumulation of

unspliced mRNAs (26, 39, 42, 44). Nothing in the present work excludes this as one

potential mode of action of KSHV ORF 57. However, our data indicate that this cannot

be the sole mode of action of ORF 57, since (i) its augmentation of ORF 50's activity

operates at a post-translational level (Fig. 7); and (ii) nut-1 RNA, which is localized

entirely to the nucleus, is also strikingly upregulated by the ORF 57 protein (Fig. 5)

The effects of the intron in the nut-1 –promoted reporter are interesting in this

regard. Despite the fact that the ORF 50/57 synergy is expressed primarily by effects at

the nut-1 promoter, expression of the intronless version of the gene is upregulated 10-fold

more efficiently than its intron-bearing derivative (Fig 8A). This is consistent with the

notion that some intronless mRNAs might be preferentially stabilized or exported for

translation in the cytosol. Interestingly, the fact that identical mRNAs do not behave

similarly when directed by the kaposin promoter (Fig 8B) suggests that events at the

promoter can also influence processing and transport of the RNAs. While incompletely

understood, such effects have been observed in other systems – for example, 3'

processing of some cellular RNAs is strongly influenced by the promoter used to drive

expression of the RNA (29). Clearly, transcriptional and post-transcriptional events are

often coupled (4), and we should not be surprised that regulators acting principally by

post-transcriptional mechanisms can sometimes be influenced by earlier events in the

biogenesis of the target RNA.

We do not yet understand the basis for the remarkable synergy between the products of ORFs 50 and 57, nor why it is so promoter-selective. In part this reflects our incomplete understanding of the mechanism of activation by ORF 50. In other studies, we have genetically identified ORF 50 response elements in several different KSHV promoters and have found that such elements display considerable sequence heterogeneity. We note with interest that the two promoters (nut-1 and kaposin) that are subject to ORF 50/57 synergy share ORF 50 response elements that are identical in sequence and in their position relative to the start site. By contrast, the ORF 50 response element in the TK promoter is completely divergent from that in nut-1 and kaposin. (J. Chang, D. Lukac and D. Ganem, unpublished). This heterogeneity might be one factor contributing to the promoter-selectivity of the effect. For example, if ORF 57 expression were to modify the DNA binding activity of ORF 50, such a change might lead to enhanced binding to some but not all recognition sites. Clearly, fuller understanding of this process must await a clearer definition of the biochemistry of ORF 50's DNA binding and its interactions with the basal transcription machinery.

Although synergy with IE regulators has not previously been observed in the gammaherpesvirus homologs of ORF 57, HSV ICP27 has been demonstrated to complex with ICP4, to alter its subcellular location and to enhance its regulatory actions (31, 54). In the case of the ORF 50/ORF 57 interaction, both proteins are localized to the nucleus

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independently, and we have no evidence that nuclear subdomains to which ORF 50 is

addressed are altered by the presence of ORF 57. We have extensively searched for

evidence of a protein-protein interaction between the products of ORF 50 and 57 using

both co-immunoprecipitation assays (in vivo and in vitro) and a mammalian two-hybrid

system. To date, however, these studies have been negative.

The effects of ORF 57 on nut-1 expression described herein are dramatic and are

likely to play an important role in vivo. Nut-1 RNA is not expressed in latency, as judged

by sensitive in situ hybridization analyses (43), but is expressed with enormous efficiency

during lytic growth, generating over 10^5 molecules of nut-1 RNA /cell. We suggest that

this remarkable accumulation is due to at least two effects: (i) the ORF 50/57 synergy

that upregulates the primary transcription of the locus; and (ii) the ORF57-mediated post-

transcriptional upregulation of nuclear transcript levels. Current work is now focused

upon definition of the cis-acting elements responsible for the post-transcriptional effects

(both in nut-1 and ORF 59) with the goal of elucidating the molecular mechanism(s)

involved in these processes.

Acknowledgements

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Figure 1a
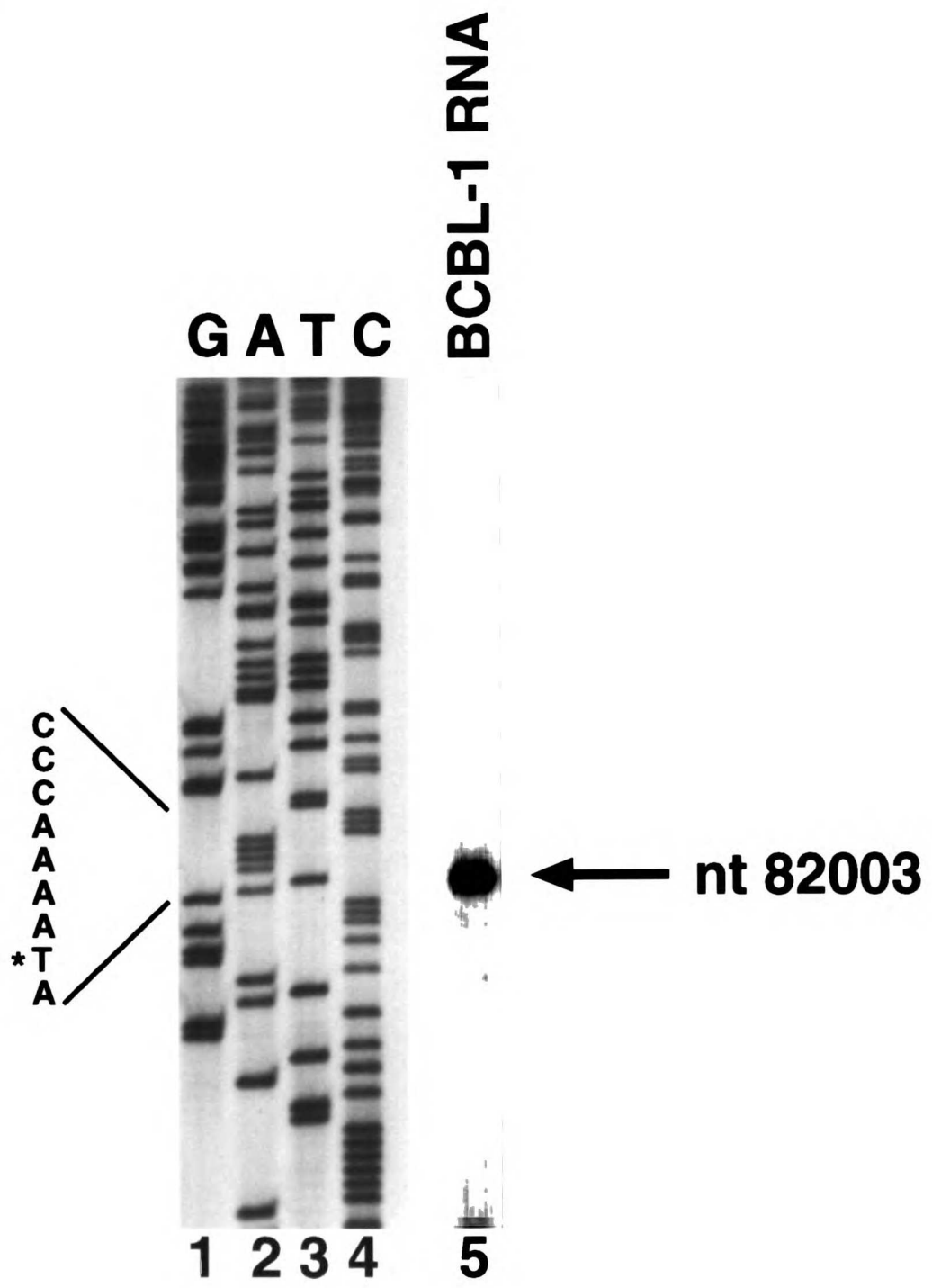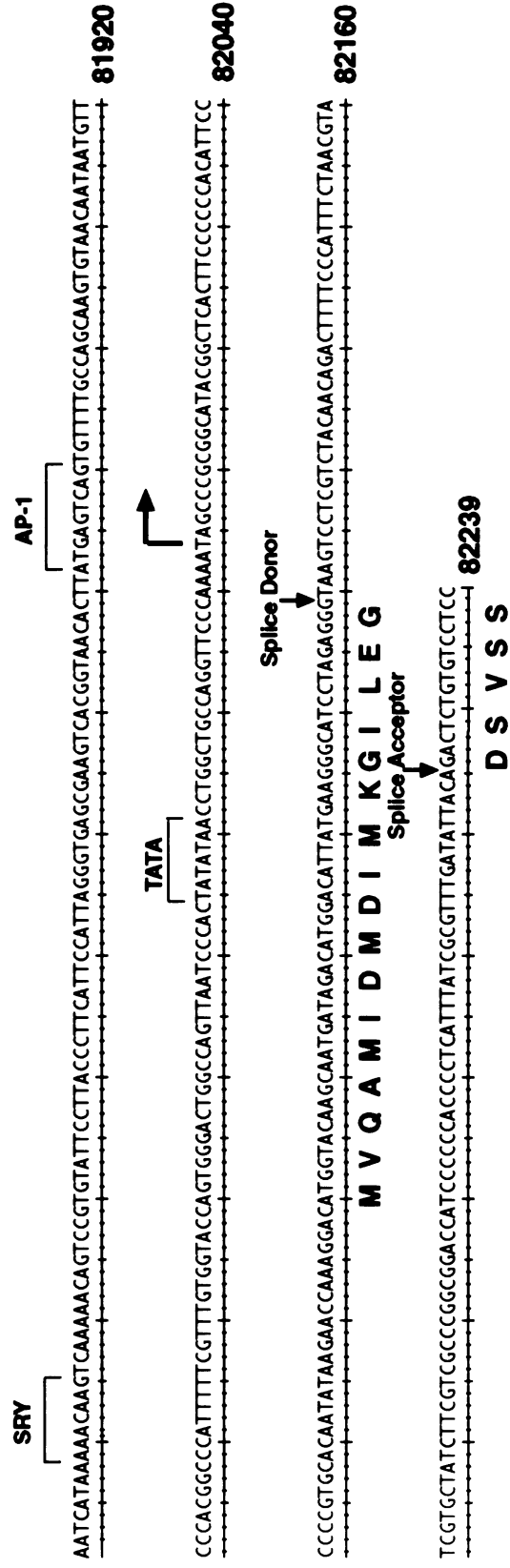
133

Figure 1b

134
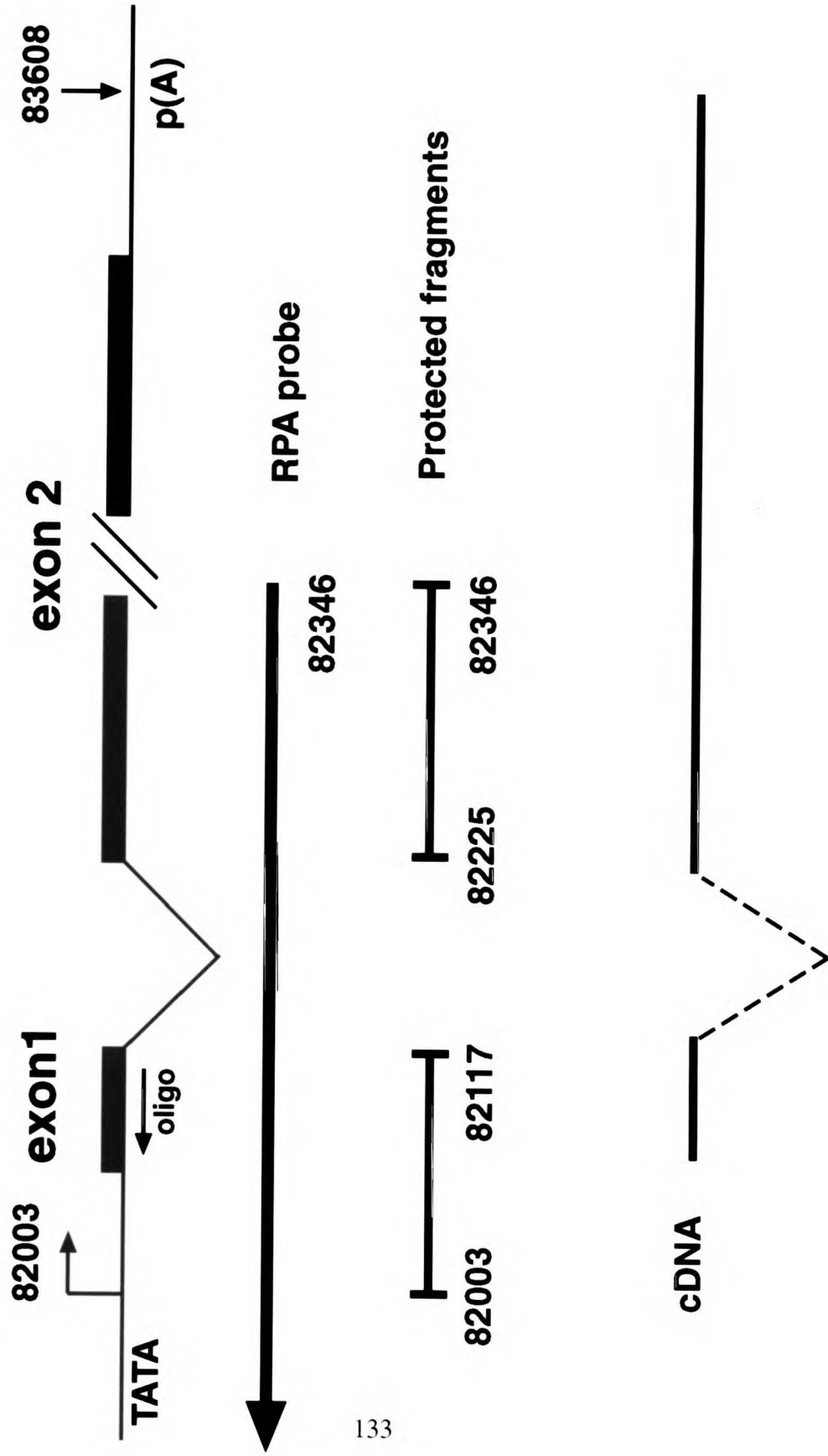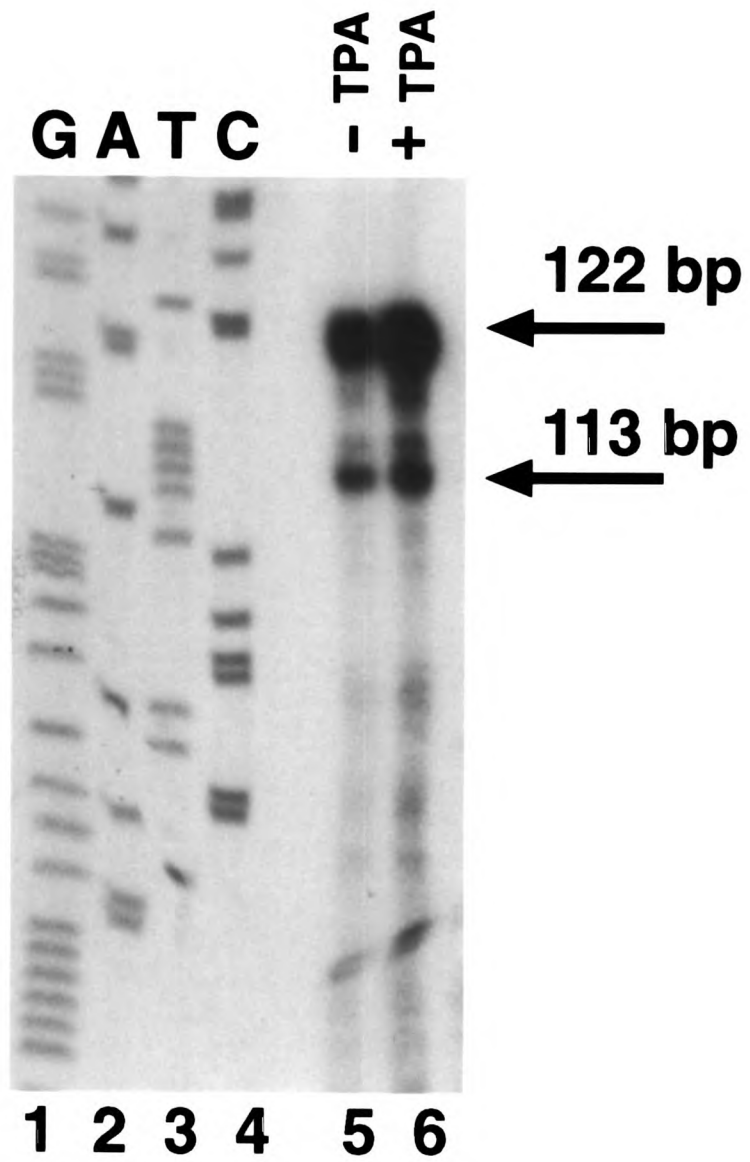
Figure 1c

Figure 1d

FIG. 1 (A): Diagram of KSHV ORF 57 mRNA structure. ORF 57 is spliced, and the transcriptional start sites and poly (A) location are shown. The position of the antisense riboprobe used for RNase protection and sizes of the resulting protected fragments are depicted, as well as the oligonucleotide used for primer extension. The longest cDNA clone is also shown below.

(B) RNase protection to identify the transcriptional start site of ORF 57 mRNA. Autoradiogram of a 6% denaturing acrylamide gel containing the protected species. Total RNA from either untreated (lane 5) or TPA-treated (lane 6) BCBL-1 cells was hybridized to the riboprobe shown in (A), digested with RNase, denatured and electrophoresed. A sequencing ladder was run on the same gel and used for sizing (lanes 1 to 4).

(C) Primer extension to confirm the start site. The extension was performed with labeled oligonucleotide (diagrammed in A) annealed to TPA-treated BCBL-1 RNA (lane 5). A sequencing ladder generated by the same primer on genomic DNA was run on the gel (lanes 1 to 4) for size comparison. The arrow indicates the extension product and its corresponding nucleotide position, also indicated by the asterix on the left. Nucleotide positions are according to the sequence of Russo et al (37).
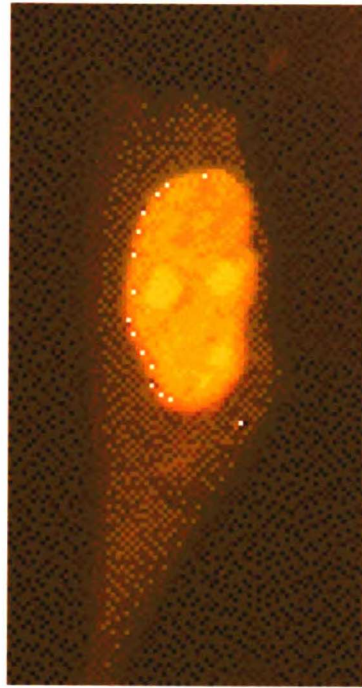
(D) Nucleotide sequence surrounding the transcription start site (arrow). Translated sequence from first AUG in mRNA is shown below the DNA sequence.

Figure 2

ORF 57

VECTOR

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FIG. 2. The C-terminally tagged version ORF 57 is localized to the nucleus. The

panels show results of immunoflouresence staining of CV-1 cells 48 hours after

transfection with either empty pcDNA 3.1 V5 vector (left panel), or pcDNA 3.1 ORF 57

V5 (right panel). Reactivity to Anti-V5 antibody (Invitrogen) was detected with a

TRITC-conjugated rabbit anti-mouse antibody.

Figure 3

Kaposin

DNA Pol

nut-1

TK

ug of ORF 57
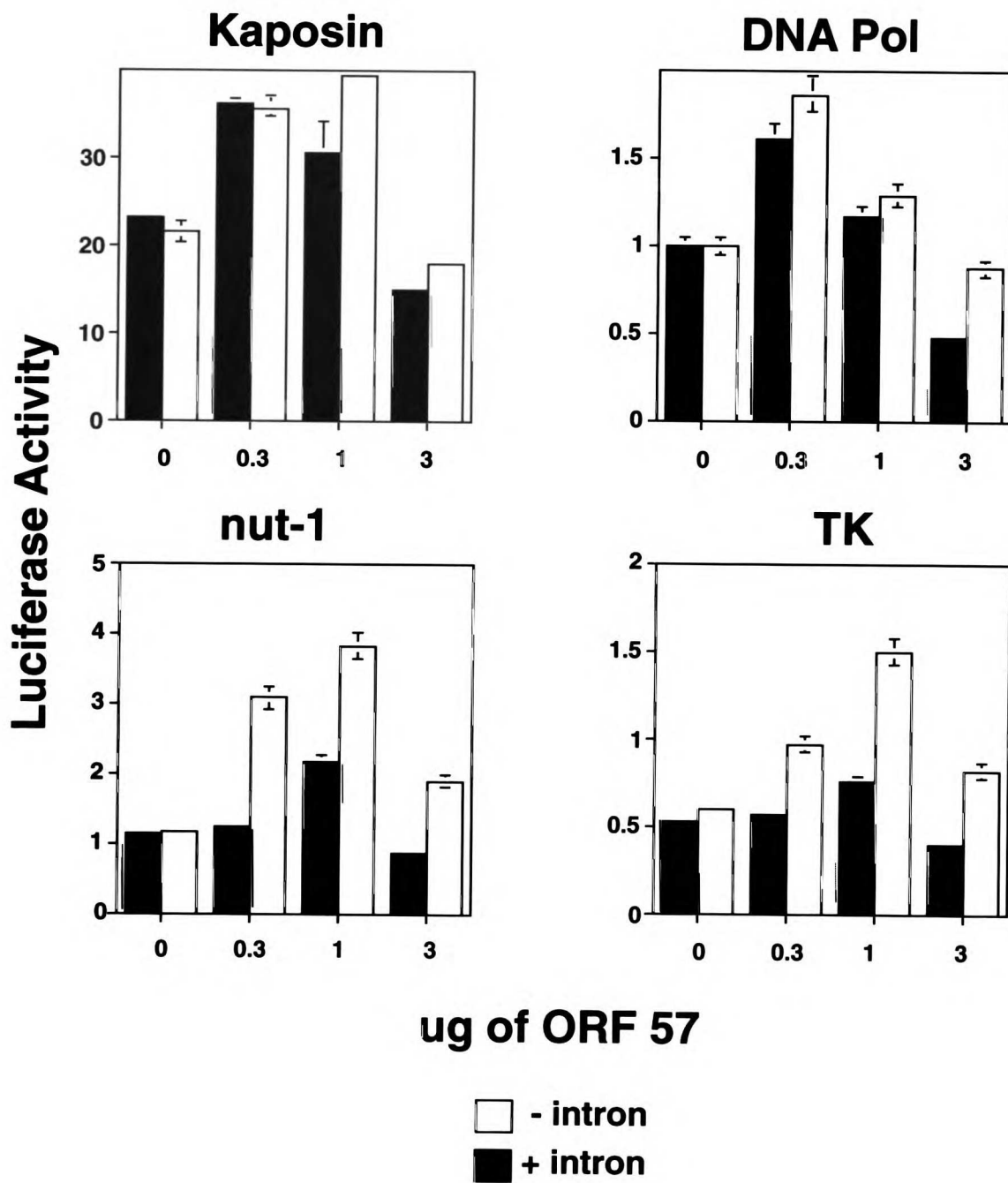
☐ - intron
■ + intron

FIG.3. Effect of ORF 57 expression on viral promoter constructs with and without introns. CV-1 cells were co-transfected with increasing amounts of pcDNA3.1 ORF 57 (0, 0.3, 1,and 3 ug) and fixed amounts of the following KSHV promoter luciferase constructs with or without an intron ; DNA Pol, Kaposin, Nut-1 and TK. Luciferase activity was measured 48 hours post-transfection. Black bars represent the effect on intron-containing reporters, white bars represent the effect on reporters without an intron. Graphs are representative examples of experiments performed in duplicate.

Figure 4a,b
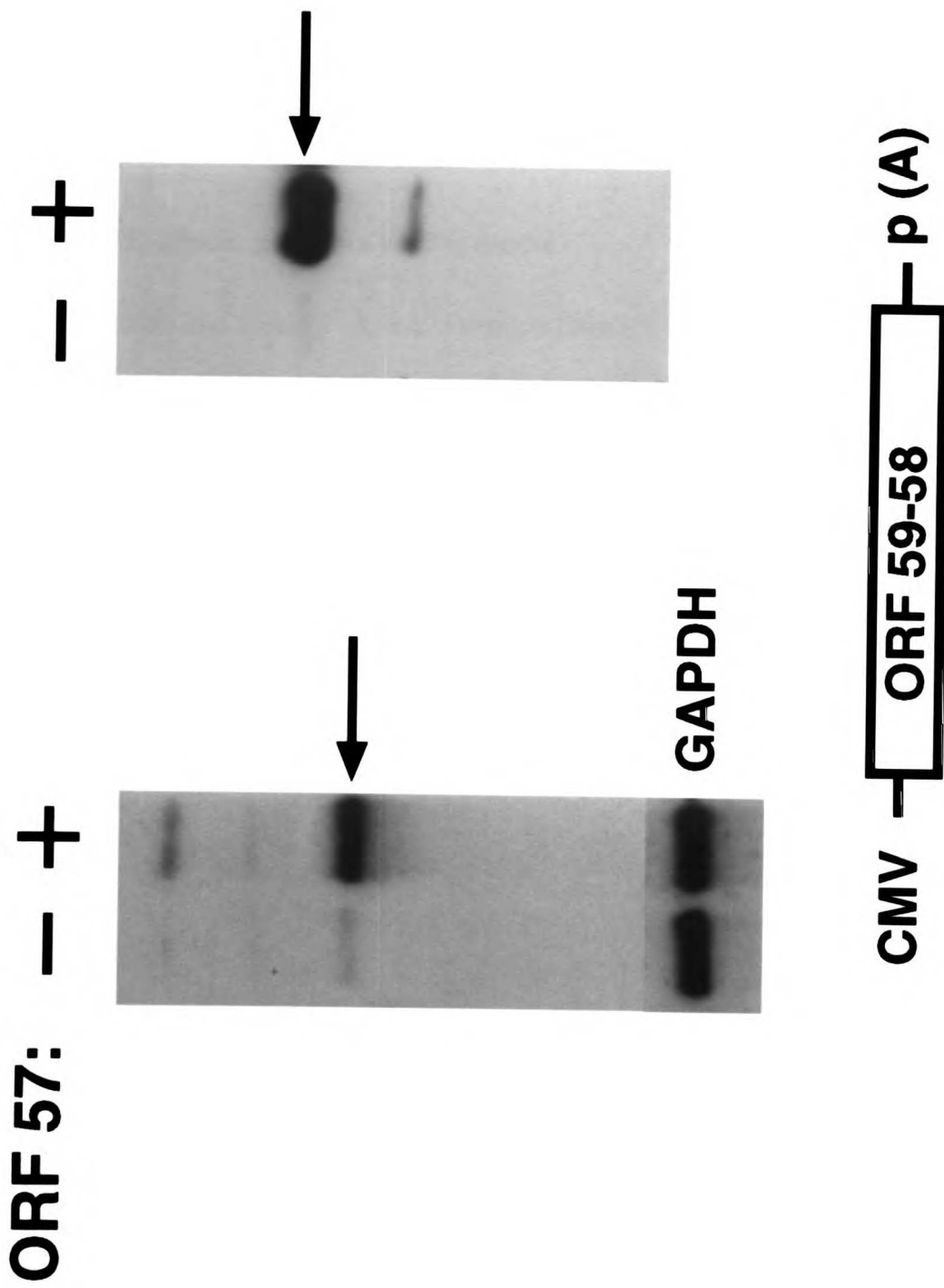
FIG.4. ORF 57 increases ORF 59/58 mRNA and protein levels.

(A). Top, autoradiogram of a Northern blot containing total RNA from CV-1 cells

transfected with a fixed amount of pcDNA 3.1ORF 59/58 in the presence (+) or absence(-

) of 0.1 ug of pcDNA3.1ORF 57. A ds DNA probe specific for ORF 59 was used.

Bottom, the blot was reprobed with a glyceraldehyde-3-phosphate dehydrogenase

(GAPDH)-specific probe as a loading control.

(B). Western blot analysis of protein extracts from CV-1 cells transfected with a fixed

amount of pcDNA 3.1 ORF 59/58 in the presence (+) or absence (-) of 1 ug of

pcDNA3.1 ORF57. The ORF59 protein was detected with a KSHV monoclonal antibody,

and then with a secondary goat anti-mouse conjugated to HRP and ECL (Amersham).

Equal amounts (10 ug) of extracts as determined by Bradford assay from each

transfection were loaded onto the gel.

Figure 5 a,b
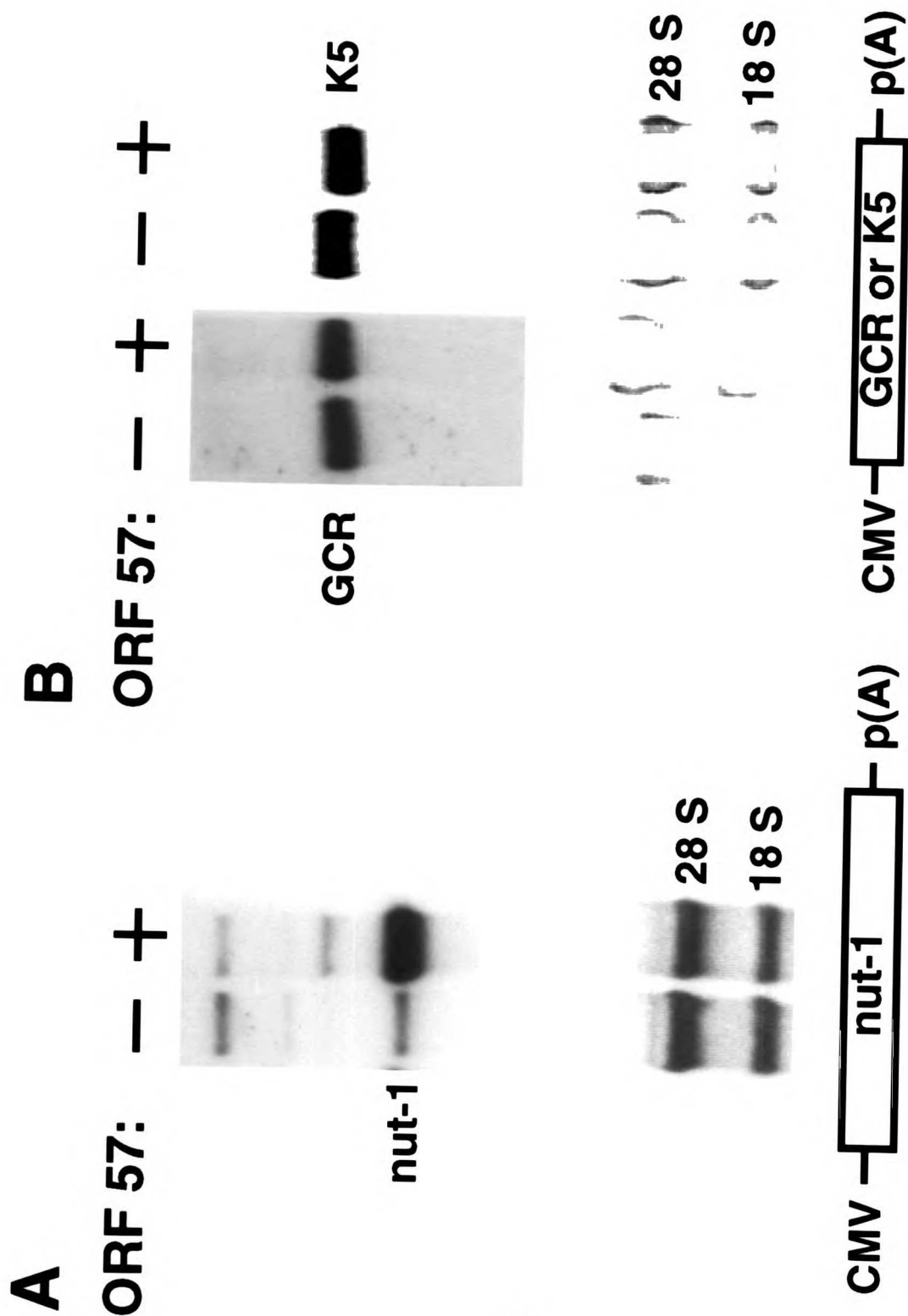
FIG. 5. Upregulation of nut-1 RNA accumulation by ORF 57 expression. The

indicated constructs (bottom) were transfected into CV-1 cells in the presence (+) or

absence (-) of 1 ug of pcDNA3.1-ORF57. 48 hours later, total RNA was prepared from

whole-cell lysates, electorphoresed through 1%agarose-formaldehyde gels, transferred to

Hybond membranes and hybridized to probes specific for nut-1 (A) or orf74/GCR (B, left

panel) or ORF K5 (B, right panel). Below each panel is shown rRNA bands in EtBr-

stained lanes as loading control.

Figure 6a

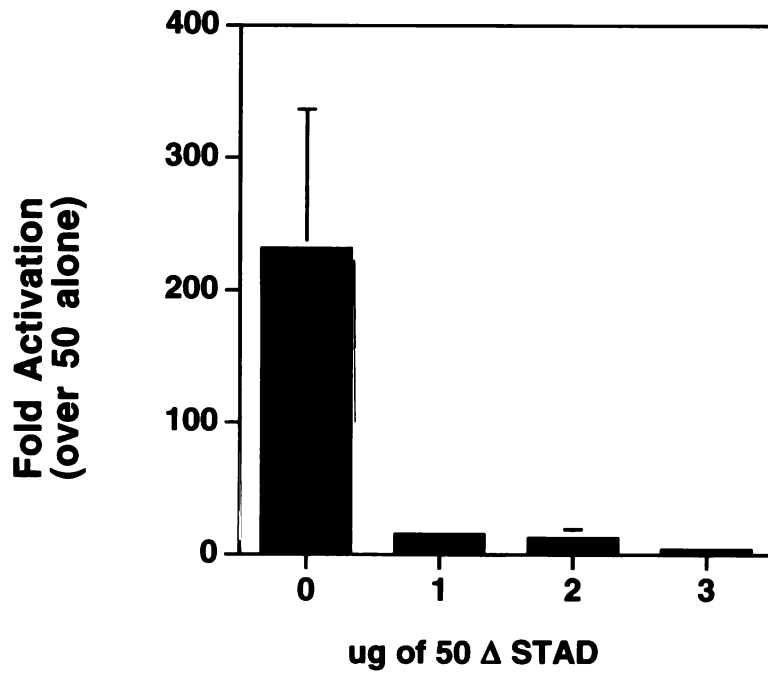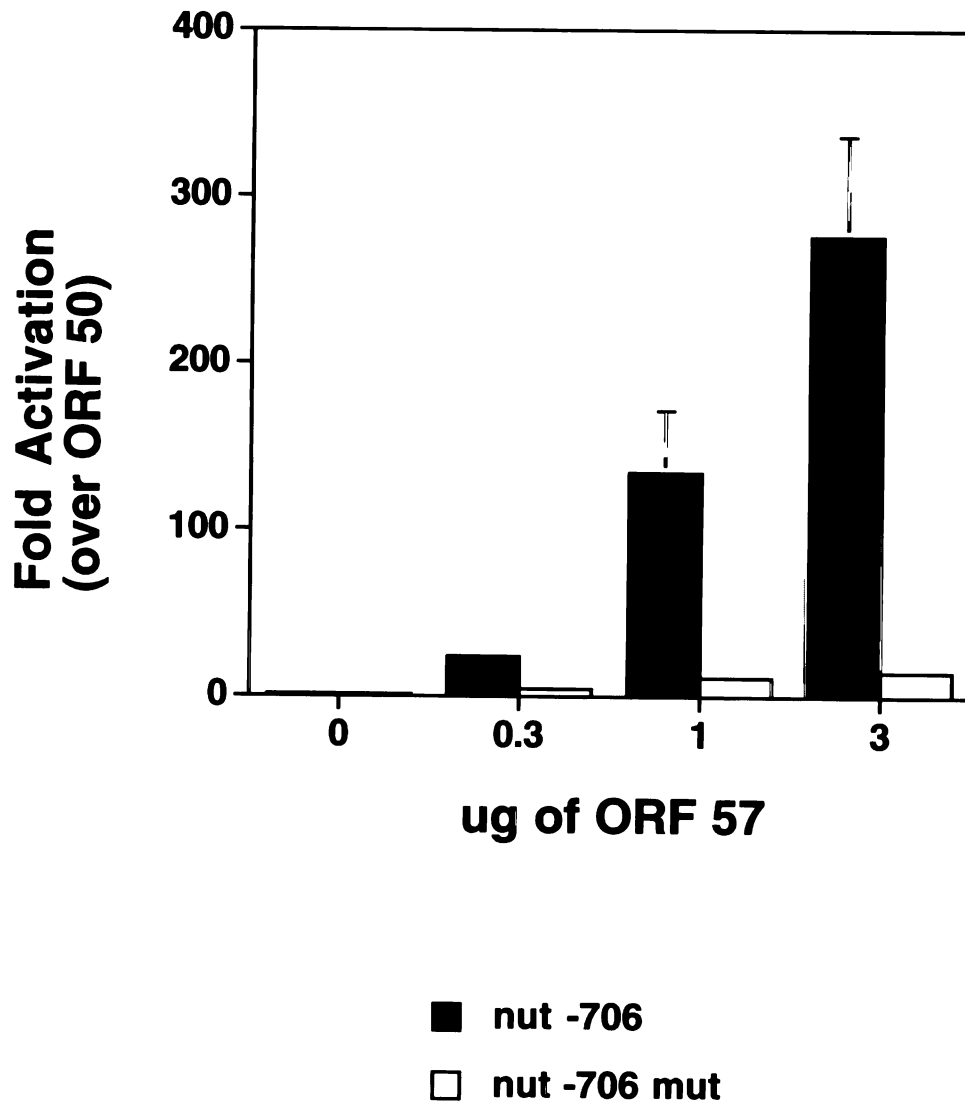Plot with x-axis labeled "ug of effector" ranging from 0 to 4, and y-axis labeled "Fold Lucifersase Activity (nut-1 promoter)" on a logarithmic scale from 0.1 to 10000. Three curves are shown: ORF 57+ ORF 50 (open circles, rising to ~4000), ORF 50 (diamonds, plateauing near 100), and ORF 57 (squares, near 1).

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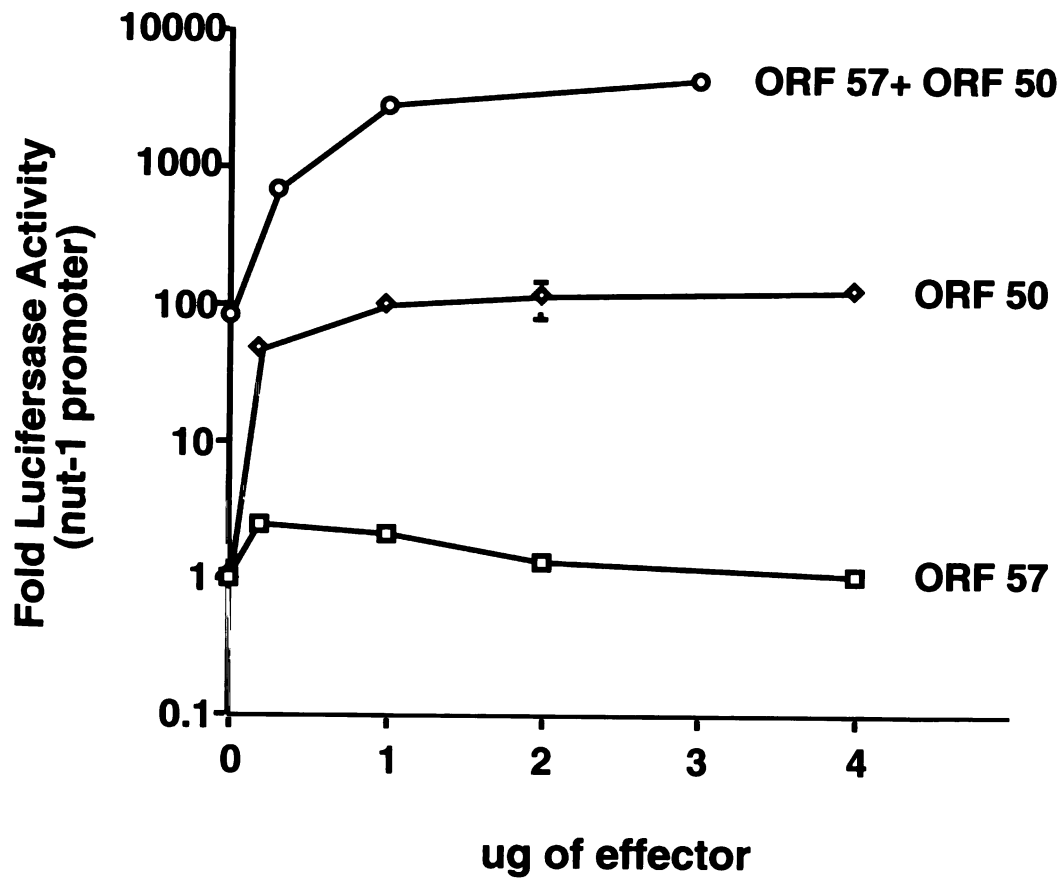
Figure 6b

Figure 6c

FIG.6. ORF 57 augments the effect of ORF 50 on the Nut-1 promoter.

(A) ORF 50/57 synergy on the nut1-reporter. CV-1 cells were transfected with a fixed

amount of the nut-1 promoter reporter (intronless), and the indicated amounts of either

pcDNA 3 ORF 50 (diamonds), pcDNA 3.1 ORF 57 (squares); or 1 ug of pcDNA3 ORF

50 plus the indicated amounts of pcDNA3.1-ORF 57 (circles). Luciferase activity was

measured 48 hours post-transfection, and plotted on the graph. Vertical bars represent

standard deviations from four experiments performed in duplicate.

(B) Dominant negative ORF 50 inhibits the augmentation of ORF 50 by ORF 57. CV-1

cells were transfected with fixed amounts of pcDNA ORF 50 (1 ug), pcDNA ORF 57 (1

ug) and the Nut-1 (0.1ug) promoter along with increasing amounts of pCMV-myc-nuc-

Δ STAD (0, 1,2 and 3 ug)(23). 48 hours post-transfection, luciferase activity was

measured and values were plotted that represented fold activation relative to that of ORF

50 alone, which was set to 1.

(C) Mutations in the nut-1 promoter abolish synergy between ORFs 50 and 57. CV-1

cells were transfected with 2 ug of pcDNA ORF 50 , increasing amounts of ORF 57 and

either I ug of the wt nut1-706 reporter or the nut –1 –706 mutant reporter. 48 hours post-

transfection, luciferase activity was measured and values plotted that represent fold

activation relative to that of 50 alone, which was set to 1.

Figure 7a,b

ug ORF 57

ORF 50

protein

ug ORF 57

ORF 50

GAPDH
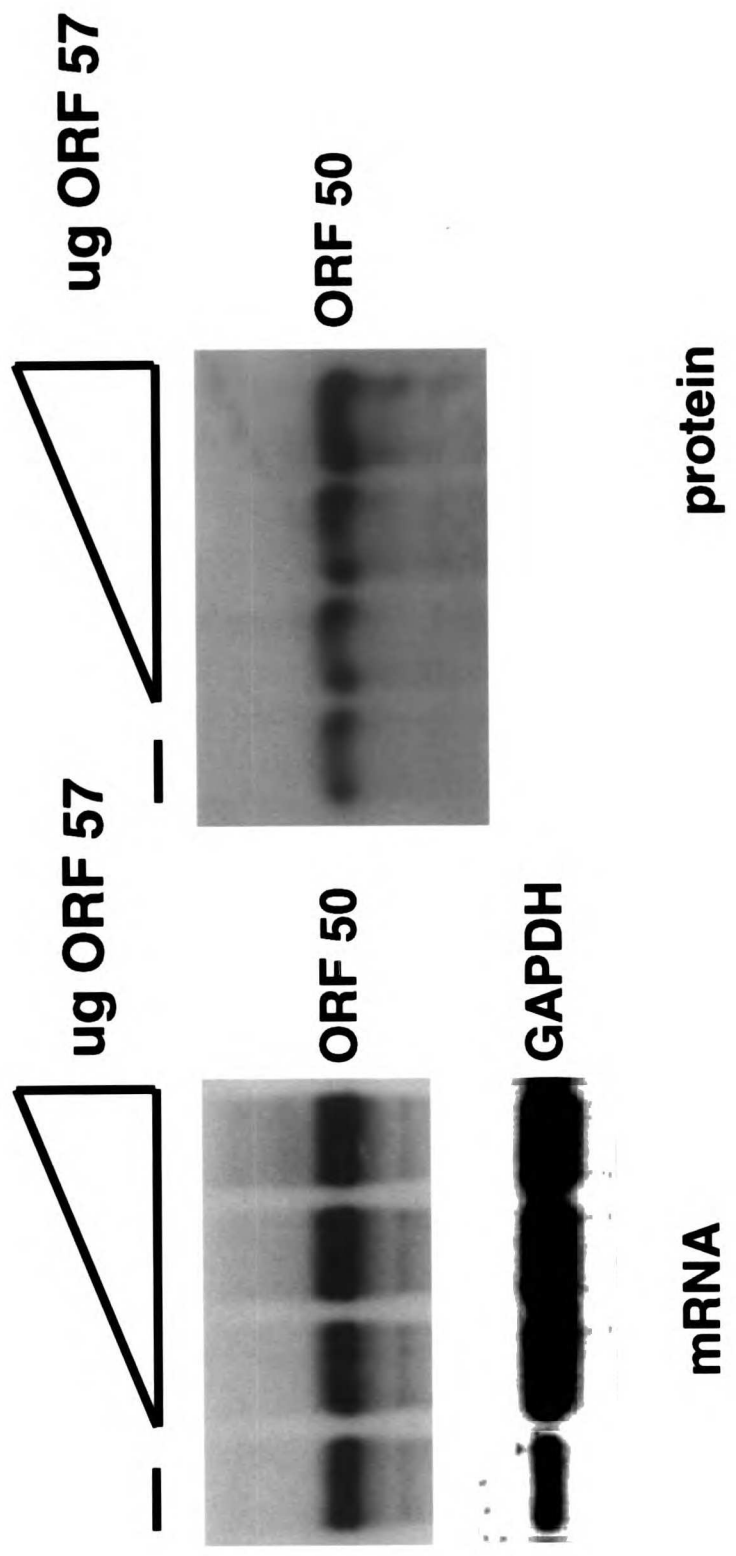
mRNA

FIG.7. ORF 50 mRNA and protein levels are not significantly increased by ORF 57 expression. (A) Top, autoradiogram of a Northern blot containing total RNA from CV-1 cells transfected with a fixed amount of pcDNA 3.1ORF 50 and the following amounts of pcDNA3.1ORF 57 : 0 ug (lane 1) 0.75ug (lane 2), 2.5 ug (lane 3), and 7.5 ug (lane 4). An antisense riboprobe for ORF 50 was hybridized to the blot. Bottom, the blot was reprobed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific probe as a loading control.

(B) Western blot analysis of protein extracts from CV-1 cells transfected with a fixed amount of pcDNA 3.1 ORF 50 and the following amounts of pcDNA3.1 ORF57: 0 ug (lane 1), 0.5 ug (lane 2) 2.5 ug (lane 3) and 5 ug (lane 4). The ORF50 protein was detected with a polyclonal antibody, and then with a secondary rabbit anti-mouse antibody conjugated to HRP and ECL (Amersham). Equal amount of extracts as determined by Bradford assay from each transfection were loaded onto the gel.
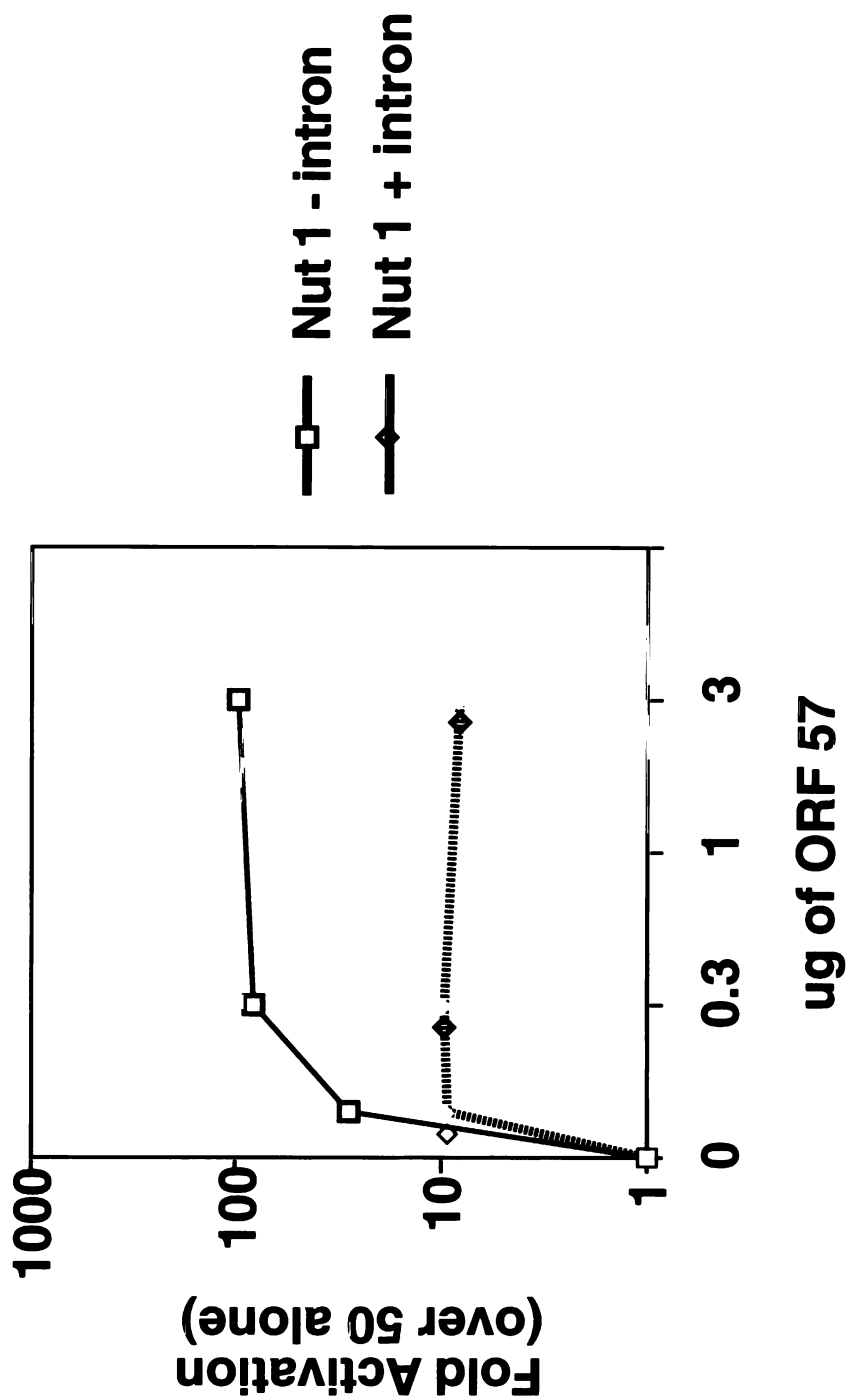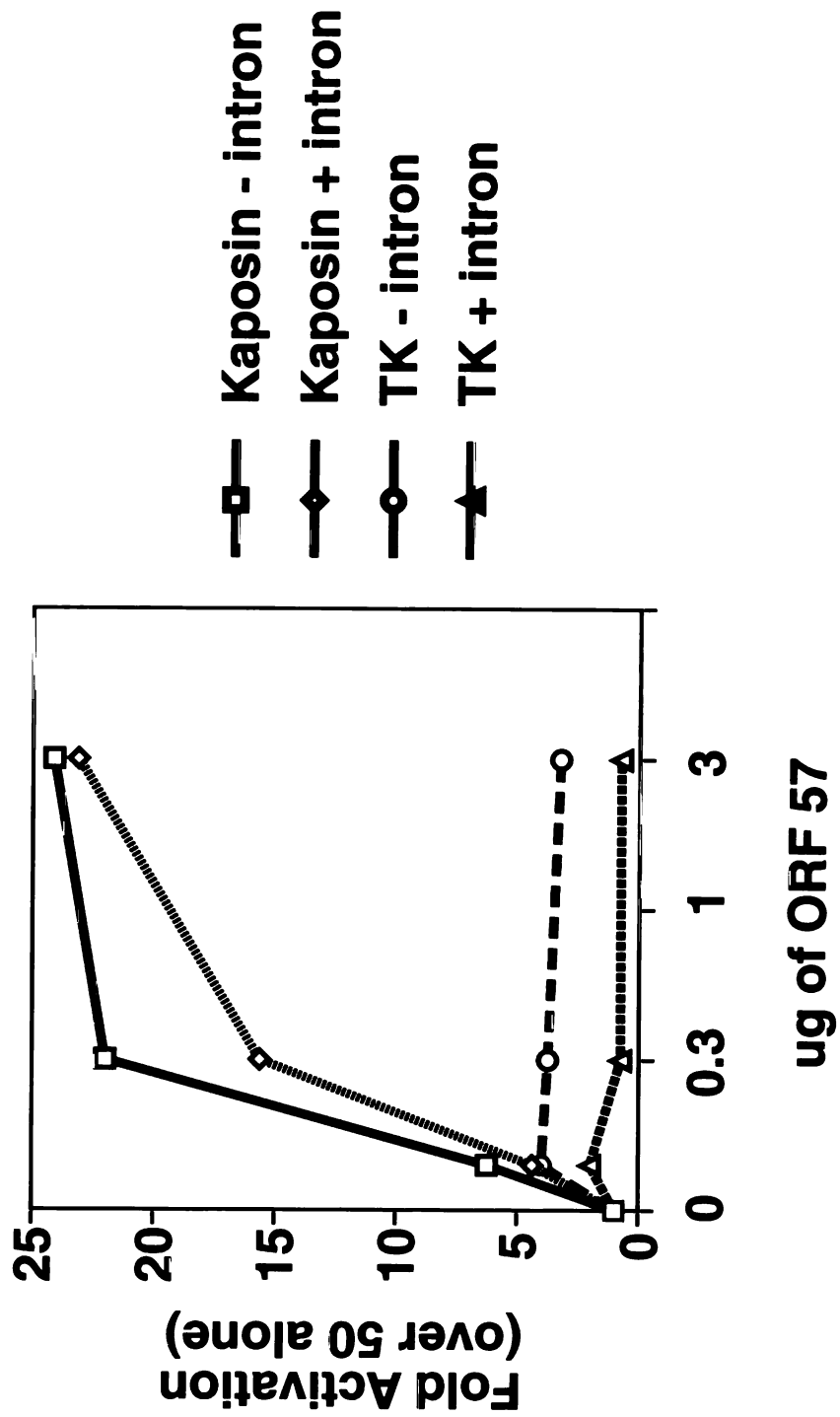
Figure 8a

Figure 8b

Legend:
- □ Kaposin - intron
- ◇ Kaposin + intron
- ○ TK - intron
- ▲ TK + intron

Y-axis: Fold Activation (over 50 alone)

X-axis: ug of ORF 57

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FIG.8. Effect of introns on synergistic activation of different promoters by the ORF 50/57 combination. CV-1 cells were co-transfected with fixed amounts of the indicated KSHV promoter constructs with or without introns (Nut 1, TK and Kaposin), a fixed amount of the transcriptional activator, pcDNA 3 ORF 50 , and increasing amounts of pcDNA 3.1 ORF 57 (0,0.3,1, and 3 ug). Luciferase activity was measured 48 hours after transfection. (A) Log scale graph displaying the effects on the Nut-1 promoter with (rectangles) and without (diamonds) an intron. (B) Graph displaying the effects on the Kaposin and TK promoters with (diamond, triangle) and without (rectangle, circle) an intron. Values are plotted as fold luciferase activity over that of ORF 50 alone, which was set to 1. Error bars representing standard deviation are from two experiments performed in duplicate. Note that activation is plotted on an arithmetic scale in B, log scale in A.

Chapter 4

Characterization of ORF 57's functional domains

INTRODUCTION

Kaposi's Sarcoma-associated herpesvirus (KSHV) is a novel lymphotropic herpesvirus that is implicated in the pathogenesis of Kaposi's Sarcoma (KS), as well as some B-cell lymphomas (5), (9), (for reviews, see references (11, 27)). KS, an endothelial tumor with neoangiogenic and inflammatory components, is the most common neoplasm of AIDS patients. KSHV DNA is found in almost all spindle (endothelial) cells of KS lesions (8, 30), as well as in circulating B-cells and possibly some tumor infiltrating monocytes (1, 6, 22, 32). KSHV infection of spindle cells is predominantly latent, but there are several pieces of evidence that the lytic cycle contributes significantly to KS pathogenesis. First, lytic replication is evident in a subset of cells within the tumor (23, 24, 30). Second, AIDS patients at risk for KS have a decreased incidence of KS when treated with ganciclovir, a drug that specifically blocks lytic viral replication (19). Third, several lytic cycle gene products (GPCR, vIL-6, v-MIPs) can stimulate inflammatory and angiogenic responses in surrounding cells and tissues (2, 3, 7). Finally, it is likely that lytic replication is also required for spread of the virus from its putative lymphoreticular reservoir to target endothelial cells. For these reasons, examination of regulation of the lytic cycle is likely to contribute importantly to our understanding of KS pathogenesis.

KSHV lytic replication follows a temporal cascade of gene expression, similar to that of all herpesviruses. IE genes are the first to be transcribed, and most encode regulators of gene expression. The expression of IE genes leads to the upregulation of DE genes, many of whose products are required for viral DNA replication. After replication, late genes, primarily encoding virion structural proteins, are expressed. In KSHV infected B-cells, ORF 50 and ORF 57 are two of the earliest genes to be transcribed. ORF 50, a potent transcriptional activator, can induce the switch from latent to lytic KSHV to replication in latently infected B cells (18), (17), (31). Recently, we (15) and others (4, 12) have demonstrated that ORF 57 is a nuclear protein that encodes a posttrancriptional regulator with multiple distinct activities. Our data showed that ORF 57 expression increased accumulation of KSHV DNA polymerase processivity factor (PF/ORF 59) and nut-1 RNA's in a specific and promoter-independent manner. Furthermore, co- expression of ORF 57 with ORF 50 results in synergistic upregulation of the nut-1 promoter, without increasing levels of ORF 50 mRNA or protein (15). ORF 57 has also been shown to enhance expression of the cellular RNA KDR/flk-1 (VEGF receptor) in a post-transcriptional manner (12). Although the steady state cellular localization of ORF 57 appears to be nuclear, Bello et al (4) have shown that ORF 57 can shuttle from the nucleus to the cytoplasm.

KSHV ORF 57 is a homologue of post-transcriptional regulators in other

herpesviruses, including ICP 27 of Herpes Simplex Virus, BMLF-1 of EBV and HVS

ORF 57. ICP 27 is a pleiotropic regulator whose functions include downregulation of

intron-containing genes, and upregulation of some late-transcripts (26, 29). Although

there are important functional differences between KSHV ORF 57 and these homologs,

they share several putative functional domains including; arginine rich regions at their

amino termini, and a leucine zipper and cysteine/histidine rich zinc finger/knuckle at the

carboxy terminus. Both arginine rich motifs and zinc finger motifs are potential RNA

binding motifs, and both ICP 27 and BMLF-1 have been shown to bind RNA; the

arginine rich regions of both ICP 27 and BMLF-1 are required for this RNA-binding (14,

28).

To determine which regions of ORF 57 were associated with the various functions

of ORF 57, we constructed a series of ORF 57 mutants affecting these conserved

domains. The mutated forms of ORF 57 were then tested in our assays for ORF 57

function: upregulation of nut-1 and ORF 59 RNA, and synergy with ORF 50 on the nut-1

promoter. The conserved zinc-finger at the C-terminus as well as an arginine rich region

at the N-terminus are necessary for augmentation of ORF 50 transactivation.

Interestingly, these mutations do not affect ORF 57's ability to increase levels of either

PF or nut-1 RNAs when normalized for comparable levels of protein expression. The

arginine rich region can also function as an NLS, but interestingly, the protein bears a

second element in its N-terminal region that can also mediate nuclear localization.

MATERIALS AND METHODS

Cell lines, plasmids and probes.

CV-1 cells were propagated and maintained in Dulbecco's modified Eagle medium H2

DME-H21 medium) supplemented with 10% fetal calf serum and penicillin-streptomycin

at 37°C in 5% CO_2. Probes for ORF 59/ DNA polymerase proccessivity factor (PF) and

nut-1 used in Northern blot analysis were created as described in (16).

pcDNA 3.1 N223 was created by digesting pcDNA 3.1 57 (18).

with Bam HI and Eco RV, and ligating the resulting fragment into pcDNA 3.1 V5B

(Invitrogen) that had also been digested with Bam HI and Eco RV. pcDNA 3.1 C223 57

was created by using PCR to amplify the 3' half of ORF 57 from pcDNA57 using the

following primers which introduced an Eco RV site at the 5' end, and a Sfu I site at the 3'

end: 57 V5 (5' GCGATATCACCATGATCACCGCTCTCATAAG) and 57V3 (5'

GCGGGTTCGAAAGAAAGTGGATAAAAG). The resulting PCR fragment was

digested with Eco RV and Sfu I and cloned into pcDNA 3.1 V5 A that had been digested

with Eco RV and Sfu I. Consecutive rounds of PCR were used to create the M4 alanine

substitution in pcDNA 3.1 57 N223 vector. First, the following primers were used to

amplify the 5' half of the desired fragment with pcDNA 3.1 57 as a template: M4-1

(contains a Bam HI site)(5' GCGGATCCATGGTACAAGCAATGATAG) and M4-2 (5'

GCGTGGAGCGGCAGCAGCGGCCTTTTCAGGGGGAGGACG). Then the following

primers were used on the same template to amplify the 3' half of the desired fragment:

M4-3 (GAAAAGGCCGCTGCTGCCGCTCCACGCGACCGCCTACAA) and M4-4 (5'

GCTCTAGAATCCCTGTCCGTAAACACCTC)(contains a Xba 1 site). These PCR

fragments were purified using QiaQuick PCR purification kit (Qiagen) and 1/50th of the

reaction from each was used as a template in a third round of PCR using the primers M4-

1, and M4-4. The resulting fragment was digested with Bam HI and Xba I and ligated

into pcDNA 3.1 V5A which had been digested with Bam HI and Xba I. To create a full

length version of ORF 57 with the M4 mutation and a V5 tag at the C-terminus, PCR

amplification using the following primers M4 5' (5'

GCGGATCCATGGTACAAGCAATG) and

M4 3'(5' GCGATATCCCTGTCCGTAAAC) was used to amplify the 5' half of ORF 57

containing the M4 mutation from pcDNA N223 M4. The resulting fragment was first

digested with Bam HI and Eco RV and cloned into pcDNA 3.1 57 V5 which had also

been digested with Bam HI and Eco RV. To create pcDNA 57 C/H, we used consecutive

rounds of PCR. The following two sets of primers were used to amplify from two

fragments containing the substitution from the template :

pcDNA3.1 57, Z1 (5' GACAGGGATATCACCGCTCTCATAAG) ,

Z2 (5' GTTTCTGGCCAAGCTGTGAGCTTCCATTACTATCACG) and Z3 (5' GCCAGAAACAGTGAAGCTGCAGCGGCAACCCGGGCC) and Z4 (5' GGCCCTCTAGACTCGAGCGGCCGCCAC). The resulting PCR fragments were purified using QiaQuick PCR purification kit (Qiagen) and 1/50th of the reaction from each was used as a template in the final round of PCR using the primers Z1 and Z4. The resulting fragment was digested with Nhe I, and Xba I, and ligated into pcDNA 3.1ORF 57 that had also been digested with Nhe I and Xba I . pRSET C57 was generated by subcloning the Eco RV – Xho fragment of ORF 57 cDNA (15) into pGEX 4T 2(Pharamacia) also digested with Eco RV and Xho I. Next, the ORF 57 fragment was released with Bam HI and Xho and cloned into the Bam HI and Xho I sites of pRSET A (Invitrogen). To create the GFP -ORF 57 series of fusion proteins, we used PCR to amplify various fragments of the ORF 57 genomic sequence with a set of primers, one with a Xho I site (for the 5' primer) and one with either a Bam HI, or Not 1 site (for the 3' primer). The PCR products were cloned into pEGFP (Clonetech) which had been digested with Xho I, and either BamHI, or Not I. To create the GFP 1-3 series, we used pcDNA 3.1 ORF 57 as a PCR template, for GFP 4, 5 and 6 used we used GFP 3 as a template and for GFP 1 M4, GFP 3 M4 we used pcDNA 57 N223 M4 as a template. The final vectors all contained ORF 57 sequences cloned in frame with the GFP open reading frame. Following is a list of primers used for each GFP-57 fusion vector.

GFP 57 1, GFP 57 1 M4 - 5' GCCTCGAGATGGACTCTGTGTCCTCC;

5' GCGGATCCCGGTATTGTAGGCGGTC

GFP 57 2 - 5' GCCTCGAGATGGGTACCCAGTCGGCCC;

5' GCGGATCCCGGTATTGTAGGCGGTC

GFP 57 3, GFP 57 3 M4 – 5' GCCTCGAGATGCAAAACACACGTCCT;

5' GCGGATCCCGTGCAGCGCGACACTG

GFP 57 4 - 5' GCCTCGAGATGGGTACCCAGTCGGCCC;

5' GCCTCGAGACCGGCGGTTCCAAAC

GFP 57 5 –5' GCGCCTCGAGGATATCATGATCCCAAAAAAATCTCA;

5' TATAGCGGCCGCTTTACTTGTACAGC

GFP 57 6 – 5' GCGCCTCGAGGATATCATGATCCAGTTTTGCTCCCC;

5' TATAGCGGCCGCTTTACTTGTACAGC

GFP 57 7 - 5' GCGCCTCGAGGATATCATGGTAAACAGGTACGGTAA;

5' TATAGCGGCCGCTTTACTTGTACAGC

All vectors were confirmed by DNA sequencing.

Transfections

All transfections were performed in CV-1 cells as in (15). For the luciferase assays, 4 ug

of total plasmid DNA was used, for amounts of effector plasmids, see figure legends. For

Northern analysis, 10 ug of plasmid DNA was transfected, see figure legends for plasmid amounts.

Western blotting and Antibodies

Western blotting was performed essentially as described in Kirshner et al (15) with the following changes; 30 ug of protein extract was loaded on the gel, and anti-V5 antibody (Invitrogen) was used at a 1:5000 dilution. Rabbit antiserum for ORF 57 was generated vs the His-tagged C-terminal half of ORF 57 expressed from pRSET C57 (Animal Pharm Services, Healdsburg, CA). Where anti-ORF 57 was used, a 1:2500 dilution of the antiserum was used in detection, and goat-anti-rabbit secondary antibody conjugated to HRP was used to visualize the protein.

Northern blotting

Transfections were performed as above in 10-cm plates, and 48 hours post-transfection, total RNA was isolated using RNAWiz (Ambion). 10 ug of RNA was separated on the gel, and randomly labeled probes for ORF 59/PF or nut-1 were used. Blots were exposed to Kodak XAR film for 2 days.

Immunofluoresence

Immunofluorescence was performed essentially as described in (15), with the following

modifications. CV-1 cells were plated to 60 % confluency on glass cover slips and

transfected with 2 ug of plasmid DNA using Superfect (Qiagen). 48 hours after

transfection, cells were washed with PBS and then fixed for 30 min in fresh 4%

paraformaldehyde. Next, the cover slips were mounted with medium containing DAPI

(Vectashield) on slides, and GFP was visualized using a fluorescence microscope.

RESULTS

Construction and expression of ORF 57 mutants

ORF 57 can affect gene expression at several levels, but the mechanism(s) by which ORF

57 functions is unknown. To further our understanding we aimed to define functional

domains of the protein. Analysis of the predicted amino acid sequence of ORF 57

revealed several regions that contain interesting potential functional motifs (Fig. 1A).

Near the amino terminus of ORF 57 is an arginine/proline rich region (aa113 to 133)

containing the sequence RPRRR (aa122-127). Such sequences are often involved in

nuclear localization and/or RNA-protein interactions. At the carboxy terminus, there is a

putative leucine zipper and cysteine-histidine rich zinc knuckle (C/H), a motif implicated

in RNA binding and splicing factors (10). To determine the importance of the arginine

rich region, we used an overlapping PCR technique to mutate the RPRRR residues to

alanines (Fig. 1 A) in pcDNA ORF 57. The most highly conserved region between ORF

57 and its herpesviral homologs is a cysteine-histidine rich putative zinc knuckle at the C-

terminus (Fig 1A). To examine the role of this element in ORF 57 function, we created a

version of pcDNA ORF 57 that substituted alanines for the conserved histidine and

cysteine residues that form the putative zinc finger (see Fig 1A).

To investigate the levels of expression of these mutated ORF 57 proteins, we transfected

CV-1 cells with equal amounts of either vector alone, ORF 57, and either ORF 57 M4 or

ORF 57 C/H (Figs 1B,C). 48 h post-transfection, whole cell extracts were harvested, and

30 ug of protein extract was examined by western blotting, using the ORF 57 antiserum.

Figures 1B and 1C (and data not shown) demonstrate that both mutants are expressed, but

at ca. 4- fold lower levels than the wild type ORF 57.

The C-terminal putative zinc finger is required for synergy of ORF 57 with ORF 50

Among all the homologs of ORF 57, the C-terminal region is the most highly conserved

functional domain. Specifically, the C-terminal zinc-finger in ICP 27 is required for its

effects on transactivation and redistribution of splicing factors (13, 20, 25). To investigate

the role of ORF 57's putative zinc finger on synergy with ORF 50, we transfected

pcDNA ORF 50 and the nut-1 promoter-driven luciferase reporter (pnut-Luc) with either

pcDNA ORF 57 wt or pcDNA ORF 57 C/H mut in CV-1 cells. Cell extracts were

collected 48 h after transfection and luciferase levels were measured. The results of this

assay are shown in Fig 2A. As expected, the wt ORF 57 is able to increase luciferase

activity 60 fold over that of ORF 50 alone. Strikingly, when the 57 C/H mutant is

expressed with ORF 50, the observed synergy is abolished. Although wild type ORF 57

is expressed 4-5 fold more efficiently than the C/H mutant, this difference cannot account

for the loss of activity of the mutant. When plasmid concentrations in the transfection are

adjusted to generate equal levels of both wild type and C/H mutant proteins, (as shown in

figure 3C), a large differential in activity continues to be observed; in fact increasing

levels of the C/H mutant above that of wt ORF 57 still do not result in synergy with ORF

50 (Fig. 2B). This indicates that the lower levels of expression of ORF 57 C/H do not

account for its inability to synergize with ORF 50, but rather that the zinc finger is

required for this function. Finally, co-expression of ORF 57 C/H with wt ORF 57 does

not impair the wild type protein's ability to enhance ORF 50 transactivation (Fig 2A),

indicating that the 57 C/H mutation is not a dominant-negative allele.

Effects of ORF 57 C/H on RNA accumulation

We next examined the ability of the 57 C/H mutant to stimulate transcript accumulation.

Equal amounts of pcDNA empty, pcDNA 57 or pcDNA 57 C/H plasmids were

transfected with pcDNA nut-1 in CV-1 cells. Total RNA was isolated 48 h post-

transfection, and Northern blotting was performed. Figure 3A is an autoradiogram of a

Northern blot probed with a ds DNA probe for nut-1. While ORF 57 wt is able to

increase nut-1 RNA levels compared to vector alone (left panel, lanes 1,3), ORF 57 C/H

seemed to be impaired in this ability (lane 2). Similar results were obtained when the

same experiment was performed using the PF/ORF 59 as a target RNA (3A, right panel).

However, in these experiments, although eqivalent effector plasmid levels were used, the

protein levels were not equal: there was 4-5 fold more wild type ORF 57 than mutant (see

Fig.1C). To determine whether the disparity in protein amount was the cause of the

reduced activity of the mutant, we repeated the experiment, but this time adjusted the

plasmid concentrations to obtain equal levels of protein expression(see Fig. 3C). Shown

in Figure 3B, when plasmid levels are adjusted (1.5 ug of wt ORF 57 vs. 7.5 ug of 57

C/H), the mutant protein is able to result in accumulation of both nut-1 and PF RNAs at

wt levels. This indicates that the putative zinc finger is not required for ORF 57's ability

to increase levels of target RNAs, and suggests that this function is genetically separable

from that required for synergy with ORF 50.

Effect of ORF 57 arginine/proline mutations on synergy with ORF 50

ORF 57 could be augmenting ORF 50 transactivation of the nut-1 promoter by several

different mechanisms, including but not limited to: interaction between ORF 50, ORF 57

and another viral, or cellular factor, and stability of unknown factors. In a further effort

to understand how ORF 57 and ORF 50 function together, we tested the ability of the

amino terminal mutant, M4, of ORF 57 to augment ORF 50's activation of the nut-1

promoter. We co-expressed either pcDNA empty, pcDNA 57, or pcDNA 57 M4 with the

nut-1 promoter luciferase reporter in CV-1 cells, using a transcriptionally saturating

amount of ORF 50 plasmid. 48 hours post-transfection, cell extracts were analyzed for luciferase activity. Fig. 4A shows the results of an experiment in which the results are plotted as fold luciferase activity over that of ORF 50 alone, which was set to 1. Again we observed that co-expression of ORF 57 and ORF 50 results in a high level of luciferase activity from the nut-1 promoter. The M4 mutant reduces 50/57 synergy by ca.90% . As with the C/H mutant above, these results cannot fully be accounted for by the 4 fold reduction of expression of this mutant (Fig 1C). When we repeated the experiment, and adjusted the plasmid concentrations to obtain equal levels of protein expression (Figs 4B,4C), synergy did not occur, even at ORF 57 M4 expression levels that are 10 fold over that of wild type ORF 57. Therefore the M4 region is also necessary for ORF 57's ability to augment ORF 50's activation.

Effect of ORF 57 amino terminal mutant M4 expression on RNA accumulation

Possibilities for the mechanism by which ORF 57 expression results in accumulation of both ORF 59 and nut-1 RNA include increased RNA stability and transport of RNA from the nucleus to the cytoplasm, both of which might require RNA binding. Since ORF 57 contains a region that resembles the arginine rich regions in the 5' end of HSV ICP 27 and EBV Mta (which are known to bind viral RNA) we wanted to investigate whether

that region in ORF 57 was involved in this effect. To this end, we transfected CV-1 cells

with pcDNA ORF 57, pcDNA ORF 57 M4 or vector alone with either pcDNA ORF

59/PF or pcDNA nut-1, and after 48 hours we isolated total RNA. Northern blotting

analysis was performed with 7.5 ug of RNA and the blots were probed with either PF or

nut-1 DNA probes. Figure 5A, left panel, shows the effects of ORF 57 and ORF 57 M4

expression on nut-1 RNA accumulation and Fig 5A ,right panel, shows their effects on

PF mRNA accumulation. In these experiments , where we have transfected equal

amounts of ORF 57 and ORF 57 M4 plasmids, it appears that the M4 mutant is unable to

increase accumulation of RNA. However, when the plasmid levels are adjusted to

generate equal levels of 57 and 57 M4 protein, (Fig 5B) we observed that the M4 mutant

is capable of increasing both nut-1 and PF RNA levels, though the accumulation is

slightly (ca. 2 fold) lower than that directed by wt ORF 57.

Expression of the C-terminal deletion mutants of ORF 57

In a further attempt to decipher the importance of putative functional domains at N and C

terminal halves of the protein, we created a series of deletions. First, we bisected ORF 57

using the EcoRV site at nt 82842 (aa.223), as this separated the putative functional

motifs. The 5' half(called N223) and 3'(C233) half were tagged with a V5 epitope to

enable us to monitor levels of protein expression using an anti-V5 antibody. We could

not use our antibody to ORF 57 to detect the N-terminal fragment as this antiserum was raised against the C-terminal half of the protein. We used an overlapping PCR technique to mutate the RPRRR residues to alanines (Fig. 1 A) in the context of the 5' half of the molecule. The resulting clone was called N223 M4. To examine the expression of these deletions/mutations, we transfected CV-1 cells with 4 ug each of pcDNA 57 V5, pcDNA N223, pcDNA C223 and pcDNA N223 M4. Whole cell extracts were isolated 48 hrs post-transfection and quantified by Bradford assay. Next, 30 ug of extract from each were examined by immunoblotting and detected using anti-V5 (Invitrogen) (Fig.6A). Interestingly, the N223 and N223 M4 proteins were expressed at a much higher level than full length ORF 57. We were unable to detect expression of the C223, even when 50 ug of extract was immunoblotted (data not shown). Interestingly, the C-terminal half of ICP 27 is also poorly expressed (33). To determine the relative amounts of expression of full length ORF 57 versus the mutants, we transfected cells with a 10-fold range of N223 and N223 M4 plasmid DNA. The results (data not shown) demonstrate that 10 fold less plasmid DNA is required for the mutants to be expressed at the same level as full length. As the various mRNA's are expressed from identical plasmids and promoters, it is likely that the accumulation is due to increased protein stability.

Effect of ORF 57 C- terminal deletions on synergy with ORF 50 and RNA accumulation

Next, we tested the ability of C-terminal deletion of ORF 57 to augment ORF 50's activation of the nut-1 promoter. We co-expressed them with the nut-1 promoter luciferase reporter in CV-1 cells, using a transcriptionally saturating amount of ORF 50 plasmid. Fig. 6B shows the results of an experiment where full length 57, N223 and N223 M4 were expressed with ORF 50 and the nut 1 reporter. 48 hours post-transfection, cell extracts were analyzed for luciferase activity. While 57 can significantly increase luciferase levels, N223 and N223 unable to synergize with ORF 50. This experiment was also performed with the V5 tagged versions of these mutants, and similar results were obtained (data not shown). However, the V5 tagged wt ORF 57 protein was less active than untagged ORF 57 reducing the dynamic range of the assay.

Effect of ORF 57 C- terminal deletions on RNA accumulation

CV-1 cells were transfected with plasmids expressing full length ORF 57, N223, N223 M4 or pcDNA 3.1 alone with pcDNA ORF 59/PF or pcDNA nut-1, and after 48 hours total RNA was examined by Northern blotting with the PF probe. Consistent with the phenotype of the 57 C/H mutant, the N223 fragment (Fig. 6C) retains significant ability to upregulate PF mRNA. This upregulation, however, is strongly impaired by the M4

mutation. Compared to the null phenotype of the full length M4 protein on PF mRNA accumulation (Fig. 5B), this suggests that the N-and C-termini contain redundant functions, both of which must be ablated to impair PF transcript accumulation.

Identifying the Nuclear Localization Signals

The relative preservation of the RNA accumulation function of ORF 57 was unexpected, given the hypothesized roles of arginine rich regions in RNA binding and nuclear localization. To better define the determinants of nuclear localization, we examined the ability of 57-GFP fusions to be transported to the nucleus. First, we bisected ORF 57 using the EcoRV site at nt 82842, and tagged the N-terminal half with a V5 epitope to enable us to monitor levels of protein expression using an anti-V5 mAb (our antibody to ORF 57 was raised against the C-terminal half of the protein). This protein was expressed in CV-1 cells and its localization determined by immunofluoresence. Both this protein (N223) and a derivative of it bearing the M4 mutation, (N223 M4) were efficiently localized to the nucleus (Fig 7). Thus, all the information needed for nuclear targetting resides in the N-terminus of the molecule. Next, we fused sequential amino terminal fragments to GFP (Fig. 8A) in the parent vector pEGFP and examined their subcellular distribution on transfected CV-1 cells. As expected, wild type GFP is dispersed throughout the nucleus and cytoplasm (Fig.8A, bottom panel). Adding various in-frame

fusions of ORF 57 re-localized the majority of the GFP to the nucleus. Fig 8A. shows that residues 17-133, when fused to GFP, (construct GFP 1), direct nuclear expression of GFP and a version of GFP 1 bearing the M4 mutation (GFP 1 M4) displays a similar distribution. Smaller fragments, GFP 2, (aa. 56-133) and GFP 3 (aa. 113-146) also directed GFP to the nucleus. However, when GFP 3 was re-created with the M4 mutation (GFP 3 M4), GFP expression was not limited to the nucleus, but was also present in the cytoplasm. This indicated that the M4 region is required for nuclear localization in this specific fragment of ORF 57. But since a longer version (GFP 1 M4) was nuclear, we presumed the existence of a second NLS in the region upstream of aa.113. To localize this NLS, we fused aa. 56-113 of ORF 57 to GFP to create GFP 4; as predicted this construct resulted nuclear fluorescence of GFP. A series of smaller sequential deletions of that region; GFP 5 (aa 76-113), GFP 6 (aa 84-113) and GFP 7 (aa 99-113) were constructed to map the second NLS of ORF 57, this is shown in figure 8B. GFP 5 and GFP 6 expression resulted in primarily nuclear localization of GFP. However, GFP 7 expression results in both nuclear and cytoplasmic expression of GFP similar to that of GFP alone. Thus, the second NLS at the amino terminus of ORF 57 is the proline/arginine rich region between aa 84 and 99: IQFCSPLSRPRSPSP. Other proteins with multiple NLSs have been identified, and in fact the ORF 57 homolog ICP 27 of HSV contains multiple basic NLSs (21). It is possible that the distinct NLSs in ORF 57

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operate at various efficiencies, or at different times, perhaps in combination to achieve

maximal efficiency, or perhaps they function in the different cell types that KSHV

infects. In fact, the second NLS appears to be less effective at relocating all of the GFP

to the nucleus than that defined by the M4 mutation.

DISCUSSION

ORF 57 is a posttranscriptional regulator with multiple activities. Here we have shown that the C-terminal zinc-knuckle region and the arginine rich N-terminal element are each required for synergy with ORF 50. Interestingly, these mutations singly do not impair ORF 57's ability to increase accumulation of either PF or nut-1 RNA. This suggests the presence of redundancy in this function which was further borne out by examination of the phenotype of the N223 fragment. As expected from the above, this fragment was unable to synergize with ORF 50, but still retained significant activity in transcript accumulation. The latter could be ablated by elimination of its arginine rich region. Redundancy is also present in the NLS function, with at least 2 functional NLSs residing in the N-terminal region alone.

In all the homologs of ORF 57, the C-terminal region is the most highly conserved functional domain, implying its functional importance. Consistent with this, the C-terminal zinc-finger in ICP 27 is required for its effects on transactivation and redistribution of splicing factors (13, 20, 25). Our findings are consistent with the importance of the C-terminus for at least one key function of ORF 57:synergy with ORF 50, but do not allow us to determine the underlying biochemical mechanism. Synergy with ORF 50 involves activation of that protein's transactivation activity by post-

translational means (15). One model would be complex formation between ORF 50 and ORF 57. To date, however, we have been unable to detect such complexes (15). Alternatively, ORF 57 expression might result in post-translational modification of ORF 50, and efforts to detect such modification are underway. Similarly essential but as yet undefined, the RPRRR motif in the N-terminus also plays a role in synergy between ORF 57 and ORF 50. Further definition of the biochemical function(s) of these elements will likely require the development of <u>in vitro</u> assays in which the multiple effects of ORF 57 on RNA metabolism can be reproduced and studied.

Acknowledgements
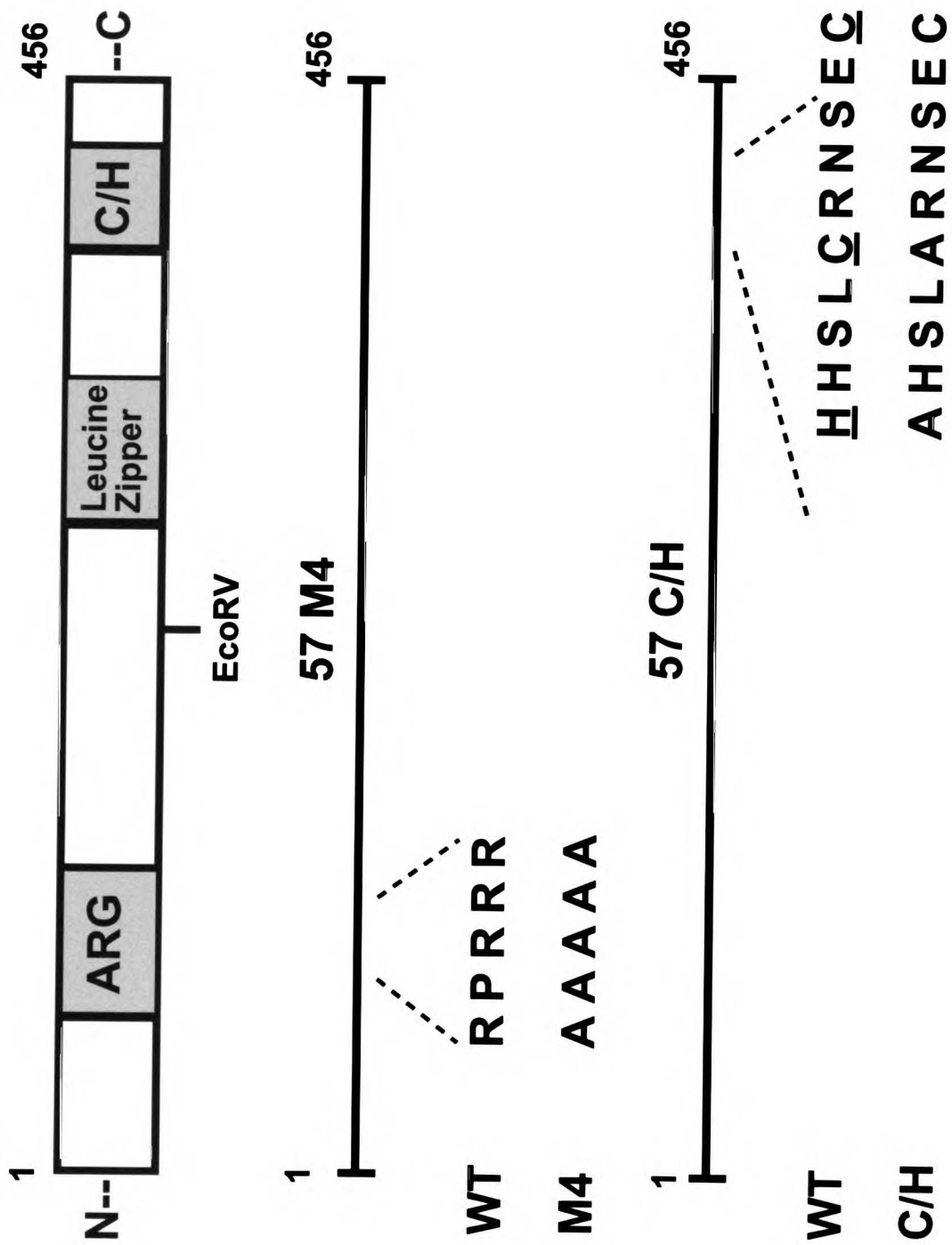
Figure 1a

Figure 1 b,c
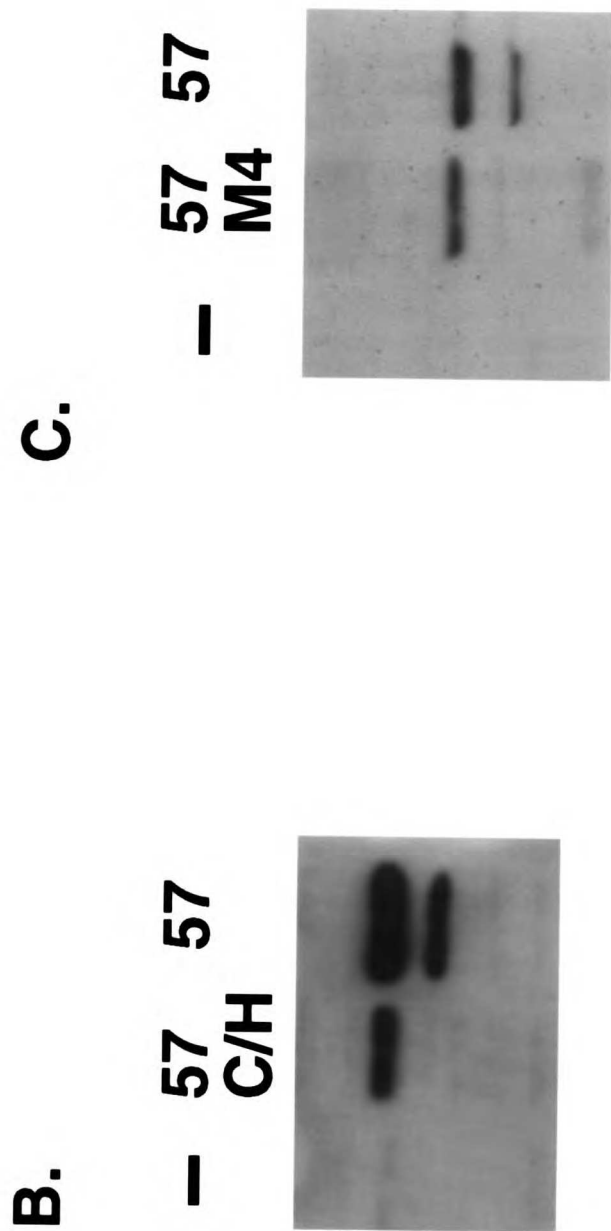
FIG.1.A. Diagram of ORF 57 putative domains and mutations. ORF 57 has several regions conserved among its herpesviral homologs. There is an arginine rich region at the amino terminus (ARG), and a putative leucine zipper and zinc-finger (C/H) region at the c-terminus. ORF 57 5' and ORF 57 3' truncations are shown. Below, the M4 mutation, which substituted alanines for the WT sequence, and the C/H mutation, which substituted alannes for key conserved residues (C,H), are indicated.

1 B,C. Western blot analysis of ORF 57 mutant protein expression levels. 30 ug of whole cell extract from CV-1 cells transfected with ORF 57 (B, C lane 3), ORF 57 C/H (B,lane 2), ORF 57 M4 V5 (C, lane 2) or pcDNA empty (B,C lane 1) was examined by immunoblotting. The proteins were detected by antisera to the C-terminus of ORF 57. And then with a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase and ECL (Amersham-Pharmacia). Equal amounts of extracts as determined by Bradford assay from each transfection were loaded onto the gel.

Figure 2a
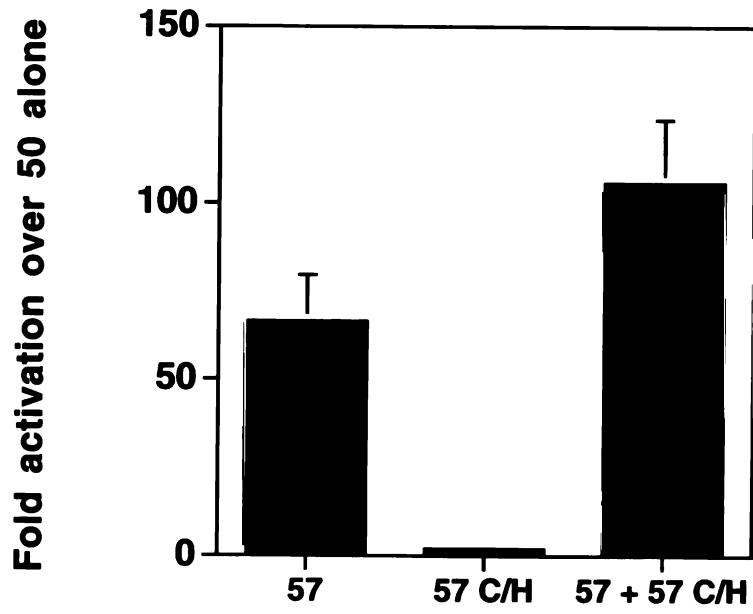
Figure 2b
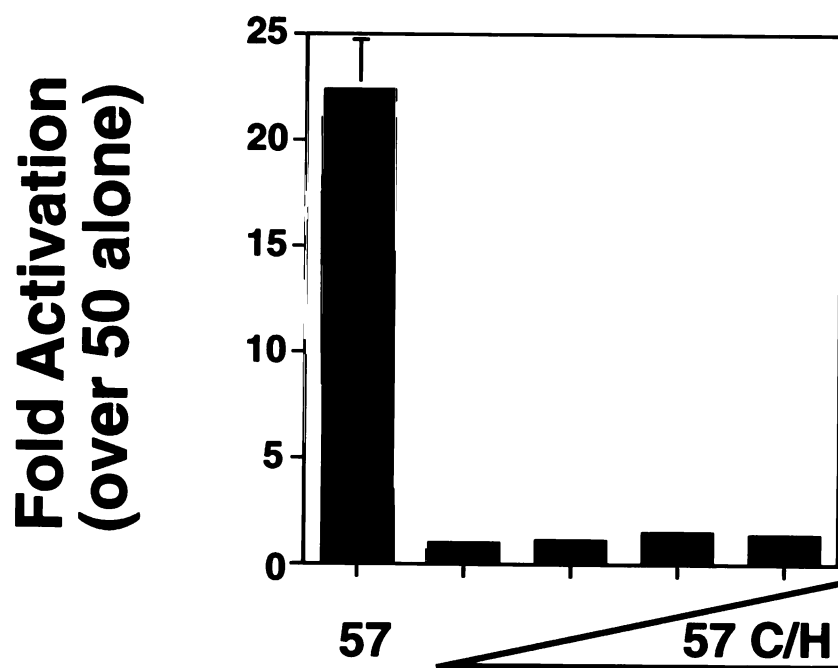
FIG.2 A,B, ORF 57 C/H mutant is severely impaired in its ability to synergize with ORF 50.

A. CV-1 cells were transfected with a fixed amount of the nut-1 promoter reporter (0.1 ug), ORF 50 (1 ug), and 1 ug of either pcDNA 3.1 57 (lane 1), pcDNA 3.1 57 C/H (lane 2), or 1 ug of each (lane 3). Luciferase activity was measured 48 h posttransfection and the values that represent fold activation relative to that of ORF 50 alone (which was set to 1) were plotted. Error bars represent the standard deviations from two experiments performed in duplicate.

B. CV-1 cells were transfected with a fixed amount of the nut-1 promoter reporter (0.1 ug), ORF 50 (0.5 ug), and either 0.3 ug of pcDNA 3.1 57 (lane 1), or 0.3 (lane 2), 1 ug (lane 3), 2 ug(lane 4) or 3 ug of pcDNA 3.1 57 C/H (lane 5). Luciferase activity was measured 48 h posttransfection and the values that represent fold activation relative to that of ORF 50 alone (which was set to 1) were plotted. Error bars represent the standard deviations from two experiments performed in duplicate.
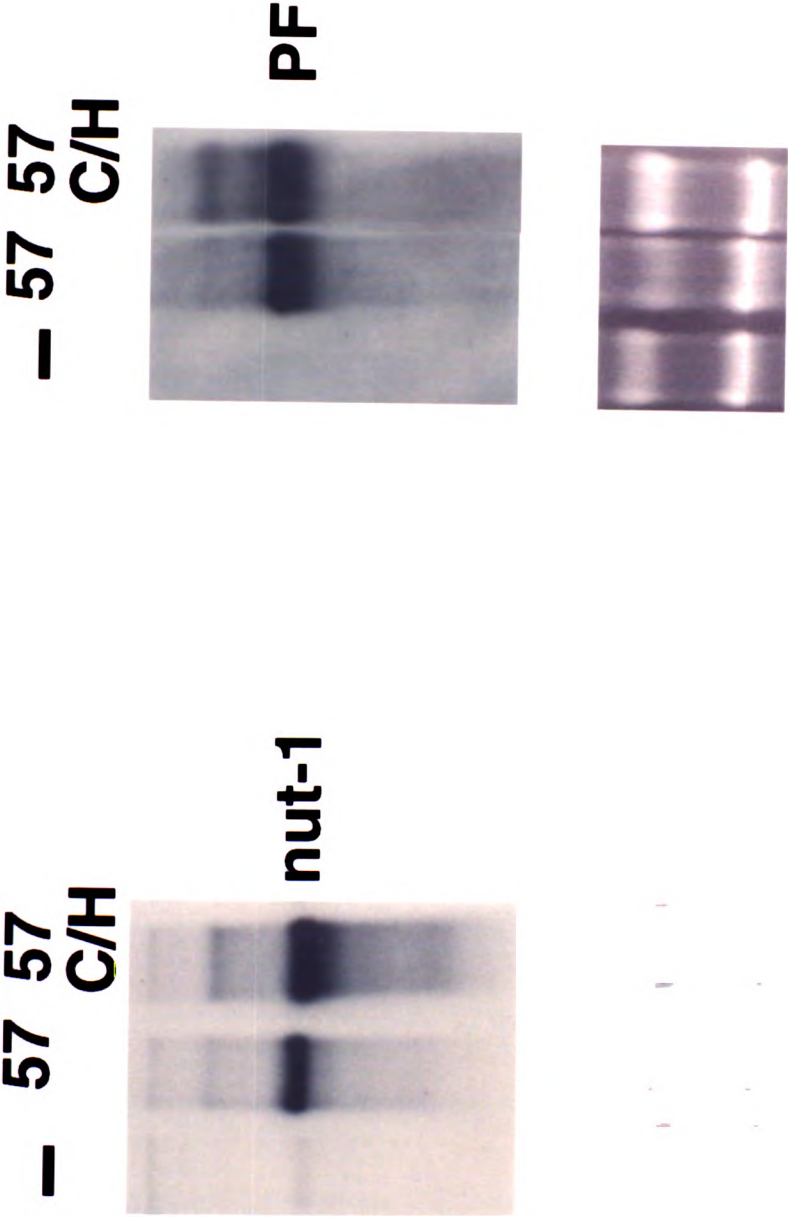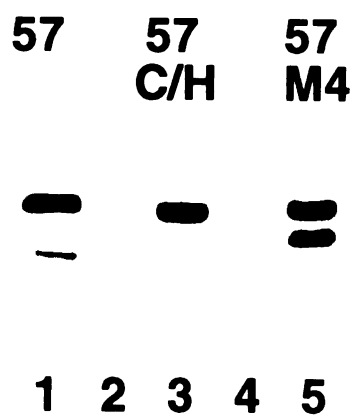
Figure 3a
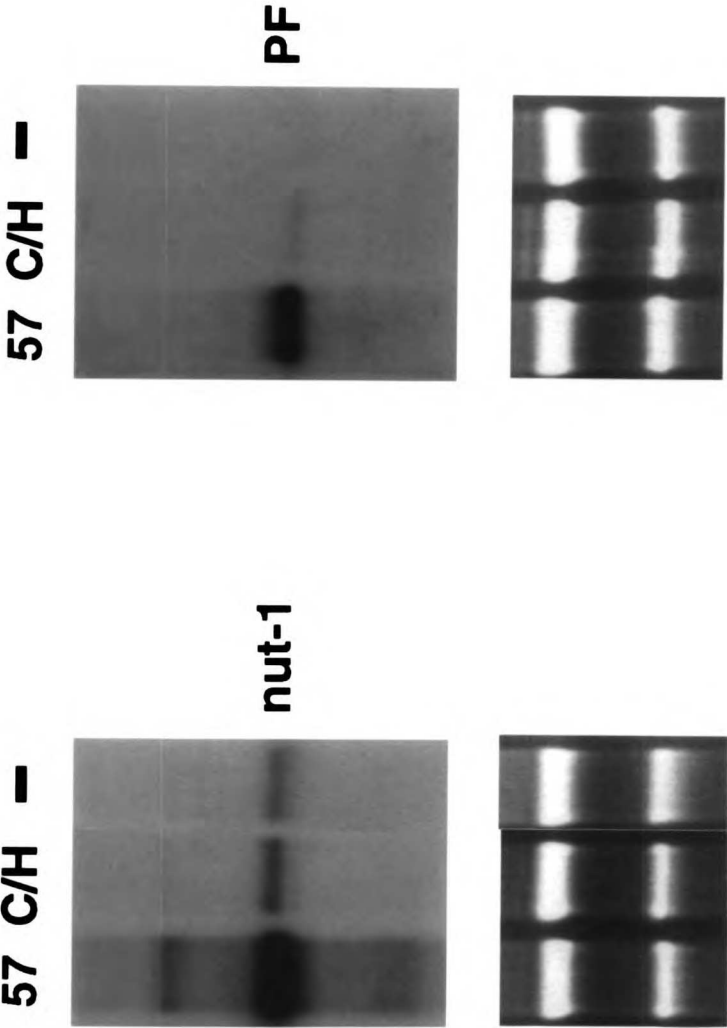
Figure 3b

Figure 3c

57   57   57
       C/H   M4

1 2 3 4 5

FIG.3 A,B,C. ORF 57 C/H mutant promotes accumulation of RNA.

A. Autoradiogram of a northern blot containing total RNA from CV-1 cells transfected

with 3 ug of (A: pcDNA nut-1 or B: pcDNA ORF 59) and either 3ug of pcDNA empty

(lane 1), pcDNA 57(lane 2) or pcDNA 57 C/H (lane 3). A ds DNA probe for nut-1 was

used; below, Et-Br stained ribosomal bands indicate amount of RNA loaded on gel. .

B. Autoradiogram of a northern blot containing total RNA from CV-1 cells transfected

with 2 ug of (left: pcDNA nut-1 or right: pcDNA ORF 59) and either 3ug of pcDNA

empty (lane 1), 1.5 ug pcDNA 57(lane 2) or 7.5 ug pcDNA 57 C/H (lane 3). ds DNA

probes for nut-1, PF were used; below, Et-Br stained ribosomal bands indicate amount of

RNA loaded on gel.

C. . Western blot of equal levels of mutant protein expression. CV-1 cells were

transfected as above (3b), and whole cells extract (40 ug) was examined by

immunoblotting. Lanes 1 and 3 correspond to lanes 1,2, in 3b, and lane 5 corresponds to

lane 3 in Fig 5b. The proteins were detected by antisera to the C-terminus of ORF 57. A

secondary goat anti-rabbit antibody conjugated to horseradish peroxidase and ECL

(Amersham-Pharmacia) was used. Equal amounts of extracts as determined by Bradford

assay from each transfection were loaded onto the gel.
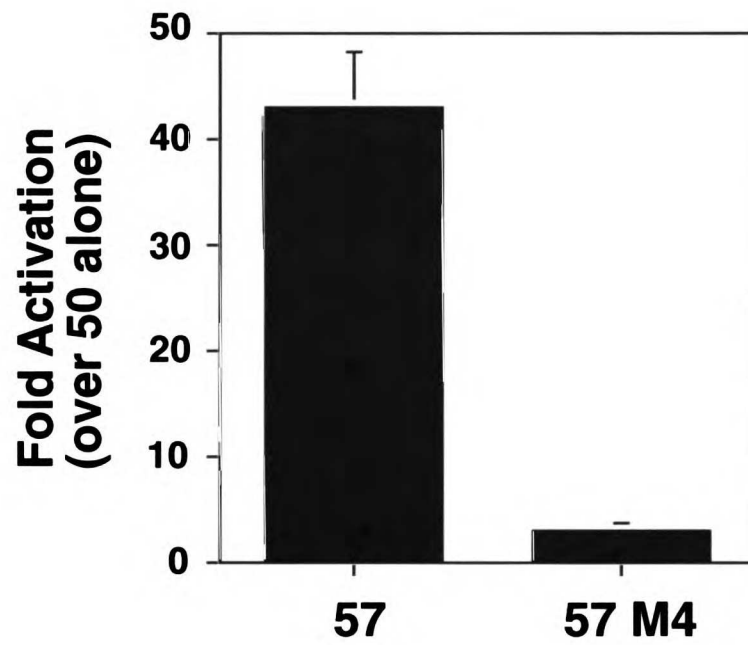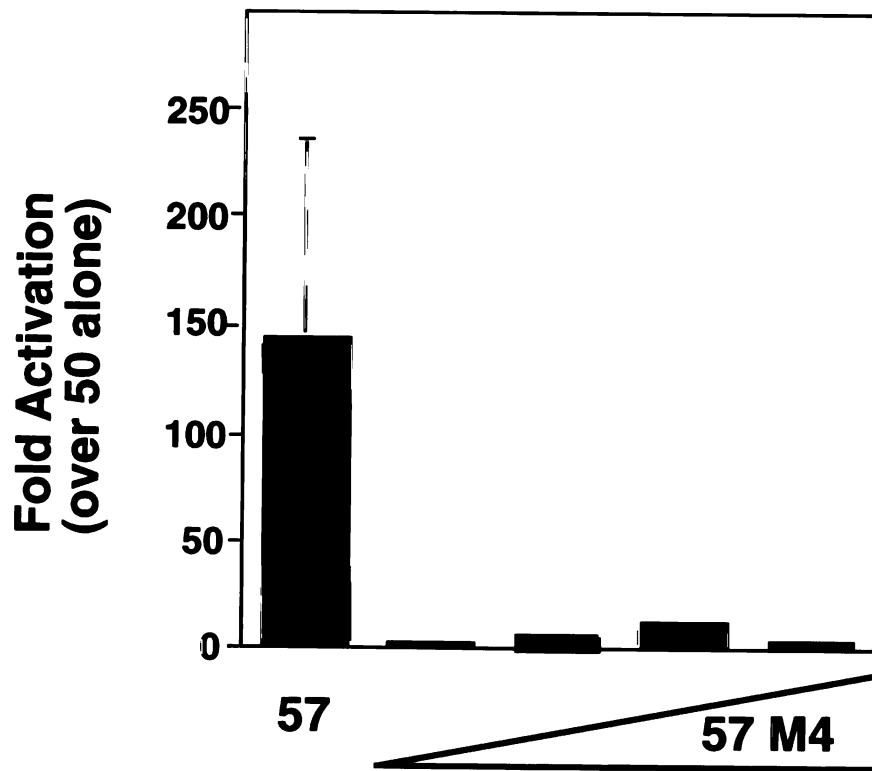
189

Figure 4a

Figure 4b

FIG.4A,B. ORF 57 M4 mutant is also impaired in its ability to synergize with ORF

50.

A. CV-1 cells were transfected with a fixed amount of the nut-1 promoter reporter (0.1

ug), ORF 50 (1 ug), and 1 ug of either pcDNA 3.1 57 (lane 1), pcDNA 3.1 57 M4 V5

(lane 2), or 1 ug of each (lane 3). Luciferase activity was measured 48 h posttransfection

and the values that represent fold activation relative to that of ORF 50 alone (which was

set to 1) were plotted. Error bars represent the standard deviations from two experiments

performed in duplicate.

B. CV-1 cells were transfected with a fixed amount of the nut-1 promoter reporter (0.1

ug), ORF 50 (0.5 ug), and 0.5 ug of either pcDNA 3.1 57 (lane 1), or 0.5 ug (lane 2) , 1

ug (lane 3), 2 ug (lane 4), or 4 ug (lane 5) of pcDNA 3.1 57 M4 V5 . Luciferase activity

was measured 48 h posttransfection and the values that represent fold activation relative

to that of ORF 50 alone (which was set to 1) were plotted. Error bars represent the

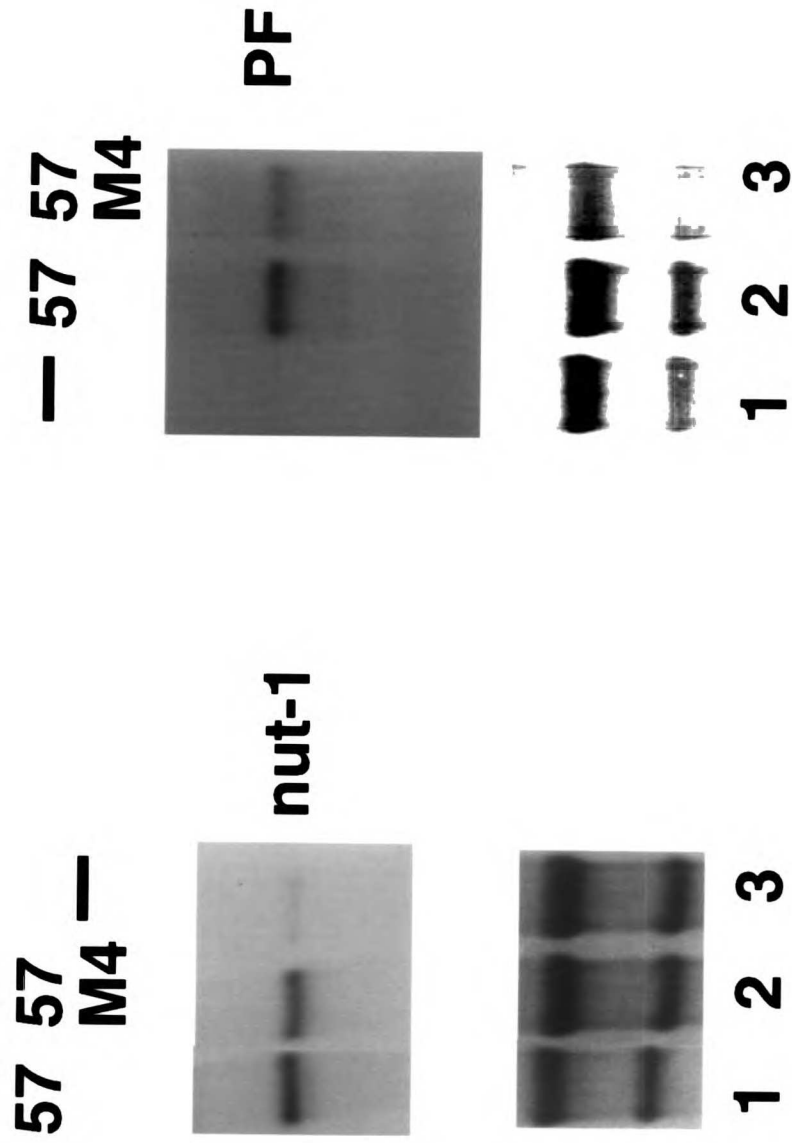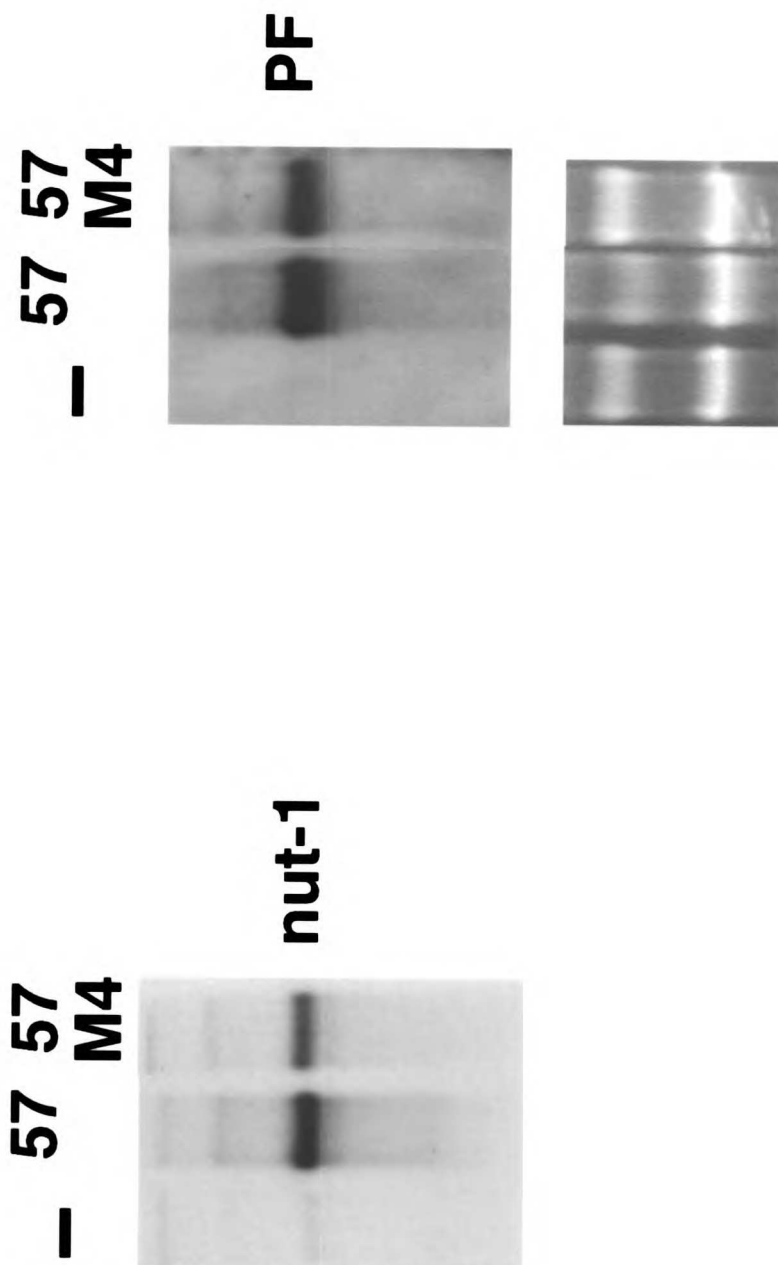standard deviations from two experiments performed in duplicate.

Figure 5 a

Figure 5b

FIG. 5 A,B. The ORF 57 M4 mutant promotes accumulation of RNA

A. Autoradiogram of a northern blot containing total RNA from CV-1 cells transfected with 3 ug of (A: pcDNA nut-1 or B: pcDNA ORF 59) and either 3 ug of pcDNA empty (lane 1), pcDNA 57(lane 2) or pcDNA 57 M4 V5(lane 3). Ds DNA probes for nut-1, and PF were used; below, Et-Br stained ribosomal bands indicate amount of RNA loaded on gel.

B. Autoradiogram of a northern blot containing total RNA from CV-1 cells transfected with 2 ug of (left panel: pcDNA nut-1 or right panel: pcDNA ORF 59) and either 3 ug of pcDNA empty (lane 1), 1.5 ug pcDNA 57(lane 2) or 6 ug of pcDNA 57 M4 V5 (lane 3). ds DNA probes for nut-1 and PF were used; below, Et-Br stained ribosomal bands indicate amount of RNA loaded on gel.
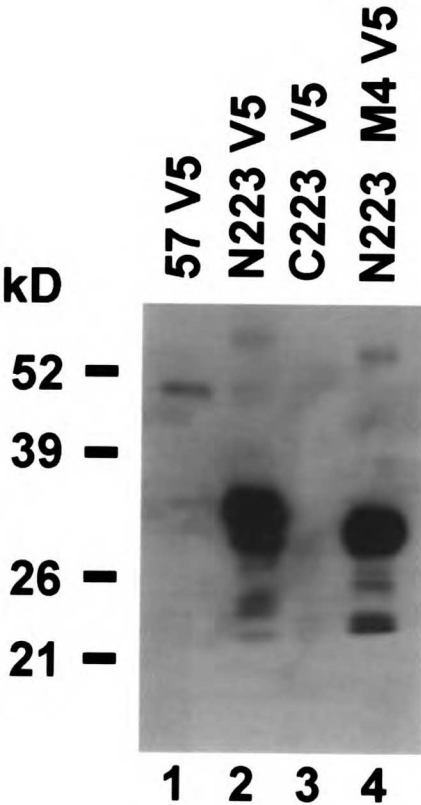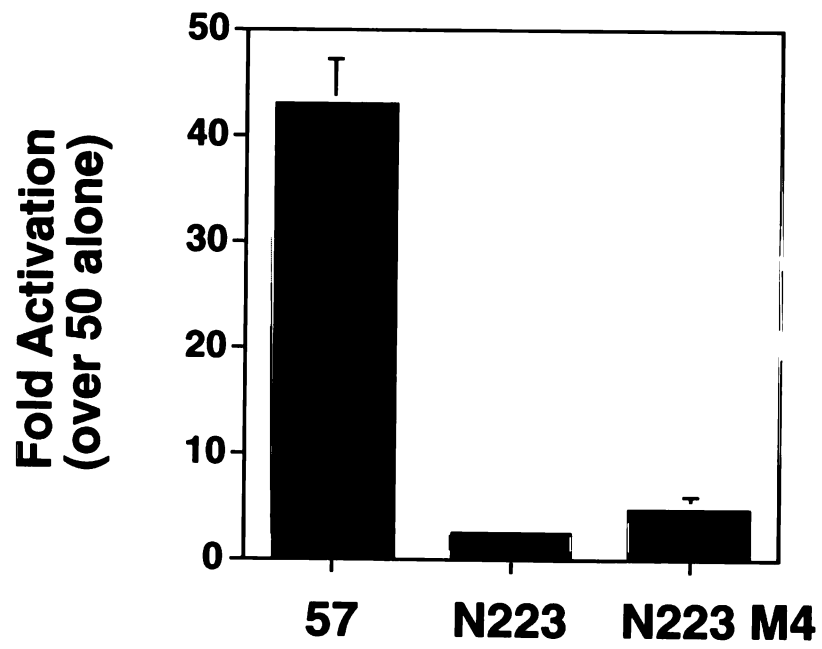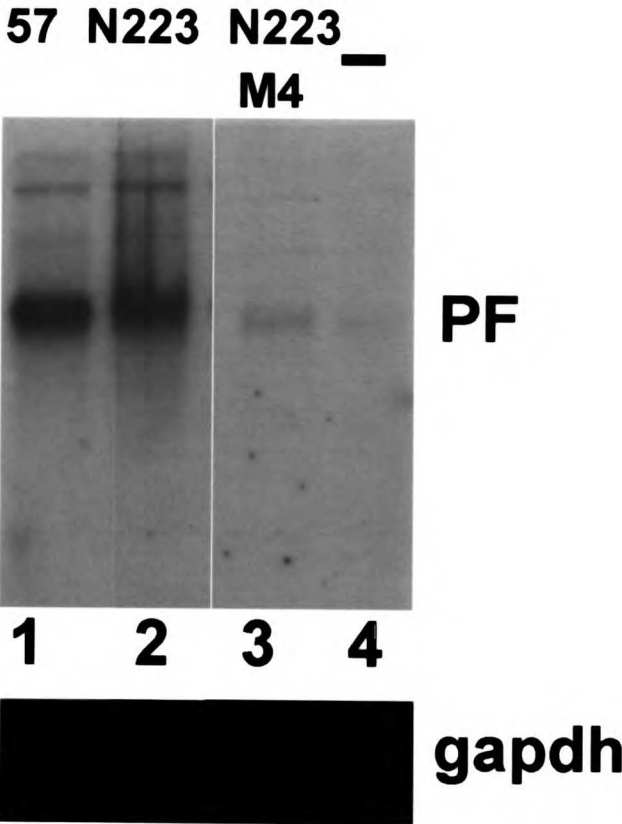
Figure 6a

Figure 6b

Figure 6C

FIG.6 A,B,C

Examination of C-terminal deletions of ORF 57

**A. Western blot analysis of ORF 57 truncation and mutant protein expression levels.**

30 ug of whole cell extract from CV-1 cells transfected with ORF 57V5 (lane 1), ORF 57

N223V5 (lane 2), ORF 57 C223 V5 (lane 3), or ORF 57 N223M4 V5 (lane 4) was

examined by immunoblotting. The V5-tagged forms of ORF 57 were detected by anti-

V5 antibody (Invitrogen), and then with a secondary rabbit anti-mouse antibody

conjugated to horseradish peroxidase and ECL (Amersham-Pharmacia). Equal amounts

of extracts as determined by Bradford assay from each transfection were loaded onto the

gel.

B. CV-1 cells were transfected with a fixed of the nut-1 promoter reporter (0.1 ug), and a

transcriptionlly saturating amount of ORF 50 (2 ug), and 2 ug of pcDNA 57, pcDNA 57

N223, or pcDNA N223 M4. Luciferase activity was measured 48 h post-transfection and

the values that represent fold activation relative to that of ORF 50 alone (which was set to

1) were plotted. Error bars represent standard deviation from three experiments

performed in triplicate.

C. Autoradiogram of a Northern blot containing total RNA from CV-1 cells transfected

with a fixed amount of either pcDNA 3.1 ORF 59/58(PF) with pcDNA ORF 57 V5 (lane

1), pcDNA ORF 57 N223 V5 (lane 2), pcDNA 57 N223 M4 V5 (lane 3) or pcDNA 3.1

empty (lane 4). A ds probe for PF was used; bottom panel, the blot was reprobed with a

probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading

control.

Figure 7

57 V5

N223 V5
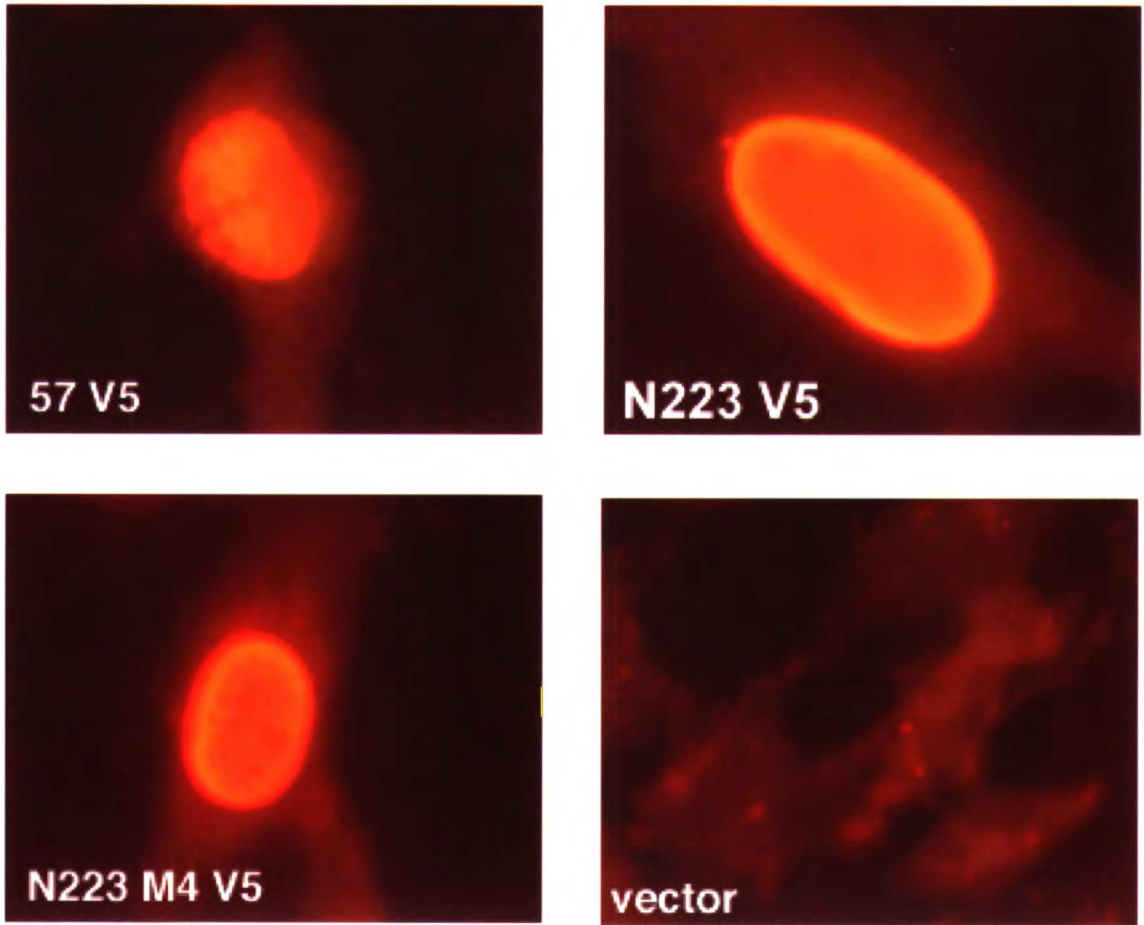
N223 M4 V5

vector

FIG.7. Localization of ORF 57 truncations and mutations.

The panels show immunoflouresence staining of CV-1 cells 48 h after transfection with

pcDNA 57 V5, pcDNA 57 N223 V5, pcDNA 57 N223 M4 V5 or vector alone. Reactivity

to anti-V5 antibody (Invitrogen) was detected with a TRITC-conjugated rabbit anti-
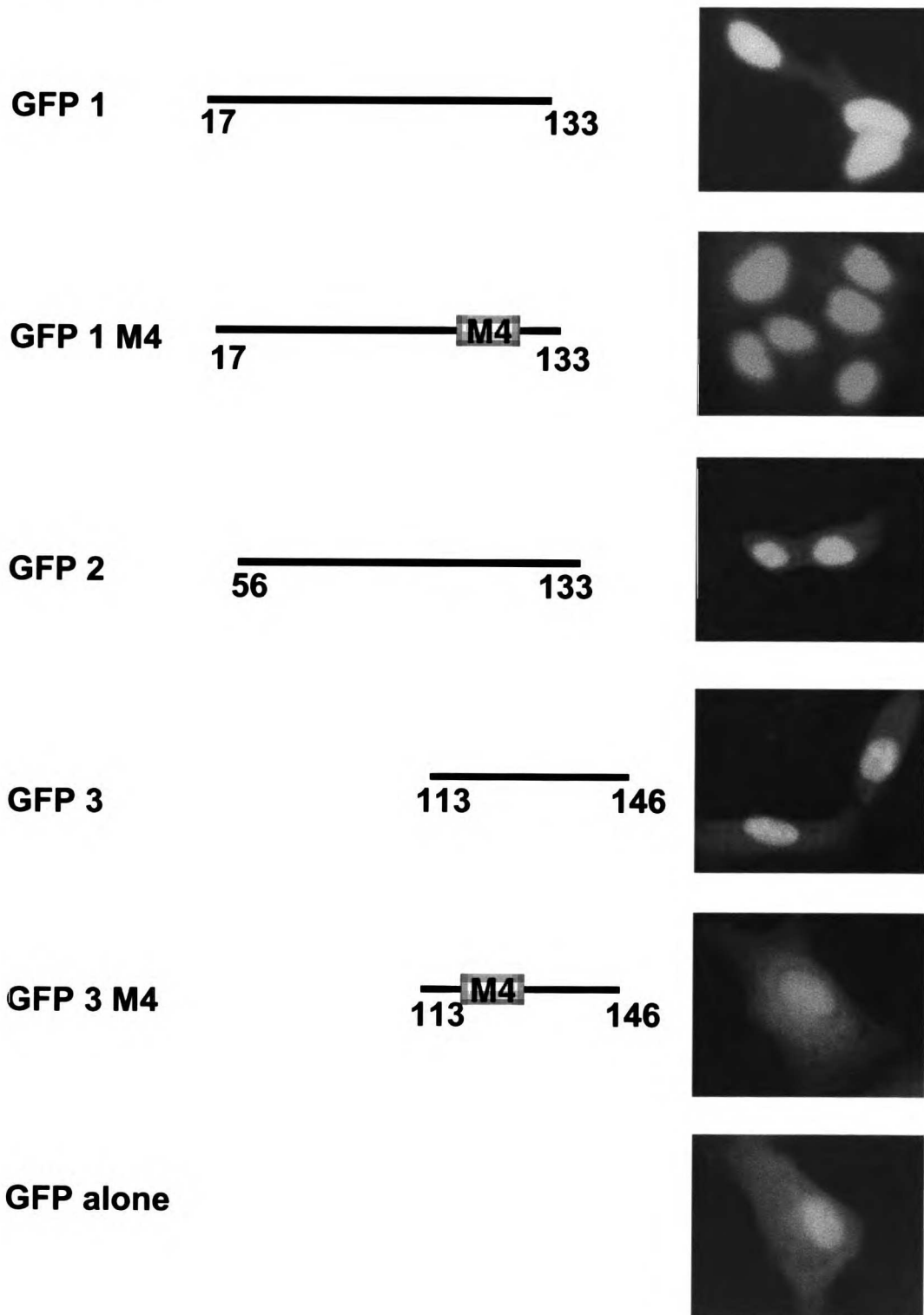
mouse antibody.

Figure 8a

GFP 1

17 _____ 133

GFP 1 M4

17 _____ M4 ___ 133

GFP 2

56 _____ 133

GFP 3

113 _____ 146

GFP 3 M4

113 __ M4 ___ 146

GFP alone

203

Figure 8b

GFP 4   ——————————————
         56   113

GFP 5   ——————————————
             76   113

GFP 6   ——————————
                84   113

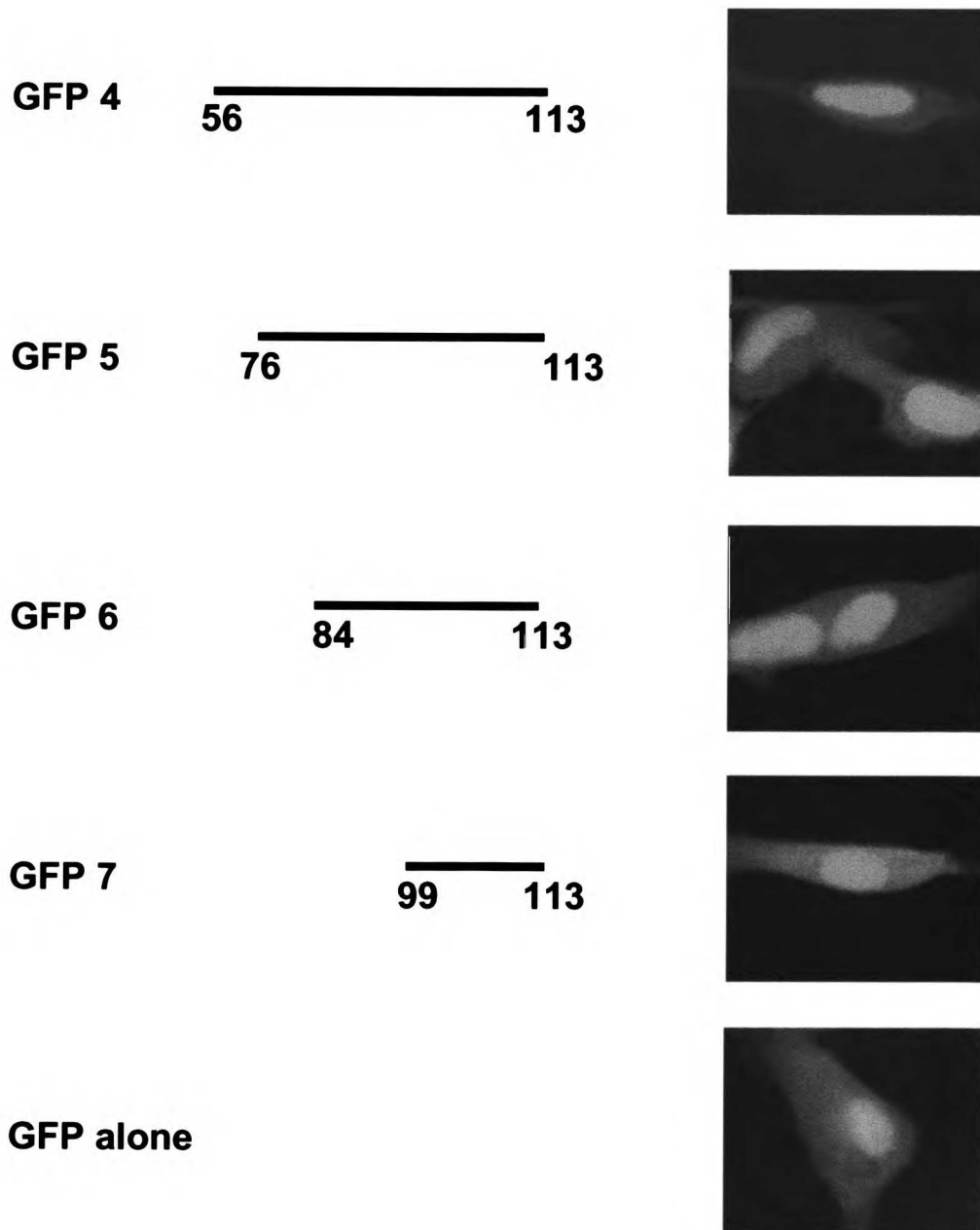GFP 7   ————————
                   99   113

GFP alone

FIG.8.A, B. Identification of ORF 57 nuclear localization signals.

Portions of ORF 57 were fused to GFP in the vector pEGFP (Clonetech). The resulting

vectors were transfected into CV-1 cells, and 48 h later, cells were fixed in 4%

paraformaldehyde. GFP was visualized by flouresence microscopy. The figure shows

the name of the vector, the residues of ORF 57 that were fused to GFP, and a

representative example of the cells after visualization. Some vectors contained the M4

mutation of ORF 57, which is indicated by the box containing M4. GFP when expressed

alone is localized diffusely in both the nucleus and the cytoplasm.

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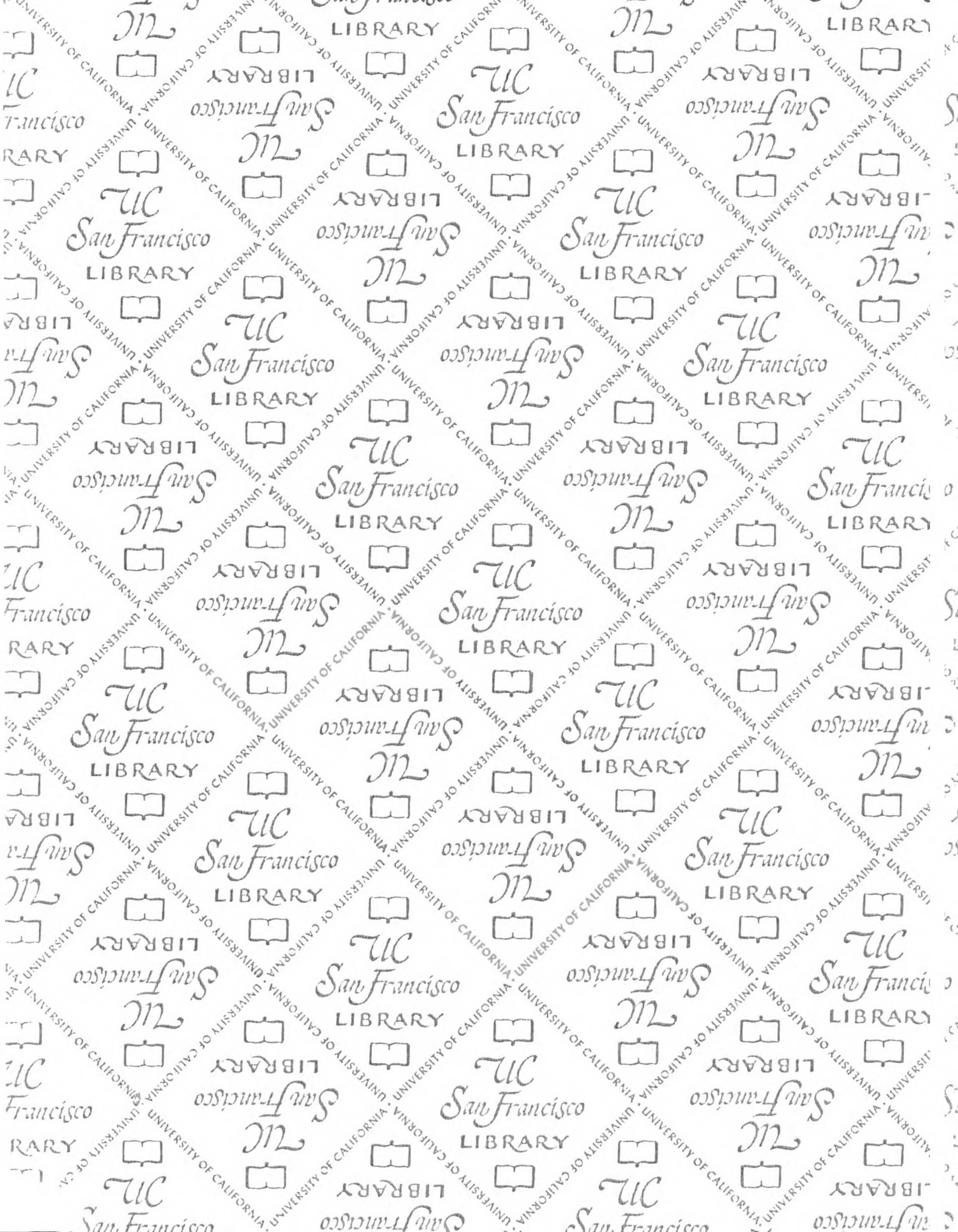
Chapter 5

CONCLUSION

CONCLUSION

KSHV lytic replication likely plays a significant role in KS and PEL development. Growing evidence implicates lytic viral genes as drivers of pathogenesis and clinical studies show that drugs which halt lytic replication can slow progression to disease. The investigations in this thesis were undertaken in an effort to understand both the regulatory mechanisms at work during lytic replication, as well as one of the potent lytic genes responsible for angiogenesis and cell proliferation.

KSHV GCR is a protein which has been demonstrated to be an inducer of both proliferation and angiogenesis. We demonstrate that this vGCR is expressed during the early lytic phase of viral replication. Furthermore, it is expressed as part of a bicistronic transcript containing the K14 open reading frame and is likely to be translated by unconventional mechanisms. For vGCR to play a role in KS and PEL development, it may be part of a paracrine signalling cascade, as the cells in which it is expressed are destined to produce virions and subsequently lyse.

KSHV lytic replication itself is central to pathogenesis as the virus must spread throughout the body to target tissues. The regulation of this phase therefore is a

fascinating topic of study. The work in this thesis was performed to understand the role

of ORF 57, which we have discovered to be a potent post-transcriptional regulator, in

promoting viral replication and subsequent pathogenesis. ORF 57 expression alone can

increase viral RNA levels significantly. ORF 57 and ORF 50 appear to work together to

regulate expression of some RNAs. There are several mechanisms by which ORF 57

may exert its effects on gene expression. It may bind in a complex with ORF 50 and/or

other viral or cellular factors to upregulate the nut-1 promoter. The biochemical

mechanisms underlying ORF 57 functions as well as its larger role in the induction

pathway of viral lytic replication are yet to be discovered. However, our investigations

have provided the foundation for further studies into ORF 57's possible mechanisms.