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Modulation of Kaposi's Sarcoma-associated Herpesvirus (KSHV/HHV8) origin-dependent DNA replication by K-bZIP, K-Rta, and LANA

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Abstract

There are eight Kaposi Sarcoma-associated Herpesvirus (KSHV) viral-encoded proteins that are necessary for lytic origin-dependent DNA replication. Six of the eight proteins have a high level of homology to other herpesvirus proteins necessary for oriLyt-dependent DNA replication. There are two proteins involved in oriLyt DNA replication that are unique to KSHV, the major transactivator K-Rta and the relatively functionally unknown protein K-bZIP. During the course of our investigation on the role of K-Rta and K-bZIP in DNA replication we discovered that the major Latency Associated Protein (LANA) also plays a role in oriLyt-dependent DNA replication.

To determine which K-bZIP protein domains contribute to oriLyt-dependent DNA replication and facilitate suppression of K-Rta-mediated transcriptional activation, we generated a series of deletion constructs and site-directed mutations in the K-bZIP ORF. Mutation of key leucine residues within the putative leucine zipper (LZ) motif eliminated the ability of the protein to homodimerize and complement oriLyt-dependent DNA replication. Deletion of the basic amino acid region (BR) or LZ domain did not affect the ability of K-bZIP to bind to K-Rta indicating that either region contributes to heterodimerization with K-Rta. However, deletion or mutations introduced into both the LZ and BR resulted in elimination of the suppressive activity of K-bZIP even in the presence of a K-bZIP—K-Rta interaction. Interestingly, mutants that lacked the ability to suppress K-Rta transactivation were still capable of complementing oriLyt-dependent DNA

replication, indicating that this activity does not contribute to the DNA synthesis-related activity of K-bZIP.

To determine the role of K-bZIP in the context of the viral genome, we generated a recombinant KSHV bacterial artificial chromosome (BAC) with a deletion in the K-bZIP open reading frame. This BACmid, BAC36ΔK8, displayed an enhanced growth phenotype with respect to virus production and accumulation of virus-encoded mRNAs measured by real-time PCR under conditions where K-Rta was used to induce the virus lytic cycle. Induction of the virus lytic cycle using tetradecanoyl phorbol acetate/n-butyrate (TPA/NaBut) resulted in no virus production and an aberrant gene expression pattern from BAC36ΔK8-containing cells compared to wild-type (wt) BAC. Immunofluorescence staining revealed that subcellular localization of K-Rta was unchanged but there was a disruption of LANA subcellular localization in cells harboring BAC36ΔK8, suggesting that K-bZIP influences LANA localization. Coimmunoprecipitation experiments confirmed the K-bZIP interacts with LANA in BCBL-1 cells and in contransfection assays.

In an effort to understand the consequences of a K-bZIP—LANA interaction we developed a modification of the transient cotransfection replication assay where both lytic (oriLyt) and latent (terminel repeats-TR) DNA replication are evaluated simultaneously. LANA repressed oriLyt-dependent DNA replication in a dose dependent manner when added to the cotransfection replication assay. This repression of oriLyt-dependent DNA replication was overcome by increasing amounts of a K-bZIP expression plasmid in the contransfection mixture of by

dominant-negative inhibition of the interaction of LANA with K-bZIP by the over expression of the K-bZIP—LANA interaction domain. Using the chromatin immunoprecipitation assay (ChIP) we show that LANA interacts with oriLyt in the region associated with K-bZIP binding suggesting suppression of lytic replication by LANA is mediated by direct binding. These data suggest that the interaction of LANA with K-bZIP modulates lytic and latent replication.

LIST OF FIGURES AND TABLES

Chapter 3

Introduction and Background

Herpesvirus Classification

Herpesviruses constitute a class of double-stranded DNA viruses. The hosts of herpesviruses range from reptiles, amphibians, to birds and mammals. The classification of Herpesviridae consists of three subfamilies (27), alphaherpesvirinae, betaherpesvirinae, and gammaherpesvirinae. For humans there are eight known herpesvirus. Alphaherpesviruses include Herpes Simplex-1 (HSV-1 or Human Herpesvirus-1, HHV-1), Herpes Simples-2 (HSV-2 or HHV-2), and Varicella Zoster Virus (VZV or HHV-3). Betaherpesvirus include Cytomegalovirus (CMV or HHV-5), HHV-6 and HHV-7. Gammaherpesvirus includes Epstein-Barr virus (EBV or HHV-4) and Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8). Primary criterion for inclusion in the Herpesvirus family is based on virion morphology. Herpesvirus virions are approximately 200nm in diameter (73). Herpesvirus virions are typically spherical and contain a core, capsid, tegument and envelope (86). The core consists of a linear double-stranded DNA molecule, surrounding the core is the capsid, the tegument surrounds the capsid, and the lipid envelope completes the virion.

Herpesviruses are highly adapted to their host and severe symptoms from infection are usually limited to young children or immunosuppressed patients. Natural transmission routes range from aerosol spread to mucosal contact and seminal fluid (2, 6, 13, 68). Herpesviruses have elaborate means of modulating the

host responses to infection and are able to establish lifelong latent infections (3).

For each of the three sub-families of herpesviruses a generalization can be made in

the type of cells where they establish latency. Alphaherpesvirus establish latency in

the neurons, for betaherpesvirus latency is in cells of the monocyte lineage, and for

gammaherpesvirus the site of latency is in lymphocytes. Although in healthy

immune competent individuals herpesviruses rarely cause severe disease, there is

increasing evidence demonstrating that herpesviruses can cause certain types of

cancer. Recently human cytomegalovirus has been implicated as the cause of

glioblastomas in some cases (89). Epstein-Barr virus (EBV) has also been found to

be the cause of some nasopharyngeal carcinomas, Burkitt's lymphomas and gastric

adenocarcinoma (31, 64).

All herpesviruses have six conserved core viral encoded replication proteins

that are required for lytic DNA replication. In the case of KSHV there are eight

virally encoded proteins that are required for DNA replication. These are ORF9

encodes the polymerase, ORF59 encodes the processivity factor, ORF44 encodes the

helicase, ORF56 encodes the primase, ORF40/41 encodes the primase-associate

factor, ORF6 encodes the single-stranded DNA binding protein, ORF50 encodes K-

Rta the major tranactivator, and K8 encoded K-bZIP. Beta and gammaherpesviruses

rely on DNA-binding transactivators to increase transcription across the oriLyt

region that opens the DNA and allows interaction with replication machinery, for

KSHV these proteins are K-Rta and K-bZIP (109).

Clinical and pathological aspects of KSHV

KSHV is the most recently identified human herpesvirus, first isolated and described as the causative agent of Kaposi's sarcoma by Yuan Chang and Patrick Moore in 1994 (20). KSHV genome spans 140 kbp flanked by multiple 801 bp terminal repeats, and has approximately 86 genes (Fig. 1) (80). Kaposi's Sarcoma is a multifocal malignancy usually affecting elderly Mediterranean men or patients with immune disorders including those with organ transplants or infected with Human Immunodeficiency Virus (HIV) (21, 33). KSHV is the cause of primary

Figure 1. The KSHV genome spans 140 kbp and is flanked by multiple 801 bp terminal repeats. There are approximately 86 open reading frames (ORFs) (80).
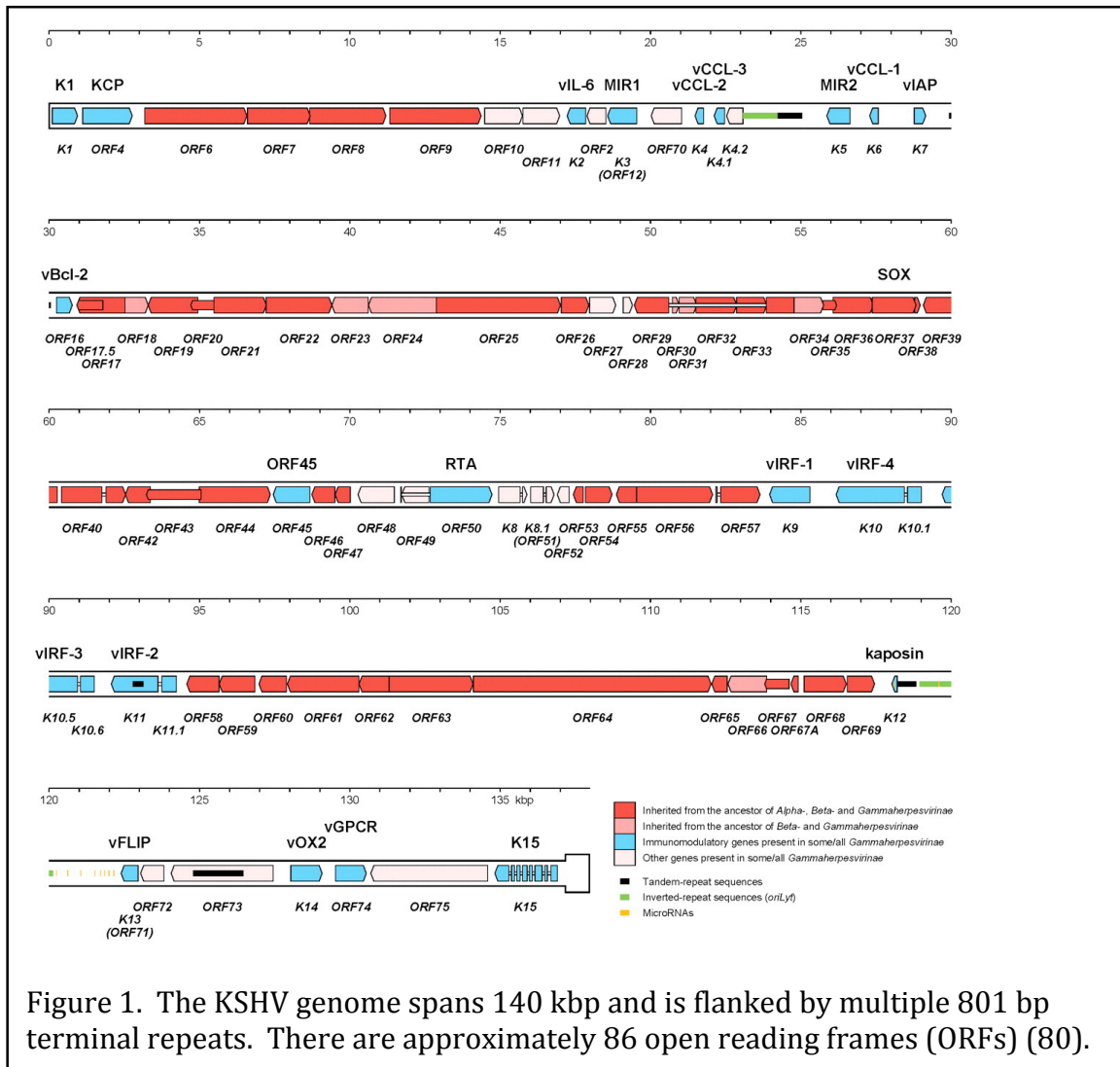
effusion lymphoma (PEL), or body cavity lymphoma and multicentric Castlemens disease (MCD) (85). Lytic infection or reactivation appears to lead to disease and considerable pathogenesis (51, 92). Suppression of lytic infection with the use of anti-herpesvirus drugs such as Ganciclovir or Foscarnet can slow the progression of KS(82). Globally KS represents ~1% of cancers attributed to infection (25). KSHV is endemic is sub-Saharan Africa and regions near the Mediterranean sea (30-50% seropositive), KSHV-seropositive is rare (>5%) in most other parts of the world (21, 32).

Naturally occurring KSHV is found primarily in CD19-positive peripheral blood mononuclear cells (47, 70). In experimental tissue culture conditions KSHV has been found to be able to infect various human cell lines including, primary fibroblasts, 293 (human embryonic kidney) cells, and human primary vascular endothelial cells (11, 53, 78).

K-Rta the major transactivator in KSHV

The major transactivator for KSHV replication and transcription is K-Rta (replication and transcription activator), encoded by the ORF50 gene (23, 66, 96). The full-length K-Rta contains 691 amino acids, and is predicted to have a molecular mass of 73.7 kDa, but it is usually seen to migrate around 101 and 119 kDa suggesting that K-Rta is post-translationally modified (18, 66). The K-Rta protein has a N terminal DNA binding domain, a central dimerization domain, a C terminal acidic activation domain, and two nuclear localization signals (23, 66).

In the lifecycle of KSHV, consisting of latent and lytic phases, there are four distinct stages of viral gene expression: latent, immediate-early (IE), early, and late genes (37). The IE genes are made immediately after primary infection or during reactivation from latency, the IE genes do not require de novo protein synthesis (29). In general the IE genes encode for regulatory proteins, and are critical for initiating viral transcription (71). One of the most significant IE gene products in KSHV is K-Rta. Expression of K-Rta is necessary and sufficient to trigger lytic reactivation resulting in the cascade expression of viral proteins, and if the lytic cycle is completed there is host cell lysis and the release of viral progeny (36, 67, 96, 109). After chemically inducing lytic replication in BCBL-1 cells, ORF50 mRNA can be detected within one hour after treatment with TPA or four hour after treatment with sodium butyrate (67, 97, 114).

It has been demonstrated that K-Rta has the ability to activate transcription of several viral genes, these include ORFK1 (14, 15), ORFK2 (28, 94), ORFK3 (81), ORFK5 (40), ORFK6 (19), PAN RNAs (93-95), ORF35 (69), ORF49 (35), ORF50 itself (22, 30, 87), ORFK8 (65, 91, 104, 107), ORF57 (16, 94, 103), ORFK9 (23, 100), ORF74 (59), ORFK12 (94) and ORFK15 (108). More recently, using a ChIP-on-ChIP assay, it was identified that K-Rta can also transactivate ORFK4.1, ORF16, ORF29, ORF45, ORFK10, ORF59, and KSHV miRNA cluster (24). K-Rta also binds to the left and right origin of lytic replication (oriLyt-L and oriLyt-R) (105, 106). K-Rta can transactivate by either direct binding to RTA-responsive elements (RREs) or by indirect binding to other cellular factors (19). A KSHV bacterial artificial

chromosome (BAC36) with a deletion of ORF50 confirmed that K-Rta is the major lytic switch protein when this mutant virus was unable to reactive under chemical treatment but latent genes were still expressed at wild type levels (109). The K-Rta DNA binding domain is located between amino acid 1-530, and studies have shown that a mutant K-Rta containing only this DNA binding domain and lacking the transactivation domain can act as a dominant-negative mutant of K-Rta transactivation (66). K-Rta is a component of KSHV virions (10), which would theoretically ensure lytic replication upon primary infection, but this might not be the case as herpesviruses have a some what casual assembly and egress process that may capture a wide variety of nonstructural proteins and mRNAs (12, 54, 56).

The ability of K-Rta to act as a transactivator is mitigated by its interaction with K-bZip and LANA. The protein-protein interactions between K-Rta and K-bZIP lead to repression of K-Rta's ability to transactivate in vitro (60, 61). K-bZIP is able to repress K-Rta's transactivation of the ORF57 and K8 promoters but it has no effect on the PAN promoter, this demonstrates promoter specific repression by K-bZIP (60). The ability of K-Rta to autoactivate its own promoter is modulated by LANA, which is able to bind to the ORF50 promoter and repress transcription (55, 56).

LANA, Latency-Associated Nuclear Antigen

Latency-associated nuclear antigen (LANA) protein, encoded by ORF73, contains 1,162 amino acids and has a predicted size of 222-234 kDa. LANA is the

major latent protein found in KSHV and is primarily a nuclear protein that interacts with many viral and cellular proteins (50, 52, 76). LANA protein is generally divided into three distinct parts. LANA has an N terminal 337 amino acid domain, a hydrophilic central domain of 585 amino acids consisting of multiple repeat elements with a large number of charged polar amino acids glutamine, glutamic acid and asparagine; and a C terminal 240 amino acid domain (84). LANA also contains a unique nuclear localization sequence (NLS) mapping to amino acids 24-30 (74, 90, 99). LANA was first characterized by its distinctive punctate locations within the nucleus when visualized by immunofluorescent staining (57). LANA has also been described as an oncogenic protein since it is known to disregulate cellular tumor suppressor pathways associated with pRb and p53 (75).

LANA tethers the viral episomal DNA to the host chromosome by directly binding to sequences within the terminal repeat (TR) region of the KSHV genome through its C terminal end and to the host chromosome through its N terminal end (Fig. 2). LANA tethering to the host chromosome allows for efficient partitioning of the KSHV episome in dividing cell. Multiple studies have confirmed that LANA localizes to the nucleus during latency and interacts with the border of heterochromatin during interphase and with chromosomes during mitosis (7, 26, 98, 99). The major chromosome binding region of LANA was mapped to the N terminal end between amino acids 1-32 (90).

LANA is thought to play a major role in maintaining KSHV latency (7, 8, 26). During latency only a small number of genes are expressed, most predominately is

LANA. One theory is that the virus limits gene expression during latency in order to

minimize host immune response (113). LANA transcription has been shown to be

under the control of the LANA promoter (LANAp) (46, 77, 88) and LANA protein can
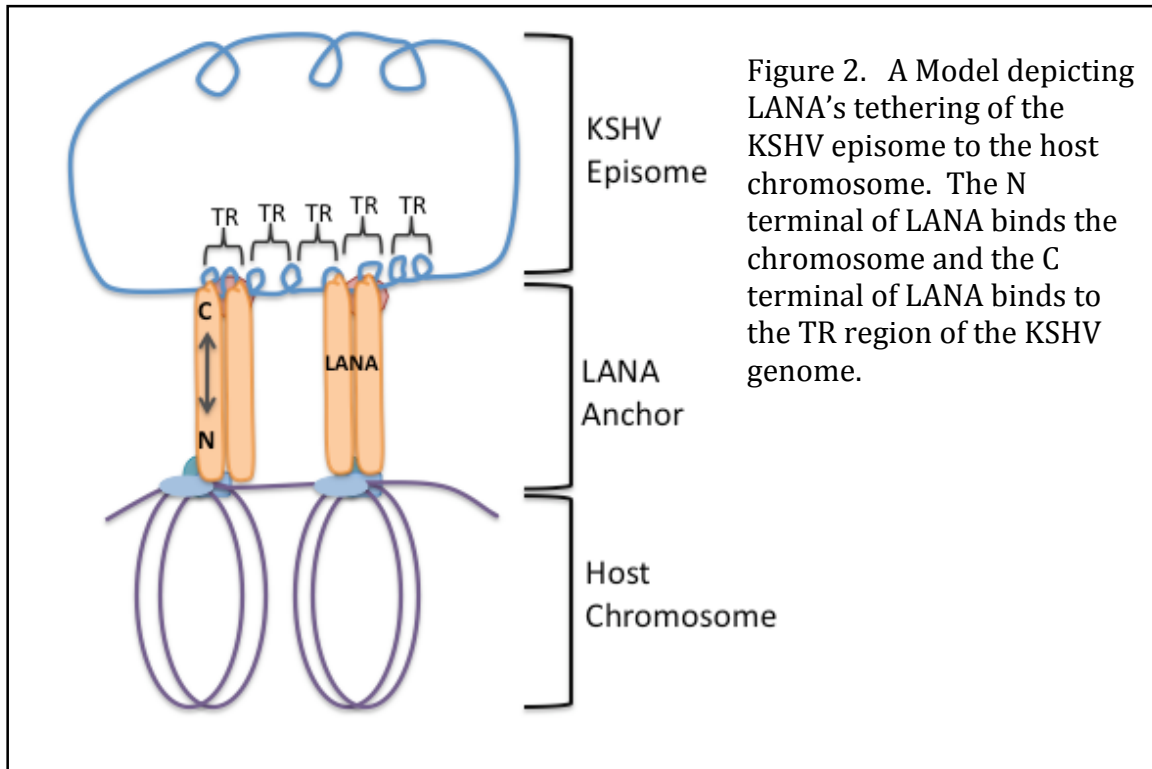
Figure 2. A Model depicting LANA's tethering of the KSHV episome to the host chromosome. The N terminal of LANA binds the chromosome and the C terminal of LANA binds to the TR region of the KSHV genome.

autoactivate its own promoter during latent infection to ensure that levels of LANA

are adequate to maintain latency (46, 48).

It has been well documented that LANA can repress as well as activate

transcription (1, 38, 43, 46, 62, 77). LANA has been shown to block the expression

of K-Rta, which is critical for the latency to lytic reactivation, by blocking the

expression of K-Rta LANA maintains viral latency (55). Not only has it been shown

that LANA can down regulate the expression of K-Rta in transient reporter assays,

but also LANA physically interacts with the K-Rta protein in vitro and associates

with K-Rta in KSHV-infected cells (55). These results taken together show that

LANA uses a two-pronged approach to down-regulate K-Rta, by repressing transcription of K-Rta and also by antagonizing the function of K-Rta protein itself.

Two different experimental approaches, one using siRNA to knock-down LANA and one using a mutant KSHV virus with a disrupted LANA gene, showed that in the absence of LANA, KSHV can undergo spontaneous lytic replication and fails to go into latency (34, 58, 111). The mechanism that LANA employs to maintain latency also explains why KSHV is predominantly maintained in the latent state in infected cells and why after primary infection KSHV rapidly establishes latency. Interestingly, K-Rta can activate the LANA promoter and up-regulate LANA expression in transient transfection experiments, and the transcription of K-Rta has been correlated with the expression of LANA during the early stages of de novo infection (56). K-Rta itself helps to establish latency through the activation of LANA expression during the early stages of infection.

K-bZIP, an unique viral protein

K-bZIP is a multi-functional protein involved in lytic DNA replication. K-bZIP, encoded by K8, contains 237 amino acids and belonging to the basic-leucine zipper family of transcription factors (63). K-bZIP is partial homologous to the Epstein-Barr virus (EBV) protein Zta, encoded by BZLF1 and was thought to have a function similar to Zta in lytic replication and reactivation (63). However, in contrast to EBV Zta, K-bZIP has no intrinsic transactivation activity and no direct binding to DNA has been demonstrated. K-bZIP is expressed through a K-Rta responsive promoter and

is a phosphorylated and SUMO-modified protein (44). K-bZIP can suppress the transactivation capability of K-Rta on certain promoters, notable the K-bZIP promoter and ORF57 promoter but not the PAN promoter (45, 60, 61). Studies have also found that K-bZIP can dimerize with K-Rta and LANA (45, 83), as well as homodimerize (63).

Initial studies of KSHV lytic replication determined that K-bZIP interacted with oriLyt and it was proposed that K-bZIP would play a key role in the initiation of lytic DNA replication. Studies have shown that K-bZIP interacts with regions of oriLyt that contain C/EBPα transcription factor binding sites (102, 103). Despite the evidence for a direct involvement in lytic DNA replication, recent studies clearly show that K-bZIP is not required in the context of the viral genome for virus replication or in the transient cotransfection replication assay when K-Rta is over-expressed (49). These new data have shifted the hypothesis from one that postulated that K-bZIP is the key factor for the initiation of lytic DNA synthesis to the role of facilitator of lytic DNA replication. Evidence suggests that at low concentrations of K-Rta, K-bZIP may aid or facilitate lytic DNA synthesis, but is not required when adequate levels of K-Rta are present.

KSHV DNA replication, cis- and trans-acting factors

Specific cis- and trans-acting factors are required for lytic and latent DNA replication. Cis-factors for DNA replication include specific DNA sites within the genome that the trans-factors can interact with to facilitate replication (Fig. 3).

Trans-acting factors include virally encoded proteins and cellular proteins that

function to replicate the viral DNA. There are eight virally encoded proteins

required for KSHV lytic DNA replication (Table 1). There is only one protein, LANA

that is required for latent DNA replication (9).

KSHV Core Replication Protein

KSHV gene	Protein	Function
ORF6	SSB	Single-stranded DNA binding protein
ORF9	POL	DNA polymerase
ORF40/41	PAF	Primase associated protein
ORF44	HEL	Helicase subunit
ORF56	PRI	Primase subunit
ORF59	PPF	Polymerase processivity factor
ORF50	K-Rta	Major transactivator
K8	K-bZIP	Origin binding protein

Table 1. The eight virally encoded proteins required for KSHV lytic
DNA replication.

The cis-regions where lytic DNA replication originates are termed oriLyt.

Two lytic origins (oriLyt-L and oriLyt-R) were identified using the transient

replication assay (5). These lytic origins for KSHV are located between ORFs K4.2

and K5 for oriLyt-L and ORF69 and vFLIP for oriLyt-R. These two lytic origins are

almost exactly identical with respect to DNA sequence with one major exception;

oriLyt-R encodes 10 micro RNAs that span both the upstream and downstream

regions of oriLyt-R. Several essential elements within oriLyt-L were identified

including two A+T rich sequences, C/EBPα binding motifs, AP-1 binding sites and a

K-Rta binding element (5). Many mutations have been introduced into oriLyt and

these experiments have established that the A+T rich regions cannot be mutated or

deleted (4). In addition, the C/EBPα transcription factor binding sites within oriLyt

also act as an essential substrate for protein binding (105).   Other essential regions

include the RRE (Rta-response element) and AP-1 sites. Previous studies that

deleted either oriLyt-L or oriLyt-R (and the double deletion), demonstrated that

only oriLyt-L could support viral DNA replication (110).

Figure 3. A model showing the protein-protein interactions and protein-
DNA interactions for DNA replication.
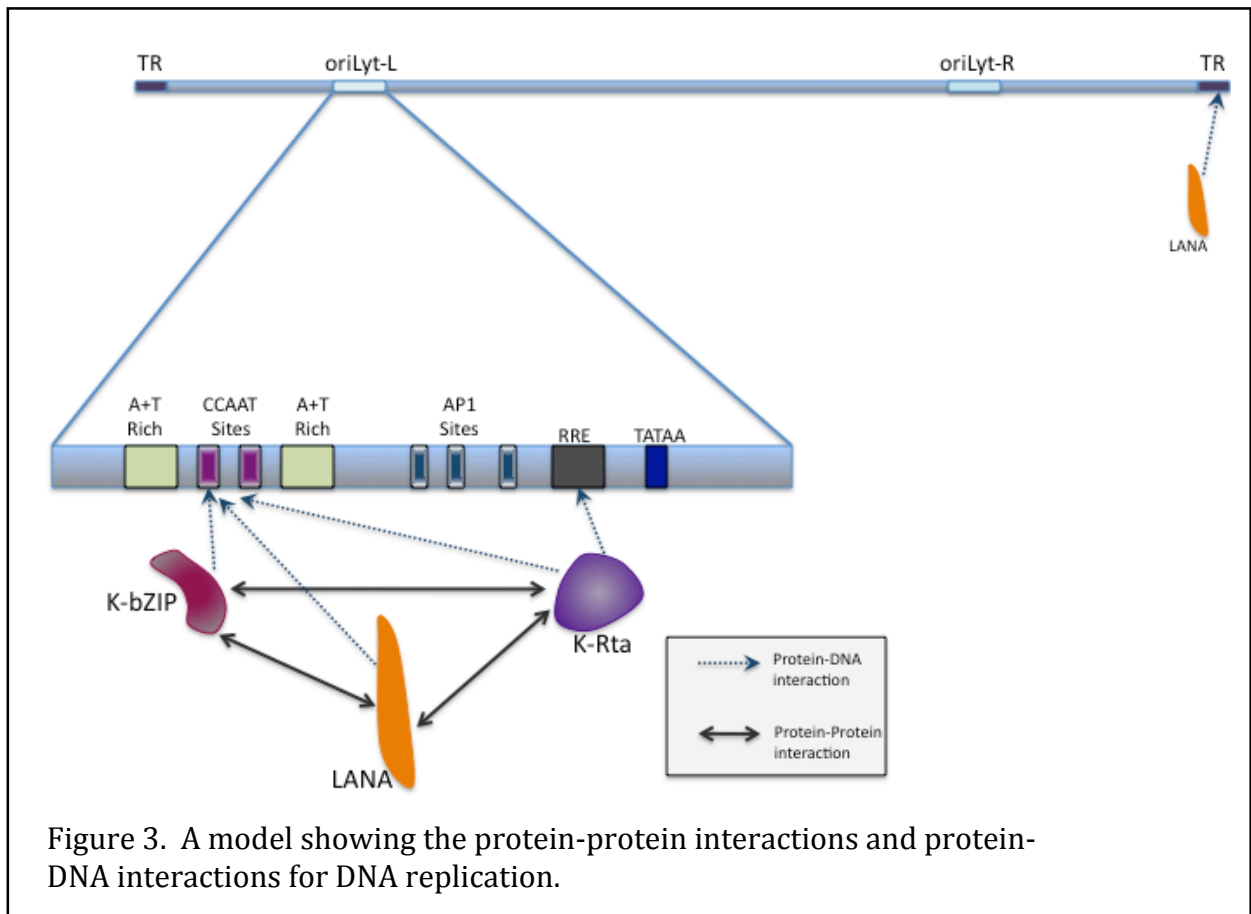
Latent DNA replication has significantly less factors to consider compared to

lytic DNA replication. LANA protein has been shown to be necessary and sufficient

for latent DNA replication (7, 26). Plasmids that contain only the terminal repeat

(TR) element have been shown to replicate in LANA-expressing cells (39, 41, 42,

62). Within the TR two cis regions have been identified, one is LANA binding sites

(LBS1/2) and the other is LANA replication element (LRE) (101). It has been shown

that a 71 bp minimal replicator region, containing both the LBS1/2 and LRE, was

able to support transient DNA replication in the presence of LANA (42).

Studying Latency and Lytic Reactivation

KSHV has two distinct lifecycles, latency and Lytic reactivation. Like all

herpesviruses, KSHV infection persists for the life of the host as either a latent or

lytic infection. The distinction between latent and lytic cycle is based on the

transcripts that are produced by the virus. During latency only the minimum

number of viral genes are expressed to maintain the episomal viral genome in

dividing cells, the low amount of viral gene expression is also helpful for immune

evasion from host defenses. Lytic reactivation occurs when the virus enters into

productive replication to generate new virus, lysing the host cell in the process.

One of the problems in studying latency or lytic reactivation KSHV is

obtaining a population of virus that is exclusively latent or exclusively lytic. To

study KSHV most researchers use a B-cell line that has been derived from body-

cavity lymphoma (BCBL-1) and is known to be positive for KSHV, but tested

negative for another gamma-herpesvirus Epstein-Barr Virus (EBV). In these

cultures of BCBL-1 every cell carries 10-50 copies of the viral episome, and

expresses the latency-associated nuclear antigen (LANA/ORF73) (7, 34). There are two common ways to induce lytic reactivation in BCBL-1 cells, one method is by chemical induction with the addition of 12-O-tetradecanoyl phorbol-13 acetate (TPA), or valproic acid (2-propylpentanoic acid) (112). The second way to induce lytic reactivation is by exogenous addition of the major transactivator K-Rta (67). When chemical induction is used only a small percentage (25-30% or less) of latently infected B-cells respond and support lytic reactivation (17), and less than a quarter of those that even start the lytic cycle go on to finish and produce virus (79). Inducing lytic reactivation by chemicals is also difficult because there is a fine line between induction and chemical toxicity. Adding the major transactivator K-Rta leads to a slightly higher proportion of cells initiating the lytic cycle. The frequent incomplete lytic reactivations coupled with the cells that stay in latency makes the study of the lytic cycle very complicated and unclear. The same can also be said for studying a purely latent system since B-cells infected with KSHV show 2-5% spontaneous lytic reactivation without any exogenous stimulation (72, 113).

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Transcriptional Repression of K-Rta by Kaposi's Sarcoma-Associated

Herpesvirus K-bZIP is Not Required for OriLyt-Dependent DNA Replication

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Abstract

Kaposi's sarcoma-associated herpesvirus origin-dependent DNA replication requires the core replication proteins plus K-Rta and K-bZIP. To determine which K-bZIP protein domains contribute to oriLyt-dependent DNA replication and facilitate suppression of K-Rta-mediated transcriptional activation, we generated a series of deletion constructs and site-directed mutations within the K-bZIP ORF. Mutation of key leucine residues within the putative leucine zipper (LZ) motif eliminated the ability of the protein to homodimerize and complement oriLyt-dependent DNA replication. Deletion of the basic amino acid region (BR) or LZ domain did not affect the ability of K-bZIP to bind to K-Rta indicating that either region contributes to heterodimerization with K-Rta. However, deletions or mutations introduced into both the LZ and BR resulted in elimination of the suppressive activity of K-bZIP even in the presence of a K-bZIP-K-Rta interaction. Interestingly, mutants that lacked the ability to suppress K-Rta transactivation were still capable of complementing oriLyt-dependent DNA replication, indicating that this activity does not contribute to the DNA synthesis-related activity of K-bZIP.

Introduction

Kaposi's sarcoma-associated herpesvirus or human herpesvirus 8 (HHV8)

lytic DNA replication requires 6 core replication proteins common to the

herpesvirus family. These proteins: ORF6 (single-stranded DNA binding protein),

ORF9 (DNA polymerase), ORF40/41 (primase-associated factor), ORF44 (helicase),

ORF56 (primase), ORF59 (polymerase processivity factor) provide the enzymatic

machinery for replication of viral DNA (AuCoin et al., 2004a). In addition to these

core proteins, two other viral-encoded proteins are required for origin-dependent

DNA replication. These proteins, ORF50 (K-Rta) and K8 (K-bZIP), supply an as yet

undetermined activity.

K-Rta is the major transactivator for HHV8 and is responsible for the switch

from latency to lytic replication (Byun et al., 2002; Gradoville et al., 2000; Lukac et

al., 1999; Wang et al., 2001). K-Rta has autoregulatory properties, with respect to

DNA binding and its own expression, and was shown to activate both immediate

early as well as early genes, including K8 (K-bZIP) (Byun et al., 2002; Chang et al.,

2004; Deng et al., 2000; Lukac et al., 2001). Recent studies indicated that the

expression of K-Rta in the context of the viral genome is required for lytic

replication and activation of several viral encoded proteins (Xu et al., 2005). With

respect to the role of K-Rta in DNA replication, current data indicates that K-Rta

activates a K-Rta-responsive promoter within oriLyt and suggests that initiation of

DNA synthesis is triggering by a transcription event. This mode of initiation of lytic

DNA synthesis would be consistent with that proposed for both Epstein Barr Virus

(EBV) and human cytomegalovirus (HCMV) (Hammerschmidt et al., 1988; Schepers

et al., 1996; Schepers et al., 1993).

The role of K-bZIP in DNA replication is less clear. K-bZIP interacts with K-

Rta and suppresses the transactivation activity of K-Rta on some K-Rta-responsive

promoters (Izumiya et al., 2003a; Liao et al., 2003). K-bZIP was also implicated in

controlling the cell cycle by directly binding to cyclin-CDK2 (Izumiya et al., 2003b).

Since K-bZIP has no reported transactivation function, the assumption is that a

direct or indirect interaction with oriLyt provides an enzymatic activity or some

other role that orchestrates initiation of DNA synthesis. Consistent with this model,

K-bZIP was shown to interact with oriLyt via CCAAT enhancer binding sites within

oriLyt through an indirect or "piggyback" interaction with CCAAT enhancer binding

protein alpha (C/EBPα) in BCBL-1 cells (Wang et al., 2003a; Wang et al., 2003b).

Our previous studies demonstrated that the oriLyt-dependent DNA

replication-associated activity of K-bZIP requires the presence of an intact leucine

zipper motif (AuCoin et al., 2004a). Apparently, the region containing the leucine

zipper is required for multimerization, but does not form the traditional coiled-coil

structure (Al Mehairi et al., 2005). The BR plus the leucine zipper motif were

demonstrated to comprise the region of interaction between K-bZIP and C/EBPα

(Wang et al., 2003a). Consequently, the K-bZIP leucine zipper and BR amino acid

motifs are key protein domains essential for the interaction with cellular and other

viral encoded proteins. This suggests that the replication function supplied by K-

bZIP may be distinct from that supplied by the closely related Zta protein of Epstein

Barr Virus (EBV). In EBV, Zta provides a transactivation function as well as a

replication activity within oriLyt by interacting with several defined Zta response

elements (ZREs) (Fixman et al., 1992; Sarisky et al., 1996).

There is an apparent discrepancy surrounding which motif is required for

interaction with, and for suppression of, K-Rta-mediated transcriptional activation.

Two contradicting reports show that the either the BR or the LZ region is the point

of interaction between K-bZIP and K-Rta (Izumiya et al., 2003a; Liao et al., 2003).

Liao et al reports that a deletion of the leucine zipper region of K-bZIP abrogates its

suppressive affects; however, Izumiya et al report that deletion of the BR region

resulted in a loss of suppression of K-Rta-mediated transactivation (deletion of the

LZ had no effect). This controversy served as a major impetus for our investigation

of the binding domains of K-bZIP and how repression of K-Rta-mediated

transactivation relates to DNA synthesis.

In this report we defined the K-bZIP point of interaction required for both

homodimerization of K-bZIP and heterodimerization with K-Rta. We show that

point mutations introduced into the leucine zipper motif disrupt the ability of K-

bZIP to homodimerize and fail to complement oriLyt-dependent DNA replication. In

addition, K-bZIP LZ mutants that fail to homodimerize no longer inhibit the

transactivation activity of K-Rta. Deletion of both the LZ and BR domains of K-bZIP

is required to eliminate a K-bZIP-K-Rta interaction, indicating that either structure

can interact with K-Rta. Mutations introduced into the BR region of K-bZIP did not

interfere with binding to K-Rta, but affect the ability of K-bZIP to repress K-Rta-mediated transactivation and oriLyt amplification. Finally, we show that the suppression of K-Rta transactivation by K-bZIP does not contribute to oriLyt-dependent DNA replication.

Results and discussion

The K-bZIP leucine zipper and the basic amino acid region contribute to homodimerization. The first step to defining a region within the K-bZIP protein that contributes to a self-interaction is to identify a deletion mutant that no longer forms a dimer. We generated 7 HA-tagged expression plasmids each with a different 33 amino acid deletion in the K-bZIP ORF (Fig. 1A). Deletion plasmids were each cotransfected with a full-length wt K-bZIP expression plasmid that was FLAG tagged. Protein extracts were prepared and immunoprecipitated with anti-FLAG antibody. An interaction between the two K-bZIP species was detected by reacting Western blot of the immunoprecipitated protein with an anti-HA antibody. All of the K-bZIP deletion proteins interacted with wt K-bZIP except deletion number 6 (D6) (Fig. 1B, lane 6). K-bZIP D6 has a 33 amino acid deletion that removed the entire BR region and most of the LZ motif, leaving leucine residues 205, 212 and 219 of the LZ domain. All FLAG and HA-tagged K-bZIP proteins were similarly expressed (Fig. 6B, see FLAG and HA Lysates) and all FLAG-tagged protein species were efficiently immunoprecipitated (Fig. 6B, see FLAG IP FLAG West). These results show that the region between amino acids 169 and 202 of the K-bZIP ORF contribute to homodimerization and confirm earlier reports implicating the LZ and BR domains.

A

B

1. K-bZIP protein lacking the BR and LZ domains fail to homodimerize. (A) Genomic arrangements of nucleotide coordinates for ORF50 and K-bZIP and a schematic of the K-bZIP ORF showing the basic amino acid region (BR) and leucine zipper motif (LZ) and HA tag. Also shown are the 7 in frame deletion mutants generated for K-bZIP and the designated amino acids that were deleted. **(B)** Western blot of co-immunoprecipitation assay of K-bZIP deletion mutants and wt K-bZIP. Cells were transfected and treated as described in materials and methods. Lanes: wt-K-bZIP-FLAG, transfection and immunoprecipitation of control K-bZIP-FLAG protein; wt K-bZIP-FLAG and wt K-bZIP-HA, cotransfection control plasmids and immunoprecipitation of FLAG and HA tagged wt K-bZIP protein; 1, protein samples from cotransfection of wt K-bZIP-HA and D1; 2, protein samples from cotransfection of wt K-bZIP-HA and D2; 3, protein samples from cotransfection of wt K-bZIP-HA and D3; 4, protein samples from cotransfection of wt K-bZIP-HA and D4; 5, protein samples from cotransfection of wt K-bZIP-HA and D5; 6, protein samples from cotransfection of wt K-bZIP-HA and D6; 7, protein samples from cotransfection of wt K-bZIP-HA and D7. FLAG Lysate: protein samples from transfections reacted with anti-FLAG antibody, HA Lysate: protein samples from transfections reacted with anti-HA antibody, FLAG IP HA West: cotransfections were immunoprecipitated with anti-FLAG antibody and western blots were reacted with anti-HA antibody, FLAG IP FLAG West: cotransfections were immunoprecipitated with anti-FLAG antibody and western blots were reacted with anti-FLAG antibody.

The leucine zipper domain of K-bZIP mediates homodimerization. The

co-immunoprecipitation experiment showed that the region of K-bZIP containing

the LZ and BR domains is required for homodimerization. In addition, since K-bZIP

is known to exist as a dimer in transfected and infected cells (Al Mehairi, Cerasoli,

and Sinclair, 2005; Izumiya et al., 2003a) we wanted to investigate if dimerization of

K-bZIP was involved in, or required for, the DNA replication function conferred by

the protein.

Figure 2A is a schematic of the ORF50 and K-bZIP genomic region. Also

shown are the domains of interest within the K-bZIP ORF (Fig. 2A). The putative

basic region (BR) for K-bZIP was identified as containing six basic amino acids (Fig.

2A) and the putative leucine zipper (LZ) encompasses leucine residues and an

isoleucine residue at amino acids 191, 198, 205, 212 and 219 (Fig. 2A). In order to

study the contribution of these domains to homodimerization we altered several

leucine residues within the LZ using site-directed mutagenesis and assayed the

ability of these mutants to interact with wt K-bZIP using the co-

immunoprecipitation assay (Fig 2C).

293T cells were cotransfected with FLAG tagged LZ mutants (Fig. 2B) along

with wild type HA tagged K-bZIP. The differentially tagged proteins allowed us to

detect binding between mutant and wild type K-bZIP species upon co-

immunoprecipitation by anti-FLAG antibodies followed by Western blot analysis

using HA specific antibodies. Under these cotransfection conditions leucine zipper

mutants LZ-1 and LZ-2 failed to interact with K-bZIP-HA (Fig. 2C). Control lysates

A

B

C

2. K-bZIP homodimerization requires an intact LZ. (A) Schematic showing the K-bZIP ORF and amino acid residues comprising the BR (basic region) and LZ (leucine zipper). **(B)** Amino acid sequence of the leucine zipper motif and the location of mutations introduced into key leucine residues to generate K-bZIP mutants LZ-1, LZ-2 and LZ-3 and LZ-205. **(C)** Cotransfection and immunoprecipitation of wt K-bZIP with leucine zipper mutants. Assays were performed as described previously.

showed that all proteins were expressed efficiently and at similar levels (Fig. 2C, FLAG and HA lysates). These results indicated that the LZ motif of K-bZIP, especially residues 198 and 205, is essential for efficient homodimerization.

K-bZIP requires an intact BR/LZ region to interact with K-Rta   Since it was previously reported that there is an interaction between K-bZIP and the viral transactivator ORF50 (K-Rta), we next wanted to evaluate the ability of the K-bZIP deletion mutants to interact with K-Rta. Cells were cotransfected with plasmids expressing each K-bZIP deletion mutant (from Fig. 1A) and a K-Rta expression plasmid containing an in-frame FLAG epitope. Co-immunoprecipitation assays were performed and precipitated protein complexes were analyzed by Western blot. Consistent with the K-bZIP homodimerization results, K-bZIP deletion mutant D6 failed to interact with K-Rta (Fig. 3C).

The next step was to determine which specific K-bZIP domain(s) were responsible for interacting with K-Rta. It was previously reported that the BR of K-bZIP alone could interact with K-Rta (Izumiya et al., 2003a). However, another report found that only the K-bZIP leucine zipper motif was the point of interaction between the two proteins (Liao et al., 2003). Both of these studies used peptide fragments of K-bZIP protein corresponding to the LZ or BR regions to analyze these interactions. We were interested in the binding characteristics of K-bZIP with K-Rta in the context of the complete protein coding region minus the BR, LZ or other protein domains. As stated earlier, the BR region of K-bZIP consists of six amino acids just upstream of the LZ motif (Fig. 1A). We used site-directed mutagenesis to
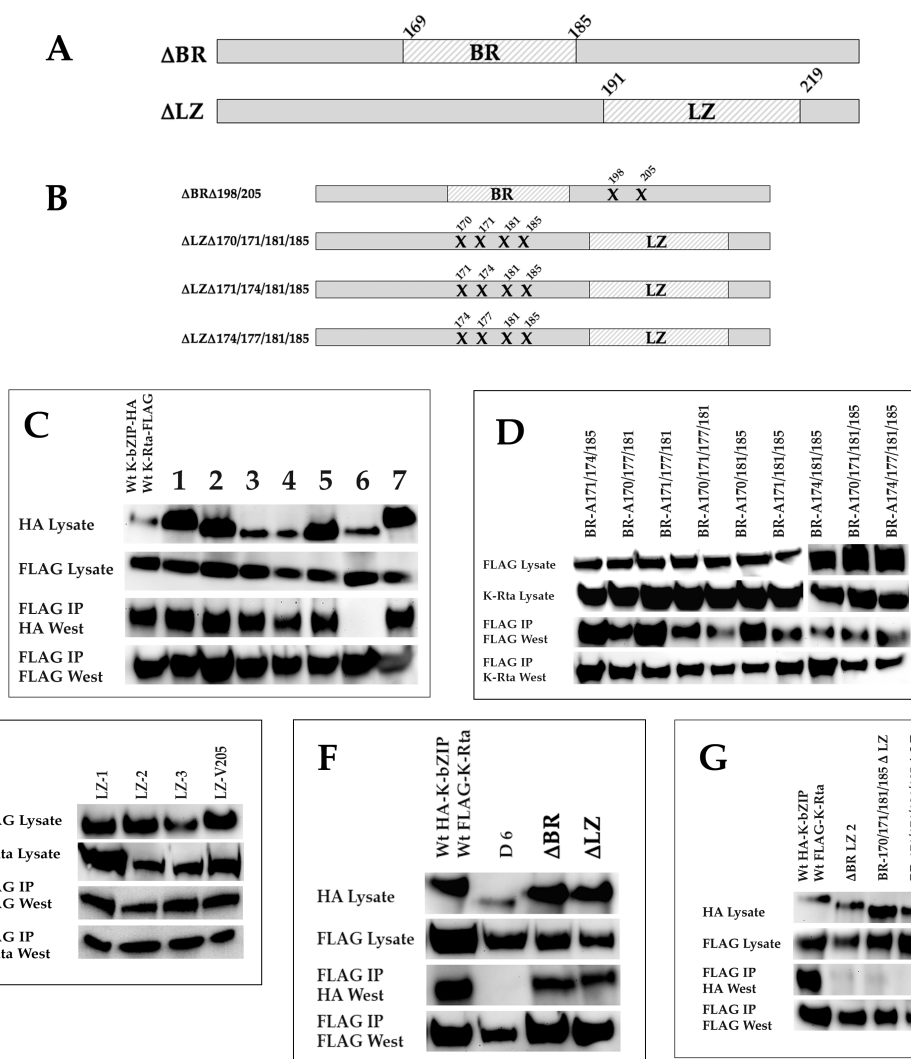
3. Interaction of K-bZIP BR and LZ mutants with K-Rta. **(A)** Schematic of expression plasmids where either the LZ or the BR of K-bZIP was deleted from the ORF and **(B)** expression plasmids where amino acid residue changes were introduced into the K-bZIP BR or LZ domains in the backbone of BR or LZ deletion mutants. **(C)** Co-immunoprecipitation of K-Rta with K-bZIP deletion mutants. Lanes: Wt K-bZIP-HA Wt K-Rta-FLAG, cotransfection and immunoprecipitation of Wt K-bZIP and wt K-Rta; 1, cotransfection and immunoprecipitation of D1 and wt K-Rta; 2, cotransfection and immunoprecipitation of D2 and Wt K-Rta; 3, cotransfection and immunoprecipitation of D3 and Wt K-Rta; 4, cotransfection and immunoprecipitation of D4 and wt K-Rta; 5, cotransfection and immunoprecipitation of D5 and Wt K-Rta; 6, cotransfection and immunoprecipitation of D6 and wt K-Rta; 7, cotransfection and immunoprecipitation of D7 and wt K-Rta. **(D)** Amino acid residue mutations introduced into the BR domain of K-bZIP have no effect on K-Rta binding. Western blot of co-immunoprecipitated protein-protein interactions between various K-bZIP BR mutants and wt K-RTA. **(E)** K-bZIP leucine zipper mutants interact with K-Rta. Western blot of co-immunoprecipitated protein samples from cotransfections of K-bZIP mutants lacking either the BR or LZ, plus D6 as negative control, and wt K-Rta. **(F)** BR or LZ regions of K-bZIP can interact with K-Rta. FLAG Lysate: protein samples from transfections reacted with anti-FLAG antibody, HA Lysate: protein samples from transfections reacted with anit-HA antibody, FLAG IP HA West: cotransfections were immunoprecipitated with anti-FLAG antibody and western blots were reacted with anti-HA antibody, FLAG IP FLAG West: cotransfections were immunoprecipitated with anti-FLAG antibody and western blots were reacted with anti-FLAG antibody. **(G)** Mutations in the LZ and BR abrogate the K-bZIP-K-Rta interaction. Western blot of co-immunoprecipitated protein samples from cotransfection of various K-bZIP BR-LZ mutants and wt K-Rta. FLAG Lysate: protein samples from transfections reacted with anti-FLAG antibody, HA Lysate: protein samples from transfections reacted with anit-HA antibody, FLAG IP HA West: cotransfections were immunoprecipitated with anti-FLAG antibody and western blots were reacted with anti-HA antibody, FLAG IP FLAG West: cotransfections were immunoprecipitated with anti-FLAG antibody and western blots were reacted with anti-FLAG antibody.

generate 9 K-bZIP expression plasmids with multiple mutations in the BR region.

Each of these expression plasmids was used in the cotransfection co-immunoprecipitation assay with a K-Rta expression plasmid. All BR mutated K-bZIP proteins species were able to interact with K-Rta (Fig. 3D). We also performed K-bZIP-K-Rta co-immunoprecipitations using the K-bZIP leucine zipper mutants, two of which, LZ-1 and LZ-2, failed to interact with wt K-bZIP. Again all LZ mutants were capable of interacting with K-Rta (Fig. 3E).

Since all of the K-bZIP proteins with amino acid residue changes in either the BR or LZ domains were capable of interacting with K-Rta we next decided to evaluate the ability of LZ or BR K-bZIP deletion mutants to interact with K-Rta in the co-immunoprecipitation assay. We generated expression plasmids where either the entire BR or LZ regions were deleted (Fig. 3A). These plasmids were co-transfected with a K-Rta expression plasmid and immunoprecipitations were preformed. Surprisingly, both of these K-bZIP mutant proteins could interact with K-Rta (Fig. 3F, lanes ΔBR and ΔLZ). The control plasmid, where the entire BR/LZ region was removed from the K-bZIP protein, failed to interact with K-Rta. This result indicated that either the LZ or the BR domains could interact with K-Rta. In order to confirm this finding we used plasmid clones that had either the LZ or BR domain deleted and then introduced several point mutations into the remaining LZ or BR region. These expression plasmids were cotransfected along with a wt K-Rta expression plasmid and co-immunoprecipitations were performed. An expression plasmid encoding a K-bZIP protein species that lacked the entire BR and had leucine

residues 198 and 205 mutated interacted weakly with K-Rta (Fig. 3G). A weak interaction was also observed when using a plasmid clone that expressed a K-bZIP species that lacked the entire LZ region and included mutations in amino acid residues 170/171/181/185 (Fig. 3G). No interaction was detected in cotransfected cells using K-bZIP expression plasmids that lacked the entire LZ domain and contained mutations in residues 171/174/181/185 or 174/177/181/185 (Fig. 3G). These experiments showed that both the BR and LZ of K-bZIP can interact with K-Rta and that several amino acid residues within the BR domain contribute to binding. Residues within the LZ region that confer homodimerization also contribute to heterodimerization.

The basic region, as well as leucine zipper region, contributes to suppression of K-Rta mediated transactivation. K-bZIP suppresses the transactivation function mediated by K-Rta. This suppression appears to be promoter specific in that two early promoters, those regulating the expression of K-bZIP and ORF57, are down regulated in the presence of K-bZIP (and K-Rta), whereas the promoter responsible for the expression of PAN is unaffected by K-bZIP. We wanted to evaluate the ability of some of our LZ or BR mutants to suppress K-Rta mediated transactivation. Vero cells were transfected with a K-bZIP promoter luciferase reporter plasmid (gift from H-S Kung, UC Davis) along with plasmids expressing either wt K-bZIP or LZ or BR mutants, and a K-Rta expression plasmid. Cells were harvested 24 h post transfection and assayed for luciferase activity. All luciferase activity comparisons were made against basal K-bZIP promoter activity.

As expected, wt K-bZIP efficiently suppressed K-Rta-mediated transactivation of the K-bZIP promoter (Fig. 4A, wt-K-bZIP). In addition, complete removal of the LZ and BR domains resulted in a loss of repressive function by K-bZIP (Fig. 4A). Some of the K-bZIP BR mutants failed to suppress K-Rta mediated activation of the K-bZIP promoter (Fig. 4A, BR 171/181/185 and BR 170/171/181/185). The K-bZIP recombinant with mutations in amino acid residues 170/181/185 retained its ability to suppress K-Rta mediated transactivation similar to wt K-bZIP (Fig. 4A). However, when the LZ domain was removed, this species lost its ability to repress (Fig. 4A, BR 170/181/185 ΔLZ). This data demonstrated that even in the presence of a protein-protein interaction between K-bZIP and K-Rta, suppression of transcriptional activation was dependent upon the retention of at least some basic amino acid residues within the BR of the K-bZIP protein.

Interestingly, one BR mutant, BR-171/177/181, increased the transactivation activity of K-Rta on the K-bZIP promoter (Fig. 4A). This increased activity was over 5-fold higher than that observed for K-Rta alone and over 100-fold higher than the suppression mediated by wt K-bZIP (Fig. 4A). This positive affect on K-Rta transactivation was also observed when using a promoter (PAN) not normally regulated by K-bZIP (Izumiya et al., 2003a). Nevertheless, when the K-bZIP mutant BR-171/177/181 was cotransfected along with a K-Rta expression plasmid and a PAN luciferase reporter plasmid, the activity of the PAN promoter was shown to increase over 500-fold when compared to PAN promoter alone and over 10-fold higher than when K-Rta was present, with or without a wt K-bZIP expression

4. Mutations introduced into the BR or LZ regions of K-bZIP affect suppression of K-Rta mediated transcriptional activation. (A) Vero cells were cotransfected with either a wt K-bZIP expression plasmid or various BR-or LZ-mutated K-bZIP expression plasmids, a K-Rta expression plasmid and a luciferase reporter plasmid that has the K-bZIP promoter driving the expression of the luciferase gene. Data is shown as relative fold activation over luciferase activity measured in cells transfected with only the K-bZIP luciferase reporter plasmid. Total DNA for all transfection was equal and maintained by using pGEM as filler DNA, except where noted. **(B)** K-bZIP BR-171/177/181 activates the PAN promoter. BR-171/177/181 was cotransfected with a PAN promoter luciferase reporter plasmid along with a K-Rta expression plasmid. Increasing amounts (2μg, 5μg and 10μg) of the BR-171/177/181 plasmid were added to the transfection mixture.

plasmid (Fig. 4B). As expected, the addition of a wt K-bZIP expression plasmid had no effect on luciferase activity from the PAN promoter (Fig. 4B).

Leucine zipper mutant LZ-2 also failed to suppress activation of the K-bZIP promoter (Fig. 4A). This species contained mutations in leucine residues 198 and 205. This result could suggest that this region of the protein is important for binding with K-Rta or may indicate that a K-bZIP-K-bZIP interaction is a prerequisite for suppression of K-Rta mediated activation. This set of experiments showed that the BR of K-bZIP could serve to regulate the activity of K-Rta in a negative, or in one case, a positive manner.

The leucine zipper domain contributes to K-bZIP replication function. . Since it was previously demonstrated that K-bZIP supplied an essential function in DNA replication, we next wanted to identify important domains within the protein that contributed to its function. It is known that mutations introduced into two of the leucine residues within the putative leucine zipper abolished the DNA replication function of K-bZIP (AuCoin et al., 2004b), hence we sought to investigate the physical parameters behind this observation. We next wanted to evaluate the ability of K-bZIP mutants to complement oriLyt-dependent DNA replication. We previously demonstrated that the core replication proteins plus K-Rta and K-bZIP were required for efficient amplification of HHV8 oriLyt in the transient replication assay (AuCoin et al., 2004a). We cotransfected core replication proteins plus K-Rta, oriLyt and the various K-bZIP mutants and examined the ability of several mutants to complement oriLyt amplification. Since it was demonstrated that K-bZIP was one

of the non-core proteins required for oriLyt-dependent DNA replication, we wanted

to investigate the role of the specific protein domains of K-bZIP with respect to

oriLyt-dependent DNA replication. As mentioned earlier, it was previously reported

that K-bZIP is capable of forming both a homodimer and heterodimer with K-Rta (Al

Mehairi, Cerasoli, and Sinclair, 2005; Izumiya et al., 2003a; Liao et al., 2003). It was

shown here and previously that the LZ domain of K-bZIP is required for interaction

with K-Rta and a mutant lacking the LZ domain no longer repressed K-Rta mediated

transactivation (Liao et al., 2003). Further, it was also demonstrated that

multimerization of K-bZIP is also conferred through the LZ domain (Al Mehairi,

Cerasoli, and Sinclair, 2005). In addition, we have demonstrated that 2 key leucine

residues contribute to homodimerization and mutations of leucine residues within

the LZ region of K-bZIP inactivated the DNA replication activity of the protein in the

transient cotransfection replication assay (AuCoin et al., 2004a). Our goals now

were to narrow down which leucine residues directly contributed to DNA

replication and determine if multimerization of K-bZIP is necessary to confer the

DNA replication activity of the protein.

Plasmids encoding mutated K-bZIP proteins were used in the cotransfection-

replication assay in place of wt K-bZIP. All of the other required core replication

proteins as well as K-Rta were included in the mixture along with HHV-8 oriLyt. As

a control for demonstrating authentic amplification of oriLyt, we treated all samples

with either DpnI (and EcoRI), which cleaves unmethylated/replicated DNA, or MboI,

which has the same base sequence recognition as DpnI but cleaves both methylated

and unmethylated DNA. Therefore any amplified oriLyt plasmid will be cleaved

when treated with MboI ensuring that amplification of oriLyt occurred. Also, we ran

duplicate gels of cotransfected DNA that was cleaved with EcoRI alone and

performed Southern blots on these samples to show that an equal amount of input

oriLyt was in the transfection mixture and in gel samples (Fig. 5B). K-bZIP species

that lacked the entire LZ or the BR region failed to complement oriLyt-dependent

DNA replication (Fig. 5A, lanes 3, 3* and 4, 4*). Also, the K-bZIP LZ-1 and LZ-2

mutants failed to complement oriLyt amplification indicating that these leucine

residues are required for the replication function and suggests that

homodimerization is essential (Fig. 5A, lanes 5, 5* and 6, 6*).

**Repression of K-Rta mediated transcriptional activation by K-bZIP is
not necessary for complementation of oriLyt-mediated DNA replication.** We

also used the replication assay to evaluate K-bZIP protein species with mutations in

the BR domain. The K-bZIP protein species lacking the entire BR failed to

complement oriLyt amplification in the transient cotransfection replication assay

(Fig. 5A, lane 3 and 3*). However, those K-bZIP mutants with mutations in the BR

domain were still capable of complementing oriLyt amplification, even those

mutants that failed to repress K-Rta-mediated transactivation (Fig. 5A, lanes 8-11

and 8*-11*). This result strongly suggests that the repressive function of K-bZIP

does not participate in DNA synthesis. Interestingly, the K-bZIP mutant that

conferred an increase in K-Rta transcriptional activation also showed an increase in

oriLyt amplification indicating that increased activity of K-Rta can lead to a highe

A

1 1* 2 2* 3 3* 4 4* 5 5* 6 6*   7 7* 8 8* 9 9*10 10*11 11*12 1

B 1 2 3 4 5 6 7 8 9 10 11 12

5. Amino acid changes within the BR and the LZ motif affect the ability of K-bZIP to complement oriLyt-dependent DNA replication. Southern blot of DNA from cotransfection-replication assay using Vero cells transfected with various K-bZIP mutants. All cotransfections included the core replication proteins, a K-Rta expression plasmid, cloned oriLyt and wt K-bZIP or various mutant K-bZIP expression plasmids as indicated. **(A)** Cells were cotransfected with K-bZIP expression plasmids that produced mutated protein with amino acid changes in the BR. (* lanes were treated with MboI) Lanes: 1, wt K-bZIP; 2, K-bZIP D6; 3, K-bZIP ΔBR; 4, K-bZIP ΔLZ; 5, K-bZIP LZ-1; 6, K-bZIP LZ-2; 7, wt K-bZIP; 8, BR 171/177/181; 9, BR 170/171/181/185; 10, BR 170/181/185; 11, BR 171/181/185; 12, No K-bZIP. **(B)** OriLyt input control. Southern of total cell DNA extracted from cotransfected Vero cells cleaved with EcoRI only and hybridized with a probe specific for HHV8 oriLyt sequences.

accumulation of replicated oriLyt. Both replication controls (MboI cleavage) and input control show that the replicated product is authentic and similar amounts of input oriLyt DNA were transfected.

Previously we demonstrated that the leucine zipper region of K-bZIP was essential for conferring a replication function to the protein (AuCoin et al., 2004a). We have now extended those previous findings by showing that mutations introduced into key leucine residues within the zipper motif profoundly affect the activity of K-bZIP in the origin-dependent replication assay. The results presented here show that leucine residue 205, along with residue 198 from the previous studies, are key amino acids with respect to DNA replication and homodimerization. One ramification of mutations within the leucine zipper region is that these mutations no longer allow for a K-bZIP-K-bZIP interaction. This suggests that dimerization of K-bZIP is essential for complementation of DNA replication.

Dimerization appears not to be necessary for the interaction of K-bZIP with K-Rta. K-bZIP mutant LZ-2 still interacted with K-Rta although it no longer complemented oriLyt-dependent DNA replication. This result can be interpreted two ways: i) it could be that K-bZIP interacts with K-Rta as a monomer or ii) it is essential that K-bZIP be in a dimer or multimer state when binding to K-Rta and it is this structure that contributes to activity. In a previous report, the BR of K-bZIP was implicated as the domain that interacts with K-Rta (Izumiya et al., 2003a). It also appears that the regulatory function of K-bZIP is distinct from the replication function. This is exemplified by the fact that a K-bZIP BR mutant was still capable of

complementing oriLyt-dependent DNA replication but was not able to suppress K-Rta-mediated transactivation.

Our results show that both the LZ and BR domains can interact with K-Rta. This may explain the apparent conflicting results from studies seeking to identify the K-bZIP domain that interacts with K-Rta (Izumiya et al., 2003a; Liao et al., 2003). It is clear from the results shown in this report that both structures contribute to the interaction of K-bZIP with itself, to the suppression of K-Rta, and to DNA replication.

Mutations introduced into the BR did not affect K-bZIP homodimerization but this domain still may be required for an as yet unidentified DNA-protein interaction. Indeed the complete removal of either the BR or LZ regions resulted in a loss of replication function. Interestingly, even though efficient binding was observed in co-immunoprecipitation experiments, mutations in the BR locus still affected the ability of K-bZIP to suppress the transactivation activity of K-Rta. This strongly suggests that inhibition of transactivation is not exclusively due to a protein-protein interaction. While others have investigated the role of specific elements in the repression of transcriptional activation by K-Rta, ours is the first study to evaluate the effects of specific amino acid residue changes within K-bZIP, particularly in the BR. Recently, it was shown that SUMO modification was partly responsible for transcriptional repression activity of K-bZIP (Izumiya et al., 2005). Sumoylation was shown to occur at lysine residue 158, a residue that was not altered in our studies. In addition, phosphorylation also plays a role in the repressive activity of K-bZIP on K-Rta mediated transactivation (Izumiya et al.,

2007). These data and the results presented here suggest that regulation of repression activity of K-bZIP is controlled by multiple elements and mechanisms. It is worth noting that another splice variant of K-bZIP exists in cells harboring viral DNA. This splice variant, K8β, contains the upstream basic region but not the C-terminal leucine zipper domain (Yamanegi et al., 2005). The K8β variant apparently serves to antagonize the activity of K-bZIP. The studies presented here involving the leucine zipper domain of K-bZIP would not affect the antagonistic function of K8β.

One BR mutant, BR-171/177/181, surprisingly increased the activity of K-Rta with respect to transcriptional activation of both the K-bZIP and PAN promoters. This result may be due to a change in the mode in which K-bZIP interacts with K-Rta, which may subsequently alter the binding of K-Rta, directly or indirectly, to K-Rta-binding sites within these promoters, leading to increased activity. This effect was also confirmed in the cotransfection replication assay where the addition of this BR mutant in the cotransfection mixture led to an increased intensity of amplified oriLyt. HHV8 oriLyt contains an ORF50 Response Element (RE) that is an almost exact copy of the PAN promoter ORF50RE. The apparent reason for an increased amplification signal for oriLyt is probably due to an up-regulation of transcriptional activation, from K-Rta, within oriLyt similar to what was observed with the K-bZIP promoter. Recently, it was postulated that the ORF50REs in K-Rta-responsive promoters fall into two groups (Chang et al., 2005). The interaction of K-Rta with the K-bZIP and ORF57 promoter may be indirect, in

that cellular transcription factors may be involved and may indicate an increased affinity for RBP-Jκ or increase the binding of the K-Rta complex for DNA.

A recent study suggested that K-bZIP may serve to recruit the core DNA replication enzymes and K-Rta to oriLyt (Wang et al., 2006). This recruitment could be in conjunction with transcription factors such as C/EBPα and the viral transactivator K-Rta. It is clear from the data presented here that the leucine zipper and basic regions contribute to the activity of K-bZIP and K-bZIP may aid in the function of K-Rta within oriLyt. Another scenario is that, in the context of the viral genome, K-bZIP may serve a dual role: i) as a negative regulator of DNA replication by controlling the effects of K-Rta, and ii) supplying an as yet undefined enzymatic function to lytic DNA replication, independent of its observed suppression activity.

Materials and Methods.

  Plasmids. The set of plasmids expressing the HHV8 core replication genes,

K-Rta and K-bZIP was previously described (AuCoin et al., 2004a).

  K-bZIP site-directed mutagenesis. A parent K-bZIP expression plasmid

was generated by subcloning the K-bZIP ORF into pCMV Xi (Genlantis) such that

either an in frame HA or FLAG tag was added. Site-directed point mutations were

introduced into the K-bZIP ORF using QuickChange mutagenesis kit (Stratagene)

according to manufacture's instructions. For the introduction of several different

base pair changes we subjected plasmids to multiple rounds of mutagenesis.

Primers were used such that amino acids were changed to alanine residues in the

basic regions and to valine residues in the leucine zipper region. The individual

plasmid names within the figures indicate which amino acid changes were made; all

plasmids were sequenced and the entire ORF was examined to ensure that the

proper mutation was made. For leucine zipper mutants: LZ-1, primer 5'-

GATGCACAAGTATGTTTCGTAGCGGCGAGATTGGAGGCACATAAG-3, was used and the

resulting mutant was used with primer, 5'-

AGGAACAGATTATTTTCCGTCGCGACATGCTGATGCGAATGTGCCAGC-3'; LZ-2, primer

5'-GAAAAGGATGCACAAGTATGTTTCCTAGCGGCGAGAGTGGAGGCACATAAGGAACAG-

3' was used; for LZ-3, primer 5'-

AAGGATGCACAAGTATGTTTCGTAGCGGCGAGATTGGAGGCACATAAG-3' was used.

Primers used to generate BR mutants and other mutants are listed in Table 1.

Cotransfection replication assay. Vero cells were transfected with the complete set of core replication proteins and wild type or mutated K-bZIP expression plasmids. A K-Rta expression plasmid was included in the transfection mixture when using wild type oriLyt but was omitted when using oriLyt plasmids containing the HCMV MIEP. Assays were performed as previously described (AuCoin et al., 2004a). To test K-bZIP BR or LZ mutants, the compete set of replication plasmids, along with a K-Rta expression plasmid and pDA15 were included in the transfection mixture. Mutant K-bZIP-encoding plasmids were substituted for a wt K-bZIP expression plasmid.

Antibodies. Mouse monoclonal anti-FLAG antibody (Sigma, St. Louis, MO) and mouse monoclonal anti-HA antibody (Sigma) were used as primary antibodies for immunoprecipitation and immunoblotting analysis; horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were used as secondary antibodies. M2 monoclonal mouse anti-FLAG-conjugated agarose beads (Sigma, St. Louis, MO) were used for the immunoprecipitation of FLAG-tagged K-bZIP proteins and complexes. A K-Rta antibody (gift from D. Ganem, UC San Francisco) was also used in co-immunoprecipitations. The anti-K-bZIP antibody was generated at the University of Nevada Monoclonal Antibody Facility using antigen purified from mammalian (Vero) cells.

Co-immunoprecipitation and Western analysis. Ten-centimeter dishes of 293FT cells were transfected with 5 μg of wild-type HA-tagged K-bZIP expression plasmid and 5 μg of FLAG-tagged K-bZIP mutant expression plasmid using the

TransIt LT1 Transfection Reagent (Mirus, Madison, WI). Protein was harvested from the transfected 293FT cells 24-48 hours after transfection. The transfected 293FT cells were rinsed twice with ice-cold PBS (phosphate-buffered saline solution, 10 mM $NaPO_4$, 137 mM NaCl, 2.5 mM KCl, pH 7.5) and lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X, 0.1% Tween 20, pH 7.5) with 10μl/mL protease inhibitor cocktail for mammalian tissues (Sigma, St. Louis, MO). DNA was sheared by passing the lysate three times through a 22-gauge needle, and cell debris was cleared by centrifugation at 12 000xg for 10 min at 4oC. The K-bZIP-K-bZIP immunocomplex was captured by addition of 40 μl of anti-FLAG M2 mouse monoclonal antibody-conjugated agarose beads (Sigma, St. Louis, MO) and incubation overnight at 4oC with gentle rotation. The beads were washed three times for 5 min each in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and then boiled for 5 min in 70 μl of Laemli sample buffer-5% β-mercaptoethanol (Bio-Rad, Hercules, CA) to denature the anti-FLAG antibodies and elute the K-bZIP-K-bZIP immunocomplex. Protein samples from the cell lysate and the immunoprecipitated protein were separated by 10%-SDS polyacrylamide gel electrophoresis and then transferred onto 22 μm polyvinyldene difluoride membrane (Bio-Rad, Hercules, CA) using a semidry transfer apparatus (Bio-Rad, Hercules, CA). After blocking non-specific antigen binding for 15 min at room temperature in TBST (20 mM Tris-HCl, 137 mM NaCl, 0.05% Tween 20, pH 7.6)-5% skim milk, the membranes were incubated with primary antibodies for 1hr at room temperature or overnight at 4oC with gentle rotation. Protein species within each immunoprecipitate were detected

using anti-HA antibody and separately using anti-FLAG antibody, to detect the

presence of both wild-type (HA-tagged) and mutant (FLAG-tagged) K-bZIP. The final

dilution of the M2 mouse monoclonal anti-FLAG primary antibody used was 1:5000.

After incubation with primary antibody the membranes were washed with TBST for

15 min at room temperature, with rotation. The membranes were then incubated

with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary

antibody (1:5000 dilution) for 30 min at room temperature, then washed with TBST

for 15 min at room temperature. The membranes were exposed for 5 min using a

chemiluminescent HRP substrate (SuperSignal West Femto Maximum Sensitivity

Substrate, Pierce, Madison, WI), and visualized using a Bio-Rad Chemi Doc.

Luciferase Reporter Assay. Vero cells were cotransfected with pGL3-K-

bZIP promoter (provided by H-S Kung, UC Davis), a K-Rta expression plasmid

(AuCoin et al., 2004a) and plasmids expressing K-bZIP BR or LZ mutants. Cells were

lysed and standard luciferase detection was performed (Promega). Each assay was

measured in triplicate and each experiment was repeated three times. A

representative experiment is shown.

Table 1. Primers used to generate site-directed K-bZIP mutants.

Mutant		Oligonucleotide Sequence
BR-A171/174 /185	F	BR-A171/174 Template + Primer for BR-A185
	RC	BR-A171/174 Template + Primer for BR-A185
BR-A170/177 /181	F	BR-A177/181 Template + Primer for BR-A170
	RC	BR-A177/181 Template + Primer for BR-A170
BR-A171/177 /181	F	BR-A177/181 Template + Primer for BR-A171
	RC	BR-A177/181 Template + Primer for BR-A171
BR-A170/171 /177/181	F	BR-A177/181 Template + Primer for BR-A170/171
	RC	BR-A177/181 Template + Primer for BR-A170/171
BR-A171/174 /185	F	BR-A181/185 Template + Primer for BR-A170
	RC	BR-A181/185 Template + Primer for BR-A170
BR-A171/181 /185	F	BR-A181/185 Template + Primer for BR-A171
	RC	BR-A181/185 Template + Primer for BR-A171
BR-A174/181	F	BR-A181/185 Template + Primer for BR-A174

/185	RC	BR-A181/185 Template + Primer for BR-A174
BR-A170/171 /181/185	F	BR-A181/185 Template + Primer for BR-A170/171
	RC	BR-A181/185 Template + Primer for BR-A170/171
BR-A171/174 /181/185	F	BR-A181/185 Template + Primer for BR-A171/174
	RC	BR-A181/185 Template + Primer for BR-A171/174
BR-A174/177 /181/185 (BR-A174/177 Template)	F	5'- GGCGCCGTGTCATCGGCAGCATACACAGCACAGCTGCAGCAG - 3'
	RC	5' - GCCTGCTGCAGCTGTGCTGTGTATGCTGCCGATGACACGGCGC - 3'
Deletion 1	F	5' – AGCTCAAGCTTCGAATTCATGTTTTTTACGGACAATACTG – 3'
	RC	5' – CAGTATTGTCCGTAAAAAACATGAATTCGAAGCTTGAGCT – 3'
Deletion 2	F	5' – AGCCTCAACGGGCAACCAGAAACGGTCATTGACCTTACAG – 3'
	RC	5' – CTGTAAGGTCAATGACCGTTTCTGGTTGCCCGTTGAGGCT – 3'
Deletion 3	F	5' – CCCGGCCATACCGGTCTGTCCTTCCTGGACGCTCTCTC – 3'
	RC	5' – GAGAGAGCGTCCAGGAAGGACAGACCGGTATGGCCGGG – 3'
Deletion 4	F	5' – TAAATTCCACATCCCCGATGAACGCTTATGCACTAAGG – 3'

	RC	5' – CCTTAGTGCATAAGCGTTCATCGGGGATGTGGAATTTA – 3'
Deletion 5	F	5' – ACATAGAAAGTTTGAAGAGCAAGGCAGATACGGCCGCG – 3'
	RC	5' – CGCGGCCGTATCTGCCTTGCTCTTCAAACTTTCTATGT – 3'
Deletion 6	F	5' – GTCACATTCTCCCACGCGAAAGGAGGCACATAAGGAACAGA – 3'
	RC	5' – TCTGTTCCTTATGTGCCTCCTTTCGCGTGGGAGAATGTGAC – 3'
Deletion 7	F	5' – CCTAGCGGCGAGATTGGATTACAAGGATGACGACGATAAGTG – 3'
	RC	5' – CACTTATCGTCGTCATCCTTGTAATCCAATCTCGCCGCTAGGA – 3'
NO BR	F	5' – GTATGTGATCAGTCACATTCTCCCCAGCTGCAGCAGGCATTA – 3'
	RC	5' – TAATGCCTGCTGCAGCTGGGGAGAATGTGACTGATCACATAC - 3'
NO LZ	F	5' – AAGACAGCTGCAGCAGGCAATGCGAATGTGCCAGCAGCCAG – 3'
	RC	5' – CTGGCTGCTGGCACATTCGCATTGCCTGCTGCAGCTGTCTTGT – 3'
LZ-1 (LZ-3 Template)	F	5' – CATAAGGAACAGATTATTTTCGTTCGCGACATGCTGATGCGA – 3'
	RC	5' – TCGCATCAGCATGTCGCGAACGAAAATAATCTGTTCCTTATG – 3'
LZ-2	F	5' – GATGCACAAGTATGTTTCCTAGCGGCGAGAGTGGAGGCACAT – 3'

	RC	5' – ATGTGCCTCCACTCTCGCCGCTAGGAAACATACTTGTGCATC – 3'
LZ-3	F	5' – GAAAAGGATGCACAAGTATGTTTCGTAGCGGCGAGATTGGAG – 3'
	RC	5' – CTCCAATCTCGCCGCTACGAAACATACTTGTGCATCCTTTTC – 3'
NO BR – V198/205	F	LZ-2 Template + Primer for NO BR
	RC	LZ-2 Template + Primer for NO BR
NO LZ – A170/171 /181/185	F	BR-A170/171/181/185 Template + Primer for NO LZ
	RC	BR-A170/171/181/185 Template + Primer for NO LZ
NO LZ – A171/174 /181/185	F	BR-A171/174/181/185 Template + Primer for NO LZ
	RC	BR-A171/174/181/185 Template + Primer for NO LZ
NO LZ – A174/177 /181/185	F	BR-A174/177/181/185 Template + Primer for NO LZ
	RC	BR-A174/177/181/185 Template + Primer for NO LZ

F= Forward Primer   RC=Reverse Complement Primer

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Over-Expression of The Kaposi's Sarcoma-Associated Herpesvirus Transactivator K-Rta can complement a K-bZIP deletion BACmid and yields an enhanced growth phenotype

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ABSTRACT

Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8

(KSHV/HHV8) ORF50 encodes a transactivator, K-Rta, which functions as the switch

from latent to lytic virus replication. K-bZIP interacts with K-Rta and can repress its

transactivation activity for some viral promoters. Both K-Rta and K-bZIP are

required for origin-dependent DNA replication. To determine the role of K-bZIP in

the context of the viral genome, we generated a recombinant HHV8 BAC with a

deletion in the K-bZIP ORF. This BACmid, BAC36ΔK8, displayed an enhanced

growth phenotype, with respect to virus production and accumulation of viral

encoded mRNAs measured by real-time PCR (qPCR), when K-Rta was used to induce

the virus lytic cycle. Conversely, induction of the virus lytic cycle using TPA/n-

butyrate resulted in no virus production and an aberrant gene expression pattern

from BAC36ΔK8 containing cells compared to wtBAC. This null virus phenotype

was efficiently complemented by the expression of K-bZIP in *trans* restoring virus

production to wtBAC levels. Immunofluorescence staining revealed that subcellular

localization of K-Rta was unchanged, however a disruption of LANA subcellular

localization was observed in cells harboring BAC36ΔK8 suggesting that K-bZIP

influences LANA localization. Co-immunoprecipitation experiments confirmed that

K-bZIP interacts with LANA in BCBL-1 cells and in cotransfection assays. Lastly, the

ChIP assay revealed that, in an environment where K-Rta is over-expressed and in

the absence of K-bZIP, K-Rta binds to C/EBPα sites within oriLyt suggesting that it is

K-Rta that supplies an essential replication function and K-bZIP may serve to

augment or facilitate the interaction of K-Rta with oriLyt.

INTRODUCTION

Transient assays have shown that Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 (KSHV/HHV8) lytic origin-dependent DNA replication requires the gene products of open reading frames (ORFs) 6, 9, 40/41, 44, 56 and 59 (1). It was also shown that two additional ORFs, 50 and K8 were necessary for efficient replication. The requirement for ORF50 (K-Rta), with respect to oriLyt function, is linked to the presence of an ORF50-responsive promoter present within oriLyt (1, 34). In addition K-Rta was required in the context of the viral genome for the activation of the entire lytic cycle as demonstrated by experiments using an HHV8 BAC recombinant with a deletion of the ORF50 gene (37).

K-bZIP is the proposed homolog of Epstien-Barr virus (EBV) Zta and was shown to interact with oriLyt through a piggyback conformation using the transcription factor CAAT enhancer binding protein α (C/EBPα) (20, 33). In EBV, Zta is required for DNA replication and is the lytic switch protein with transactivation activity (6, 7, 9, 19, 28). However, no associated transactivation activity has been found for K-bZIP. In contrast, transient assays demonstrated that K-bZIP interacts with K-Rta and suppresses the transactivation activity of K-Rta of some viral promoters (10, 11, 18). This repression effect may serve to modulate the activity of K-Rta during a lytic infection. K-bZIP contains a basic amino acid region and a putative leucine zipper that is essential for the associated replication function

in transient assays (1). K-bZIP also interacts with several cellular factors such as

transcription factors and proteins that control cell cycle (12, 13, 18, 26).

Recently, it was shown that K-Rta interacts with oriLyt in a region of HHV8

that contains C/EBPα transcription factor binding sites presumably through a

protein-protein interaction with K-bZIP (35). K-Rta was also shown to interact with

members of the core replication complex. These data suggest that K-Rta plays a role

in lytic replication in addition to that of a transcriptional activator.

To date most of the data collected, with respect to the activity of K-bZIP, is in

the context of transient assays. These assays, for example the cotransfection

replication assay, are invaluable with respect to initial characterization of protein

function and interaction with cellular and viral factors. Transient assays, however,

are limited in that they cannot address the activity of viral proteins in the context of

the viral genome in a dynamic cellular environment. To this end, we generated an

HHV8 BAC recombinant that has the entire K-bZIP ORF deleted. This recombinant,

BAC36ΔK8, was used to produce stable latently infected Vero cell lines. BAC36ΔK8

and BAC36 (wild type)-containing cells lines treated with TPA/n-butyrate, to induce

viral lytic replication, revealed that the BAC36ΔK8 was incapable of reactivation and

did not produce infectious virus, indicating that K-bZIP is required for virus

replication. In addition, although sucellular localization of ORF50 was unchanged,

BAC36ΔK8 cell lines displayed a non-punctate pattern for LANA. Co-

immunoprecipitation experiments showed that K-bZIP directly interacted with

LANA suggesting a role for K-bZIP in latency and/or reactivation. Trans expression

of K-bZIP in BAC36ΔK8 containing cells restored virus production to wt BAC levels

and partially redistributed LANA nuclear localization pattern those observed in wt

BAC cell lines indicating that the null virus phenotype was due to lack of expression

of K-bZIP. In contrast, when lytic reactivation was induced using a recombinant

adenovirus that over-expressed K-Rta, BAC36ΔK8 produced infectious virus at

levels many fold higher than those observed with BAC36 using the same conditions.

Analysis of gene expression revealed an increase in accumulation of mRNA encoding

immediate early, early and late viral proteins. These data strongly suggest that K-

bZIP serves to regulate viral gene expression in the context of the viral genome and

that over-expression of K-Rta in BAC36ΔK8-containing cells can compensate for the

replication associated properties for K-bZIP. A ChIP assay performed using

BAC36ΔK8 containing cell lines in an environment where K-Rta was over-expressed

clearly demonstrated that K-Rta can interact with oriLyt at the C/EBPα sites in the

absence of K-bZIP strongly suggesting that K-Rta and not K-bZIP supplies an

essential replication function. Hence, K-bZIP may act to amplify or augment the role

of K-Rta in initiation of lytic DNA synthesis, but K-bZIP itself is not required for lytic

DNA replication under conditions where K-Rta is over-expressed.

MATERIALS AND METHODS

Cells and plasmids. Vero and Ad293 cells were maintained in Dulbecco's

modified Eagle medium (DMEM) supplemented with 10% bovine growth serum

(BGS). BCBL-1 cells were maintained in RPMI supplemented with 10% fetal bovine

serum. BAC36, the wild-type HHV8 BACmid, was provided by Dr. S. Gao (University

of Texas). Vero cells containing the BAC constructs were maintained in DMEM

supplemented with 10% BGS (Hyclone, Logan, UT) and 250 μg/ml hygromycin.

Plasmid pCMVK8 was generated using forward primer: 5'-

cgacttaacagatctcgagctcaagcttcgaattcATGCCCAGAATGAAGGACATACCTACTAAGAGTT

C -3' and reverse primer: 5'

cccgggcccgcggtaccgtcgactgcagaattcTCACTTATCGTCGTCATCCTTGTAATCACATGGTG

GGAGTGGCGCGTC 3' in a PCR reaction using K-bZIP cDNA clone pcDNA3.1-K-bZIP

and the LANA expression plasmid pGTR4-ORF73 LANA were supplied by D. Ganem

(UC San Francisco).

Construction of a KSHV K8 (K-bZIP) deletion mutant, BACΔK8.

Mutagenesis of the BAC36 was performed using the Red Recombination method as

described previously (38). The forward PCR primer: 5'-

gtgtaggctggagctgcttcttcctccgttgtcgactataacctggcgtgtaaacgtgtaaccctgccaaat-3' and

reverse primer: 5'-

ctatacctgctgcagctgtcttgtgtatgctttcgatgacacgcggccgtatctgtcaaacatgagaattaa-3' were

used to amplify a Kanamycin resistance cassette flanked by BAC sequences. This

cassette was transformed into BAC36-containing bacteria and the resulting colonies

were plated on Kanamycin plates and subsequently screened by Southern blot. Purified BACΔK8 DNA was transfected into Vero cells using Lipofectamine 2000 (Invitrogen) according to the manufacture's instructions. Individual cell colonies were selected and expanded and cells stably harboring the BACΔK8 DNA were selected with the 1mg/ml hygromycin and propagated in the 250μg/ml hygromycin. Once cells were expanded, the BACmid was isolated from Vero cells and re-transformed into bacteria and analyzed for the presence of the deletion by sequence analysis and Southern blot.

High titer recombinant adenovirus 50 stock preparation. A recombinant adenovirus expressing ORF50, Ad50, was provided by Dr. Ganem (UCSF) and was expanded in Ad293 cells (Stratagene). The infected Ad293 cells were harvested and centrifuged (5000xg) for 10 min to remove the media. The Ad293 cell pellet was washed with the hanks' balanced salt solution, recentrifuged and resuspended in 8 mls of the salt solution. The virus was released through 4- freeze/thaw/vortex cycles. The cell debris was removed by centrifugation at 1500 xg for 5 min. The recombinant adenovirus was purified by cesium chloride density gradient centrifugation for 18 hours at 100,000 xg. The virus fraction was collected with a syringe and a needle and stored in the 2X storage buffer (10mM Tris pH8.0, 100mM NaCl, 0.1% BSA, & 50% glycerol). For control infection experiments, AdTrack (expresses EGFP) viral stocks were prepared as described above.

Construction of a recombinant adenovirus expressing K-bZIP-FLAG. AdEasy Adenoviral Vector System (Stratagene) was used for generation of the AdK8.

The K-bZIP cDNA ORF was amplified from pTARGET K-bZIP-FLAG and ligated into AdEasy pShuttle vector (Stratagene). Recombinant adenovirus was generated as per manufacture's instructions.

Induction of lytic replication and detection of supernatant virus. Vero cells stably harboring BAC36 or BAC36ΔK8 were treated with either TPA (25ng/ml)/n-butyrate (0.3 mM) and high-titer recombinant adenovirus ORF50 at the MOI of 4500 or cells were treated with the TPA/n-butyrate mixture only. To compliment K-bZIP, both BAC36 and BAC36ΔK8-containing mutant cell lines were infected with a recombinant adenovirus expressing K-bZIP or transfected with a plasmid encoding K-bZIP under the control of the PAN promoter. Media was harvested and centrifuged twice at 1000 xg for 10 min. Then, supernatant was centrifuged for 1 hour at 100,000 xg using a SW41Ti rotor. Supernatant was discarded and the virus pellet was kept at $4°C$ overnight. The pellet was resuspended in media and the virus was used to infect fresh Vero cells. The presence of infectious virus was observed through the expression of GFP or quantitated using qPCR. For qPCR, pelleted virus was DNAase treated (Turbo DNA, Ambion) to remove any contaminating DNA and then viral DNA was extracted from the purified virus pellet as previously described (38). Briefly, one equal volume of the DNA cell extraction buffer (2% SDS, 10 mM EDTA and Tris-HCL pH 8.0) with proteinase K (150 µg/ml) was added to the virus suspension. DNA was extracted using phenol:choloform:isoamyl alcohol (24:24:1) followed by ethanol precipitation. Equal volume of DNA was used for qPCR analysis. BAC36 DNA was utilized for a

standard curve for the analysis. Each experiment was performed a minimum of three times.

RNA purification and quantitation of the viral transcripts by qPCR. Vero cells containing BAC36 or BAC36ΔK8 were treated with TPA (25ng/ml)/n-butyrate (0.3 mM) and high-titer recombinant adenovirus ORF50 or TPA/n-butyrate alone to induce lytic replication. The cells were harvested at various time points and total RNA was extracted with the PureLink total RNA purification system kit (Invitrogen) according manufacture's instructions. cDNA was synthesized from 2μg of total RNA in the presence of random hexamers, dNTPs and superscript III reverse transcriptase (Invitrogen). Data was analyzed as previously described (38).

Generation of a monoclonal antibody against K-bZIP (K8 Mab). A monoclonal antibody for K-bZIP was produced at the University of Nevada Monoclonal Antibody Core Facility. Vero cells infected with AdK8 were prepared using lysis buffer (50mM Tris-HCL, pH 7.4, 150 mM NaCl, 1 mM EDTA 1% Triton X 100 and 0.1% Tween) and recombinant K-bZIP-FLAG protein was affinity purified using FLAG-affinity beads. The antibody is an IgG_1 isotype.

Detection of viral proteins with immunofluorescence assay. Vero cells containing BAC36 or BAC36ΔK8 were plated on coverslips and reacted with anti-HHV8 specific antibodies as previously described (37). Cells were imaged using a Olympus Fluoview FV1000 microscope using FV10-ASW 1.5 viewer software. The ORF50 antibody used was a gift from D. Ganem. For complementation of LANA

subcellular distribution, cells were trasnsfected with pCMVK8 at various concentrations and imaged as above.

Co-immunoprecipitation assay. 2×10^8 BCBL-1 cells were treated with TPA (25ng/ml) and NaB (0.3mM) for four days, nuclear extract protein was prepared according to Sigma Cellytic NuCLEAR Extraction Kit protocol without the use of a detergent. K-bZIP, K-Rta or LANA (ABi cat #13-210-100) specific antibodies (10 µl) was added to 400 µl of BCBL-1 nuclear extract and the mixture was rotated at 4° C for 2 h followed by the addition of 50 µl of protein G beads. This mixture was further rotated overnight at 4° C. The beads were washed 4 times with 1 ml of TBS (Tris-HCL, pH 7.4, 150 mM NaCl) rotating for 10 min each at 4° C between every wash. Thirty microliters of the immunoprecipitated protein was separated through a 10% SDS PAGE gel which was subsequently transferred to Immobilion P membrane (Millipore). After an initial blocking step (30 min with TBS + 5% non-fat milk), blots were reacted with anti-LANA, anti-K-Rta or anti-K-bZIP antibody overnight at 4° C followed by washing and incubation with secondary antibody anti-IgG HRP conjugated. Protein bands were visualized using a chemiluminescence substrate (Pierce Fempto) and a CCD camera.

For cotransfections, 293FT (2×10^6 10 cm dish) cells were transfected with LANA, K-bZIP and/or K-Rta expression plasmids using TransIT LT1 (Mirrus). Fourty eight hours post transfection protein extracts were prepared using lysis buffer (50 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Trition X100 and 0.1 % NP40) and anti-LANA specific antibody was added and co-

immunoprecipitations were done as describe above. For control immunoprecipitation experiments, the monoclonal antibody Mab84 was used and was previously described (4).

Chromatin immunoprecipitation assay (ChIP). Vero cell lines (4 X 10⁷) containing BAC36ΔK8 were infected with Ad50 (MOI 4500) for 4 days. Samples were fixed for 10 min with 1% formaldehyde, washed twice with 1X PBS and lysed for 15 min in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton-X 100, 0.1% Tween 20, 1mM EDTA). Samples were sonicated to shear DNA (~500 bp) and fragments were diluted six-fold with ChIP buffer (12.5 mM Tris pH 8, 200 mM NaCl, 1% Triton-X 100) and precleared with mouse IgG-AC (Santa Cruz Biotechnology) at 4°C for 30 min. For each immunoprecipitation, 2 μg of antibody (anti-ORF50 or anti-Kb-ZIP) was incubated with the lysate at 4°C overnight. A no antibody and antibody isotype control immunoprecipitation was also performed. Protein G-Plus agarose beads (Santa Cruz Biotechnology) were blocked with varying amounts of sheared salmon sperm DNA and BSA at 4°C overnight then washed with ChIP buffer. The blocked and washed protein G-plus beads were incubated with the lysate at 4°C for 1 h. The beads were washed once with low salt buffer (0.1% SDS, 0.1% Triton-X 100, 2mM EDTA, 20 mM Tris pH 8, 150 mM NaCl), once with high salt buffer (0.1% SDS, 0.1% Triton-X 100, 2mM EDTA, 20 mM Tris pH 8, 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 10 mM Tris pH 8) and twice with TE. Beads were resuspended in TE, incubated with RNase A at 37°C for 30 minutes, then incubated with proteinase K and 10% SDS at 37°C for 4 h, followed

by incubation at 65°C overnight. For input control samples, NaCl was added to the

sonicated lysate to a final concentration of 0.3 M, and incubated at 65°C overnight.

After the antibody, no antibody and isotype control immunoprecipitated samples

were filtered through a 0.45 μm filter to remove the agarose beads, they were

extracted with phenol-chloroform and ethanol precipitated. Primers used for PCR

amplification 5'- AATCCCCCATAATCCTCTGC-3' and reverse 5'-

GGAAAAATCAAAACAAAACTC-3' correspond to nucleotides 23,326-23,572 of HHV8

oriLyt. Control primers: 5'-ACGTCCGGAGAGTTGGAACTGTCA-3' and 5'-GGGGTC

CATGGGATGGGTTAGTCA-3', are complementrary to the ORF45 region.

RESULTS

Deletion of the K8 (K-bZIP) locus from BAC36. K-bZIP is the product of a

spliced transcript that originates just downstream of the ORF50 loci (Fig. 1A). Using

the wild-type BAC36 as template we designed PCR primers such that the entire K-

bZIP ORF was removed from the genome. This construction did not disrupt the

downstream K8.1 ORF or the polyadenlylation signal at nt 76,713, which is

presumably used by both K8.1 and K-Rta (ORF50) (29, 30). The final BAC mutant

also left 328 nts upstream of the start of the K8.1 mRNA (22, 31). Figure 1 shows a

schematic for the generation of the recombinant, BAC36ΔK8, using PCR primers that

integrated the Kanamycin resistant gene marker (Kan+) into the HHV8 BAC genome

replacing the entire K-bZIP ORF. Since we designed primers to contain the substrate

sequence for FLP recombianase (FRT sites), the Kan+ marker was subsequently

removed by expressing FLP recombinase in bacteria (Fig. 1A). This excision of the

Kan+ cassette retained only the 15 bp FRT site, which we engineered to contain a

Hind III recognition sequence, therefore the function of any promoter or other cis

acting element upstream or downstream of the K-bZIP ORF was not affected.

Resulting BAC recombinants were screened and analyzed by agarose gel

electrophoresis and subsequently by Southern blot analysis to show that the K-bZIP

ORF was removed from the HHV8 genome (Fig. 1B and C). BACΔK8 *Hind* III

cleavage results in a 2.2 kb band along with a 5 kb band which is generated from

further downstream, upon removal of the K8 locus (and Kan+ cassette) (Fig 1B)

indicating that the K-bZIP ORF was deleted. A Southern blot showed the presence of

a 2.2 kb band in the recombinant BACΔK8 when hybridized with a probe from

within the ORF50 locus (Fig. 1A and C). Subsequently, the recombinant was

sequenced to ensure that the proper insertion/deletion was made.

Figure 1. Generation of a recombinant HHV8 BAC with a deletion in the K8 (K-bZIP) open reading frame. (A) Schematic diagram showing the ORF50 K8 genomic BAC36 locus that was targeted to produce a recombinant with a deletion of the K-bZIP ORF by replacing it with the gene cassette that confers kanamycin resistance flanked by FRT sites. Also shown is the final gene configuration after removal of the Kan cassette using FLP recombinase and the location of the hybridization probe used for Southern blot confirmation. **(B)** Ethidium bromide-stained gel BAC36 (lane 1), and the mutated BACmid, BAC36ΔK8 (lane 2) cleaved with HindIII. Shown are the resultant 5.0 and 2.2 kb bands in the BAC36ΔK8 recombinant due to introduction of an additional Hind III site within the FRT recognition sequence. **(C)** Southern blot of BAC36 and BAC36ΔK8 purified DNA cleaved with HindIII. Shown are the expected bands produced from both BACmid DNAs. The probe used for the experiment is shown in (A).

BAC36ΔK8 harboring cell lines produce 30-fold more virus than BAC36

containing cells when treated with a K-Rta expressing adenovirus. We used the

BAC recombinant, BAC36ΔK8, and BAC36 to generate Vero cell lines that harbored

either latent BAC36ΔK8 or BAC36 DNA. Once these cell lines were established, the

first step was to induce the lytic cycle in each BACmid-containing cell line and

compare virus production and mRNA accumulation levels. We initially chose to

induce lytic replication by infecting the BACmid-containing cells with a recombinant

adenovirus expressing (Ad50) the major viral transactivator the lytic switch protein

K-Rta. Previous studies demonstrated that this treatment, using concentrated Ad50,

resulted in an extremely efficient reactivation of latent BAC36 from Vero cell lines

(8).

BAC36 or BAC36ΔK8 containing cell lines were infected with Ad50 and

supernatant virus was concentrated as previously described (38). The supernatant

virus was used for qPCR analysis of viral DNA and for infection of fresh Vero cells to

evaluate the number of green cells (infectious virus). qPCR performed on the

supernatant virus revealed that an approximately 30-fold increase in supernatant

viral DNA was produced from BAC36ΔK8-containing cells treated with Ad50 when

compared to similarly treated BAC36 containing cells (Fig. 2A). This observation

was confirmed when equal volumes of supernatants from each BAC containing cell

line was incubated with fresh Vero cells. We also measured the relative levels of K-

Rta protein present in each cell line. Cellular protein lysates were prepared from

each Ad50-infected cell line and Western blots were performed using an anti-K-Rta

specific antibody. The K-Rta protein levels were similar in each cell line

demonstrating that Ad50 efficiently infected both cell lines. In order to rule out

possible complementation of BAC36ΔK8 by an adenovirus protein we transfected
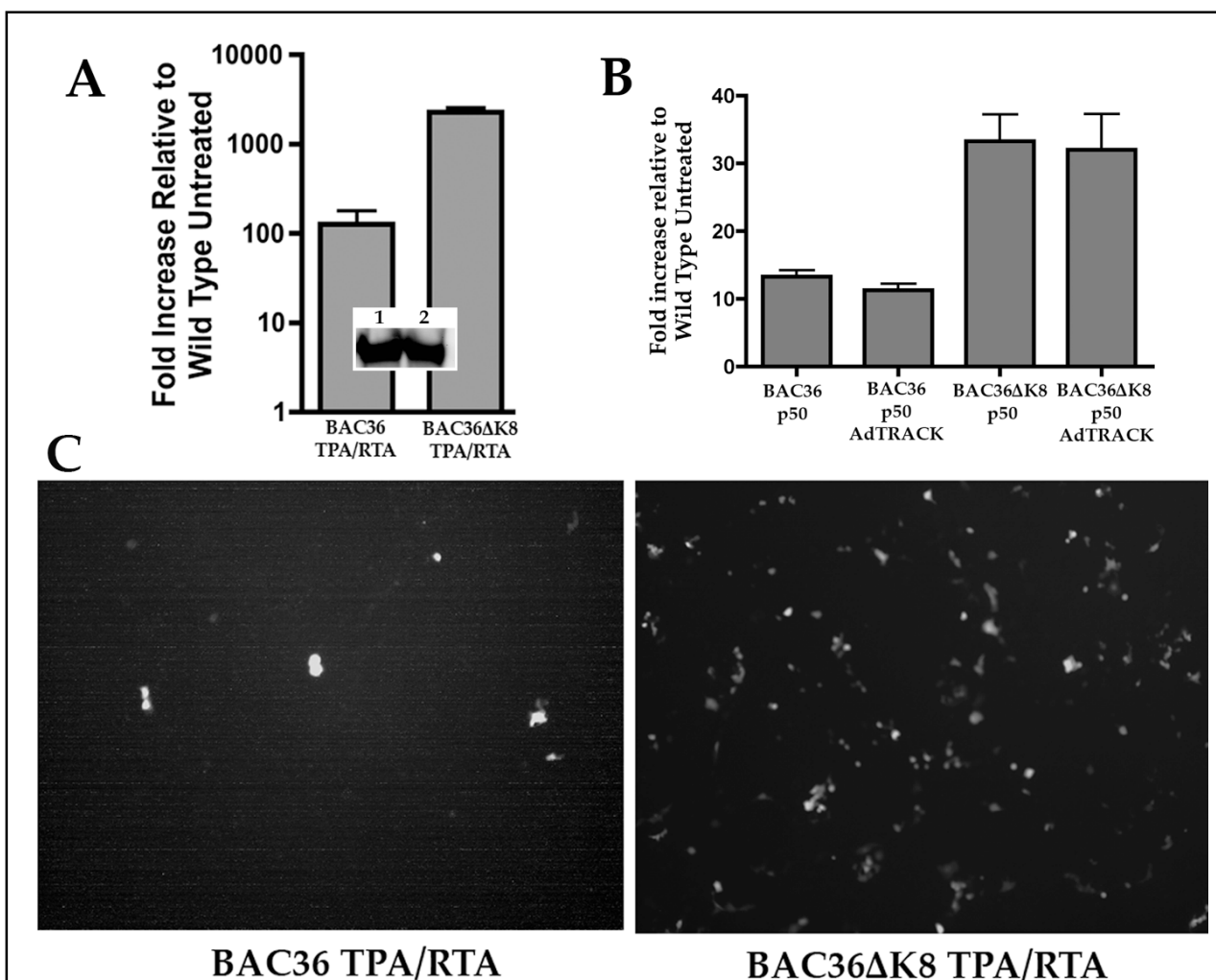
Figure 2. BAC36ΔK8 displays an enhanced growth phenotype upon lytic cycle induction with Ad50. Vero cell lines stably carrying BAC36 or BAC36ΔK8 were treated with TPA (25ng/ml) and Ad50 (MOI 4500) at 24-h post plating and incubated for 5 days. Supernatant virus was harvested as described in materials and methods and total cellular RNA was extracted and subjected to qPCR analysis. **(A)** Quantification of the viral DNA by qPCR. Viral DNA was extracted from supernatant virus and qPCR was performed using ORF59 primers/probe. The relative increase in viral DNA was determined and expressed as a fold increase compared to the untreated BAC36 supernatant virus. **Inset**: Western blot reacted with anti-K-Rta antibody from protein lysates from Ad50-infected BAC-containing cell lines. Lanes: 1, protein lysate from BAC36 cell line infected with Ad50; 2, protein lysate from BAC36ΔK8-containing cells infected with Ad50. **(B)** Addition of adenovirus has no effect on HHV8 BAC virus production. Vero cell lines containing either BAC36 or BAC36ΔK8 were transfected with a K-Rta expression plasmid (pTarget K-Rta-FLAG) and subsequently infected with the control adenovirus AdTRACT. Viral DNA was extracted from the supernatants and analyzed as described in A. **(C)** Ad50 induced BAC36ΔK8 results in an increase in infectious virus. Concentrated supernatant virus from BAC36ΔK8 or BAC36 cell lines was used to infect fresh Vero cells. Cells were imaged 3-5 days post infection for the presence of EGFP positive cells.

cell lines with a K-Rta expression plasmid and subsequently infected with the control adenovirus AdTRACK (Clonetech). The addition of a control adenovirus had no affect on the level of supernatant virus produced in BAC36 or BAC36ΔK8 containing cell lines indicating that an adenovirus encoded protein did not account for the increase in virus production (Fig. 2B). This control experiment demonstrated that over expression of K-Rta, by plasmid transfection, also resulted in an increase in virus production in the BAC36ΔK8 containing cell line, however the response was not as dramatic as that observed in Ad50 infected samples.

In Ad50-treated cell lines, microscopic examination revealed the presence of many more green (virus infected) cells from BAC36ΔK8 supernatant virus compared to cells incubated with BAC36 supernatant virus (Fig. 2C). These results suggested that under conditions where an excess of K-Rta was present, the BACmid that lacked the ability to express K-bZIP produced a much higher amount of infectious virus than K-bZIP-competent BACmid.

Increased accumulation of viral-specific transcripts from cells containing BAC36ΔK8 treated with Ad50 and TPA/n-butyrate. Since we observed an increase in infectious virus and viral DNA in supernatants from cells containing BAC36ΔK8 treated with Ad50 (MOI 4500) we investigated the relative accumulation levels of viral specific mRNAs from both BAC36 and BAC36ΔK8 cell lines. Cell lines were induced with Ad50, total cellular RNA was harvested at five days post induction and qPCR was performed. We measured mRNA accumulation

for transcripts ORF40/41, ORF44, ORF59, K8.1 and LANA. The overall level of

mRNA accumulation was about 2-6 fold higher in cells harboring BAC36ΔK8 when

compared to BAC36 containing cells (Fig. 3). The largest difference was observed in

the mRNA encoding K8.1, which encodes a late protein that is structural component

of the HHV8 particle (17, 27). This is interesting since it was suggested that the K8.1

promoter lies within the K8 ORF but apparently the upstream region in the K8-

deleted BAC was sufficient for K8.1 expression (31). Overall, the transcription data

suggests that an increase in accumulation of all kinetic classes of RNA occurred in

the cell line containing BAC36ΔK8 when compared to BAC36 cell lines treated with

K-Rta/TPA.

Figure 3. qPCR analysis of several viral encoded mRNAs from Ad50-induced BAC-containing cell lines. Vero cell lines harboring BAC36ΔK8 or BAC36 were treated with TPA and Ad50 (MOI 4500) and total cellular RNA was harvested and following cDNA synthesis was analyzed by qPCR using primers and probes specific for several viral transcripts. Cyclophilin was used as an internal standard and data was normalized and analyzed using the comparative Ct method. The data is reported as relative fold increase over untreated BAC36. Each analysis was done 6 times.

We also evaluated the mRNA accumulation of ORF57 in Ad50-treated BAC36

and BAC36ΔK8 containing cell lines. In transient assays, ORF57 expression was

transactivated and upregulated by K-Rta and the two proteins were shown to

interact in infected and transfected cells (3, 5, 21, 24, 32). In addition, K-bZIP

suppresses the K-Rta-mediated transcriptional activation of the ORF57 promoter

(11). We infected BAC36ΔK8 cell lines using several different Ad50 MOIs and

measured the relative amounts of ORF57 mRNA accumulation in each cell line.

ORF57 accumulation, at the highest MOI (4500), resulted in an approximately 1000-

fold difference in ORF57 mRNA accumulation in BAC36ΔK8-containing cells (Fig. 4).

To ensure that the level of K-Rta expression was the same in both Ad50-infected cell

lines, the relative amount of K-Rta expression was measured by western blot in both

Figure 4. Ad50 treatment induces a marked increase in the accumulation of ORF57 mRNA in BAC36ΔK8. BAC36 and BAC36ΔK8 cell lines were treated with TPA (25ng/ml) and increasing amounts of Ad50. Total cellular RNA was extracted and ORF57 mRNA accumulation was measured at 5 days post treatment by qPCR. Data is shown as fold increase over untreated BAC36. Error bars are the standard deviations from three separate experiments.

cell lines at the three highest MOIs used. No difference in K-Rta expression was observed hence the increase in ORF57 mRNA was not due to an increase in K-Rta protein levels in BAC36ΔK8-containing cells (data not shown). These results indicated that in the absence of K-bZIP and under conditions where K-Rta is over-expressed, both virus replication and gene expression are greatly enhanced. This suggests that the replication function associated with K-bZIP is dispensable and can be compensated for by K-Rta.

K-bZIP expression is required for viral replication when lytic cycle induction is performed with TPA/n-butyrate. In light of the results from experiments where BAC-containing Vero cell lines were induced to enter the lytic cycle with the adenovirus Ad50, we wanted to investigate the growth characteristics under conditions using TPA/n-butyrate to induce the lytic cycle. In this case the lytic cycle would be induced by the up regulation K-Rta instead of the epigenetic expression of the transactivator. BAC36 and BAC36ΔK8-containing cell lines were treated with TPA/n-butyrate for 5 days and supernatant virus was harvested and subjected to qPCR evaluation. No viral DNA was detected in supernatants harvested from BAC36ΔK8 cell lines by qPCR, whereas supernatants from BAC36 (wild-type) containing cells showed over 2.5×10^{10} copies of viral DNA (Fig. 5). This experiment demonstrated that under the conditions traditionally used to induce HHV8 lytic replication, where much less K-Rta is produced, BAC36ΔK8 failed to replicate, indicating that K-bZIP was essential for virus growth under these treatment conditions.

Figure 5. BAC36ΔK8 is replication deficient when lytic replication is induced with TPA/n-butyrate. BAC36ΔK8 and BAC36 cell lines were treated with TPA/n-butyrate and supernatant virus was collected, concentrated and used for qPCR analysis as described in materials and methods. Shown is a representative graph from one experiment. Error bars are the standard deviations of viral copy numbers from three separate samples.

BAC36ΔK8 displays an aberrant gene expression pattern upon

induction with TPA/n-butyrate. Once it was determined that virus production

was impaired from BAC36ΔK8 harboring cell lines, when the lytic cycle was induced

using only TPA/n-butyrate, we next examined the gene expression pattern from the

mutant virus under these conditions. BAC36ΔK8 and BAC36 cell lines were induced

with TPA/n-butyrate and mRNA accumulation was measured for several HHV8-

specific genes at 5 days post treatment. The level of ORF50 (K-Rta) mRNA was

slightly higher in TPA/n-butyrate induced BAC36ΔK8 cell lines and there was a

small but measurable accumulation of K-Rta mRNA in uninduced cells verses those

observed in BAC36 (Wt) containing cells (Fig. 6, ORF50 graph). Despite this

apparent level of ORF50 mRNA accumulation, transcripts originating from the

ORF57 locus were undetectable from BAC36ΔK8 cell lines (ORF57 graph). In

addition, RNA transcripts from the PAN locus were approximately 20-fold higher in

TPA/n-butyrate treated cells carrying BAC36ΔK8 than BAC36 (PAN graph). Upon
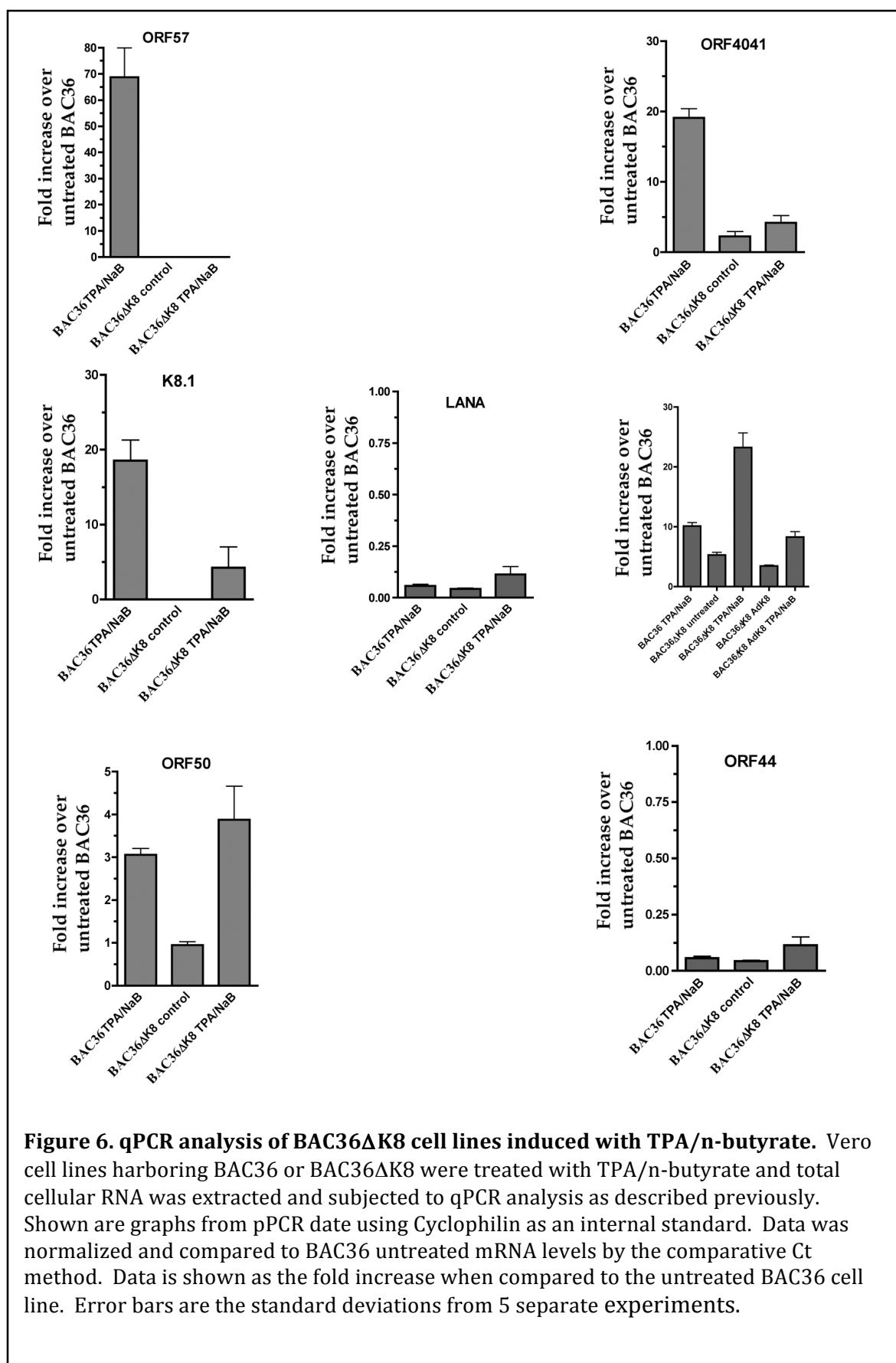
Figure 6. qPCR analysis of BAC36ΔK8 cell lines induced with TPA/n-butyrate. Vero cell lines harboring BAC36 or BAC36ΔK8 were treated with TPA/n-butyrate and total cellular RNA was extracted and subjected to qPCR analysis as described previously. Shown are graphs from pPCR date using Cyclophilin as an internal standard. Data was normalized and compared to BAC36 untreated mRNA levels by the comparative Ct method. Data is shown as the fold increase when compared to the untreated BAC36 cell line. Error bars are the standard deviations from 5 separate experiments.

 treatment with the AdK8 adenovirus the PAN mRNA levels in BAC36ΔK8 returned to levels similar to those observed in BAC36 cell lines (PAN graph). BAC36 displayed a 5-fold increase in PAN RNA upon lytic cycle induction, consistent with an increase in K-RTA expression. mRNA accumulation of other viral encoded transcripts, for example ORF40/41 and K8.1, were much lower from BAC36ΔK8 than those observed from BAC36 containing cells (Fig. 6, ORF40/41 and K8.1 graphs). Levels of LANA mRNA were approximately the same in BAC36ΔK8 and BAC36 containing cells lines (Fig. 6, LANA graph). These results suggested that the lack of K-bZIP lead to a general decrease in gene expression most strikingly in ORF57 even in the environment where K-Rta was abundant, however mRNA originating from the PAN locus accumulated to higher levels in BAC36ΔK8 cell lines with treatment, suggesting a deregulation of the PAN promoter when K-bZIP is not expressed.

Expression of K-bZIP in trans complements restores virus production in BAC36ΔK8 containing cells. Since no virus replication was observed under conditions where the virus lytic cycle was induced using TPA/n-butyrate, we next sought to complement the replication deficient mutant virus with the exogenous expression of K-bZIP. AdK8 was used to complement BAC36ΔK8 in the presence of TPA/n-butyrate. Under these conditions, wild-type virus production, as measured by qPCR, was observed when BAC36ΔK8-containing cells were treated with both TPA/n-butyrate and AdK8 (Fig. 7). This experiment strongly suggested that the null phenotype observed for BAC36ΔK8 was due to the lack of K-bZIP expression and

Figure 7. Restoration of wild-type virus production by complementation of BAC36ΔK8 with the epigenetic expression of K-bZIP. BAC36ΔK8 and BAC36 cell lines were TPA/n-butyrate and AdK8 (MOI 1700) for 5 days. Supernatant virus was collected, concentrated and used for qPCR analysis. Shown is a graph reporting the viral DNA copy number from supernatant virus. A representative experiment is shown. Error bars are the standard deviation from three sample replicates.

supplying K-bZIP in trans was sufficient to overcome the defect. This data indicated that K-bZIP can modulate gene expression in the context of the viral genome.

Disruption of the subcellular localization of LANA in the BAC36ΔK8 cell line. In order to further investigate the observed phenotype of the BAC36ΔK8 BACmid we examined the subcellular distribution of LANA and K-Rta (ORF50) in BAC36ΔK8 and BAC36-containing cell lines. qPCR data suggested that K-Rta protein levels should be similar in both BAC36ΔK8 and BAC36 cell lines given that mRNA accumulation was similar between the two BACmids. No K-Rta-specific signal was detected in uninduced cell lines (Fig. 8A). However when cell lines were induced with TPA/n-butyrate, a strong K-Rta specific signal was detected within the nucleus of BACmid-containing cells at 5 days post induction (Fig. 8B). No apparent difference in subcellular localization or intensity of signal was observed between the two BACmid-containing cell lines (Fig. 8). We also measured the protein levels

Figure 8. K-Rta subcellular localization is unchanged in BAC36ΔK8 cell lines. BAC36 and BAC36ΔK8 containing cell lines were fixed and reacted with anti-K-Rta antibody followed by detection with Alexa Fluor 555. Panel A, uninduced cell lines reacted with anti K-Rta. Panel B, TPA/n-butyrate induced cell lines reacted with anti-K-Rta. (C) Levels of K-Rta protein in TPA/n-butyrate induced cell lines. Western blot of protein extracts from cell lines containing either BAC36 or BAC36ΔK8 DNA reacted with anti-K-Rta specific antibody. K-Rta protein levels were evaluated between 72 and 120 h post induction.

 of K-Rta in each cell line treated with TPA/n-butyrate. Consistent with the qPCR

data, similar K-Rta protein levels were observed in each BAC-containing cell line

(Fig. 8C).

Uninduced cell lines were also subjected to IFA using the anti-LANA

antibody. Cell lines harboring BAC36 displayed a typical punctate nuclear staining

of the LANA protein (Fig 9A). However, in the cell line harboring BAC36ΔK8, LANA

displayed a more diffuse pattern of staining and was not entirely localized within

the cell nucleus (Fig. 9B). Transfection of the BAC36ΔK8 cell line with the K-bZIP

expression construct, pCMVK8 was able to partially restore LANA subcellular

localization to a more punctate nuclear staining suggesting that, even in cells that

were not treated with TPA/n-butyrate, K-bZIP may influence the activity or

localization of LANA (Fig. 9C see arrows). Examination of LANA protein levels by

Western blot showed similar protein expression levels of LANA was observed in

both BAC36ΔK8 and BAC36-containing cell lines (Fig. 9D, lanes 1 and 2,

respectively).

To show that K-bZIP can interact with LANA directly (in the absence of any

other viral protein) we performed a series of experiments where a LANA expression

plasmid was cotransfected along with a K-bZIP expression plasmid with or without

a K-Rta expression plasmid. Protein extracts were immunoprecipitated with anti-K-

bZIP antibody and western blots of immunoprecipitated protein was reacted with

anti-LANA or anti-K-bZIP specific antibodies. Immunoprecipitation of K-bZIP

revealed that K-bZIP interacted with LANA directly and did not require the presence

Figure 9. LANA is dispersed in uninduced BAC36ΔK8 cell lines. BAC36 and BAC36ΔK8 containing cell lines were fixed and reacted with anti-LANA antibody followed by detection with Alexa Fluor 555. The left column shows the cell nucleus stained with DAPI. **Panel A**, Vero cell line harboring BAC36 DNA reacted with anti-LANA antibody (80X magnification); **Panel B**, Vero cell line harboring BAC36ΔK8 DNA reacted with anti-LANA antibody (80X magnification); **Panel C**, Vero cell line harboring BAC36ΔK8 DNA and infected with AdK8 (MOI 1800) and reacted with anti-LANA antibody (80X magnification). The arrows show the punctate localization pattern of LANA protein. **(D)** LANA protein levels in BAC36 and BAC36ΔK8 containing cell lines. Lanes: 1, Protein lysate from BAC36 containing cell lines, 2, Protein lysates from BAC36ΔK8 containing cell lines. The arrow indicates the LANA protein band. **(E)** Co-immunoprecipitation of LANA and K-bZIP in transfected 293FTcells. Cell were transfected with a LANA expression plasmid and a K-bZIP expression plasmid. Protein complexes were co-immunoprecipitated using an anti-K-bZIP specific antibody. Immunoprecipitated protein complexes were separated on an SDS-PAGE gel and transferred to Immobilon-P and reacted with either anti-LANA or anti-K-bZIP antibody. Lanes: 1, Immunoprecipitation from cells transfected with a
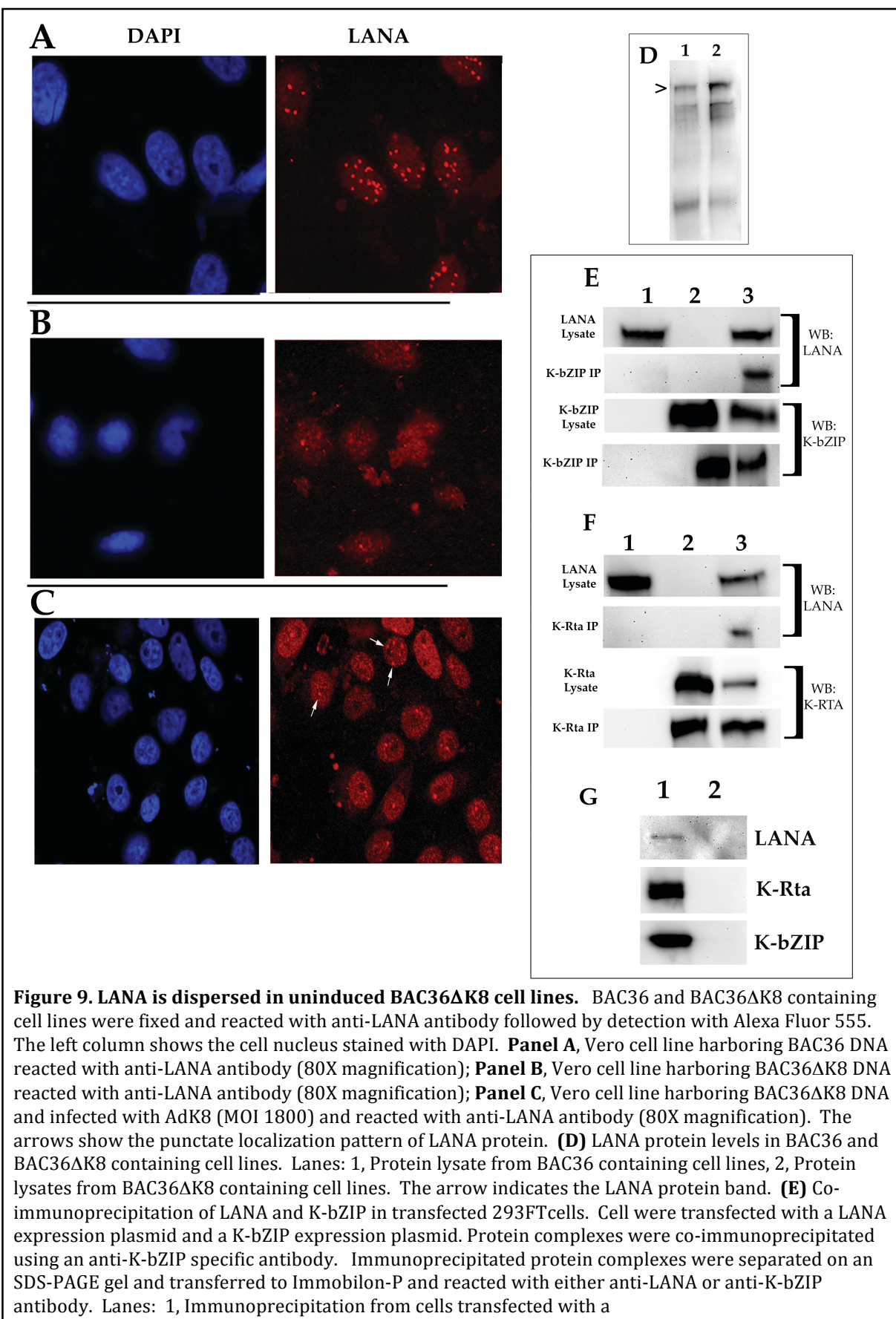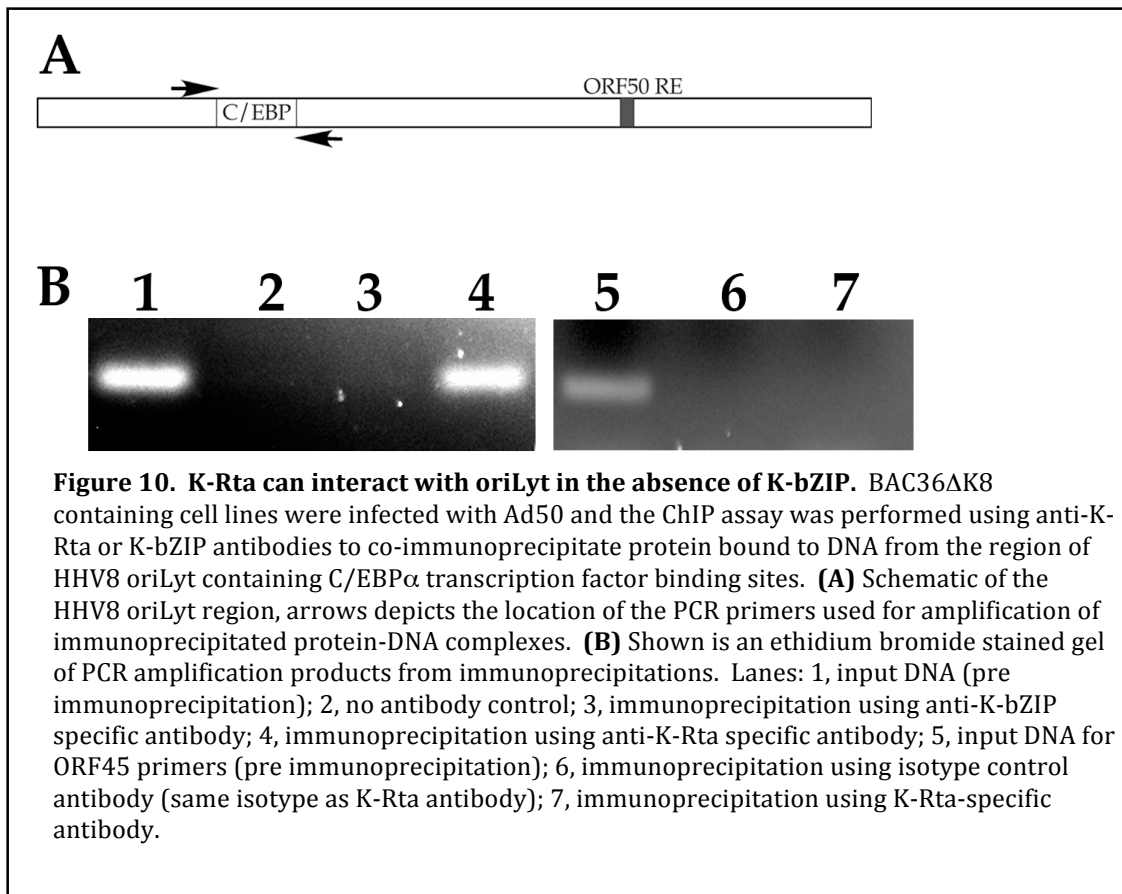
Fig. 9 con't. LANA expression plasmid; 2, Immunoprecipitation from cells transfected with a K-bZIP expression plasmids; 3, Immunoprecipitation from cells transfected with a LANA and K-bZIP expression plasmids. Blots were reacted with antibodies as indicated on the right side of the figure. **(F)** Co-immunoprecipitation of LANA and K-Rta in transfected 293FTcells. Cell were transfected with a LANA expression plasmid and a K-Rta expression plasmid. Protein complexes were co-immunoprecipitated using an anti-K-K-Rta specific antibody. Immunoprecipitated protein complexes were separated through an SDS-PAGE gel and transferred to Immobilon-P and reacted with either anti-LANA or anti-K-Rta antibody. Lanes: 1, Immunoprecipitation from cells transfected with a LANA expression plasmid; 2, Immunoprecipitation from cells transfected with K-Rta expression plasmid; 3, Immunoprecipitation from cells transfected with a LANA and K-Rta expression plasmids. Blots were reacted with antibodies as indicated on the right side of the figure. **(G)** Non-specific antibody control. 293FT cells were co-transfected with plasmids expressing K-Rta, K-bZIP and LANA and protein lysates were prepared. Lysates were incubated with the anti-UL84 specific antibody Mab84. Immunoprecipitated protein was separated on an SDS PAGE gel and western blots were prepared. Bolts were reacted with either anti-K-Rta, anti-K-bZIP or anti-LANA specific antibodies. Lanes: 1, protein lysates prior to immunoprecipitation; 2, immunoprecipitated protein using Mab84.

of K-Rta (Fig. 9E, lane 3 panel K-bZIP WB:LANA). Control lanes where K-bZIP

antibody was used to IP LANA alone transfected cells failed to reveal a detectable

band (Fig. 9E lane 1 WB:LANA and WB:K-bZIP). As shown previously, K-Rta was

also efficiently pulled down along with LANA protein (Fig. 9F, lane 3 WB:LANA).

These experiments demonstrated that LANA and K-bZIP can interact in the absence

of K-Rta and K-bZIP may influence the localization and activity of LANA. As an

additional control to ensure that there no non-specific interaction in our co-

transfection immunoprecipitation assays, we used an unrelated antibody (Mab84)

in an immunoprecipitation experiment with K-Rta, K-bZIP and LANA co-transfected

protein lysates. In all cases, no protein was pulled down when using an unrelated

antibody (Fig. 9G, lane 2). Protein lysates shown that K-Rta, K-bZIP were all

expressed efficiently (Fig. 9G, lane 1).

K-Rta can interact with oriLyt in the absence of K-bZIP. The current

thinking on the dynamics of initiation of lytic DNA replication in HHV8 involves the

Figure 10. K-Rta can interact with oriLyt in the absence of K-bZIP. BAC36ΔK8 containing cell lines were infected with Ad50 and the ChIP assay was performed using anti-K-Rta or K-bZIP antibodies to co-immunoprecipitate protein bound to DNA from the region of HHV8 oriLyt containing C/EBPα transcription factor binding sites. **(A)** Schematic of the HHV8 oriLyt region, arrows depicts the location of the PCR primers used for amplification of immunoprecipitated protein-DNA complexes. **(B)** Shown is an ethidium bromide stained gel of PCR amplification products from immunoprecipitations. Lanes: 1, input DNA (pre immunoprecipitation); 2, no antibody control; 3, immunoprecipitation using anti-K-bZIP specific antibody; 4, immunoprecipitation using anti-K-Rta specific antibody; 5, input DNA for ORF45 primers (pre immunoprecipitation); 6, immunoprecipitation using isotype control antibody (same isotype as K-Rta antibody); 7, immunoprecipitation using K-Rta-specific antibody.

interaction of K-bZIP with oriLyt. Recently it was shown that K-Rta could also interact with oriLyt in the region where K-bZIP is known to bind (35). This region contains several C/EBPα consensus-binding motifs. The interaction of K-Rta with oriLyt was mediated by K-bZIP. However, those studies used fragments of oriLyt DNA that contained mutations within the C/EBPα binding motifs, hence a direct interaction of K-Rta with oriLyt at those sites could not be assessed. Consequently, it is not known whether K-Rta can interact with oriLyt directly through the C/EBPα sites in the context of the mutant virus that lacks K-bZIP and under conditions where K-Rta is abundant. We wanted to understand the reason why an environment where K-Rta is over-expressed is conducive to the complementation of

BAC36ΔK8. The fact that we observed that the BACmid without K-bZIP could

replicate when K-Rta is over expressed suggests that K-Rta can perform a required

replication function and, therefore, interact with oriLyt without the aid of K-bZIP.

To test this theory, we infected the BAC36ΔK8 cell line with Ad50 and performed

the ChIP assay using primers flanking the previously identified C/EBPα consensus-

binding motifs within oriLyt known to be a substrate for K-bZIP. Figure 10A is a

schematic of the HHV8 oriLyt region showing the relative location of the consensus

C/EBPα sites and the ORF50 RE. The arrows show the location of PCR primers used

in the ChIP assay. The ChIP assay revealed that K-Rta could interact with oriLyt in

the absence of K-bZIP when immunoprecipitations were performed using anti-K-Rta

specific antibody as shown by the presence of a PCR amplified product (Fig. 10B

lane 4). Whereas the control PCR reactions consisting of no antibody or an anti-K-

bZIP specific antibody, failed to show any amplified PCR product (Fig. 10B, lanes 2

and 3 respectively). Control primers were used that are complementary to the

ORF45 locus. No PCR product was detected when PCR primers were used that

amplified a region of DNA outside of oriLyt known not to interact with K-Rta (Fig.

10B, lanes 6 and 7). This experiment strongly suggests that K-bZIP may facilitate an

interaction between K-Rta and oriLyt and this ancillary function may be overcome

by high concentrations of K-Rta, which provides and essential replication function

without K-bZIP.

DISCUSSION

The HHV8 K8 gene product, K-bZIP, is the proposed homolog to EBV Zta. On a functional level, however, the two proteins do not appear to have much in common. For EBV, Zta is the major transactivator and can reactivate latent virus, whereas K-bZIP is incapable of activating the lytic cycle and does not have a transactivation activity. One similarity is that Zta, like K-bZIP, is required for oriLyt-dependent DNA replication (1). Efficient oriLyt amplification using the cotransfection replication assay for HHV8 also required K-Rta, whereas in EBV, no such additional transactivator protein was needed (6). The most striking difference between Zta and K-bZIP is the association of K-bZIP with K-Rta and the repressive effects by K-bZIP on K-Rta-mediated transcriptional activation of certain HHV8 promoters. As for the replication function for K-bZIP, to date no know enzymatic or direct DNA binding activity has been identified for the protein. Apparently, K-bZIP interacts with oriLyt through binding with CAAT enhancer binding protein-α (C/EBPα) and this protein complex binds to C/EBPα consensus binding sites within oriLyt (33). Therefore, it is logical to study the effects of a recombinant virus that lacks the K8 gene and is deficient for K-bZIP expression.

The most interesting observation, from the study presented here, was that K-bZIP was not required for virus gene expression, viral DNA replication or virus production under the conditions where K-Rta was over-expressed from an adenovirus vector. Surprisingly, the amount of virus produced was several fold higher than that observed from the parent BAC36 under the same conditions. We

initially used Ad50 to induce the lytic cycle because it was reported that this was a

very efficient method to reactivate virus from cell lines harboring BACmid DNA (8).

The BAC recombinant BAC36ΔK8 showed a marked increase in virus production as

measured by the appearance of green cells indicative of infectious virus, as well as

an increase in viral supernatant DNA. The growth-enhanced phenotype was

unexpected since K-bZIP is the proposed initiator protein for lytic DNA synthesis

and therefore should be indispensable for growth. The use of a control adenovirus

(AdTRACK) had no effect on BAC36ΔK8 gene expression or virus production, ruling

out any complementation by an adenovirus protein (data not shown). Although

viral mRNA accumulation for specific transcripts was somewhat higher in

BAC36ΔK8, the increase in virus production could be due disregulation at the

protein level. For example the activity or protein half-life of K-Rta may be affected

by the absence of K-bZIP. One viral transcript that was markedly upregulated was

ORF57. The ORF57 protein, which also interacts with K-Rta, is associated with

regulation of gene expression via export of viral transcripts from the cytoplasm to

the nucleus; all of this data is consistent with it being the proposed homolog to EBV

MTA (24, 25). It was recently reported that expression of ORF57 was required for

viral growth as demonstrated using an ORF57 deleted BACmid (8, 23). Over-

expression of the ORF57 protein could account for an enhanced growth phenotype

given that its role is to enhance the expression of intron-less viral mRNAs.

Independent of the role of ORF57 in BAC36ΔK8 infected cells, it is apparent that the

over-expression of K-Rta, can compensate, directly or indirectly, for K-bZIP and complement a K-bZIP deficient virus.

K-Rta was recently implicated as having a dual role in lytic DNA replication, in that it was shown to act as a transactivator (binding to an ORF50 response element) as well as interacting directly with oriLyt in a region that has not been identified as a K-Rta responsive promoter (36). Also, K-Rta was shown to be part of the replication complex and it is proposed that it could act as a replication initiator protein for lytic DNA synthesis. The data presented here shows that K-Rta is sufficient for initiating lytic virus replication in the absence of K-bZIP when K-Rta is present at high concentrations. It was previously shown that K-Rta binds to oriLyt via an interaction with K-bZIP, which subsequently binds to C/EBPα sites. This interaction is obviously not required in the context of the viral genome since our data indicate the K-Rta can still mediate lytic DNA synthesis in the absence of K-bZIP in an environment where K-Rta is over-expressed. The ChIP assay confirmed that K-Rta interacts with oriLyt at the C/EBPα sites in the absence of K-bZIP. Therefore another mechanism must exist by which K-Rta can interact with oriLyt or it could be the case that K-Rta transactivation of the oriLyt promoter is sufficient to trigger lytic DNA synthesis. One other plausible explanation is that K-bZIP serves to amplify, augment or target K-Rta to oriLyt and consequently it is K-Rta which performs an essential replication function. Hence, when the artificial condition of K-Rta over-expression is present, the ancillary role of K-bZIP is no longer needed. This would certainly explain why in the absence of high amounts of K-Rta, for example when

TPA/n-butyrate was used to induce the lytic cycle, there was a lack of detectable virus production observed in the K-bZIP negative mutant BACmid. One other scenario is that K-bZIP has a negative effect on initiation of DNA synthesis and "occupies" C/EBPα sites in oriLyt until a high concentration of intra nuclear K-Rta displaces it and subsequently lytic replication ensues due to both the absence of K-bZIP and the presence of K-Rta interacting with the lytic origin.

Under conditions where lytic replication was induced using TPA/n-butyrate treatment the BAC36ΔK8 virus failed to replicate or produce infectious virus. One striking observation was the fact that PAN mRNA accumulation was approximately 25-fold higher in TPA/n-butyrate induced BAC36ΔK8-containing cells compared to untreated and 5-fold higher in TPA/n-butyrate treated BAC36 samples. This is interesting since in transient assays, K-bZIP was shown to have no influence on the PAN promoter with respect to repression of K-Rta mediated transactivation. This suggests that, in the context of the viral genome, other factors may contribute to PAN mRNA expression or RNA half-life and K-bZIP may play some role in PAN regulation. Expression of K-bZIP *in trans* was able to restore PAN mRNA and virus replication in BAC36ΔK8-containing cell lines. This is clear evidence that the nature of the null virus phenotype for BAC36ΔK8 is due to the lack of K-bZIP expression.

BAC36ΔK8 preserved the polyadenlyation site at nts 76714 and therefore transcription of K-Rta and K8.1 were not disrupted. The deletion of the K-bZIP ORF left approximately 300 bp upstream of the K8.1 ORF. Although the promoter region

has not been defined for the K8.1 ORF, there is apparently enough regulatory

sequence present for adequate expression of this protein. The K8.1 protein is not

required for viral replication and it was postulated that the cis regulatory region

controlling expression may be located within the K8 locus (22, 31). Our data here

suggests that the cis regulatory region within the context of the viral genome for

K8.1 expression is either controlled by the immediate upstream region from the

ORF or by sequences that control the expression of ORF50 much further upstream

from the K8.1 ORF. Another explanation for increased expression from this ORF

could be due to a disruption of the native regulatory element which led to an over

expression of K8.1 and the obvious over expression of K-Rta.

We also investigated the subcellular distribution of K-Rta and LANA in

BAC36ΔK8 and BAC36-containing cells. Since it is know that K-Rta interacts with

LANA we assumed that K-bZIP is a part of this complex and LANA was shown to

have the ability to down regulate K-Rta-mediated transcriptional activation and the

early expression of K-Rta was shown to contribute to the establishment of latency

(15, 16). Hence, we were interested in the localization of LANA and K-Rta in cell

containing BAC36ΔK8. The subcellular localization of K-Rta was apparently

unchanged in BAC36ΔK8 cell lines when compared to BAC36-containing cells.

However, although only a slight increase in accumulation of LANA mRNA in the

BAC36ΔK8 cell line was observed, there was a distinct difference in the subcellular

distribution of LANA in the K-bZIP mutant BAC cell line compared to BAC36. This,

however, did not translate into any apparent difference or difficulty in the

establishment of latency with the mutant BAC. The change in subcellular localization of LANA in BAC36ΔK8 cell lines suggests that K-bZIP plays a role in latency along with K-Rta. We did observe that K-bZIP was part of a complex with K-Rta and LANA in BCBL-1 cells. We further investigated if K-bZIP could directly interact with LANA by using cotransfection assays. The results of these assays revealed that LANA can directly interact with K-bZIP in the absence of any other viral protein. This is the first indication that K-bZIP is associated with LANA and may contribute to the activity of LANA.

It might at first seem unlikely that a protein that is known to be present during the lytic phase of replication can have an effect on LANA, a latent protein. However, the fact that K-bZIP was shown to be a component of the virion suggests that this lytic protein is present during initial and later stages of infection (2). Also, it was described that the initial stages of virus infection appear to be lytic, with the expression of many lytic genes including K-bZIP, followed by the subsequent establishment of latency (14, 16). This may also be the case where the initial stages following BAC transfection may result in a short lytic replication cycle followed by a latent infection. It should be noted that neither K-Rta nor K-bZIP was shown to be expressed or present in our latently infected cell lines.

Another plausible explanation as to how a lytic protein can affect the distribution of LANA, is that the initial expression of K-bZIP, early in infection (or in this case BACmid transfection), may influence the subcellular localization and activity of LANA. Therefore, the localization of LANA can be impacted by the

expression of K-bZIP at very early times after virus infections as well as, in our BACmid-harboring cell lines, eliminating the need for continual expression during the latent phase. The fact that K-bZIP interacts with LANA allows for the possibility that K-bZIP could modify or "target" LANA within the cell. The exact mechanism by which K-bZIP influences the subcellular localization LANA is disrupted in cells containing the mutant BAC is unknown and warrants further investigation of early events in the virus lytic cycle.

This is the first report to describe the function of K-bZIP in the context of the viral genome. K-bZIP is intriguing since it appears to be a multifunctional protein but with no clear enzymatic or transactivation activity. The viral mutant described here, BAC36ΔK8, demonstrates that K-bZIP is dispensable under certain conditions and suggests that its role may be more regulatory with respect to modulating the activity of K-Rta. It appears that K-Rta is the elusive origin binding protein in HHV8 and K-bZIP is needed to facilitate this role. These results also emphasize the importance of evaluating replication associated viral factors in the context of the viral genome since transient assays like the cotransfection replication assay, powerful as they may be, should be used as a springboard to studies using whole virus in a cellular environment.

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Kaposi's Sarcoma-Associated Herpesvirus/Human Herpesvirus 8 (KSHV/HHV-8) K-bZIP modulates LANA's suppression of lytic origin-dependent DNA synthesis

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Unpublished Data

Abstract

The original cotransfection replication assay identified eight HHV8 viral-encoded proteins required for origin-dependent lytic DNA replication. Two of these virus-encoded proteins, K-Rta (ORF50) and K-bZIP (K8), interact with oriLyt together and separately. It was demonstrated that under conditions where K-Rta is over-expressed, a K-bZIP deficient BACmid had an enhanced growth phenotype and an aberrant subcellular localization pattern for the latency associated nuclear protein (LANA) indicating that K-bZIP does not directly participate in DNA replication. Studies demonstrated that K-bZIP interacts with LANA in the absence of K-Rta. In an effort to understand the consequence of a K-bZIP-LANA interaction we developed a modification of the transient cotransfection replication assay where both lytic (oriLyt) and latent (terminal repeats-TR) DNA replication are evaluated simultaneously. LANA repressed origin-dependent lytic DNA replication in a dose dependent manner when added to the cotransfection replication assay. This repression of origin-dependent lytic replication was overcome by increasing amounts of a K-bZIP expression plasmid in the cotransfection mixture or by dominant-negative inhibition of the interaction of LANA with K-bZIP by the over expression of the K-bZIP-LANA interaction domain. Using the chromatin immunoprecipitation assay (ChIP) we show that LANA interacts with oriLyt in the region associated with K-bZIP binding suggesting suppression of lytic replication by LANA is mediated by direct binding. These data suggest that the interaction of LANA with K-bZIP modulates lytic and latent replication.

Introduction

Kaposis sarcoma-associated herpesvirus or human herpesvirus 8 (KSHV/ HHV8) is a gammaherpesvirus and the cause of Kaposi sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman disease (MCD) (6). The gene expression profile of KSHV can be divided into two distinct infection phases, lytic and latent. During latent infection, there is no viral progeny produced and the HHV8 genome is maintained as multiple episomes in host cells. HHV8 viral DNA is replicated once per cell cycle and partitioned into daughter cells along with the host cell chromosomes (13, 30). Lytic replication is marked by an increase in gene expression and the production of infectious virus progeny.

Latently infected cells express only a small subset of genes thought to be critical for maintenance of the latent genome. During latent, as well as lytic infection, the latency-associated nuclear antigen (LANA ORF73) is the predominant viral antigen expressed (18). LANA facilitates latent viral DNA synthesis and tethers the KSHV episome to the host chromosome ensuring that the genome is distributed to daughter cells during each cell division. LANA is a 1,162 amino acid (aa) peptide with a calculated molecular mass of 135 kDa however, it typically migrates between 220-230 kDa on an SDS PAGE gel (8, 17). LANA contains three distinct protein domains: an N-terminal basic domain of 337-aa, a middle 585-aa hydrophilic region, and a C-terminal basic 240-aa domain (28).

LANA is localized to the nucleus of HHV8 latently infected cells and a nuclear localization sequence (NLS) between aa 24-30 was identified and is homologous to the NLS for Epstein Barr Virus (EBV) EBNA1 (29). LANA can repress as well as activate transcription (11, 14, 15, 24) (27). LANA can autoactivate transcription from its own promoter, presumably to maintain the expression of latent proteins while suppressing other viral genes (15, 27). LANA maintains viral latency by regulating the immediate-early transcriptional regulator protein K-Rta and can repress the transcriptional activity of the K-Rta promoter leading to a decrease in both HHV8 virus production and lytic cycle activation (22). Additionally, increased levels of K-Rta are observed when LANA is knocked down using siRNA (10). These studies suggest that regulation of viral latency is by the functional interaction between LANA and K-Rta which in turn appears to contribute to the switch between latent and lytic infection.

Recently, it was demonstrated that the gene product of K8, K-bZIP, interacts with LANA in transfected and infected cells (16). In the context of the virus genome, the absence of K-bZIP expression resulted in a significant increase in virus gene expression and production of virus under conditions where K-Rta was over-expressed (16). However, when TPA/*n*-Butyrate was used to induce the virus lytic cycle a marked decrease in gene expression and no virus production was observed (16). This result indicated that K-bZIP was not absolutely necessary for virus growth and K-Rta was able to compensate for the lack of K-bZIP expression. Interestingly, immunofluorescence staining revealed that the subcellular

localization of K-Rta was unchanged, however a disruption of the nuclear punctate

subcellular localization of LANA was observed in cells harboring the recombinant

BAC with K-bZIP deleted. These data indicated that K-bZIP influences LANA

localization and suggested that it may regulate the activity of LANA with respect to

controlling the switch to the lytic cycle.

In this report we define the protein binding domains for LANA and K-bZIP

and investigate the significance of the interaction between these two proteins in the

context of lytic DNA replication. Using a modification of the original transient

cotransfection replication assay we show that the interaction of K-bZIP with LANA

modulates the switch from latent to lytic infection. We cotransfected plasmids

containing HHV8 terminal repeat (TR) elements along with a plasmid containing

oriLyt to evaluate both lytic and latent replication simultaneously. This lytic/latent

replication assay was used to assess the contribution of K-bZIP and LANA towards

amplification of either replication element (13) (5, 12, 25). The presence of LANA

alone in the cotransfection mixture repressed oriLyt amplification and the

interaction of K-bZIP with LANA was essential to modulate the switch from lytic to

latent transient replication. This repression was relieved by transfection of a K-bZIP

expression plasmid or a plasmid that expressed the K-bZIP peptide that interacts

with LANA. Increasing concentrations of K-bZIP in the transfection mixture was

able to shift amplification of the TR-containing plasmid to an increase in

accumulation of replicated oriLyt. Lastly, we used the chromatin

immunoprecipitation (ChIP) assay to show that LANA binds to a region of oriLyt

that contain CCAAT/enhancer binding protein α (C/EBPα) transcription factor binding sites; the same region shown previously to interact with K-bZIP. These results implicate K-bZIP as a regulator protein that does not directly participate in lytic DNA replication but instead modulates latent and lytic replication through an interaction with LANA and oriLyt.

Materials and Methods

Cells and plasmids and BACmids. Vero and HEK293 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% bovine growth serum (HyClone, Logan, UT). BAC36, the wild type HHV8 BACmid, was provided by S. Gao (University of Texas). Vero cells containing the BAC constructs were maintained in Dulbecco's modified Eagle medium supplemented with 10% bovine growth serum and 250mg/ml hygromycin.

Plasmids. The set of plasmid that expressed the deletions of K-bZIP was previously described (1). The K-bZIP interaction domain expression plasmid, pZIP-ID, was generated by using primers that flanked the region aa 101-134 and had an in-frame ATG for proper protein translation and a 3' FLAG epitope tag. The PCR product was ligated into phCMV-xi1 (Genlantis). Forward primer: cgacttaacagatctcgagctcaagcttcgaattcATGCTGAATGCAGAAACTAAATTCCACATCCCC and reverse primer: cccgggcccgcggtaccgtcgactgcagaattcTCACTTATCGTCGTCATCCTTGTAATCGCCCTGTT TGGCCTTAGTGCATAAGCGTTC). The LANA-GFP and Terminal repeat (pTR8) plasmid constructs were a generous gift from Dr. Kenneth Kaye (Harvard University).

The KSHV wild type BAC36 and the LANA knockout BACmid have been previously described and obtained from S-J Gao (23). The KSHV recombinant

BACmids that have the K-bZIP or ORF50 locus deleted have been previously described (16, 32).

Co-immunoprecipitation assay. For cotransfection, HEK293 cells (2 x 10^6/10-cm dish) were transfected with LANA and K-bZIP expression plasmids using TransIT LT1 (Mirrus). Forty-eight hours post-transfection, protein extracts were prepared using lysis buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1% Trition X-100, and 0.1% NP-40), passed through a 23-gauge needle to shear the DNA, centrifuged at 10,000xg for 10 minutes to remove debris, lysate was precleared with mouse IgG-agarose conjugate (Santa Crus Biotechnology) at 4°C for 30 minutes then 50μl of anti-HA affinity agarose gel (Sigma) was added to the lysate. This mixture was rotated 4°C overnight. The beads were then washed four times with 1 ml of Tris-buffered saline (Tris-HCl, pH 7.4, 150 mM NaCl) each time with rotation for 10 minutes at 4°C. Twenty microliters of the immunoprecipitated protein was separated through a 10% SDS- PAGE gel, which was subsequently transferred to an Immun-Blot PVDF membrane (Bio-Rad). After an initial blocking step (15 minutes with Tris-buffered saline plus 5% nonfat milk), the blots were reacted with anti-HA, anti-LANA, or anti-K-bZIP antibodies overnight at 4°C, followed by washing and incubation with horseradish peroxidase-conjugated secondary antibody anti-IgG. Protein bands were visualized using a chemi-luminescence substrate (Femto, Pierce).

Chromatin Immunoprecipitation Assay (ChIP). For induced samples, a Vero cell line (4 x 10^7) containing wild type BAC36 was treated with TPA (25ng/ml)

for five days. For uninduced cells, cultures were grown to the same density and both samples were treated as follows: The protocol for the ChIP is modified from the Active Motif ChIP-IT express kit (cat#53008). Cells were washed once with PBS, fixed in a 1% formaldehyde/PBS solution for 10 minutes and then washed twice in PBS. 3ml of PBS were added onto the cells, the cells were scraped, spun down at 1500 x g, and resuspended in 750 µl of lysis buffer plus protease inhibitors and incubated on ice for 30 min. This solution was then sonicated 10 times for 10s each pulse. Sonicated samples were analyzed by agarose gel electrophoresis prior to further use. Prior to immunoprecipitation, 10µl was removed for the "input" sample. Sheared samples (161µl) were incubated for 15 h at 4°C with 25µl of magnetic beads, 10µl ChIP buffer-1, 3µl antibody (anti-K-LANA or Isotype control, MAB84), 1µl protease inhibitor coctail (Sigma). After incubation the magnetic beads were removed by placing on a magnetic stand and the supernatent was removed. Beads were washed once with 800µl ChIP buffer-1, followed by a 2x wash with 800µl ChIP buffer-2, rotating 10min at 4°C after the addition of each wash buffer. Beads were then resuspended with 50µl of Elution Buffer AM2 and incubated 15min room temperature. 50µl of reverse crosslink buffer was added and beads were quickly pelleted and transferred to a new tube. 88µl of ChIP buffer-2 and 2µl of 5M NaCl to was added to "input" samples. Samples were then incubated at 94°C for 15 min. 2µl of proteinase K solution was added then incubated 37°C for 1hr, and finally 2µl of proteinase K stop solution was added. This was used for PCR analysis.

The primers used for PCR amplification (5'-AATCCCCCATAATCCTCTGC-3', and reverse, 5'-GGAAAAATCAAAACAAAACTC-3') corresponded to nucleotides (nt) 23326 to 23572 of HHV8 oriLyt. The control primers, 5'-ACGTCCGGAGAGTTGGAACTGTCA-3', and reverse, 5'-GGGGTCCATGGGATGGGTTAGTCA-3', were complementary to the ORF45 region.

Transient Lytic/Latent Replication Assay. Vero cells were transfected with plasmids containing HHV8 oriLyt (2) and TR (4, 9, 25) as well as the complete set of core replication proteins, ORF6, ORF9, ORF40/41, ORF44, ORF56, ORF59, K-bZIP and K-Rta with or without a LANA expression plasmid. Cells were harvested 5-7 days post transfection and total cellular DNA was isolated and cleaved with EcoRI and DpnI. Cleaved DNA was separated using a 0.8% agarose gel and transferred to a nylon membrane and hybridized to a ^{32}P-labbled-pGEM probe.

Results

A 33 amino acid region of K-bZIP is essential for LANA binding. We

previously demonstrated that K-bZIP interacts with LANA in transfected cells. To

determine the region of the K-bZIP ORF that is mediating the binding with LANA we

generated a series of deletion mutants for K-bZIP and used these expression

plasmids in a series of cotransfection-immunoprecipitation experiments. We

generated 7 HA-tagged expression plasmids each having a different 33 amino acid

deletion within the K-bZIP ORF. Figure 1A is a schematic of the K-bZIP ORF showing

a series of K-bZIP mutants used in cotransfection co-immunoprecipitation assays.

Plasmids expressing K-bZIP fragments were each transfected along with a full-

length wt LANA expression plasmid that produced a LANA-EGFP fusion protein.

Protein extracts were prepared and protein-protein complexes were

immunoprecipitated using anti-HA antibody. Protein-protein interactions were

detected by western blot using anti-EGFP antibody. Western blots reveled that the

K-bZIP deletion mutant Δ101-134aa K-bZIP failed to interact with wt LANA whereas

all other K-bZIP deletion mutants retained their ability to interact with LANA (Fig.

1B, lane 6, WB: LANA HA-IP). As a control for expression we also evaluated protein

lysates by Western blot for both LANA and K-bZIP (Fig. 1B). All EGFP and HA

protein fusion species were expressed at similar levels and all HA-tagged protein

species were efficiently immunoprecipitated (Fig. 1B). These results show that the

region between amino acids 101 and 134 of the K-bZIP ORF contributes to the

interaction with LANA.

As can be seen in the schematic in figure 1, aa 101-134 of K-bZIP contains the

partial nuclear localization signal (NLS) for the protein. Hence the fact that we did

not observe any interaction of this deletion mutate of K-bZIP with LANA could be

due to a lack of proper subcellular localization, even though a high level of protein

expression was observed by Western blot. In order to confirm that the region of K-

bZIP from aa 101-134 was indeed involved in LANA binding, and to rule out that the

lack of protein-protein interaction was due to the fact that this region of K-bZIP has

been implicated in nuclear localization, we performed a lysate mixing experiment.

For this experiment protein lysates were prepared from either cells transfected with

K-bZIP (deletion mutants or wt) or full length LANA. K-bZIP-transfected protein

lysates were mixed with LANA prepared protein lysates and immunoprecipitations

were then performed. Under these conditions, proper subcellular localization is not

required since there is no cotransfection and protein interactions are assayed upon

mixing of the lysates. Using this protocol, wt K-bZIP interacted with full-length

LANA very efficiently (Fig. 1C, lane 2). Also, the same protein interactions that were

observed from the cotransfection immunoprecipitation assay were also observed

using the protein lysate mixing protocol. However, no interaction was observed

using Δ101-134aa K-bZIP deletion mutant (Fig. 1C lane 4, WB: LANA HP-IP). This

result is consistent with the cotransfection data and strongly suggests that the

region of interaction within the K-bZIP ORF is between amino acid residues 101-
134.

LANA amino acids 465-777 interact with K-bZIP. Figure 1 shows a
schematic of the LANA ORF. LANA contains several protein domains that contribute
to its function along with regions that are highly repetitive. To define the region of
LANA that interacts with K-bZIP we again performed the cotransfection
immunoprecipitation assay using several GFP-tagged LANA expression plasmids
each having different amino acid truncations of the LANA ORF plus the full-length K-
bZIP expression plasmid that produced an HA tagged fusion protein. Protein
extracts were prepared from cotransfected cells and interacting proteins were
immunoprecipitated using anti-HA antibody. Protein-protein interactions between
K-bZIP and LANA were detected by reacting Western blots of the
immunoprecipitated protein with and anti-GFP antibody. Protein expression in the
lysate was evaluated for both K-bZIP (Fig. 1A, top panel) and LANA truncations (Fig.
1A, bottom panel). Immunoprecipitated protein from cotransfected cells revealed
that only one of the LANA truncations was able to interact with the wt K-bZIP (Fig.
1D, Lane 6). The LANA truncations that produced a protein product that contains
amino acids 4-465 was unable to interact with wt K-bZIP (Fig. 1D, Lane 5). LANA
truncations that produced a protein from either amino acids 487-1162 or 933-1162
also failed to bind to K-bZIP (Fig. 1D, lanes 7 and 8 respectively). Based on this set
of cotransfections and immunoprecipitations we conclude that the interaction
domain for LANA with K-bZIP is contained between amino acids 465-777.

Figure 1. Identification of K-bZIP and LANA interaction domains. (A) Schematic of HHV8 K-bZIP and LANA ORFs showing the relative locations of important protein domains and subclones or deletion mutants (K-bZIP) used in cotransfections and co-immunoprecipitations. Full length and subclones of K-bZIP contained an in reading frame context HA tag for the resulting protein product. For LANA all subfragments all protein products have an in frame green fluorescent protein (GFP) tag. (B) LANA interacts with domain located within aa 101-134 of the K-bZIP ORF. HEK293 cells were cotransfected with a plasmid expressing full length LANA and various plasmids that express full length or a series of 7-33 aa deletions of the K-bZIP ORF. Protein extracts were immunoprecipitated using anti HA antibody and precipitated protein complexes were resolved through an SDS page gel subsequent to the gel being transferred to PVDF, blots were reacted with specific antibodies listed to the left of the figure. Lanes: 1, LANA expression plasmid alone; 2,
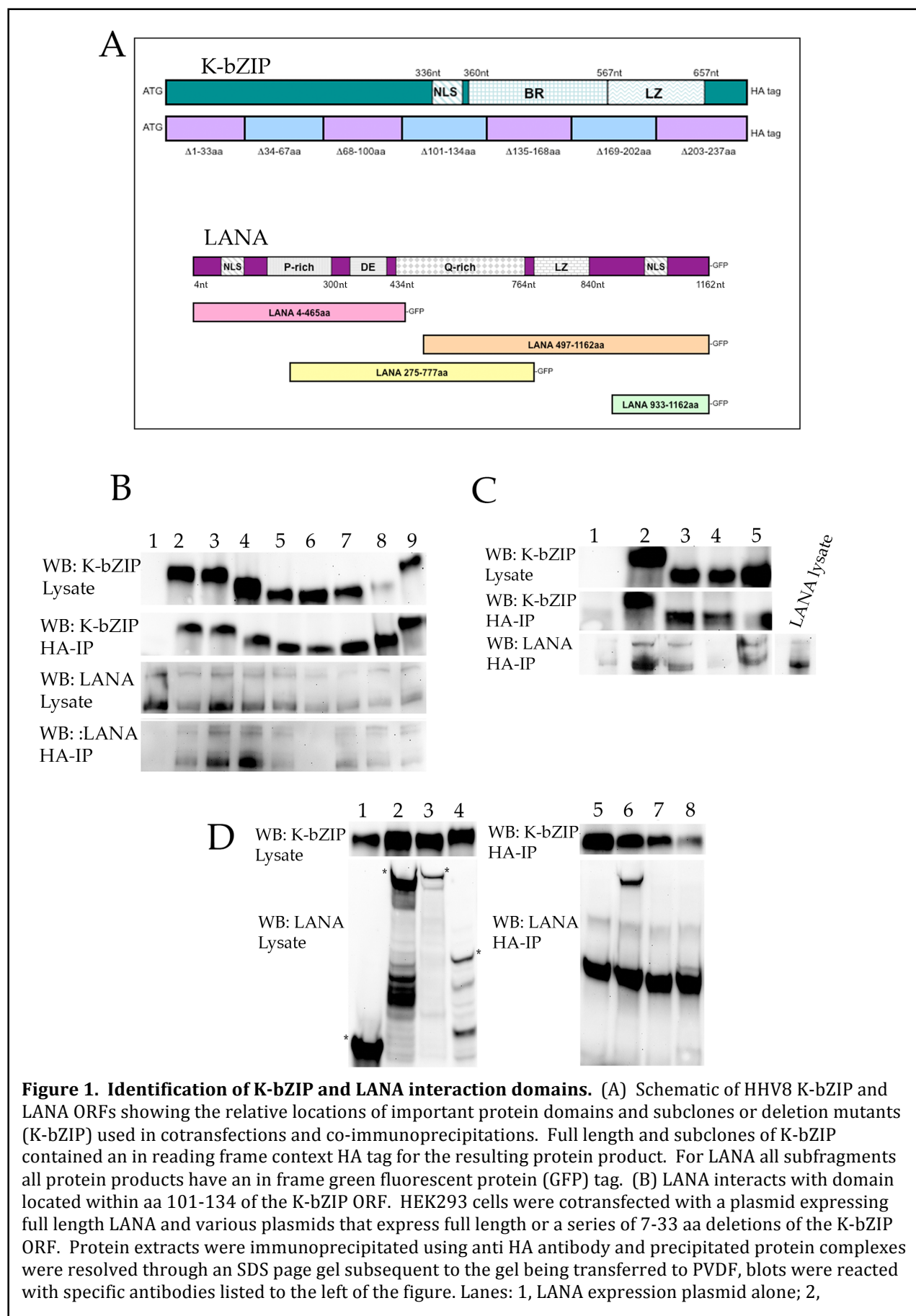
Fig. 1 con't. Cotransfection of LANA and full length K-bZIP expression plasmids; 3, Cotransfection of LANA and Δ1-33aa K-bZIP; 4, Cotransfection of LANA and Δ34-67aa K-bZIP; 5, Cotransfection of LANA and Δ68-100aa K-bZIP; 6, Cotransfection of LANA and Δ101-134aa K-bZIP; 7, Cotransfection of LANA and Δ135-168aa K-bZIP; 8, Cotransfection of LANA and Δ169-202aa K-bZIP; 9, Cotransfection of LANA and Δ203-237aa K-bZIP. (C) A K-bZIP mutant lacking aa 101-134 fails to interact with LANA. HEK293 cells were transfected with either plasmids that express various deletion mutants of K-bZIP of full-length LANA. Protein extracts were prepared and mixed together prior to immunoprecipitation with anti HA antibody. Antibodies used for Western Blots are shown at the left of the figure. Lanes: 1, Transfection of LANA expression plasmid; 2, Mixture of transfection of LANA and K-bZIP expression plasmids followed by immunoprecipitation using anti-HA antibody; 3, Mixture of transfection of LANA and Δ68-100aa K-bZIP expression plasmids followed by immunoprecipitation using anti-HA antibody; 4, Mixture of transfection of LANA and Δ101-134aa K-bZIP expression plasmids followed by immunoprecipitation using anti-HA antibody; 5, Mixture of transfection of LANA and Δ135-168aa K-bZIP expression plasmids followed by immunoprecipitation using anti-HA antibody. WB: Western Blot followed by antibody used to react with the blot, HA-IP: immunoprecipitation done using anti-HA antibody. (D) K-bZIP interacts the Q-rich region of the LANA ORF. HEK293 cotransfection of a full-length K-bZIP expression plasmid and various plasmids expression regions of the LANA ORF. Protein extracts were immunoprecipitated using anti HA antibody and resolved using an SDS page gel and blotted as above and reacted with antibodies shown to the left of the figures. Lanes: 1 and 5, Cotransfection of the K-bZIP and LANA 4-465aa expression plasmids; 2 and 6, Cotransfection of the K-bZIP and LANA 275-777aa expression plasmids; 3 and 7, Cotransfection of the K-bZIP and LANA 497-1162aa expression plasmids; 4 and 8, Cotransfection of the K-bZIP and LANA 933-1162aa expression plasmids.

LANA represses lytic DNA replication. Since we have established that K-bZIP, a protein involved in the lytic cycle, and LANA, a factor that has a clear role in latency but is present during the lytic phase of replication, interact in transfected cells we next wanted to investigate a possible role of these two proteins in lytic replication. The transient cotransfection replication assay involves the cotransfection of the set of plasmids that encode the viral proteins necessary to amplify the lytic origin of replication, oriLyt. For HHV8, oriLyt-dependent DNA replication requires the core replication proteins encoded by ORFs 6, 9, 40-41, 44, 56, 59 which are common to all herpesvirus (1). In addition, the original assay identified ORFs 50 (K-Rta) and K-bZIP as also necessary for efficient oriLyt amplification (1). We wanted to investigate the implications of adding a LANA

expression plasmid to this transfection mixture. To this end, the cotransfection

replication assay was performed as previously described except a LANA expression

plasmid was added to the transfection mixture. Amplification of HHV8 cloned oriLyt

was detected by Southern blot after cleavage of total cellular DNA with EcoRI and

DpnI. As shown before, oriLyt efficiently replicated in cells cotransfected with the

required replication protein encoding plasmids (Fig. 2A, lane, –LANA).   However,

detectable oriLyt amplification was eliminated when a LANA expression plasmid

was added to the cotransfection mixture (Fig. 2A, lane, +LANA). This result

suggested that LANA expression was capable of repressing oriLyt amplification.

Over-expression of K-bZIP can reverse LANA mediated suppression of

oriLyt amplification. The observed suppression of oriLyt amplification could be

occurring for several reasons. One possibility is that LANA is affecting the level of

protein expression particularly the level of K-Rta in the cotransfection mixture.

Although this is unlikely since all expression plasmids used in the assay use the

HCMV immediate early promoter for protein expression, we nevertheless tested this

possibility by using Western blot analysis to measure protein accumulation for K-

Rta, K-bZIP and ORF59 in the presence of LANA expression. Cells were

cotransfected with the LANA expression plasmid along with either K-Rta, K-bZIP or

ORF59 expression plasmids. Protein lysates were prepared and subjected to SDS

PAGE electrophoreses followed by Western blotting. Since all recombinant proteins

were FLAG-tagged, Western blots were reacted with anti-FLAG antibody. As an

internal control for protein loading we also reacted the same blots with antibody

specific for β-actin. LANA expression had no effect on the levels of K-Rta, K-bZIP or ORF59 protein accumulation as measured by Western blot (Fig. 2B, lanes LANA+K-Rta, LANA+K-bZIP and LANA+ORF59).

Since LANA had no effect on the level of protein accumulation in the cotransfection assay we next sought to investigate the mechanism involved in the suppression of oriLyt amplification and if it involved the interaction of LANA with any of the proteins in the replication assay. We also wanted to examine latent and lytic replication simultaneously since in the context of viral infection both LANA and lytic replication factors are present during lytic reactivation. This evaluation would give some insight into the possible switch from lytic to latent origin amplification. The latent origin of HHV8, which is located within the leftward terminal repeat (TR) region of the genome, undergoes amplification in the presence of LANA (3, 4, 7, 19). To test the implications of LANA expression on both lytic and latent replication we modified the original cotransfection replication assay to include the latent as well as the lytic origins of replication. Hence, this system would allow us to monitor the effects of various viral proteins on both lytic and latent replication in the same cells.

This lytic/latent replication assay was used to evaluate the effect of increasing levels of LANA expression on lytic replication. We first evaluated the effects of adding the plasmid containing the TR of HHV8 to the transfection mixture. The addition of this plasmid to the cotransfection mixture had no apparent effect on lytic replication as evidenced by the presence of the amplified oriLyt band (Fig. 2C, lane 4). As demonstrated above, the addition of the LANA expression plasmid to the

Figure 2. Effects of LANA on lytic DNA replication. (A) LANA represses oriLyt-dependent DNA replication. (A) Expression of LANA in the cotransfection replication assay suppresses amplification of oriLyt. Vero cells were cotransfected with plasmids that express the required replication proteins along with K-Rta and K-bZIP expression plasmids and a plasmid containing oriLyt. The cotransfection mixture in lane +LANA included a LANA expression plasmid. The arrow to the left of the figure shows the presence of amplified (DpnI-resistant) oriLyt. Bottom Panel: Southern blot showing input oriLyt DNA.
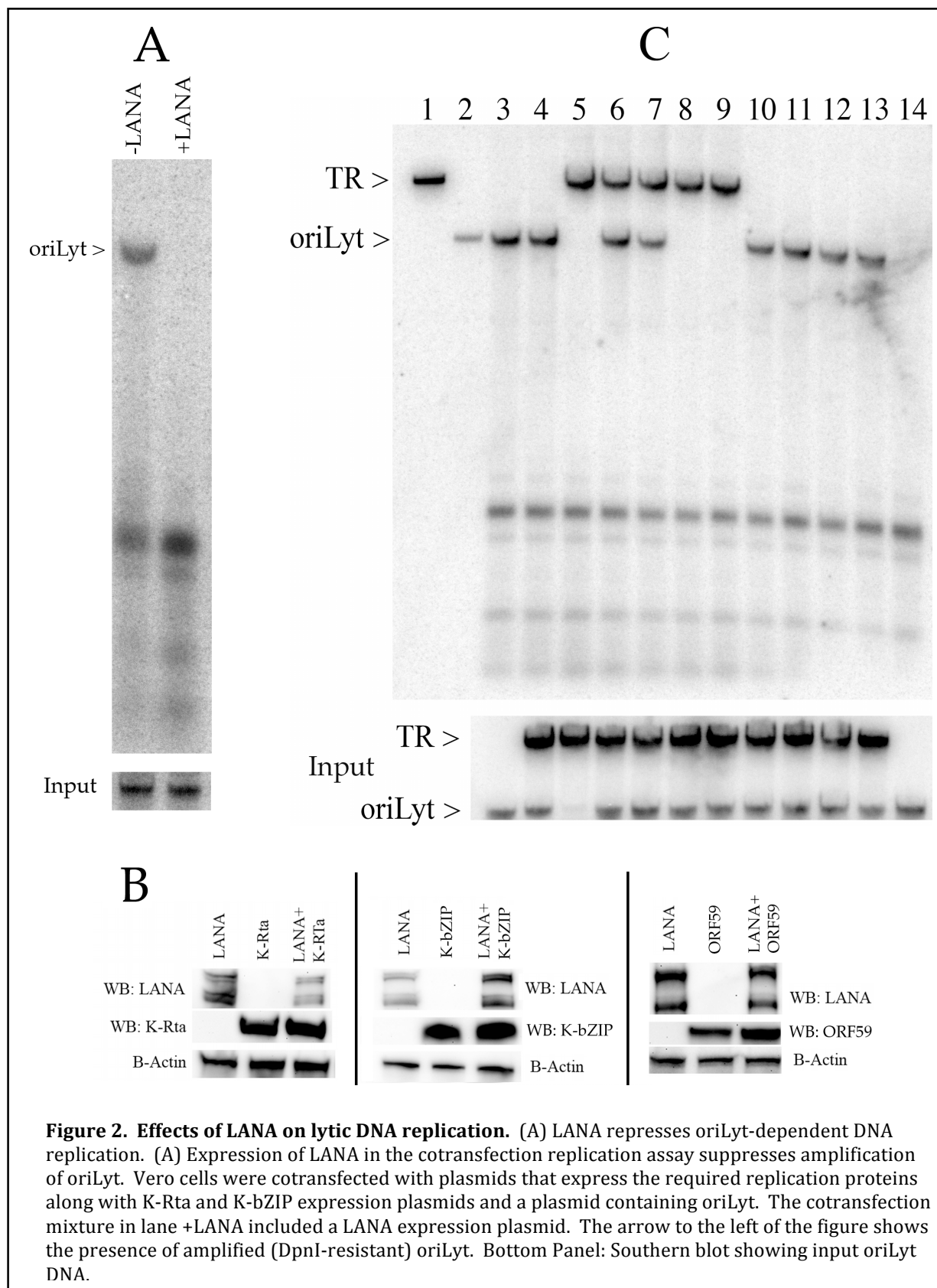
Fig. 2 con't. (B) Suppression of oriLyt amplification by LANA expression is not due to a decrease in protein production. Vero cells were transfected with a LANA expression plasmid and a K-Rta, K-bZIP or ORF59 expression plasmids. Protein lysates were prepared and protein expression was analyzed by Western blot. Blots were reacted with a LANA, K-Rta, K-bZIP or a ORF59 specific antibody. WB: antibody used for Western blot. (C) K-bZIP modulates amplification of oriLyt and the TR element. Vero cells were cotransfected with the set of plasmids expressing the required replication proteins plus plasmids containing oriLyt and the TR. Southern blot of total cellular DNA from cotransfections that contained HHV8 plasmids that encoded the required replication proteins along with oriLyt and/or TR containing plasmids hybridized with a pGEM ^{32}P-labeled probe. Cotransfections also contained a LANA expression plasmid and plasmids containing K-bZIP and K-Rta ORFs as indicated. All transfections contain 1µg of each replication plasmid, 10µg of oriLyt or TR containing plasmid and 1µg of K-bZIP and K-Rta expression plasmids except where otherwise stated. Lanes: 1, TR plasmid cleaved with EcoRI; 2, oriLyt plasmid cleaved with EcoRI; 3, cotransfection of required replication plasmids plus oriLyt; 4, cotransfection of required replication plasmids plus oriLyt and TR plasmids; 5, cotransfection of required replication plasmids plus LANA and TR plasmids; 6, cotransfection of required replication plasmids plus a LANA expression plasmid (1µg), oriLyt and TR; 7 cotransfection of required replication plasmids plus a LANA expression plasmid (2.5µg), oriLyt and TR; 8, cotransfection of required replication plasmids plus a LANA expression plasmid (5µg), oriLyt and TR; 9, cotransfection of required replication plasmids plus LANA, oriLyt and TR no K-bZIP expression plasmid; 10, cotransfection of required replication plasmids plus LANA (5µg), oriLyt and TR plasmids and a K-bZIP expression plasmid (5µg); 11, cotransfection of required replication plasmids plus LANA (5µg), oriLyt and TR plasmids and a K-bZIP expression plasmid (10µg); 12, cotransfection of required replication plasmids plus LANA (5µg), oriLyt and TR plasmids and plasmid K-bZIP aa101-134 (5µg); 13, cotransfection of required replication plasmids plus LANA (5µg), oriLyt and TR plasmids and plasmid K-bZIP aa101-134 (10µg); 14, cotransfection of required replication plasmids plus LANA (5µg), oriLyt, no ORF59 expression plasmid. Input oriLyt and TR plasmids are shown in the bottom panel.

transfection mixture resulted in the suppression of oriLyt amplification, however

due to LANA expression, the amplification of the TR plasmid can now be detected

(Fig. 2C, lane 5). When addition of lower concentrations of the LANA expression

plasmid was added to the mixture it was possible to obtain amplification of both the

TR and oriLyt plasmids in the same cotransfection sample (Fig. 2C, lane 6). The

presence of the LANA expression plasmid up to 2.5µg still allowed for simultaneous

replication of both latent and lytic cis acting elements (Fig. 2C, lane 7). Hence, when

an increasing amount of the LANA expression plasmid was added to the transfection

mixture the amplification of oriLyt was suppressed in a dose dependent manner

(Fig. 2C, lanes 6-8 oriLyt band). Interestingly, the suppression of oriLyt had no

effect on the accumulation of the latent origin plasmid which remained constant (Fig. 2C, lanes 6-8 TR band). These experiments suggested that the amount of LANA protein could influence the regulation of lytic or latent DNA replication.
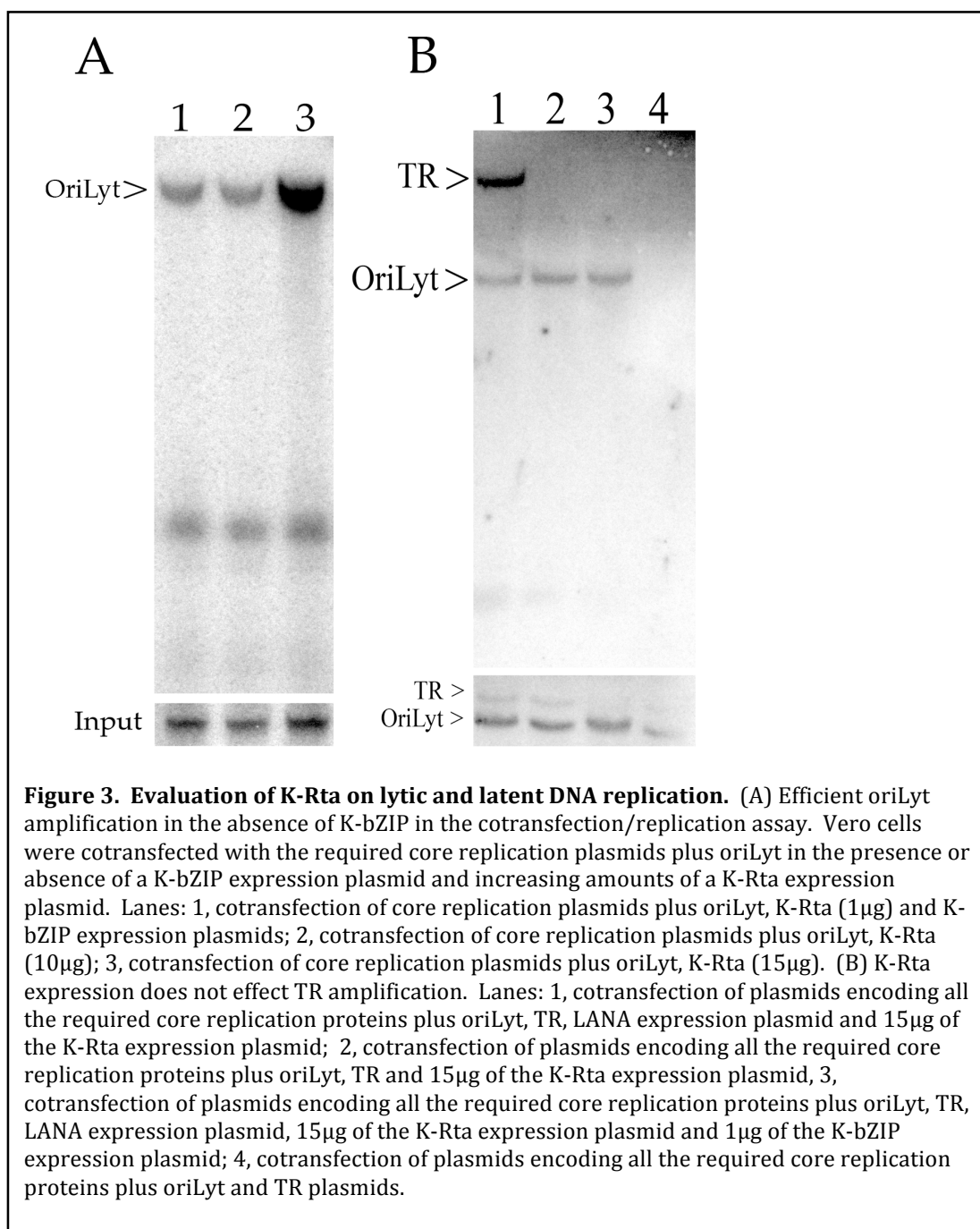
We next wanted to investigate if a protein that is known to interact with LANA, in this case K-bZIP could influence the balance between lytic and latent replication in our system. We again performed the cotransfection replication assay this time adding increasing concentrations of the K-bZIP expression plasmid to the mixture. When increasing amounts of the K-bZIP expression plasmid was added to the transfection mixture lytic replication was restored and amplification of the TR element was suppressed as shown by the appearance of the oriLyt band and the disappearance of the TR band (Fig. 2C, lanes 10 and 11 respectively). This reversal of suppression of oriLyt amplification was also observed when transfection of increasing amounts of the plasmid expressing the interaction domain of K-bZIP with LANA (Fig. 2C, lanes 12 and 13). This result strongly suggested that the interaction of K-bZIP with LANA could modulate the switch from oriLyt to TR amplification in the replication assay.

K-Rta can compensate for K-bZIP in the transient oriLyt-dependent DNA replication assay. Deletion of the K-bZIP ORF from a recombinant HHV8 BAC displayed a high level of DNA replication and virus production upon infection with Ad50, a recombinant adenovirus that over expresses K-Rta (16). These studies demonstrated that K-bZIP is not required for reactivation or lytic DNA replication under conditions where K-Rta is over expressed. We wanted to re-evaluate the
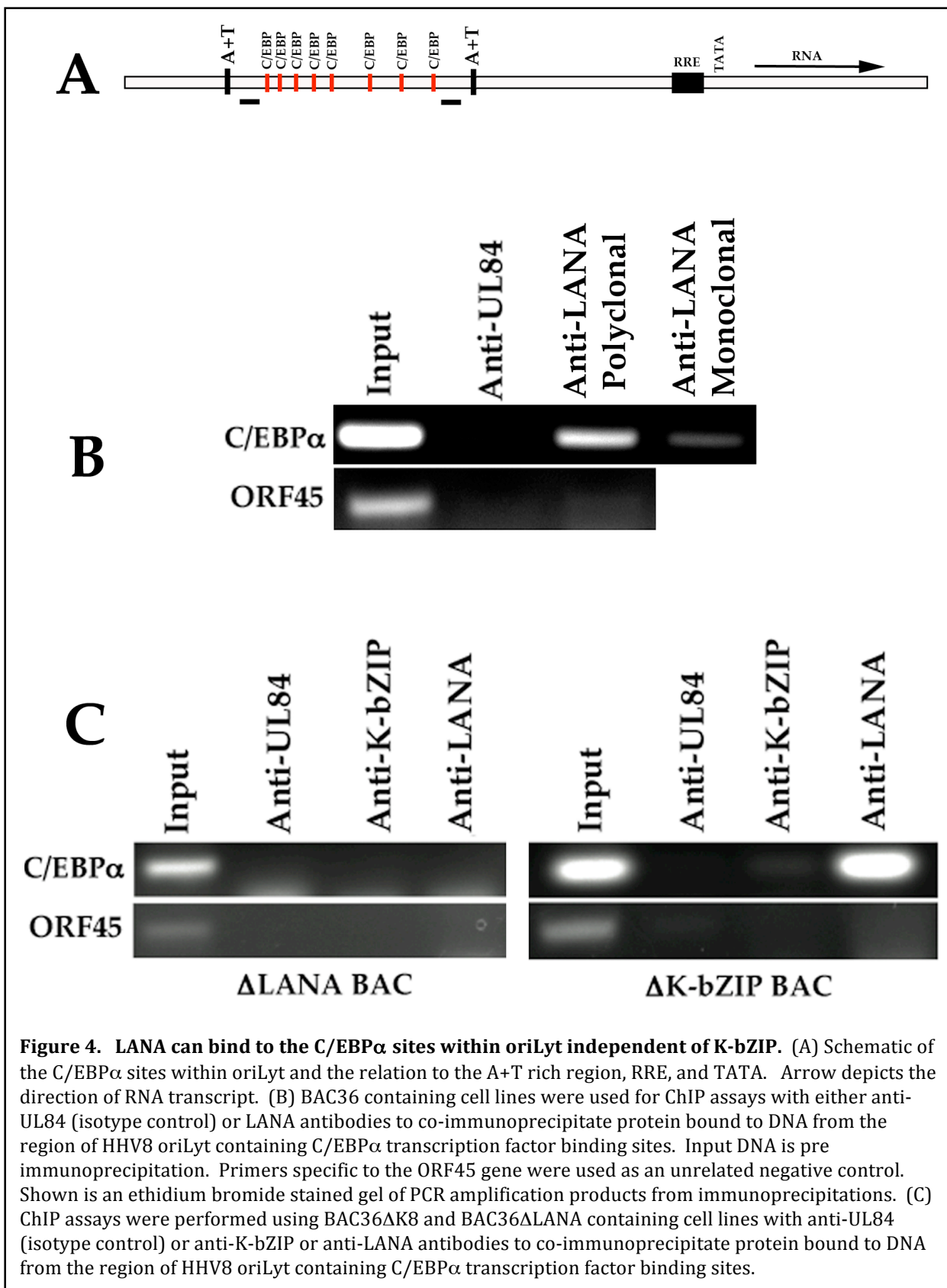
requirement for K-bZIP in the transient cotransfection replication assay under conditions where increasing concentrations of a K-Rta expression plasmid are added to the transfection mixture similar to the protocol used with the K-bZIP deficient HHV8 BACmid. Cells were cotransfected as before with the required core replication proteins, oriLyt and a K-Rta expression plasmid using concentrations from 5-15µg of K-Rta plasmid DNA which is 5-15 times more plasmid than used in the original assay. Under these conditions, oriLyt was efficiently amplified when cotransfection mixtures contained 10 or 15µg of the K-Rta expression plasmid in the absence of K-bZIP expression (Fig. 3A, lanes 2 and 3). This result confirmed the HHV8 ΔK-bZIP BACmid data showing that K-bZIP does not directly participate in lytic DNA replication and K-Rta can compensate for K-bZIP in the replication assay.

To confirm that over-expression of K-Rta did not effect TR amplification in the presence or absence of K-bZIP, we again performed the lytic/latent cotransfection replication assay under conditions where we added a high concentration of the K-Rta expression plasmid to the cotransfection mixture. Addition of the K-Rta expression plasmid in the absence of K-bZIP and in the presence of TR did not result in amplification of TR in the replication assay (Fig. 3B, lane 2). In addition, as shown in previous experiments, the addition of K-bZIP to the cotransfection mixture did not produce a TR replication signal (Fig. 3B, lane 3). The control experiment, where the K-Rta expression plasmid was omitted from the transfection mixture, failed to produce a detectable oriLyt band (Fig. 3B, lane 4). These experiments established that the over-expression of K-Rta in the

cotransfection replication assay relieved the requirement for K-bZIP and did not

influence amplification of TR in the presence or absence of K-bZIP.

Figure 3. Evaluation of K-Rta on lytic and latent DNA replication. (A) Efficient oriLyt amplification in the absence of K-bZIP in the cotransfection/replication assay. Vero cells were cotransfected with the required core replication plasmids plus oriLyt in the presence or absence of a K-bZIP expression plasmid and increasing amounts of a K-Rta expression plasmid. Lanes: 1, cotransfection of core replication plasmids plus oriLyt, K-Rta (1μg) and K-bZIP expression plasmids; 2, cotransfection of core replication plasmids plus oriLyt, K-Rta (10μg); 3, cotransfection of core replication plasmids plus oriLyt, K-Rta (15μg). (B) K-Rta expression does not effect TR amplification. Lanes: 1, cotransfection of plasmids encoding all the required core replication proteins plus oriLyt, TR, LANA expression plasmid and 15μg of the K-Rta expression plasmid; 2, cotransfection of plasmids encoding all the required core replication proteins plus oriLyt, TR and 15μg of the K-Rta expression plasmid, 3, cotransfection of plasmids encoding all the required core replication proteins plus oriLyt, TR, LANA expression plasmid, 15μg of the K-Rta expression plasmid and 1μg of the K-bZIP expression plasmid; 4, cotransfection of plasmids encoding all the required core replication proteins plus oriLyt and TR plasmids.

LANA binds to oriLyt. K-bZIP was shown to interact with oriLyt and binds to several CAAT/enhancer binding sites (C/EBPα) located within oriLyt (31). The interaction of K-bZIP with oriLyt is proposed to occur via a "piggyback" formation with the transcription factor C/EBPα making contact with oriLyt DNA. Although it was demonstrated that K-bZIP has no known intrinsic enzymatic or transactivation activity and is not required for DNA synthesis, its interaction with oriLyt does suggest some role in lytic DNA replication. Since the data presented here suggested that LANA and K-bZIP appeared to modulate oriLyt and TR amplification, we investigated the possible interaction of LANA with the HHV8 lytic origin. To identify any interaction we performed a ChIP assay using DNA extracted from BAC36-infected Vero cells and immunoprecipitating protein/DNA complexes using LANA specific antibodies. The immunoprecipitated DNA was evaluated using primers specific for the oriLyt region between nts 23326 and 23572 of oriLyt, which contains the C/EBPα transcription factor binding sites known to be a substrate for K-bZIP (Fig. 4A). A positive PCR amplification product was detected using two different LANA-specific antibodies indicating that LANA interacts with this region of oriLyt (Fig. 4B, Lanes, LANA poly and LANA mAb). Control samples using an isotype specific control antibody or primers designed to amplify a different HHV8 genomic locus failed to show any PCR signal (Fig. 4B, lane, isotype control Ab and bottom panel).

Figure 4. LANA can bind to the C/EBPα sites within oriLyt independent of K-bZIP. (A) Schematic of the C/EBPα sites within oriLyt and the relation to the A+T rich region, RRE, and TATA. Arrow depicts the direction of RNA transcript. (B) BAC36 containing cell lines were used for ChIP assays with either anti-UL84 (isotype control) or LANA antibodies to co-immunoprecipitate protein bound to DNA from the region of HHV8 oriLyt containing C/EBPα transcription factor binding sites. Input DNA is pre immunoprecipitation. Primers specific to the ORF45 gene were used as an unrelated negative control. Shown is an ethidium bromide stained gel of PCR amplification products from immunoprecipitations. (C) ChIP assays were performed using BAC36ΔK8 and BAC36ΔLANA containing cell lines with anti-UL84 (isotype control) or anti-K-bZIP or anti-LANA antibodies to co-immunoprecipitate protein bound to DNA from the region of HHV8 oriLyt containing C/EBPα transcription factor binding sites.

We also evaluated the ability of K-bZIP to interact with C/EBPα binding

motifs within oriLyt in the absence of LANA expression. For these experiments we

used the recombinant BACmid that is defective for LANA expression (23). This

BACmid is lytic upon transfection into cells but is unable to establish a latent

infection (23). Cells were transfected with the LANA knockout BACmid and ChIP

assays were performed as before. We failed to detect a positive PCR band indicating

that an interaction of K-bZIP with the C/EBPα binding motifs did not occur in the

absence of LANA expression (Fig. 4C, ΔLANA BAC panel). Control ChIP experiments

where we used a non-specific antibody (anti-UL84) or the anti-LANA antibody did

not show a specific PCR amplification product (Fig. 4C, ΔLANA BAC panel).

Amplification of an unrelated region (ORF45) also failed to produce a PCR

amplification product (Fig. 4C, ΔLANA BAC panel, ORF45). These results strongly

suggested that the interaction of K-bZIP with oriLyt was dependent upon LANA

binding to the C/EBPα binding motifs.

We also asked if LANA could interact with oriLyt C/EBPα binding motifs in

the absence of K-bZIP expression. For these experiments we used the KSHV

recombinant BACmid that had the K-bZIP ORF deleted. We again performed the

ChIP assay in BAC-infected cell lines. We were able to detect LANA binding to

C/EBPα binding motifs indicating that this interaction was not dependent upon the

expression or binding of K-bZIP to oriLyt (Fig. 4C, ΔK-bZIP panel, anti-LANA

antibody). Control ChIP experiments failed to show a positive PCR amplification

product in when using anti-K-bZIP specific antibody or amplification of the ORF45 locus (Fig. 4C, ΔK-bZIP panel).

This experiment demonstrated that LANA can bind to oriLyt in the region known to interact with K-bZIP and suggests that the interaction of K-bZIP with oriLyt is dependent upon LANA expression and interaction with oriLyt C/EBPα binding motifs. Further, LANA and K-bZIP appear to modulate DNA synthesis using a mechanism that involves direct suppression of oriLyt function.

Discussion

The regulatory processes that determine the balance between latency and lytic reactivation are complex. Typically, each viral state is studied as a separate process. Here we show the development of an in vitro system to study the regulatory and enzymatic mechanisms for both lytic and latent replication simultaneously. Clearly our results indicated that viral proteins involved in latent replication regulate the initial events in lytic DNA synthesis. HHV8 LANA plays a major role in the establishment and maintenance of the latent viral genome. Although LANA is associated with the latent state of the genome, it is expressed throughout the infectious cycle and appears to remain at a constant protein level even upon viral induction to enter the lytic cycle (20). This seemingly would present the virus with the scenario of overcoming LANA's "push" towards latency even when the expression of the viral transactivator and switch protein K-Rta is expressed and subsequently drives the virus into the lytic phase. This control mechanism appears to involve the ability of LANA to repress the expression of, and interact with K-Rta, and suggests that a change in the regulation of K-Rta can lead to the expression of lytic phase associated proteins resulting in virus production. In addition, since K-Rta is component of the virion (as is K-bZIP) it would appear to contribute to the observed initial burst of lytic replication followed by latent infection (20). In our earlier studies using an HHV8 BACmid deficient for K-bZIP expression, it was clear that K-Rta was sufficient to drive the expression of lytic genes, replicate viral DNA and produce infections virus. The ΔK-bZIP BACmid

displayed an enhanced growth phenotype when K-Rta was over-expressed

suggesting that K-bZIP had an inhibitory effect on virus growth. This modulation of

the virus lytic cycle may involve the interaction of K-bZIP with LANA given that ΔK-

bZIP BACmid-infected cells showed an aberrant subcellular pattern for LANA.

The interaction of K-bZIP and LANA involves the basic domain of the K-bZIP

protein (aa 101-134), a region of the protein that also encodes the NLS (26).

Although a K-bZIP subclone with this region deleted efficiently expressed protein,

we performed mixing experiments to show that a mutated protein with this region

deleted was unable to interact with LANA where as other mutated forms of K-bZIP

still retained their ability to bind to LANA even when lysates were mixed together

when expressed separately. The interaction domain for LANA with K-bZIP resides

in the repeat region or Q-rich domain of the protein. This region has a high degree

of glutamine amino acids.

We show here that LANA can directly suppress oriLyt-dependent DNA

replication and this suppression was not due to a general or specific decrease in

gene expression of K-Rta or essential replication protein expression. All gene

expression of replication proteins and K-Rta was driven by the HCMV immediate

early promoter and protein levels were unaffected by the presence of LANA. This

lack of an observed decrease in gene expression prompted us to investigate if the

interaction of LANA with K-Rta and/or K-bZIP may influence DNA syntheses. We

demonstrated that K-bZIP could influence the suppressive effects of LANA on lytic

DNA replication. Further, like K-bZIP and K-Rta, LANA interacts with oriLyt and the

results of the experiments presented here suggest that this interaction may suppress lytic replication directly. Additionally, K-bZIP may serve to modulate this suppression via its binding to oriLyt and LANA. Our results strongly suggest that the expression and binding of LANA to oriLyt is essential for K-bZIP binding. This presents the possible scenario of another mechanism whereby LANA maintains the virus genome in the latent state along with suppression of K-Rta gene expression. In virus infection, K-bZIP enters the cell as part of the infectious virion and this may serve to initially "orient" K-bZIP and LANA at oriLyt. Induction of the lytic cycle may displace the LANA-K-bZIP complex or allow for the binding of the protein involved in the origin recognition complex that subsequently results in lytic DNA synthesis. In this scenario, K-bZIP would not be necessary for lytic DNA replication but would act as a facilitator for DNA synthesis. In the BAC infection system, the over-expression of K-Rta may result in the displacement of LANA from oriLyt and subsequently lead to lytic DNA synthesis.

Much emphasis is placed on the association of LANA with K-Rta and the observation that LANA can regulate the expression of K-Rta in transient assays (21, 22). Previously we demonstrated that LANA interacts with K-bZIP, the putative homolog to Epstein Barr Virus (EBV) Zta. However it appears that K-bZIP has a distinctly different function in HHV8 lytic DNA replication. The evidence for the involvement of K-bZIP in the modulation of the effects of LANA suppression is based on the fact that we can achieve efficient oriLyt replication in the absence of K-bZIP when K-Rta is over-expressed in the transient cotransfection system. The over-

expression of K-Rta appears to be sufficient to overcome the suppressive effects of

LANA in both the transient system as well as in the context of the virus genome (16).

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Summary, Conclusions and Recommendations

The data presented here demonstrate the interactions between K-bZIP, K-Rta and LANA at oriLyt are involved in the modulation of lytic DNA replication. Using the transient assay, several protein motifs for K-bZIP that are essential for its facilitation of replication function were elucidated. Repression of K-Rta mediated transactivation is not required for the facilitation of replication function of K-bZIP in the transient assay. These repressive effects are not due to binding alone and are mediated through the leucine zipper (LZ) and basic regions (BR) of the protein. Initial studies of KSHV lytic replication determined that K-bZIP interacted with oriLyt at the C/EBPα site and it was suggested that K-bZIP would play a key role in the initiation of lytic DNA replication. Despite the support for a direct involvement in lytic DNA replication, the evidence presented here clearly show that K-bZIP is not required in the context of the viral genome for virus replication or in the transient cotransfection replication assay when K-Rta is over-expressed. Our evidence also indicates that K-bZIP modulates the effects of LANA at oriLyt.

We generated a recombinant KSHV virus with the K-bZIP ORF deleted, BAC36ΔK8, to study the effects of K-bZIP in the context of the viral genome. BAC36ΔK8 displayed an enhanced growth phenotype when K-Rta was over-expressed but very restricted growth when lower concentrations of K-Rta were present. This unexpected result demonstrated that K-bZIP was not required for virus replication in the context of the virus genome. Further experiments showed that even in the transient cotransfection assay, K-bZIP is not required when high a

concentration of a K-Rta expression plasmid was used. The evidence presented here suggests that at low concentrations of K-Rta, K-bZIP may aid or facilitate lytic DNA synthesis, but is not required when adequate levels of K-Rta are present.

K-Rta expression is sufficient to drive the expression of all the necessary proteins that participate in DNA replication and virus maturation to yield infectious virus. K-Rta appears to have the dual role of transcriptional activator as well as interacting with oriLyt in regions independent of the RRE. Here we demonstrated that K-Rta interacts with the same region as K-bZIP, the C/EBPα binding sites located between the A+T rich sequences. The C/EBPα transcription factor binding sites within oriLyt also act as an essential substrate for protein binding in addition to the RRE. Although is was thought that K-bZIP may recruit K-Rta to oriLyt via this interaction, our studies using the K-bZIP-deleted virus, determined that K-Rta interacts with C/EBPα binding motifs independent of K-bZIP binding or expression. The function of the interaction of K-Rta with C/EBPα binding motifs within oriLyt is presently unknown and will be the focus of future research.

Our studies on the interactions between K-bZIP, K-Rta, and LANA have lead to a better understanding on how these proteins relate to each other and how they interact with the cis-acting regions involved in DNA replication. Several proteins that participate in lytic DNA replication were shown to be components of the virion, including K-Rta and K-bZIP. Although the implications of these proteins in packaged virions are as yet unknown, it could reflect the ability of the virus to generate an initial lytic infection upon introduction into cells, this hypothesis would benefit from

future investigation. The modulations between latent and lytic KSHV DNA replication are complex and while this study has furthered our understanding on the impact of K-bZIP, K-Rta, and LANA in DNA replication there are still many facets of this topic to be explored.