SEX HORMONE MODULATION OF HHV-8 TRANSCRIPTION

by

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Human herpesvirus 8 (HHV-8) seroprevalence is similar in men and women, but Kaposi's sarcoma (KS) is mostly seen in men, suggesting that sex hormones play a role in KS development. Previous work from our laboratory has identified potential estrogen response elements (EREs) within several regulatory genes' promoters such as K8 (KB-Zip), ORF50 and ORF49. The HHV-8 EREs were found to bind the alpha estrogen receptor. The functionality of the K8 EREs were demonstrated using transient expression assays.

In this study, we tested the hypothesis that estrogen modulates the transcription of HHV-8 during latency or reactivation.

BCBL-1 cells are a B established from a pleural effusion lymphoma that is HHV-8 positive and Epstein-Barr virus (EBV) negative were used in the current study. Trex-BCBL-1 cells contain a doxycycline inducible ORF50 gene. MCF-7 cells are well studied breast cancer cell line. Production of infectious virus was determined using a $TCID_{50}$ assay. Transcription of individual viral genes was measure by RT-PCR using specific primers. Measurement of cellular transcription following estrogen treatment was performed using estrogen signaling PCR arrays.

Treatment of BCBL-1 or TRex-BCBL-1 cells with estrogen did not result in viral reactivation as measure by flow cytometry. Estrogen treatment did not prevent TPA-induced reactivation of BCBL-1 cells or doxycycline-induced reactivation of TRex-BCBL-1 cells.

Treatment of BCBL-1 cells with estrogen alone resulted in a decrease of constitutive viral transcription. BCBL-1 express estrogen receptor beta (compared to MCF-7 cells which express estrogen receptor alpha).Treatment of MCF-7 cells with estrogen result in an increase in transcription of cellular genes while treatment of BCBL-1 resulted in a decrease in cellular transcription.

Our results suggest that estrogen can down-regulate both cellular and viral transcription in B cells. This down regulation may be involved in the lack of KS development in women.

Statement of Public Health relevance: Reactivation of HHV-8 has been shown to be associated with the advent of Kaposi's sarcoma. By determining the role of estrogen in the transcription of viral genes and the estrogen receptors involved, potential therapeutic target can be found. The results from these experiments will help understand the biology of HHV-8, and pathology of Kaposi's sarcoma.

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1.0 INTRODUCTION

1.1 HUMAN HERPESVIRUS-8 SEROPREVALENCE

The human herpesvirus 8 (HHV-8) or Kaposi's sarcoma-associated herpesvirus (KSHV) is a member of the family of Herpesviridae, subfamily Gammaherpesviridae, genera Rhadinovirus. HHV-8 is the only known human Rhadinovirus[1]. HHV-8 was discovered in 1994 by Chang and coworkers and is the etiological agent of Kaposi's sarcoma [2]. HHV-8 is found in all forms of KS including classical, endemic, AIDS-related and iatrogenic. It is also the causative agent of pleural effusion lymphomas and some forms of multicentric Castleman's disease [3]. The seroprevalence of HHV-8 is not uniform in the world with less than 5% in the Unites States and Northern Europe, 10 to 20% in Italy and Greece and 50% or greater in some parts of Africa. The mode of infection of HHV-8 appears to be different between low and high endemic areas [4].

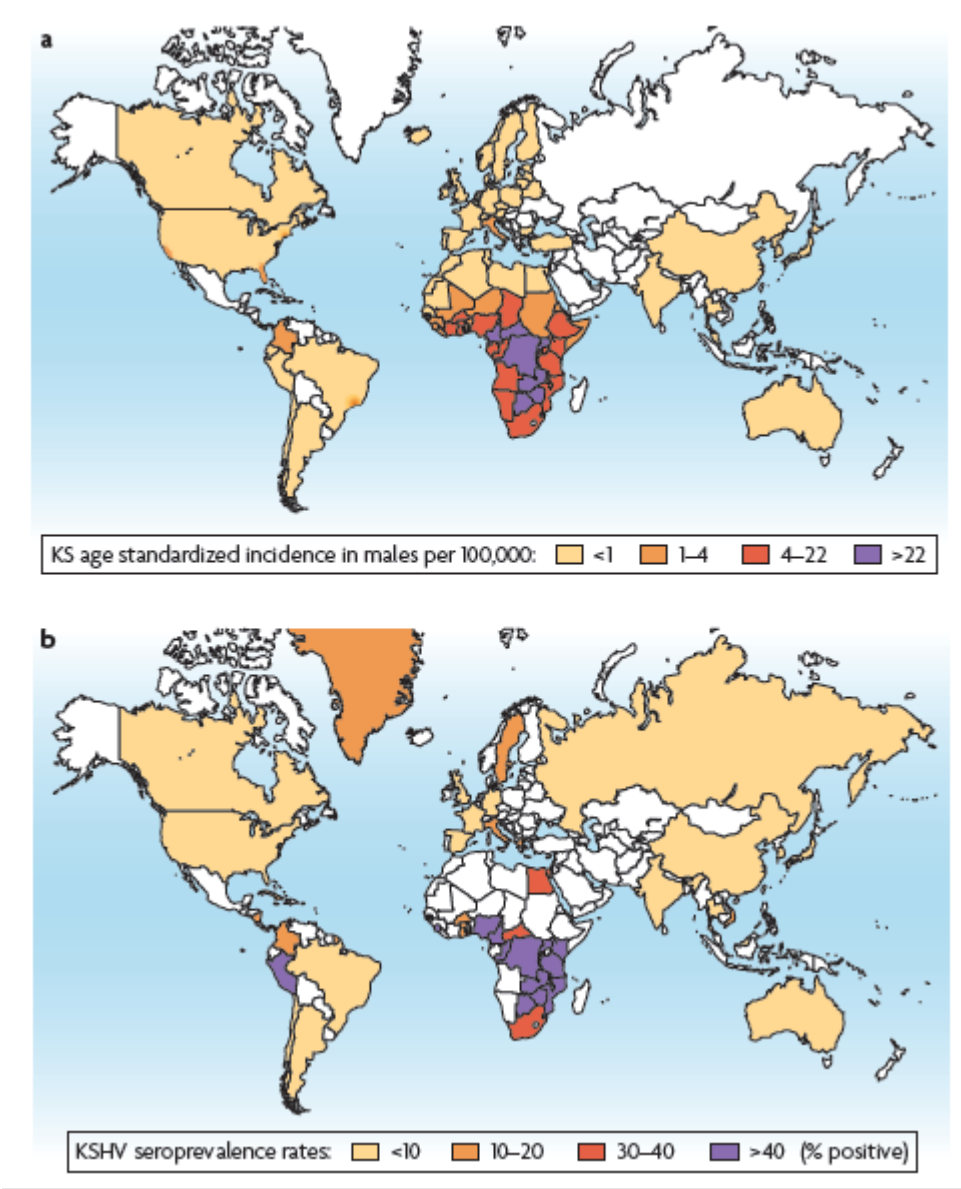


Figure 1 Geographical Seroprevalence of KS and Seroprevalence of HHV-8. *(A), the age-standardized incidence of Kaposi's sarcoma (KS) is depicted for males. (B) seroprevalence rates of HHV-8 compiled from multiple studies. (Figure reprinted with permission from Mesri, E. A., E. Cesarman, et al. (2010). "Kaposi's sarcoma and its associated herpesvirus." Nat Rev Cancer 10(10): 707-719.)*

1.2 KAPOSI'S SARCOMA

Kaposi's sarcoma was named after Moritz Kaposi who first published a description of the diseases in 1872 [5] Kaposi's sarcoma is an angioproliferative tumor of vascular origin that arises from multiple lesions of the skin. The lesions evolve from flat maculae or patches (early/patch stage) to plaques and subsequently nodules can merge (late/nodular stage) [6]. It was first described in Eastern Europe in late 19th century. KS is seen in four forms, classical, iatrogenic, endemic and AIDS-related.

1.2.1 Classical Kaposi's sarcoma

Classical KS has the greatest frequency, occurring primarily among elderly males of Mediterranean, Southern-Europe and Middle-Eastern decent [6]. Classic KS appears in an indolent form in these men and is usually found in lower limbs. It rarely affects the visceral organs [5]. Most importantly for the purposes of the present study, classical KS is more frequent in men than women with sex ratios evaluated to be from 3:1 to 10:1 [7].

1.2.2 Iatrogenic Kaposi's sarcoma

The iatrogenic form of KS represents a less common form that occurs in persons with solid organ transplants. The development of iatrogenic KS is directly associated with induced immunosuppression due to the solid organ transplantation. Immunosuppression in these patients results in the reactivation of several herpesviruses including HHV-8 [8]. The reactivation of

HHV-8 which results in the development of KS are more common in patients from Italy, Saudi Arabia or who are of Jewish descent according to a study of 20 cases [6].

1.2.3 Endemic Kaposi's Sarcoma

In the 1950's the form of KS seen in some regions of Africa was described as endemic. This form is frequently found in East and Central Africa and represents 1% to 10% of all cancers seen in these regions. In fact, KS was reported to represent 10% of all malignancies in Uganda with a male to female sex ratio of 15 to 1 [9]. KS can appear as an indolent form, similar to classical KS or as an aggressive form with disseminated nodules and infiltrated visceral lesions in men from their 40s. Occasionally, endemic KS will develop in young children causing severe granulomas [4].

1.2.4 AIDS-Related Kaposi's Sarcoma

AIDS-related KS is also described as the epidemic form. In 1981, with the onset of the AIDS epidemic, the incidence of Kaposi's sarcoma rose steeply [10]. Kaposi's sarcoma is an AIDS-defining illness and also represents the most common malignancy seen in AIDS patients [11]. A high HIV viral load and steep decline of CD4+ cells increases the risk of developing Kaposi's sarcoma in HHV-8 seropositive men [12] [13].

Homosexual men with AIDS have a greater risk of developing Kaposi's sarcoma than other populations including homosexual women. Similar to most forms of KS, the AIDS-related form occurs predominately in men [14].

1.3 PLEURAL EFFUSION LYMPHOMAS

HHV-8 is also the causative agent of pleural effusion lymphomas (PELs), also termed body-cavity-based lymphomas (BCBL). PELs harbor HHV-8 DNA and many also contain EBV [15]. They have several unique pathological characteristics: they do not display the same morphology as Burkitt-lymphomas and they do not have c-myc gene rearrangements. PEL are comparable to large-immunoblastic lymphomas. They appear first as lymphomateous effusions and stay localized to the body cavity of origin. They do not express regular B-cell associated antigens but do express CD45. PELs are more frequent in men than in women [3] and contain about 50-150 copies of HHV-8 episomal DNA per cell [15]. In the context of HIV, PEL represent only 0.13% of all AIDS-related malignancies in the USA [16].

1.4 HUMAN HERPES VIRUS 8: THE GENOME

HHV-8 is an enveloped, double-stranded linear DNA virus. The HHV8 genome is 170 kilobases in length [17], with at least 87 open reading frames (ORF) (as shown in Figure 2), and contains a long unique region flanked by long terminal repeats [18]. The HHV-8 genome has similar properties as other herpesviruses such as encoding for numerous enzymes involved in nucleic acid metabolism, DNA synthesis, and protein processing. HHV-8 DNA replication and capsid formation occur in the nucleus of the cell and the viral envelope is made from the nuclear membrane. HHV-8 replication results in the destruction of the infected cell. The virus (like all

herpesviruses) can establish a latent infection in which the viral DNA exists as a circular, double stranded DNA episome. During latency, HHV-8 expresses a small set of proteins associated with latent infections. The virus can be reactivated from Pleural Effusion Lymphoma (PEL) cells (immortalized B cells) in vitro by the addition of 12-O-tetradecanoyl phorbol-13 acetate (TPA) [1].

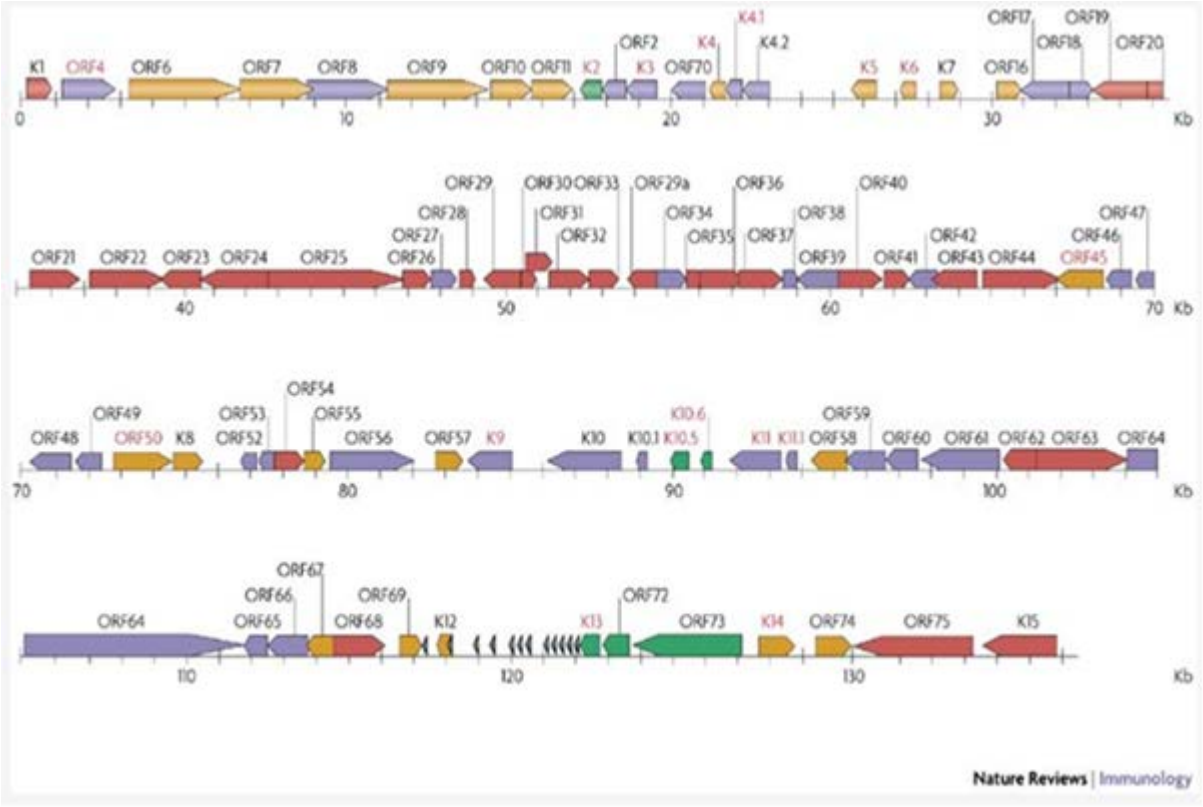


Figure 2. HHV-8 Genome Map. *Figure reprinted with permission from Laurent Coscoy, Immune evasion by Kaposi's sarcoma associated herpesvirus. Nat Rev Immunol, 2007. 7(5): p. 391-401.*

1.4.1 Latency Phase

Like other herpes viruses, HHV-8 has a latency phase, during which latency genes are expressed and the lytic genes are silenced. The major latency HHV-8 genes are ORF73,

encoding the latency-associated nuclear antigen or LANA-1, OR72, encoding v-cyclinD which promotes the passage through G1 phase of the cell cycle and K13, encoding vFLIP. All of these genes are expressed in a single locus and regulated by the same promoter [19], [20], [21].

1.4.2 Lytic Phase

Lytic replication of HHV-8 has been associated with the pathogenesis of Kaposi's sarcoma[22], [23] It is theorized that immunosuppression (such as AIDS) can cause the latent virus to reactivate (presumably from B cells), replicate and infect endothelial cells (which are implicated as the KS tumor cell) causing them to become transformed and proliferate. . One of the genes associated with the lytic cycle is the ORF50 gene which encodes the replication and transcription activator (RTA) protein. RTA is necessary and sufficient to activate the entire lytic phase [24], [25]. K-bZIP or ORF-K8 encodes a protein expressed early during the lytic cycle that acts as a transcription repressor, negatively regulating RTA-mediated transactivation [26]. Of the 80+ proteins encoded by the HHV-8 genome, the vast majority are expressed during the lytic cycle of replication. It can be assumed that expression of these genes is similar to other herpesviruses in that transcription occurs in a regulated, cascade fashion [23].

1.5 ESTROGEN

According to the Merriam-Webster medical dictionary [27], the female sex-hormone estrogen is accountable for female secondary sex characteristics. In humans, estrogen is

produced by both males and females, with the levels significantly higher in women compared to men. Estrogen can be found in the ovary, adrenal cortex, and testis. Estrogen includes estradiol, estriol and estrone.

1.5.1 Estrogen Receptors

The estrogen receptor is defined as a ligand-activated enhancer protein [28] and is part of the steroid nuclear receptor superfamily. Originally it was believed that there was only one estrogen receptor: estrogen receptor alpha, but in 1995, a group of researchers reported that they cloned a second estrogen receptor: estrogen receptor beta, from a rat prostate cDNA library. Estrogen alpha (Eα) and beta (Eβ) share similar structural architecture: they are made of three independent but interacting functional domains, the A/B domain or NH2 terminal, the DNA-binding domain or C domain, and the D/E/F or ligand-binding domain. Estrogen receptors alpha and beta can be expressed in cells as homodimers or as heterodimers. Estrogen can modulate gene transcription by three separate methods as shown in Figure 3. Estrogen binds to its receptors alpha or beta, changes its conformation to activate it forming a dimer. The complex enters the nucleus where it binds to specific DNA sequences, the estrogen response elements that are located in or near estrogen-responsive genes. Binding of the estrogen/estrogen receptor complex to EREs can result in either activation or repression of the associated cellular gene. In addition to regulating cellular transcription via EREs, the estrogen/estrogen receptor complex can also interact with the transcription factor site SP1 or the transcription factor complex fos/jun on AP1 sites to modulate gene expression. In the presence of Eα, estrogen functions as an agonist while in presence of Eβ, estrogen functions as an antagonist in the AP1 pathway [28], [29], [30],[31] [32], [33]. Previous studies have identified SP-1 and AP-1 sites in the HHV-8

genome [34]. Analyses from our laboratory have also found several AP-1 sites within the HHV-8 genome. Pertinent to this study are several found within the K8 promoter (Figure 4) [35].

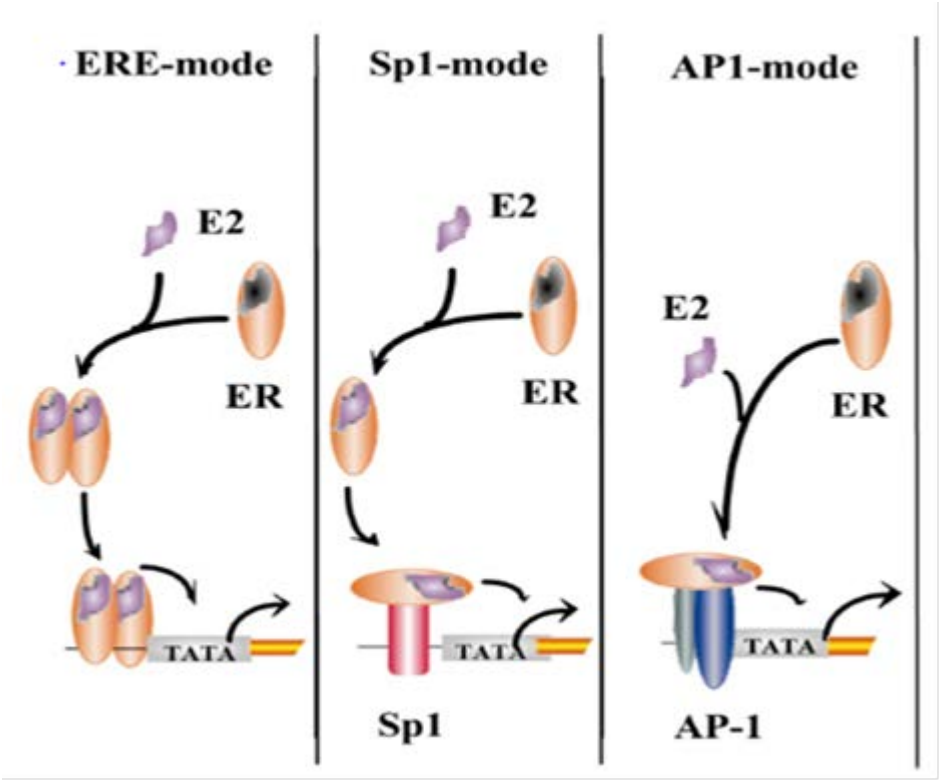


Figure 3. Model Representing the Various Modes through which Estrogen Receptors can Modulate Transcription of Genes. *(Reprinted with permission from Nilsson, S., S. Makela, et al. (2001). "Mechanisms of estrogen action." Physiol Rev 81(4): 1535-1565.)*

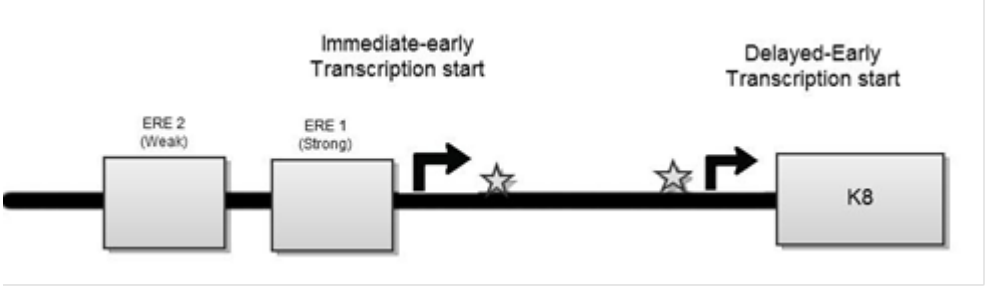


Figure 4. AP-1 Sites in K8 Promoter Regions. *Known transcription start sites are shown by the arrows and AP-1 sites are represented by the stars. From the dissertation: Identification and Functional Characterization of Estrogen Response Elements in the Human Herpesvirus-8 Genome by Patrick R. Shea 2007.*

1.5.1.1 Estrogen Alpha Receptor

The first estrogen receptor, estrogen receptor alpha (ERα), was identified in the 1980s and was thought to be responsible for the mediation of all the physiological effects of natural estrogens [29]. While ERα has been shown to be activated by phosphorylation in different cell lines even in the absence of estrogen, in the presence of physiological doses of estrogen, the phosphorylation of the receptor is enhanced. The estrogen- dependent phosphorylation of estrogen receptors is caused by many enzymes. These enzymes include the estrogen-dependent casein kinase II in MCF7 cells (a breast cancer cell line that express the alpha receptor) which phosphorylates the estrogen receptor at serine residue, Ser-167. There are also several other serine residues that are phosphorylated following estrogen activation that does not contain casein II binding sites [29], [36],[37], [38].

1.5.1.2 Beta Receptor

Estrogen receptor beta was identified in 1995 and is still understudied. Few studies have investigated the phosphorylation of that receptor compared to estrogen receptor alpha [39]. In order to study how the estrogen receptor beta was activated, Tremblay et al. showed the phosphorylation of recombinant Eβ when it was expressed in human embryonic kidney cells (293 T). Epidermal growth factor (EGF)-induced phosphorylation of ERβ's target sites are two serine residues at positions 106 and 124 in consensus sequences for MAPK [29, 39].

Relevant to the present study, western blot analyses performed in our laboratory have shown that MCF-7 cells express the estrogen alpha receptor and the BCBL-1 cells express the estrogen receptor beta (Figure 5) [35] .

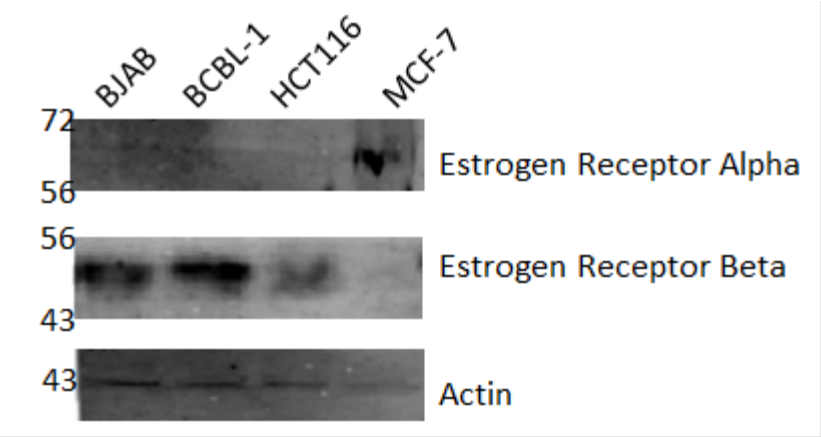


Figure 5. Western Blot Depicting the Estrogen Receptor Alpha and the Receptor Beta in BCBL-1 Cell Lines. *Jenkins, FJ, unpublished data*.

1.5.2 Estrogen Response Elements

Estrogen response elements (EREs) are a specific sequence in a genome to which estrogen/estrogen receptors' complex bind to. The minimal consensus sequence of EREs is 15 base pairs long with palindromic repeats: 5'-AGGTCAnnnTGACCT-3', where n stands for any nucleotide [40],[41], [42]. Contact between the estrogen/estrogen receptor complex and the estrogen response elements' sugar-phosphate backbone is important for DNA sequence recognition and high affinity binding. Each monomer of the estrogen receptor complex binds the major groove, with the estrogen/estrogen receptor dimer complex situated mostly at the face of the DNA helix [28, 43].

Previous work in our laboratory screened the HHV-8 genome for potential EREs and found several as shown in Table 1. Interestingly, several of these EREs were located within a locus of regulatory genes consisting of ORF49, ORF50 (RTA) and K8 (Figure 6) [35].

Table 1. EREs Sequences within the HHV-8 Genome. *From the dissertation: Identification and Functional Characterization of Estrogen Response Elements in the Human Herpesvirus8 Genome by Patrick R. Shea. 2007*

ORF	Gene Name	ERE Sequence
ORF K8	BZLF1 homolog	GGTCAGGGCGACC
ORF K8	BZLF1 homolog	GGGGAGGGTGACC
ORF57	Transcriptional activator	GGTGAGGGGGACC
ORF53	Envelope Glycoprotein	GGTCAGGATGAAA
ORF56	DNA replication protein homolog; EBV BSLF1 homolog	GGACAACGTCACC
ORF50	Transactivator homolog; EBV BRLF1 homolog	GGTCACTGGGATC
Orf29a	Packaging protein homolog; EBV BGRF1 homolog	GGCCTGTGTGACC
ORF31	EBV BDLF4 homolog	GGTCTCGCTGACT
ORF39	Glycoprotein M	GGACACGCTGACG
ORFK14	Adhesion molecule vAdh homolog	AGTCAGCCGGACC
ORF9	DNA polymerase homolog; EBV BALF5 homolog	GGTGAAGATGATC
ORF53	Envelope glycoprotein	GGGCATGATGAAC
ORF 66	EBV GRFF2 homolog	GGGCAGGATGACT
ORF 42	EBCV BBRF2 homologue	GCTCAGCGTCACC
ORF K5	BHVA-IE1 homolog	GGACAAGTTGTCC
ORF74	G protein coupled receptor	GGGCCAGGTGACC
ORF54	dUTPase homolog, EBV BLLF3 homolog	GGCCACCGTGGAC
ORF32	DNA packaging	GGACGGCGTCACC
ORF60	Ribonucleotide reductase, small subunit	GGTCCGGGTAACC
ORF69	BFLF2 homolog	GGTCGCAGTGGAC

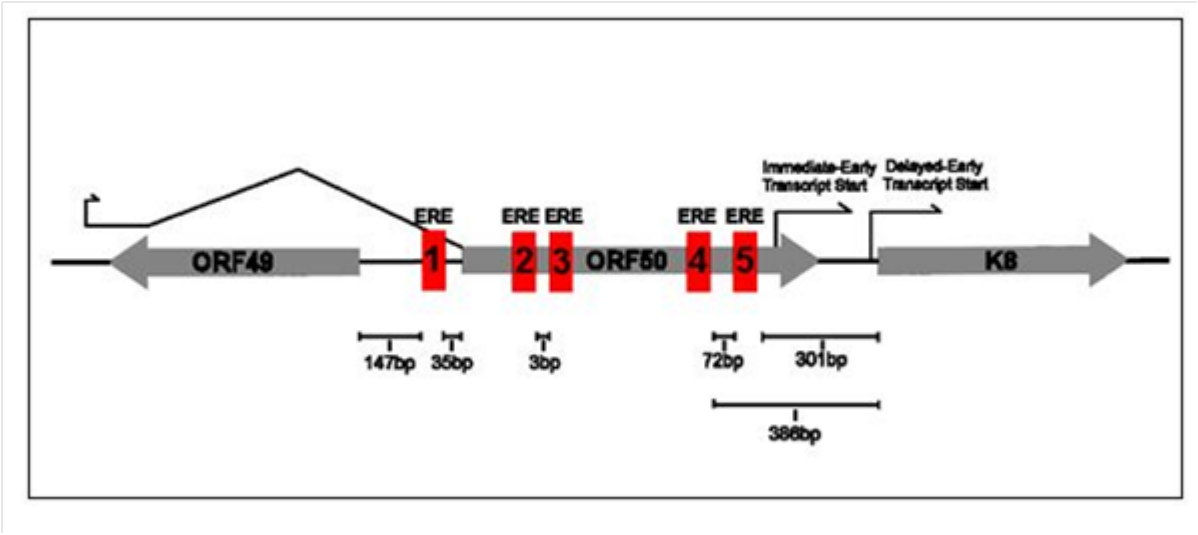


Figure 6. Map of ERE Sequences in the ORF49/ORF50/K8 Region. From the dissertation: Identification and Functional Characterization of Estrogen Response Elements in the Human Herpesvirus8 Genome by Patrick R. Shea. 2007

2.0 SPECIFIC AIMS

AIM 1: To determine if estrogen affects HHV-8 reactivation.

I. Test the hypothesis that estrogen treatment causes HHV-8 reactivation of BCBL-1 cells. BCBL-1 cells will be treated with estrogen in a dose response and time course experiment. Viral reactivation will be determined by flow cytometry using an antibody against ORF59, a HHV-8 lytic protein, and by production of infectious virus as measured by a TCID50 assay.

II. Test the hypothesis that estrogen treatment can prevent or interfere with tumor promoter agent (TPA) or replication transactivation activator (RTA)-induced HHV-8 reactivation. Time course experiment measuring viral reactivation by flow cytometry and viral replication by TCID50 in BCBL-1 or TRex-BCBL-1 cells treated with TPA or doxycycline (resulting in expression of RTA) respectively, in presence or absence of 10-6M estrogen.

AIM 2: To test the hypothesis that estrogen modulates HHV-8 and cellular transcription.

I. Test the hypothesis that estrogen modulates HHV-8 transcription.

1. Perform RT-PCR on a subset of viral genes in BCBL-1 cells treated in a dose response experiment for 4 hours with estrogen.

2. Perform RT-PCR on a subset of viral genes in BCBL-1 or TRex-BCBL-1 cells that were pretreated for 2hrs with 10-6M estrogen followed by treatment with TPA or doxycycline, respectively.

II. Test the hypothesis that modulation of cellular transcription by estrogen is dependent on the type of estrogen receptor (alpha vs beta).

1. Determine estrogen-induced transcription changes using an estrogen signaling RT-PCR array on MCF-7 cells (alpha estrogen receptor) treated with 10-6M estrogen by comparing treated to untreated cells.

2. Determine estrogen-induced transcription changes using an estrogen signaling RT-PCR array on BCBL-1 cells (beta estrogen receptor) treated with 10-6M estrogen by comparing treated to untreated cells.

3.0 MATERIALS AND METHODS

3.1 CELL LINES

BCBL-1 cells are a transformed B cell line that originated from a pleural effusion lymphoma [44]. TRex-BCBL-1 is a BCBL-1 line which contains the ORF50 gene encoding the RTA protein under the control of a doxycycline inducible promoter [45]. Both BCBL-1 and TRex-BCBL-1 cells were cultured in RPMI supplemented with 10% fetal bovine serum (FBS), 0.1% Gentamycin and grown at 37^0C and 5% CO2. MCF7 breast cancer cells were obtained from ATCC (Rockville, MD) and cultured in MEM media supplemented with 10% FBS, 0.1% Gentamycin and 1% L-Glutamine.

T1H6-DC-SIGN cell line is a 293T cell line originally developed by Naoki Inoue [46] and modified by our laboratory (Nadir and co-workers, unpublished results). This cell line contains a pBeta-GalBasic plasmid under the control of the RTA-responsive, HHV-8 T1.1 promoter and a plasmid expressing the lectin DC-SIGN under the control of the CMV immediate early strong promoter. DC-SIGN has been shown to serve as a cellular receptor for HHV-8 [47]. Infection of T1H6-DC-SIGN cells with HHV-8 results in the production of RTA (by the infecting virus) which in turn, activates the T1.1 promoter resulting in β-galactosidase

production. The T1H6-DC-SIGN cells were grown in DMEM supplemented with 10% FBS and 0.1% Gentamycin.

3.2 FLOW CYTOMETRY INTRACELLULAR STAINING

Approximately 1.5 million cells were spun down at 1800 rpm for 5 minutes washed twice in FACS (0.5% BSA in 1X PBS). 500,000 cells were split into each well of a 96-well plate or flow cytometry tubes. The cells were fixed for 10 minutes with 1ml/tube or 100 µl per well of cold fixing solution (2% Formaldehyde in 1x PBS). They were washed twice with 3ml/tube or 200 µl per well of FACS buffer and then permeabilized with 2 ml/tube or 150 µl per well of permeabilization & stain solution (0.3% Saponin/ 0.5% BSA in PBS, filtered) for 30 minutes in a 4^0C refrigerator. The permeabilized cells were spun down at 1800 rpm for 5 minutes and the excess permeabilization and stain solution was discarded.

For intracellular staining, each tube received either 5µl of 1:10 diluted ORF59 mouse monoclonal antibody, or 5µl of 1:10 diluted Mouse IgG (Isotype control) and incubated for 1.5 hours in a 4^0C refrigerator. The cells were washed twice in saponin wash solution (0.03% saponin/0.5% BSA in PBS) before the addition of the secondary antibody (PE goat anti-mouse; 5µl per tube of 1:10 dilution) to all tubes except the unstained control. The tubes were then incubated 30 minutes on ice in the dark in a 4ºC refrigerator. Following incubation, the cells were washed once in saponin wash solution, twice with FACS and re-suspended in 800 µl of FACS buffer and then analyzed using an Accuri C6 cytometer.

3.3 RNA ISOLATION METHODS

Approximately 1 million cells were spun down at 1800 rpm for 5 minutes and washed twice in 1X PBS. The cell pellets were frozen at -80^{0}C for storage prior to RNA isolation. RNA was extracted from the cell pellets using a Qiagen RNeasy mini kit according to the manufacturer's protocol.

3.4 REVERSE TRANSCRIPTION

For these experiments, two different methods of reverse transcription were used. One method used the Taqman Oligo-dt (Table 2) while the other method used the RT-cocktail from SAbiosciences according to the manufacturer's protocol. The RT-cocktail was used to make cDNA, maximizing the yields (Table 3). The cDNA was used for the estrogen signaling pathway PCR arrays.

800 ng of total RNA was reverse transcribed using the Oligo-dt Taqman kit (Table 2), according to the manufacturer's protocol. Reverse transcription was performed using a thermocycler set for 1 cycle: 30^{0}C for 15 minutes, 42^{0}C for 15 minutes, 52^{0}C for 15 minutes and 70^{0}C for 10 minutes. Following transcription, 39µl of water and 1µl of RNAse H was added to the cDNA. The cDNA was vortexed, spun down, and incubated at 37^{0}C for 30 minutes. The reaction was then heated at 95^{0}C for 5 minutes, vortexed, spun down and stored at -80^{0}C.

Table 2. Reverse Transcription Master-mix

MASTER MIX		
RT	µl	Mix
10X RT buffer	5	5X
25 Mm MgCl2	11	11X
dNTPs Mix	10	10X
oligo Dt	2.5	2.5X
RNase inhIbitor	1	1X
RNase water	0	
Total RNA	18.5	
Reverse Transcriptase	1	1X
Total		31.5µl/reaction

Total RNA was isolated using to the TRIzol Reagent (ambion) protocol, and cDNA obtained using the RT2 First Strand Kit (C-03; Table 3), according to the manufacturer's protocol.

Table 3. SAbiosciences Reverse-Transcription Master-mix

RT Cocktail for Cdna Making	
RT Cocktail	1 reaction
BC3 (5XRT Buffer 3	4µl
P2 (Primer& External Control Mix)	1µl
RE3 (RT Enzyme Mix 3)	2µl
H2O	3µl
Final volume	10 ul/reaction

3.5 REVERSE TRANSCRIPTASE-BASED REAL-TIME PCR DETECTION

Reverse transcriptase-based PCR was performed by adding 7.5µl of SYBR Green to 5 µl of primer mix [48](Table 4) and 2.5 µl of cDNA per well in duplicate in 96 well plates for a total of 12.5µl. All plates were run using the StepOnePlus Applied Biosystem PCR for 1 cycle for 10 minutes at 95^0C, 40 cycles of 15 seconds at 95^0C, 1 minute at 60^0C and a melting stage at 95^0C with 0.3^0C increments. Data was analyzed using the 2^-ΔΔCt method.

Table 4. Primers used for RT-PCR. Realt-Time Quantitative PCR Analysis of Viral Transcription.

Gene primers	Function
ORF49	Expresses protein that induce transcription of c-jun
ORF50	Replication transactivator (RTA) necessary for lytic switch
K8	K-bZIP curb ORF50/RTA expression
ORF53	Viral envelope protein
ORF57	Viral splicing factor
ORF62	Involved in assembling and DNA maturation
ORF73	Cell control and episomal persistance
ORF74	G-coupled receptor and is involved in cellular growth and stimulating factors required to maintain KS

3.6 ENHANCED LYTIC IMMUNOFLUORESCENT ASSAY

BCBL-1 cells were re-suspended at 2.5x105 cells/ml and induced with 20ng per ml (final concentration) TPA(Advanced Biotechnologies) for 5 days. The cells were then spun down at 1800 rpm for 5 minutes, washed once with equal amount of PBS, and washed twice with PBS by decreasing the volume by half. The cells were re-suspended in one tenth of the starting volume in 4% buffered Paraformaldehyde for ten minutes at room temperature. The cells were centrifuged (to pellet the cells) and the paraformaldehyde discarded. The cells were washed at least three times in 1X PBS reducing the volume by half, vortexing the cells after each wash. The cells were permeabilized for 10 minutes in 0.05% Triton-X at room temperature in one tenth of the starting volume and periodically mixed gently. After permeabilization, the cells were washed twice in 1X PBS and re-suspended in PBS at the concentration of 1.5-2x107 cells per ml then 10 µl of cell suspension was aliquoted per well of 5 mm black Teflon-coated 12-well slides (Fisher Scientific). The slides were allowed to air dry and kept at -20^{0}C.

For each batch of induced BCBL-1 cells stained by immunofluorescence assay (IFA) a known HHV-8 positive human serum was used as a positive control and normal goat serum served as a negative control. The slides were blocked with 20 µl of 10% goat serum and incubated for 30 minutes to 1 hour at 37^{0}C. Primary antibody (positive control, negative control or ORF59-specific) diluted 1:50 or 1:100 in normal goat serum was added to the appropriate wells and incubated at 37^{0}C for 1 hour. Afterward, the slides were washed twice with PBS for 5 minutes on a shaker at room temperature. Next, 20 µl of a 1:100 dilution (in normal goat serum) of FITC-labeled secondary antibody (Roche) was added per well and incubated at 37^{0}C for 30 minutes to 1 hour. The slides were rinsed twice in PBS and then washed twice for 10 minutes at room temperature in PBS in the dark. The PBS was changed one final time and the slides were

stored overnight in the dark in PBS. The slides were read the following day using a fluorescent microscope.

3.7 MEASUREMENT OF INFECTIOUS VIRUS PRODUCED: TCID50 ASSAY

Samples containing HHV-8 were sonicated, diluted 1:10 and added to T1H6-DC-SIGN cells plated at 80,000 cells per well in 96 well plates. 48 hours later, the cells were harvested, counted, spun down at 13,000 RPM for 1 minute and the supernatant was removed. The cell pellets were washed 3 times in cold PBS, spun down and re-suspended each time at 100 µl per tube. The pellet was next re-suspended in 100 µl lysis buffer (100 mM dipotassium phosphate, 100mM monopotassium phosphate, and 1M dithiothreitol). The mixture was frozen using a dry ice-ethanol bath and thawed in a 37^0C water bath three times, and spun down for 10 minutes at 13,000 RPM at 4^0C in a microcentrifuge. The supernatants were transferred into fresh tubes for storage. Measurement of β galactosidase levels was measured using the β-galactosidase detection kit from Clontech according to manufacturer's directions with some modifications. In an opaque 96-well plate (costar-black), 117.6 µl of Beta-galactosidase reaction substrate and 2.4 µl of Beta-galactosidase reaction buffer was added to 15 µl of lysate per well. All dilutions were done in triplicate and read using a luminometer (BioTek Synergy II), at 10 second integrals.

3.8 VIRAL HARVEST ASSAY

A liter of BCBL-1 cells induced for 5 days with 20ng/ml TPA was spun down at 5,000 RPM using four 250ml bottles in an SLA-1500 rotor at 4^0C. The supernatant was poured into a 1 liter bottle that was kept at 4^0C. The cell pellet was re-suspended in 5 ml of PBS with 0.1% BSA and transferred into 2 15 ml conical tubes. The re-suspended pellet was then subjected to three cycles of freeze-thaw using a dry-ice-ethanol bath and 37 ^{0}C water bath. The resulting lysate was spun down at 3,000 RPM for 10 minutes in a table-top centrifuge. The supernatant was collected and added to the previous 1 liter of supernatant. The 1 liter bottle with the total supernatant was placed in a laminar flow hood and 23 grams of NaCl added while stirring. Next, 70 grams of polyethylene glycol 8000 (PEG) was added slowly and the solution was allowed to stir overnight in a 4^0C refrigerator.

The following day, the solution was transferred into 4 new 250 ml bottles and spun in an SLA-1500 rotor at 8,000 RPM for 30 minutes at 4^0C and the supernatant was discarded. The pellet was re-suspended with 2 ml of PBS per bottle and transferred to 15 ml conical tubes, each bottle was rinsed with 1 ml of PBS that was transferred in to the 15 ml tubes. The suspension was then transferred into 12 microcentrifuge tubes and centrifuged at 13,000 RPM for 4 min. The supernatant obtained after the spin was combined in a 15 ml conical tube. Afterwards, to 4 ultracentrifuge tubes pre-absorbed with 0.1% BSA, 6 ml of 25% sucrose solution was added and 3 ml of virus solution was carefully layered on top. The ultracentrifuge tubes were spun at 28, 000 RPM at 4^0C for 2 hours using a SW28 rotor. Following centrifugation, the supernatant was discarded and 100 µl PBS and 0.1% BSA added to the pellet and allowed to soak at 4^0C

overnight. On the third day the pellet was re-suspended in PBS on ice and aliquoted into 20 µl in microcentrifuge tubes (avoiding formation of bubbles) and stored at -80 ^{0}C.

3.9 FOCUSED RT-PCR ESTROGEN SIGNALING ARRAYS

Total RNA was isolated from cells using TRIzol reagent according to the manufacturer's instructions. cDNA was obtained using the RT2 First Strand Kit (C-03) according to the manufacturer's protocol.

For the PCR reactions, 2 X SABiosceinces RT2 qPCR Master mix, a diluted First Strand cDNA synthesis reaction and water were combined as shown in Table 4 and 25µl of this cocktail was added to each well of a 96-well plate. In these experiments, the 96 well plates contain individual primer sets in each well corresponding to a different cellular gene. The PCR reactions were run using the StepOnePlus Real-Time PCR system from Applied Biosystems for 1 cycle for 10 minutes at 95 ^{0}C, 40 cycles of 15 seconds at 95^{0}C, 1 minute at 60 ^{0}C and a melting stage at 95 ^{0}C with 0.3 ^{0}C increments.

Table 5. SAbiosciences RT-PCR Mix

PCR MIX	
2X SABIOSCINCES RT2 qPCR Master Mix	1350µl
Diluted First Strand cDNA synthesis reaction	102µl
H2O	1248 µl
Total Volume	1100 µl

3.10 DATA ANALYSIS

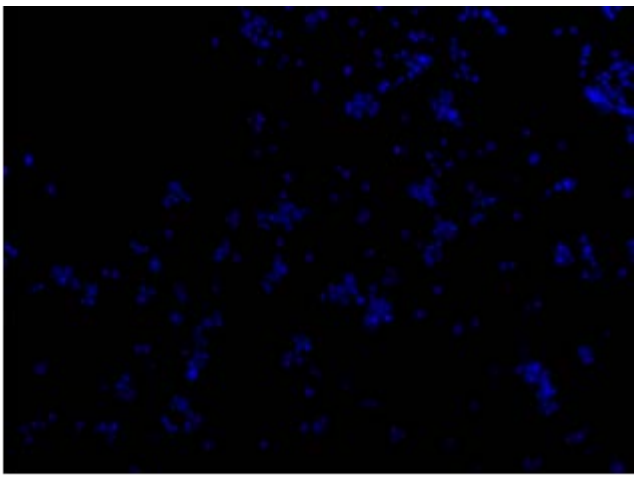
The reverse transcriptase based real time PCR data were analyzed using the 2^-ΔΔCt method, normalizing all the samples from treated cells to samples from the non-treated cells. The graphs were constructed using PRISM4 and SAbiosciences software.

4.0 RESULTS

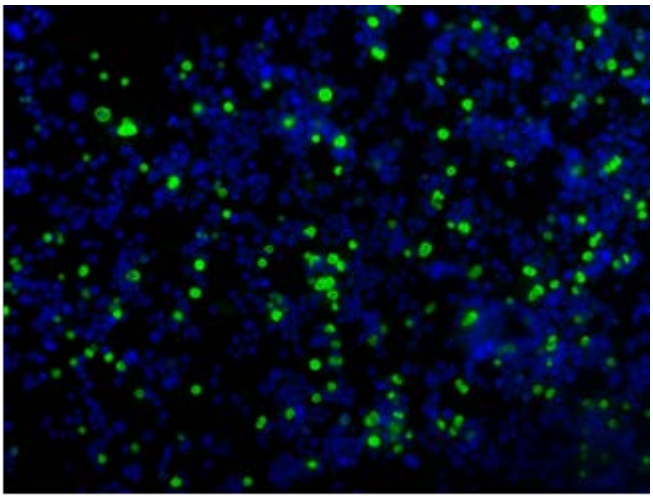
4.1 AIM # 1 TO DETERMINE IF ESTROGEN AFFECTS HHV-8 REACTIVATION

4.1.1 Induction of BCBL-1

The BCBL-1 cell line (from a B cell lymphoma) harbors the latent HHV-8 virus and when grown in cell culture approximately 5% of the cells undergo spontaneous reactivation (resulting in viral replication) while the remaining 95% of the cells stay latently infected and do not produce virus. To show that TPA could induce viral reactivation in this cell line [44], the cells were treated with TPA for 5 days. The cells were harvested and an immunofluorescence assay was performed using an antibody against the HHV-8 lytic protein ORF59. A positive control consisted of serum from an HHV-8 seropositive donor. A negative control consisted of normal goat serum. The nuclei were stained with DAPI. As seen in Figure 7, TPA induction resulted in about 60% of the cells expressing ORF59, demonstrating viral reactivation.



Negative Control. Stained with Normal Goat Serum



ORF59 Staining

Figure 7. TPA-Induced Reactivation of HHV-8 in BCBL-1 cells. *BCBL-1 cells were induced with TPA for 5 days, then permeabilized and fixed on Teflon coated 12 well slides and stained with the indicated primary antibodies. The nuclei were stained with DAPI.*

4.1.2 Time course measure of viral reactivation in TPA induced BCBL-1

To demonstrate a time course for TPA-induced HHV-8 reactivation, BCBL-1 cells were treated with 20ng/ml TPA and aliquots of the cells removed daily for 5 days. The cells were fixed, permeabilized and stained for ORF59 using a PE-labeled secondary antibody. Positive

staining was determined by flow cytometry. As shown in Figure 8, the amount of ORF59 positive cells (indicating reactivation) increased daily, reaching peak levels of approximately 50% on day 3 followed by a decline in expression due to virus-induced cell death.

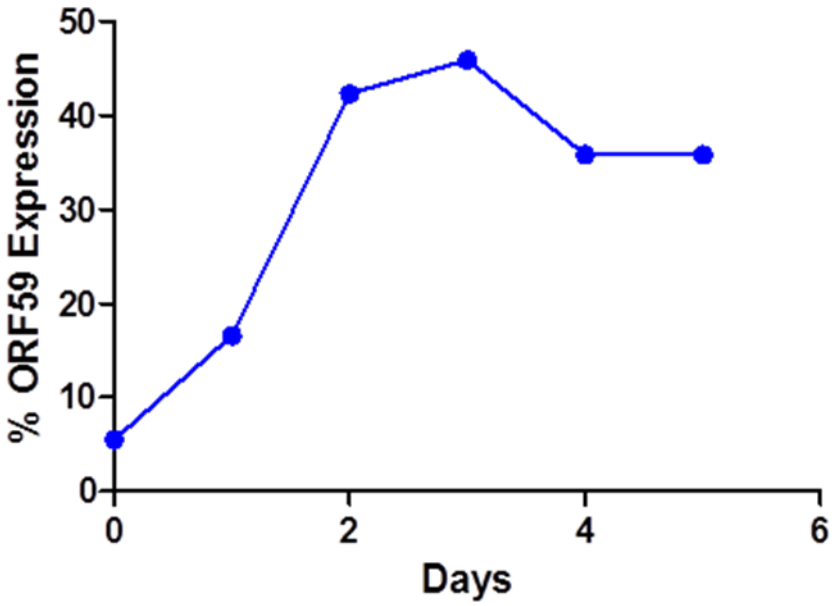


Figure 8. Time Course of TPA induced HHV-8 reactivation in BCBL-1 cells. *BCBL-1 cells were treated with 20 ng/ml TPA for 5 days and cells were sampled daily and stained intracellularly for ORF59 expression using a monocolonal antibody against ORF59 and a PE-labeled secondary antibody. The percentage of ORF59-expressing cells was measured by flow cytometry.*

4.1.3 Measure of estrogen-induced viral reactivation in BCBL-1 cells

The previous experiments demonstrated that HHV-8 can be reactivated in BCBL-1 cells by TPA. Therefore, TPA-induced reactivation of BCBL-1 cells will be used as a positive control for the following experiments.

To assess the effect of estrogen on viral reactivation in BCBL-1 cells, an estrogen dose response experiment was performed over 5 days with concentrations ranging from 1 micromolar

to 1 nanomolar with the physiological level being between 10 nanomolar and 1 nanomolar [49]. TPA-induced BCBL-1 cells were used as a positive control and ethanol as a vehicle control. The cells were grown in RPMI phenol red-free media and treated with the different doses of estrogen, TPA or ethanol. Each day for 5 days, cells were harvested, fixed, stained with an antibody to ORF59 and analyzed by flow cytometry. Estrogen did not result in viral reactivation as measured by expression of ORF59 (Figure 9).

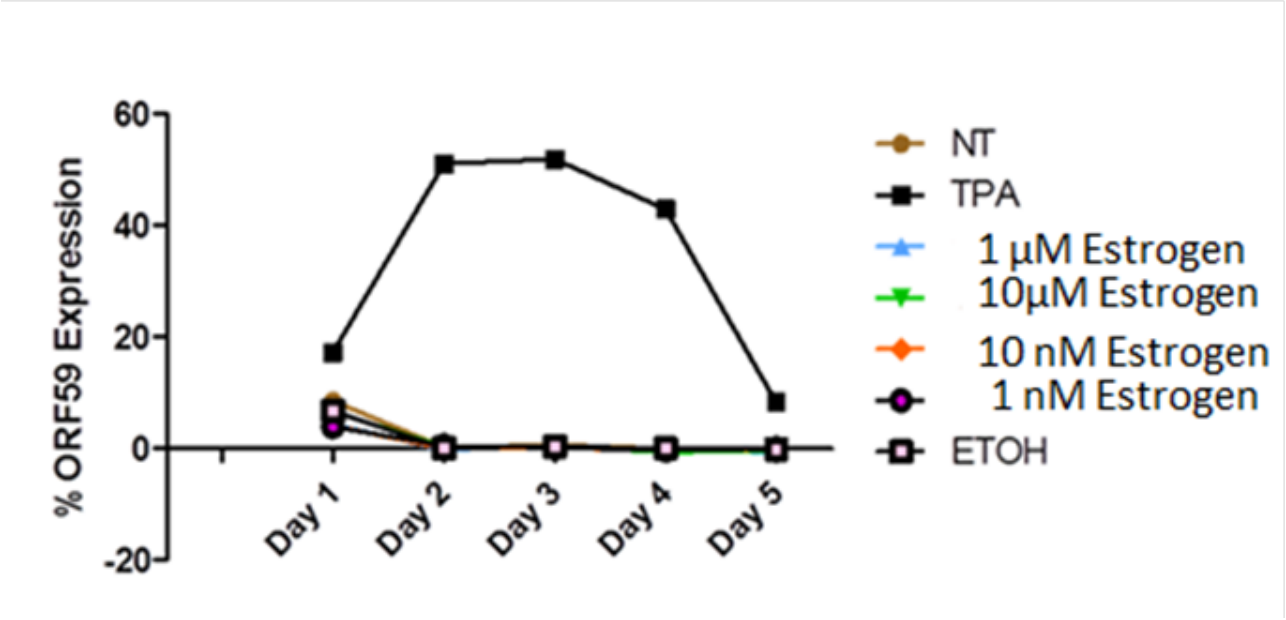


Figure 9. Estrogen Treatment does not Result in HHV-8 Reactivation in BCBL-1 Cells. *BCBL-1 cells were grown overnight in phenol red-free media, treated with various dosages of estrogen, 20ng/ml TPA or ethanol (vehicle conrol). Each day for 5 days cells were collected and stained for ORF59. Perentage of ORF59-expressing cells was determined by Flow cytometry.*

The time course experiment was repeated for confirmation, using 1 micromolar estrogen (Figure 10). Cells were grown overnight in phenol red-free media (phenol red has been shown to bind estrogen receptors in vitro [50]). TPA or estrogen was added to the cells the following day and aliquots were taken out daily for 4 days. Intracellular staining for the lytic protein ORF59 was performed and the percentage of ORF59 positive cells determined by flow cytometry. As shown in Figure 10 and in agreement with Figure 9, estrogen treatment did not result in viral

reactivation as measured by expression of ORF59. Flow cytometry analyses showed cells treated with estrogen had identical patterns to untreated cells. These results were in contrast to cells treated with TPA which demonstrated increased ORF59 expression beginning at day 3.

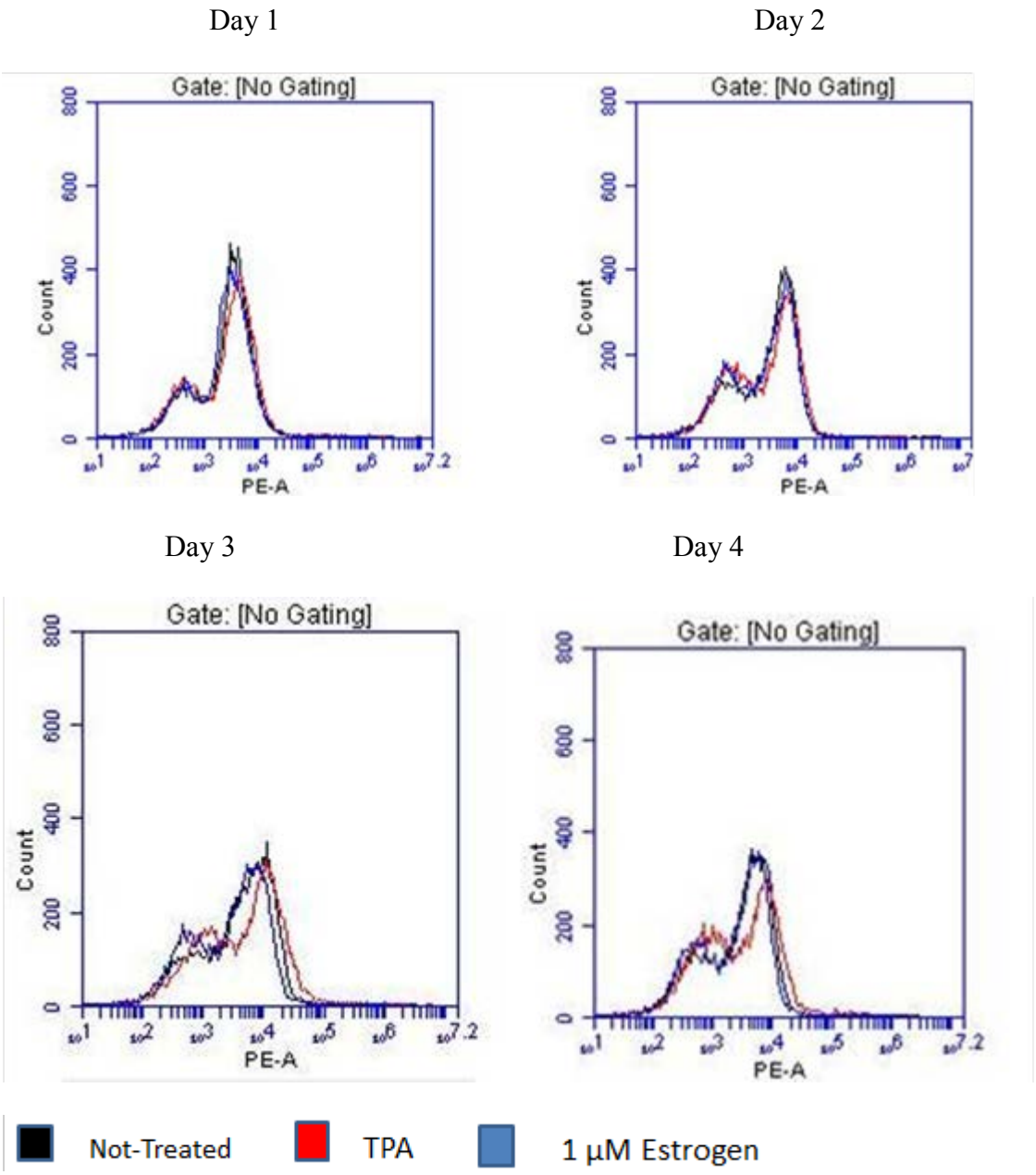


Figure 10. Determination of ORF59 Expression following Estrogen or TPA Treatment by Flow Cytometry. *BCBL-1 cells were grown overnight in phenol red free media, treated with estrogen or TPA, stained for ORF59 and analyzed by Flow cytometry.*

4.1.4 Measurement of HHV-8 Reactivation following Treatment of TRex-BCBL-1 cells with Estrogen, Doxycycline or TPA

TRex-BCBL-1 cells are a BCBL-1 cell line that contains a doxycycline - inducible ORF50 (RTA). Doxycycline treatment of TRex-BCBL-1 cells results in HHV-8 reactivation through the action of the RTA protein [45]. The RTA-induced viral reactivation in these cells is more efficient than TPA reactivation [45]. RTA is a specific HHV-8 inducing protein while TPA is non-specific, causing reactivation by binding to AP-1 sites in viral and cellular promoters [51].

TRex-BCBL-1 cells were grown overnight in phenol red-free RPMI media. The next day, the cells were treated with estrogen, TPA, or doxycycline for three days. We limited this experiment to three days as previous work in our laboratory has shown that RTA-induced reactivation is maximal at 3 days (F. Jenkins, unpublished data). Aliquots of cells were taken out daily and stained for ORF59 expression. The percentage of ORF59 positive cells was determined by FLOW cytometry. As shown in Figure 11, ORF59 expression was similar between untreated and estrogen treated cells. Doxycycline treatment resulted in increased ORF59 expression beginning 24 hrs post treatment while TPA treatment increased ORF59 expression 48 hrs post treatment.

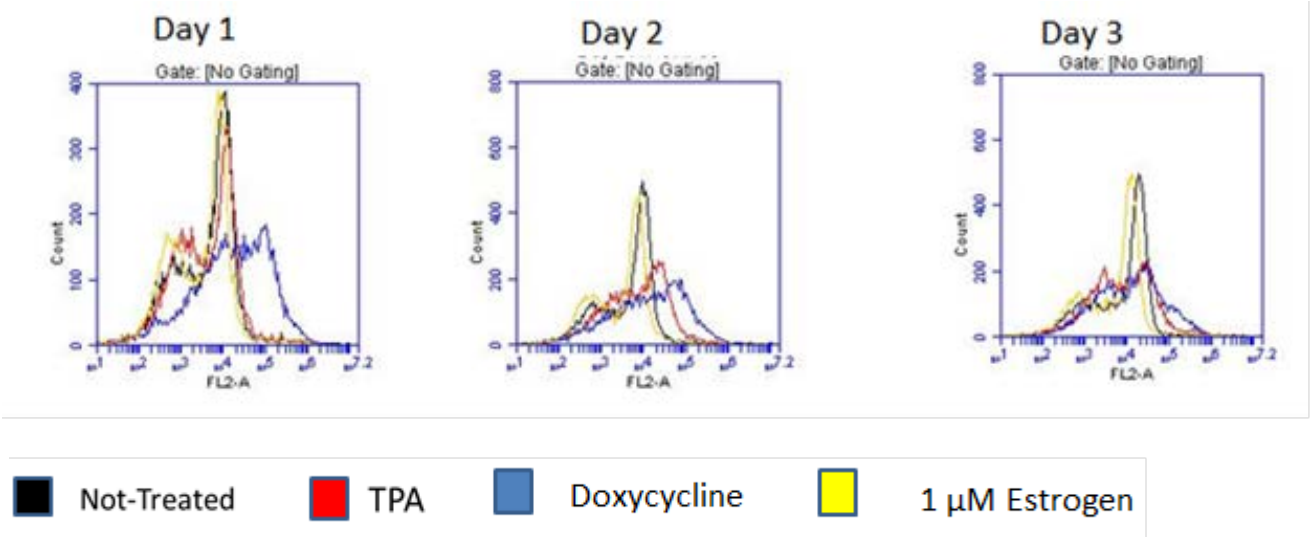


Figure 11. Time Course of Viral Reactivation in TRex-BCBL-1 cells treated with TPA, Doxycycline or Estrogen. *Cells were grown overnight in phenol red free media, treated with estrogen, TPA or doxycycline and stained for expression of ORF59 with PE as a secondary antibody, then the intensity of ORF59 expression was measure by Flow cytometry.*

4.1.5 Determination of the Effect of Estrogen on Viral Reactivation in TPA and RTA-induced TRex-BCBL-1 cells

To measure the effect of estrogen on TPA or RTA-induced viral reactivation, TRex-BCBL-1 cells were treated with TPA, doxycycline, estrogen, estrogen + TPA, or estrogen +doxycycline for 3 days. Cells treated with estrogen and TPA or doxycycline were pretreated with estrogen for 2 hrs prior to addition of TPA or doxycycline. Aliquots of cells were taken each day and stained for ORF59 and the intensity of positive cells was measured by flow cytometry. As shown in Figure 12, viral reactivation (as measured by ORF59 staining) was similar between cells treated with doxycycline alone and cells treated with estrogen and doxycycline. Similarly, viral reactivation was akin between cells treated with TPA alone and

cells treated with estrogen and TPA. Finally, estrogen treatment alone did not result in viral reactivation with ORF59 staining similar to that seen with untreated cells.

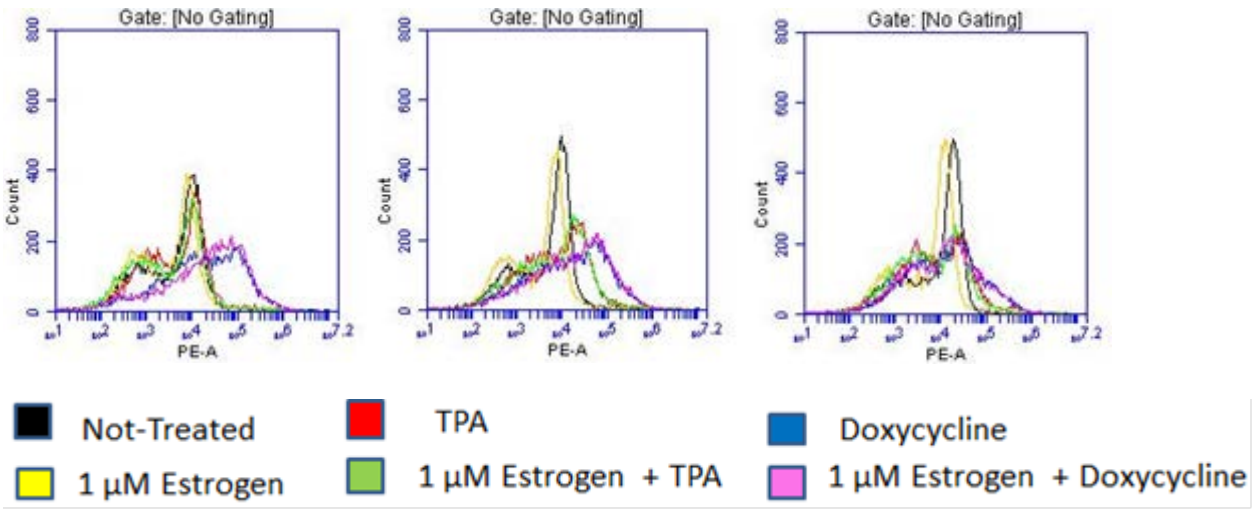


Figure 12. Time Course Experiment to Measure Viral Reactivation in TRex-BCBL-1 cells following TPA or Doxycycline with Estrogen Treatments. *Cells were grown overnight in phenol red-free media, treated with estrogen, TPA, doxycycline, estrogen plus TPA or estrogen plus doxycycline and stained for ORF59. The intensity of ORF59 staining was measured by flow cytometry.*

4.1.6 Measurement of Infectious HHV-8 virus following Treatment of BCBL-1 Cells with TPA, Estrogen or TPA+Estrogen

TCID50 assay was performed to measure the production of infectious virus in BCBL-1 cells treated for 5 days with either estrogen, TPA, or estrogen plus TPA. As shown in Table 5, compared to untreated cells, estrogen treatment increased the amount of infectious virus 3 fold while TPA treatment increased the amount of infectious virus 5 fold. In addition, cells treated with both estrogen and TPA showed a TCID50 increase of 10 fold compared to cells that were not treated. These results demonstrate that estrogen treatment increases the amount of infectious virus produced in cells undergoing viral replication.

Table 6. TCID50 Determination of Infectious Virus Produced during BCBL-1 Treatment with TPA, Estrogen or a Combination of Both. *BCBL-1 cells were grown overnight in RPMI phenol red free media, and the following day, treated with 10 E -6 M estrogen and/or 20 ng/ ml of TPA. Cells were collected 5 days post treatment, and the TCID50 assay performed.*

Treatment	Fold Increase of TCID50
Non-Treated	-
1 µM Estrogen	3
TPA	5
1 µM Estrogen +TPA	10

4.1.7 Summary of Aim 1

Aim 1 was designed to investigate the reactivation of HHV-8 in BCBL-1 and Trex-BCBL-1 cells after treatment with 10-6 M estrogen compared to TPA- and RTA-induced reactivation. Flow cytometry analysis was performed against ORF59 an HHV-8 lytic protein. A viral replication assay was also carried out to measure the amount of virus made during the different treatment using a TCID50 method developed in our laboratory.

Estrogen alone did not cause reactivation in BCBL-1 nor in TREX-BCBL-1 cells. When the cells were treated with TPA or doxycycline to induce viral reactivation in presence of estrogen, viral reactivation (as determined by ORF59 expression) did not increase but stayed similar to TPA or doxycycline induced cells. The quantity of infectious virus produced (as

measured by a TCID50 assay) increased when BCBL-1 cells were induced with TPA or doxycycline in the presence of estrogen. These results suggests that the number of cells reactivating from latent to lytic replication did not increase, but once the cells were reactivated with TPA or RTA in presence of estogen, the yield of infectious virus did increase.

4.2 AIM # 2 TO TEST THE HYPOTHESIS THAT ESTROGEN MODULATES VIRAL AND CELLULAR TRANSCRIPTION

The results in Aim 1 demonstrated that estrogen did not cause HHV-8 reactivation in BCBL-1 nor in TREX-BCBL-1 cells but when the cells were induced with TPA or doxycycline (respectively) in the presence of estrogen the production of infectious virus increased. In this aim we test the hypothesis that estrogen modulates viral and cellular transcription.

4.2.1 Determination of the Effect of Estrogen on Viral Transcription in BCBL-1 Cells

The experiments described in Aim 1 demonstrated the inability of estrogen to reactivate HHV-8 in BCBL-1 cells. Reactivation was based on expression of ORF59. The ORF59 gene does not have a potential ERE in its promoter and therefore it is possible that estrogen induced viral transcription in genes carrying EREs, without resulting in viral reactivation. Therefore we measured RNA expression by RT-PCR on cDNA from treated cells using specific primers for a subset of viral genes that have potential EREs in or near their promoters (ORF49, K8, ORF53, ORF57, ORF 62 and ORF74). Trex-BCBL-1 cells were cultured overnight in RPMI phenol red-free media, and the next day incubated for 3 hrs in the presence of estrogen, TPA or doxycycline.

Hourly, aliquots of cells were removed, RNA was isolated, cDNA made and RT-PCR performed.

ORF49 is a lytic protein that is encoded in the opposite direction of ORF50 in the HHV-8 genome. ORF49 is not able to activate any HHV-8 promoters on its own, but can reactivate several lytic promoters containing AP-1 sites by working with ORF50 [47]. Across the different time points, in cells treated with TPA, doxycycline or estrogen alone, ORF49 expression was only seen at 3hr with doxycycline treatment (Figure 13). However, when estrogen was combined with TPA or doxycycline, ORF49 expression was increased or either treatment alone (Figure 13).

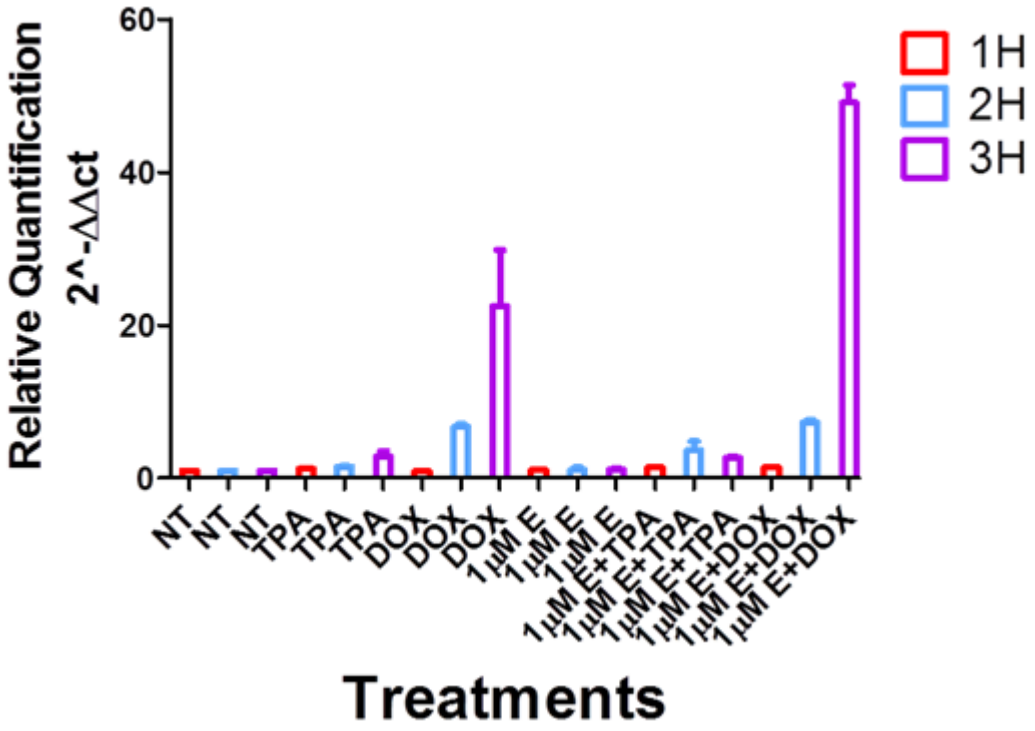


Figure 13. Time Course Measurement of ORF49 Viral Transcription in TRex-BCBL-1 following Treatment with either TPA, Doxycycline, or Both. *In experiments using combination treatments, cells were pre-treated 2h with estrogen prior to addition of TPA or doxycycline. Aliquots of the cells were taken out each hour for 3 hours, RNA isolated from the cells, cDNA made and RT-PCR performed. Results are shown as 2^ΔΔct values. NT: Non-treated, TPA (12-O-tetradecanoyl phorbol-13 acetate), Dox: doxycycline, E: Estrogen*

K8 or K-bZIP protein is a lytic protein that is required for lytic DNA replication [26]. K8 is a transcriptional repressor that binds directly to the RTA protein and curbs RTA-mediated transactivation of the K-bZIP promoter causing a negative auto-regulation system during lytic replication [48], [25]. As shown in Figure 14, while both TPA and doxycycline treatment increased expression of K8, estrogen treatment alone did not. However, in presence of both doxycycline and estrogen at 3hr, levels of K8 mRNA were higher than with doxycycline alone.

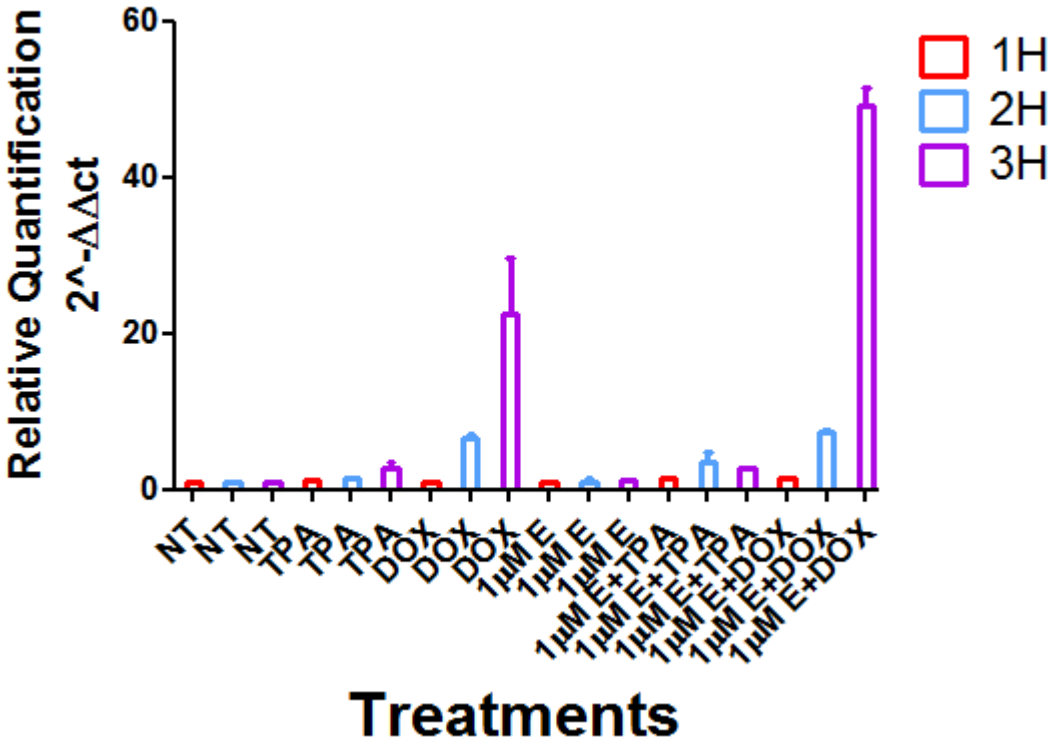


Figure 14. Time Course Measurement of K8 Viral Transcription in TRex-BCBL-1 following TPA or Doxycycline with Estrogen Treatments. *In experiments using combination treatments, cells were pre-treated 2h with estrogen prior to addition of TPA or doxycycline. Aliquots of the treated cells were taken out each hour for hourly during 3 hours,. RNA isolated from the cells, cDNA was made and RT-PCR was performed. Results are shown as 2^ΔΔct values. NT: Non-treated, TPA (12-O-tetradecanoyl phorbol-13 acetate), Dox: doxycycline, E: Estrogen.*

ORF 53 is a gene expressed during the lytic replication of HHV-8 [49]. As shown in Figure 15, the expression levels of ORF53 are generally low during the three hour time course experiment. The exceptions were 2hr treatment with both TPA and estrogen and 3hr treatment with doxycycline and estrogen.

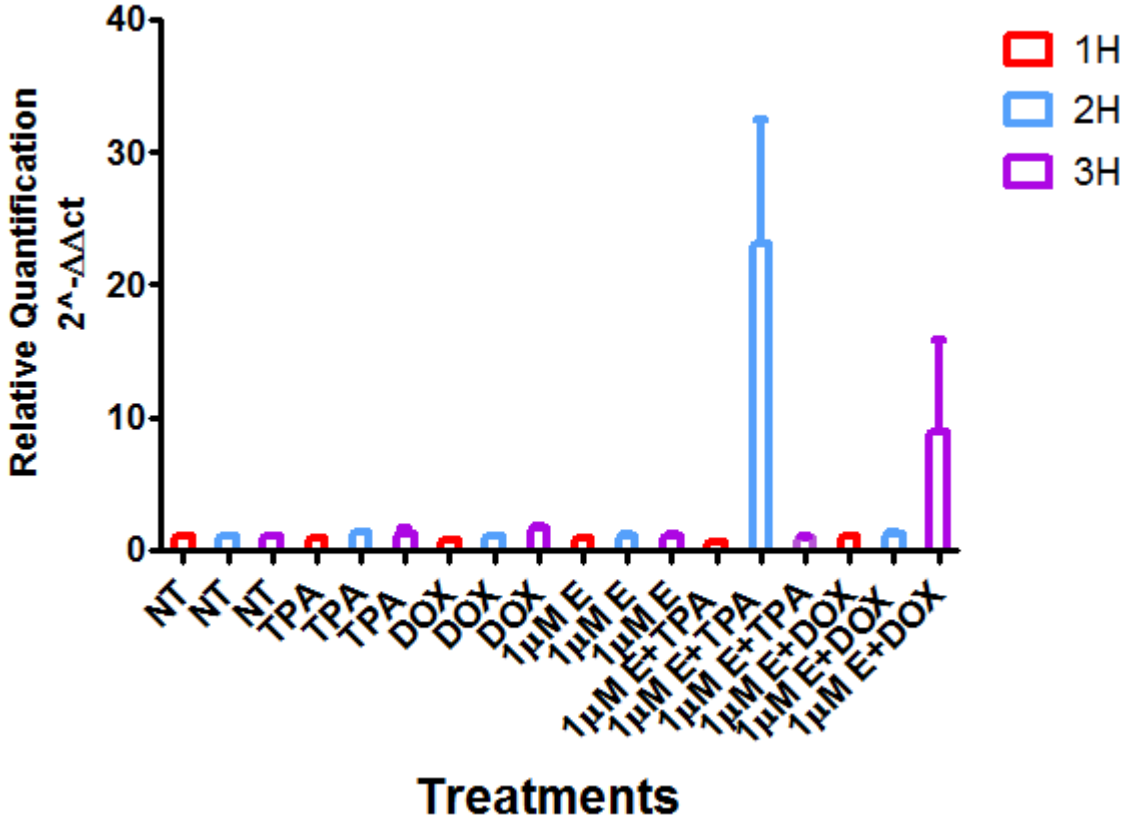


Figure 15.Time Course Measurement of ORF 53 Viral Transcription in TRex-BCBL-1 following TPA or Doxycycline with Estrogen Treatments. *In experiments using combination treatments, cells were pre-treated 2h with estrogen prior to addition of TPA or doxycycline. Aliquots of the treated cells were taken out each hour for hourly during 3 hours,. RNA isolated from the cells, cDNA was made and RT-PCR was performed. Results are shown as 2^ΔΔct values. NT: Non-treated, TPA (12-O-tetradecanoyl phorbol-13 acetate), Dox: doxycycline, E: Estrogen.*

ORF 57 gene is observed during the lytic replication of HHV8 [49]. The gene was found to function as a viral splicing factor and promote the splicing of K-bZIP transcripts. As shown in Figure 16, while ORF57 expression is not increased with estrogen treatment alone, mRNA levels

do increase over time with doxycycline treatment (representing RTA induction) and these levels are increased in the presence of estrogen.

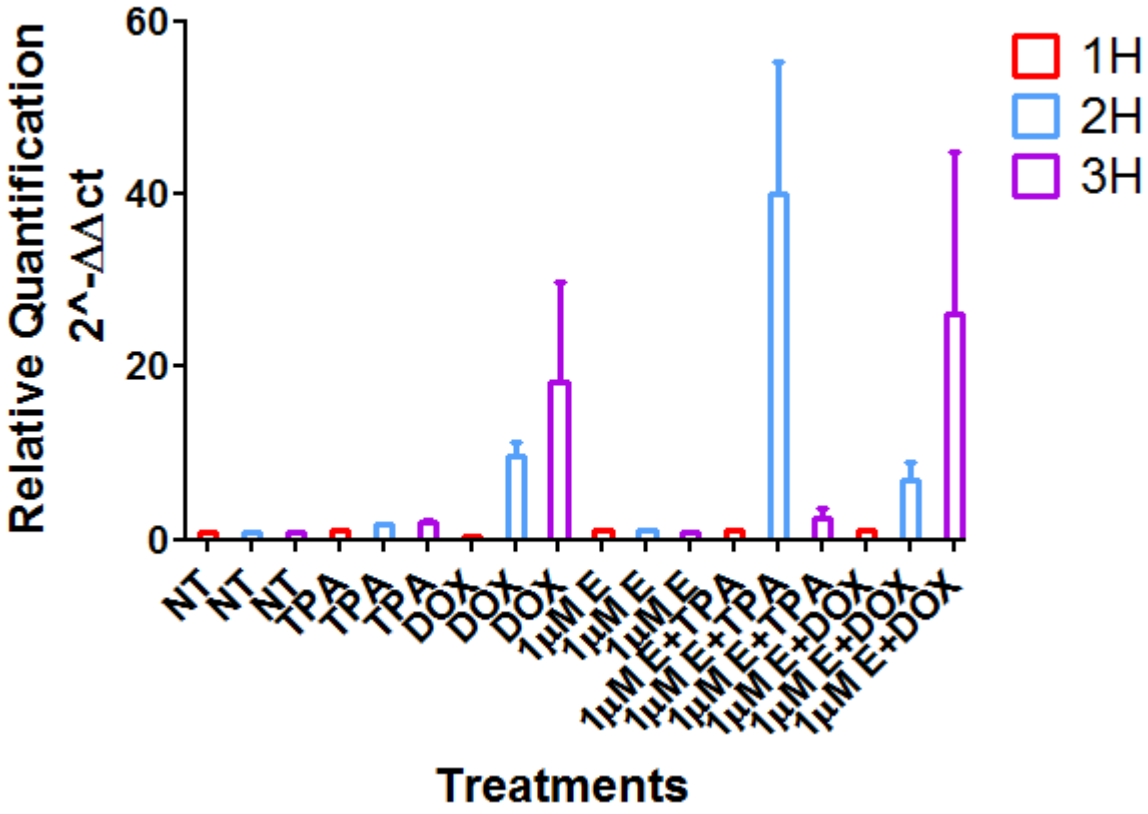


Figure 16. Time Course Measurement of ORF57 Viral Transcription in TRex-BCBL-1 Cells following TPA or Doxycycline with Estrogen Treatments. *In experiments using combination treatments, cells were pre-treated 2h with estrogen prior to addition of TPA or doxycycline. Aliquots of the treated cells were taken out each hour for hourly during 3 hours,. RNA isolated from the cells, cDNA was made and RT-PCR was performed. Results are shown as 2^ΔΔct values. NT: Non-treated, TPA (12-O-tetradecanoyl phorbol-13 acetate), Dox: doxycycline, E: Estrogen.*

The gene ORF62 is also seen during lytic replication. ORF 62 has been shown to encode a protein which is involved in assembly and DNA maturation [49]. As shown in Figure 17, expression of this gene was not detected over the three hrs time course except in cells treated with both estrogen and either TPA (2hr) or doxycycline (3hr)The levels of expression of these

genes were low overall, but doxycycline showed a relatively high expression compared to the other treatments.

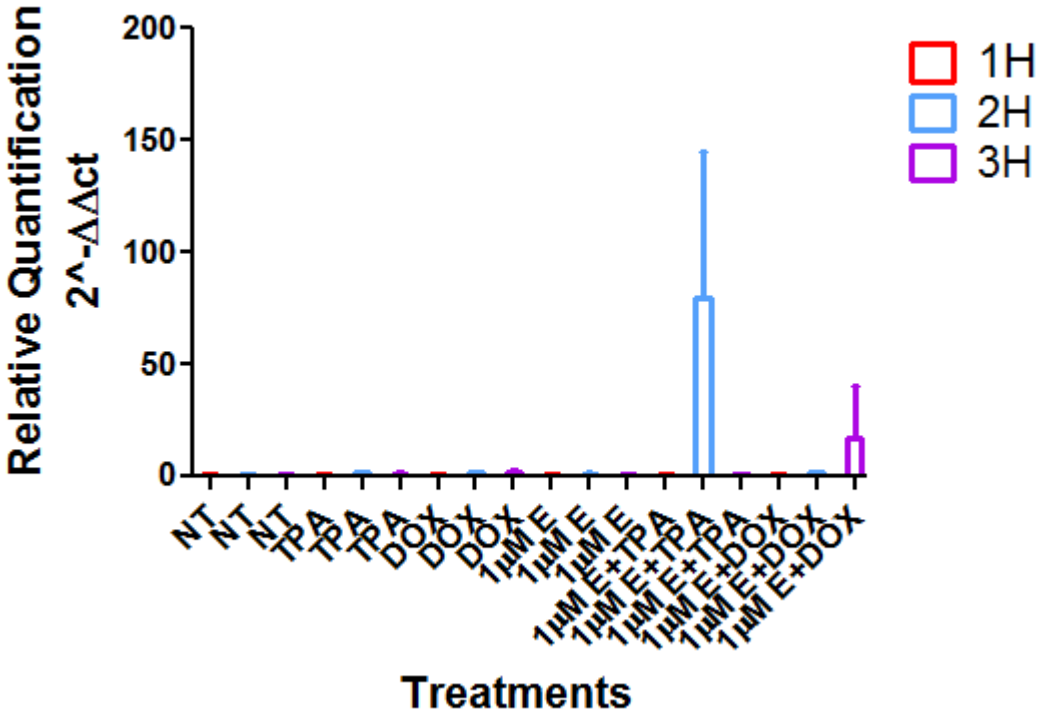


Figure 17. Time Course Measurement of ORF62 Viral Transcription in TRex-BCBL-1 following TPA or Doxycycline with Estrogen Treatments. *In experiments using combination treatments, cells were pre-treated 2h with estrogen prior to addition of TPA or doxycycline. Aliquots of the treated cells were taken out each hour for hourly during 3 hours,. RNA isolated from the cells, cDNA was made and RT-PCR was performed. Results are shown as 2^ΔΔct values. NT: Non-treated, TPA (12-O-tetradecanoyl phorbol-13 acetate), Dox: doxycycline, E: Estrogen.*

ORF 74 is member of the early HHV-8 lytic phase genes, encoding a G-protein-coupled receptor which serves as an IL-8 chemokine receptor homologue[50], [51]. As shown in Figure 18, in a fashion similar to ORF 62, expression of this gene was not detected over a three hours' time course except in cells treated with both estrogen and either TPA (2hr) or doxycycline (3hr)

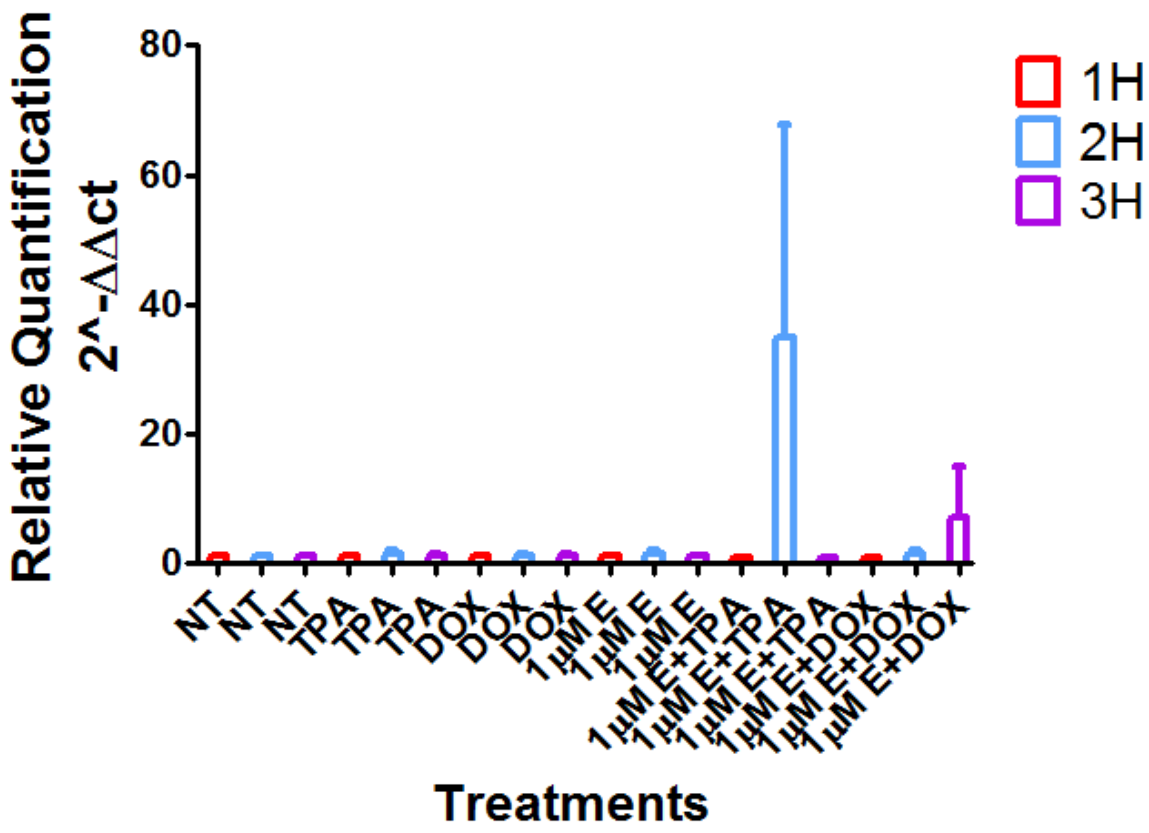


Figure 18. Time Course Measurement of ORF74 Viral Transcription in TRex-BCBL-1 following TPA or Doxycycline with Estrogen Treatments. *In experiments using combination treatments, cells were pre-treated 2h with estrogen prior to addition of TPA or doxycycline. Aliquots of the treated cells were taken out each hour for hourly during 3 hours,. RNA isolated from the cells, cDNA was made and RT-PCR was performed. Results are shown as 2^ΔΔct values. NT: Non-treated, TPA (12-O-tetradecanoyl phorbol-13 acetate), Dox: doxycycline, E: Estrogen.*

About 5-10% of BCBL-1 cells in culture are reactivating spontaneously [44]. To measure the effect of estrogen alone on viral transcription in these cells, an estrogen dose response experiment was performed in BCBL-1 cells. The cells were grown overnight in RPMI phenol red free media, the next day they were treated with the different doses of estrogen for 4 hrs. The BCBL-1 cells were harvested, and the RNA was isolated, reverse transcribed and an RT-PCR was performed. The data from Figure 19 shows that the regulatory gene ORF50 encoding RTA, was down-regulated while levels of K8, a regulatory gene known to down-regulate expression of ORF50 where induced with lower concentrations of estrogen. There were other genes such as

ORF53, ORF57, ORF62 and ORF74 that were up-regulated by estrogen treatment but they are not regulatory genes and expression is not involved in maintaining or reactivating latency.

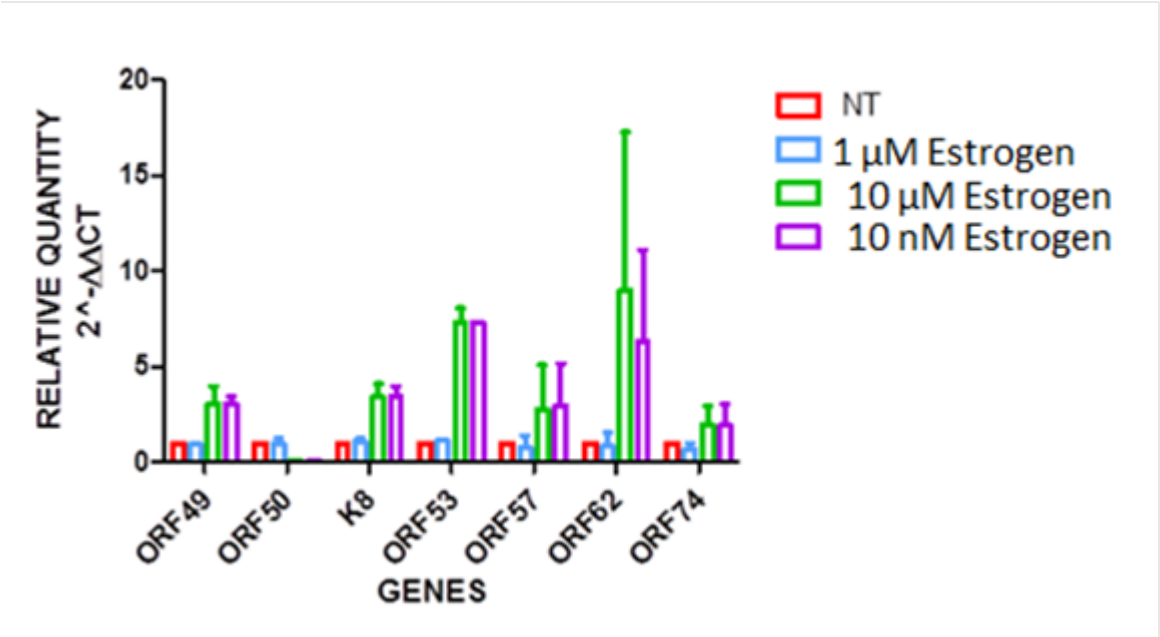


Figure 19. Estrogen Dose Response and Gene Expression in Uninduced Reactivating BCBL-1 Cells. *BCBL-1 cells were treated with 1µM, 10µM and 10 nM estrogen for 4hrs. Aliquots of cells were taken, RNA was isolated, reverse-transcribed, and then RT PCR on several viral genes was carried out.*

4.2.2 To Test the Hypothesis that Modulation of Cellular Transcription by Estrogen is Dependent on the Type of Estrogen Receptor (Alpha Vs. Beta)

From previous experiments, we observed that estrogen down-regulates some viral genes such as ORF50 while up-regulating other genes such as K8 and ORF62. To determine if estrogen affected gene transcription due to the type of estrogen receptor expressed in B cells (the beta receptor), we compared transcription of cellular genes in MCF7 and BCBL-1 cells treated with estrogen. MCF-7 is a breast cancer line extensively studied and known to predominantly

express the estrogen receptor alpha [52]. The BCBL-1 cell line was shown by previous work in our laboratory to express the estrogen receptor beta (F. Jenkins, unpublished data). The two cell lines were grown overnight in phenol red-free media, and the following day treated for 4 hours with physiological levels of estrogen (10 nanomolar). The cells were harvested, RNA was isolated, cDNA made and RT-PCR performed using SABiosciences estrogen signaling PCR array plates that contained in each well a set of primers for a single gene.

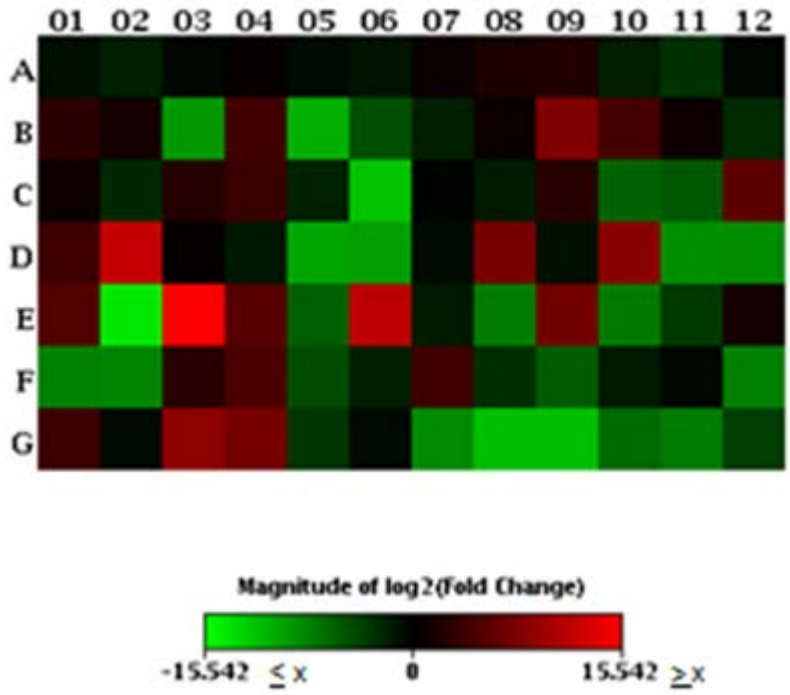


Figure 20. Heat Map of MCF-7 Cells' Genes after Estrogen Treatment Compared to Non-Treated Cells. *MCF-7 cells were grown overnight in phenol red free RPMI, treated the next with estrogen for 4 hours, aliquots of cells were taken out, RNA was isolated, cDNA was made and RT-PCR using estrogen signaling array plates were used. The genes represented in each square are shown in Table 7.*

Table 7. Representation of the Different Expression Values from MCF-7 Cells' Genes. *Each square represents a single well in the 96 well plate. The top line in each square is the abbreviation of the gene being amplified while the second line represents the fold increase or decrease in expression over that seen in untreated cells.*

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	ABCB 1 -1.04	ABCC1 -2.04	ABCC2 1.36	ABCC3 2.54	ABCC5 1.07	ABCG2 -1.28	AMR 2.59	AP1S1 5.82	APC 7.05	AR -1.96	ARNT -4.52	ATM 1.47
B	BAX 10.74	BCL2 4.29	BCL2L1 - 330.57	BLMN 28.21	BRCA1 -880.92	BRCA 2 -15.40	CCND1 -2.07	CCNE1 3.18	CDK2 407.02	CDK4 31.45	CDKN1A 3.15	CDKN1B -3.31
C	CDKN2A 3.44	CDKN2D -2.89	CLPTM1L 8.36	CYP1A1 19.96	CYP1A2 -2.34	CYP2B6 -2078.59	CYP2C19 1.57	CYP2C8 -1.63	CYP2C9 9.35	CYP2D6 -34.57	CYP2E1 -22.85	CYP3A4 93.46
D	CYP3A5 26.34	DHFR 808.5.7	EGFR 2.30	ELK1 -1.53	EPHX1 -603.11	ERBB2 -479.50	ERBB3 1.12	ERBB4 262.81	ERCC3 -1.06	ESR1 631.18	ESR2 -234.65	FGF2 -216.74
E	FOS 61.33	GSK3A -10036.6	GSTP1 -88354.2	HIF1A 68.87	IGF1R -29.99	IGF2R 5199.79	MET -1.64	MSH2 -102.52	MVP 225.08	MYC -96.65	NAT2	NFKB1 4.56
F	NFKB2 -133.72	NFKBIB -153.37	NFKBIE 10.63	PPARA 45.74	PPARD -12.97	PPARG -2.00	RARA 25.59	RARB -3.96	RARG -25.47	RB1 -1.66	RELB 1.27	RXRA -125.49
G	RXRB 24.30	SOD1 1.21	SULT1E1 728.59	TNFRSF11A 268.09	TOP1 -5.13	TOP2A 1.20	TOP2B -199.89	TP53 -1784.5	TPMT -1799.4	UGCG -47.93	XPA -98.10	XPC -7.23

As shown in Figure 20 and Table 7, estrogen treatment of MCF-7 cells resulted in up-regulation of approximately half of the genes (49%). The genes are involved mostly in cell proliferation and our results confirm what has been reported in the literature regarding estrogen regulation of cellular genes in MCF-7 cells [53, 54].

In contrast to the results obtained with estrogen treatment of MCF-7 cells, as shown in Figure 21 and Table 8, treatment of BCBL-1 cells resulted in the up-regulation of very few genes (3%) and instead resulted in down-regulation of approximately 58% of the tested genes. These results demonstrate that estrogen is differentially modulating cellular transcription depending on the estrogen receptor expressed and that in BCBl-1 cells, most cellular transcription (within the estrogen signaling pathway) is repressed.

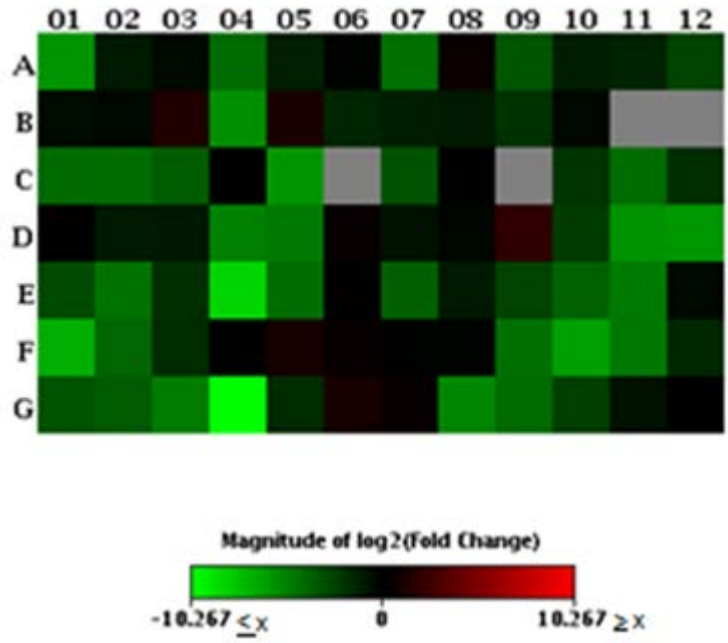


Figure 21. Heat Map of BCBL-1 Cells Genes after Estrogen Treatment Compared to Non-treated Cells. *BCBL-1 cells were grown overnight in phenol red free RPMI, treated the next with estrogen for 4 hours, aliquots of cells were taken out, RNA was isolated, cDNA was made and RT-PCR using estrogen signaling array plates were used. The genes represented in each square are shown in Table 8.*

Table 8. Representation of the Different Expression Values from the BCBL-1 Cells' Genes. *Each square represents a single well in the 96 well plate. The top line in each square is the abbreviation of the gene being amplified while the second line represents the fold increase or decrease in expression over that seen in untreated cells.*

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	AR -65.84	BAD -2.12	BAG1 -1.29	BCL2 -20.20	BCL2L2 -2.38	C3 -1.14	CCNA1 -22.73	CCNA2 1.30	CCND1 -10.92	CCNE1 -2.30	CD44 -2.73	CDH1 -6.61
B	CDKN1A -1.39	CDKN1B -1.23	CDKN2A 2.49	CLDN7 -56.43	CLU 2.04	COL6A1 -2.92	CTNNB1 -2.26	CTSB -2.11	CTSD -3.93	CYP19A1 -1.23	DLC1 -18.93	EGFR -18.93
C	ERBB2 -18.90	ESR1 -20.66	ESR2 -12.80	FAS 1.00	FASLG -66.02	FGF1 -18.93	FLRT1 -9.69	FOSL1 -1.081	GABRP -18.93	GATA3 -4.21	GNAS -20.02	GSN -3.66
D	HMGB1 1.03	HSPB1 -2.07	ID2 -1.91	IGFBP2 -38.03	ILR2A -30.53	IL6 1.24	IL6R -1.66	IL6ST -1.19	ITGA6 3.63	IGTB4 -5.47	JUN -62.79	KIT -70.94
E	KLF5 -8.31	KLK5 -23.99	KRT18 -3.91	KRT19 -385.83	MAP2K7 -20.64	MK167 1.05	MT3 -14.44	MUC1 -2.11	NFYB -6.80	NGF -15.76	NGFR -30.62	NME1 -1.30
F	PAPPA -138.75	PGR -16.95	PLAU -3.37	PTEN -1.07	PTGS2 1.97	RAC2 1.22	RPL27 -1.09	SCGB1D2 -1.12	SCGB2A1 -22.19	SERPINA3 -88.74	SERPINB5 -28.16	SERPINE1 -3.11
G	SLC7A5 -10.21	SPRR1B -12.72	STC2 -32.28	TFF1 -1231.88	TGFA -3.47	THBS1 1.931	THBS2 1.27	TIE1 -45.06	TNFAIP2 -20.23	TOP2A -5.99	TP53 -1.68	VEGFA -1.03

4.2.3 Summary of Aim 2

The hypothesis that estrogen modulates viral and cellular transcription was tested in this Aim. From the RT-PCR experiments on a subset of viral genes (after treatment of BCBL-1 with estrogen in cells reactivated by TPA or doxycycline), estrogen increased some genes while suppressing the transcription of others. Notably, estrogen treatment down-regulated transcription of ORF50 (the HHV-8 lytic switch gene) and up-regulated transcription of K8 (K-bZip; a repressor of ORF50), suggesting that estrogen is involved in maintaining viral latency.

From the estrogen signaling arrays, analyzing the effect of estrogen treatment on cellular transcription in MCF-7 cells (express the alpha estrogen receptor) and the BCBL-1 cells (expressing the estrogen beta receptor), estrogen treatment resulted in down-regulation of the majority of the tested genes in BCBL-1 cells compared to up-regulation of approximately half of the tested genes in MCF-7 cells. This experiment highlighted the difference in estrogen modulation of cellular transcription between cells expressing the alpha versus beta estrogen receptor.

5.0 DISCUSSION

In 1994, HHV-8 virus was identified in a KS lesion from an AIDS patient [2]. The biology and the pathology of the virus is still not well understood. The virus is known to be the etiological agent of all forms of KS [2] non-Hodgkin's pleural effusion lymphoma [15] and some cases of multicentric Castleman's disease. The gender difference in development of KS is glaring. In fact, classical KS is mostly almost exclusively seen in older Mediterranean men [7]. Endemic KS affects adults and children but there are reports that have shown evidence of a higher number of men with KS compared to women, with reported sex ratios of 15 men versus 1 female [9]. AIDS-related KS is also seen mainly in men [14] even though females get infected with HHV-8 as well. The development of KS related to gender difference has not been studied; a sex hormone could explain this difference. A previous study from our lab had identified several estrogen response elements in the HHV-8 genome with a locus in an area containing several viral regulatory genes. Transients expression assays, EMSAs and cold competition assays were performed to show that the viral estrogen response elements could bind specifically to estrogen receptors. These previous investigations were performed in the MCF-7 cell line which is known to express the estrogen alpha receptor. The present study is the first to analyze the effects of estrogen on the HHV-8 genome in vivo and to investigate the role of estrogen in viral reactivation, and viral and cellular gene transcription in the BCBL-1 cell line. It is important to

determine why women do not develop KS compared to men. Understanding this gender difference may lead to improved therapies designed to treat or prevent the development of KS.

To measure the ability of estrogen to induce viral reactivation, BCBL-1 or TRex-BCBL-1 cells were treated with estrogen, TPA or doxycycline and analyzed for ORF59 expression by Flow cytometry. The results demonstrated that estrogen did not cause viral reactivation. In addition, estrogen treatment also did not prevent viral reaction in BCBL-1 or TRex-BCBL-1 cells treated with TPA or doxycycline. The cells stained for ORF59 expression and cells expressing the protein were measured by Flow cytometry. The intensity cells expressing ORF59 was similar to TRex-BCBL-1 cells treated with TPA alone or with doxycycline alone. These results suggest that estrogen did not prevent TPA and doxycycline induced viral reactivation in TRex-BCBL-1 cells. They also suggest that the number of cells undergoing viral reactivation did not change. The Flow cytometry technique does not permit determination of infectious virus levels. Therefore, in order to quantify the amount of infectious virus produced during the different treatments; a TCID50 assay developed in our laboratory was used. BCBL-1 cells treated with estrogen alone or treated with estrogen and TPA were analyzed for production of infectious virus. The cells treated with estrogen and TPA had a higher TCID50 than cells treated with estrogen or TPA alone, implying that the viral replication was increased in cells treated with estrogen and reactivated by TPA. These results corroborate RT-PCR data on a subset of viral genes that have potential EREs near their promoters. In fact, we observed an estrogen-induced increase viral gene transcription. Transcription of a subset of viral genes was increased in TRex-BCBL-1 cells pretreated with estrogen followed by treatment with either TPA or doxycycline compared to cells treated with estrogen, TPA, or doxycycline alone. These studies were done using 1 micromolar concentration 17 beta-estradiol. We also showed that

among spontaneously reactivating BCBL-1 cells treated with estrogen using concentrations of 100 and 10 nanomolar, ORF50 expression (encoding the lytic switch protein RTA [24, 25]) was down-regulated compared to expression of K8, a repressor of ORF50 expression. There were other viral genes up-regulated by estrogen treatment, but they were not part of the viral regulatory genes. The lower concentrations of estrogen (100 and 10 nanomolar) were able to increase transcription of a subset of viral genes compared to 1 micromolar concentration. Previous studies by other laboratories have also shown that lower levels of estrogen could show an inverse correlation to gene transcription compared higher concentrations [49].

Herpesviruses are uniquely destructive of their host cell. Most herpes viruses, as a result of viral replication, kill the infected cell within 12-15 hrs post-infection. At the same time, all herpesviruses are capable of establishing latent infections. Latency is represented by infection without the production of infectious virus. Some herpesviruses, like HHV-8, express a small subset of viral genes during latency. Since a herpesvirus productive infection quickly results in host cell death and at the same time, the virus is capable of establishing latency, the decision to either initiate a productive infection or enter into latency must be made very early following infection. It has been reported for example, that with the herpes simplex virus in infecting neuron cells a decision between productive versus latent infection is made early after infection due to the cellular environment [55]. A recent study from our laboratory (unpublished data) has found that almost all HHV-8 genes were expressed between 12 and 15 hrs following infection or reactivation. Thus there may be cellular pressure exerted on the virus to both establish and maintain latency as a form of cell survival. The results presented in this study support the notion that estrogen is involved in maintaining viral latency by suppressing expression of ORF50 and

increasing transcription of K8. The role of estrogen in development of latency was not addressed in this study.

Part of the current study was to assess the effects of estrogen on cellular transcription in two cell types representing the two estrogen receptors, alpha and beta. HHV-8 is known to infect epithelial, endothelial and B cells in vivo [15, 56]. The latter was shown by our laboratory to express the beta receptor (unpublished data). For this experiment MCF-7 cells a breast cancer cell line of epithelial cell origin was used; it was also shown by a previous work in our laboratory to express the alpha receptor confirming what other studies have reported [52]. A RT-PCR array measuring transcription of several genes involved in estrogen signaling was used to compare RNA levels expressed in MCF-7 cells and BCBL-1 cells treated with 10 nanomolar estrogen to untreated controls. In MCF-7 cells, most of the cellular genes related with cell proliferation in this assay were up-regulated, in support with other studies reported [53, 54]. Estrogen signaling in B cells is poorly studied and as such the results from this study are one of the first to analyze the effects of estrogen on B cell signaling. The RT-PCR signaling array data using RNA from estrogen treated BCBL-1 cells showed that unlike MCF7 cells, a majority of the tested genes were down-regulated. These results imply that the estrogen receptor beta expressed in BCBL-1 cells act as a cellular gene repressor while the alpha receptor acts as an activator of cellular genes as seen in many studies showing the difference of signaling between both receptors [29, 33]. Endothelial cells have been reported to express estrogen receptors [57, 58].The MCF-7 cells and BCBL-1 treatment with estrogen's result could apply to the endothelial cells infected with HHV-8, prone to become spindle cells [59].

5.1 POTENTIAL MODEL OF ESTROGEN'S EFFECT ON HHV-8

The results from this study suggest that estrogen has a role in modulating HHV-8 transcription and in controlling the cellular microenvironment. Based on these results, the model shown in Figure 22 is proposed to explain why women are at a reduced risk of developing KS.

Upon primary infection of HHV-8 in B cells a decision is made to enter either a productive, lytic infection or to establish a latent infection. If the infection is productive, in women with high levels of estrogen, the hormone will increase the viral genes' transcription leading to higher viral titers. We hypothesize that as a result of the stronger estrogen-induced lytic replication, women would mount a stronger immune response (both humoral and cellular). This stronger immune response would help control viral replication by suppressing reactivation and as a result, reduce their risk of developing KS. In a productive viral infection in men, the low levels of estrogen would result in viral replication (compared to women) resulting in a lowered immune response. This blunted immune response would result in an increased risk of viral reactivation, therefore increasing their risk of developing KS. During a latent HHV-8 infection in B cells, higher estrogen levels in women would help suppress reactivation, lowering the risk of KS development, while in men lower levels of estrogen increase the risk of viral reactivation and therefore the development of KS. This model is supported in part by previous studies demonstrating that onset of KS is preceded by increased viral load in the blood, indicative of viral reactivation and previous studies from our laboratory demonstrating that during a primary HHV-8 infection among men, the immune response (both humoral and cellular) is less than robust [13].

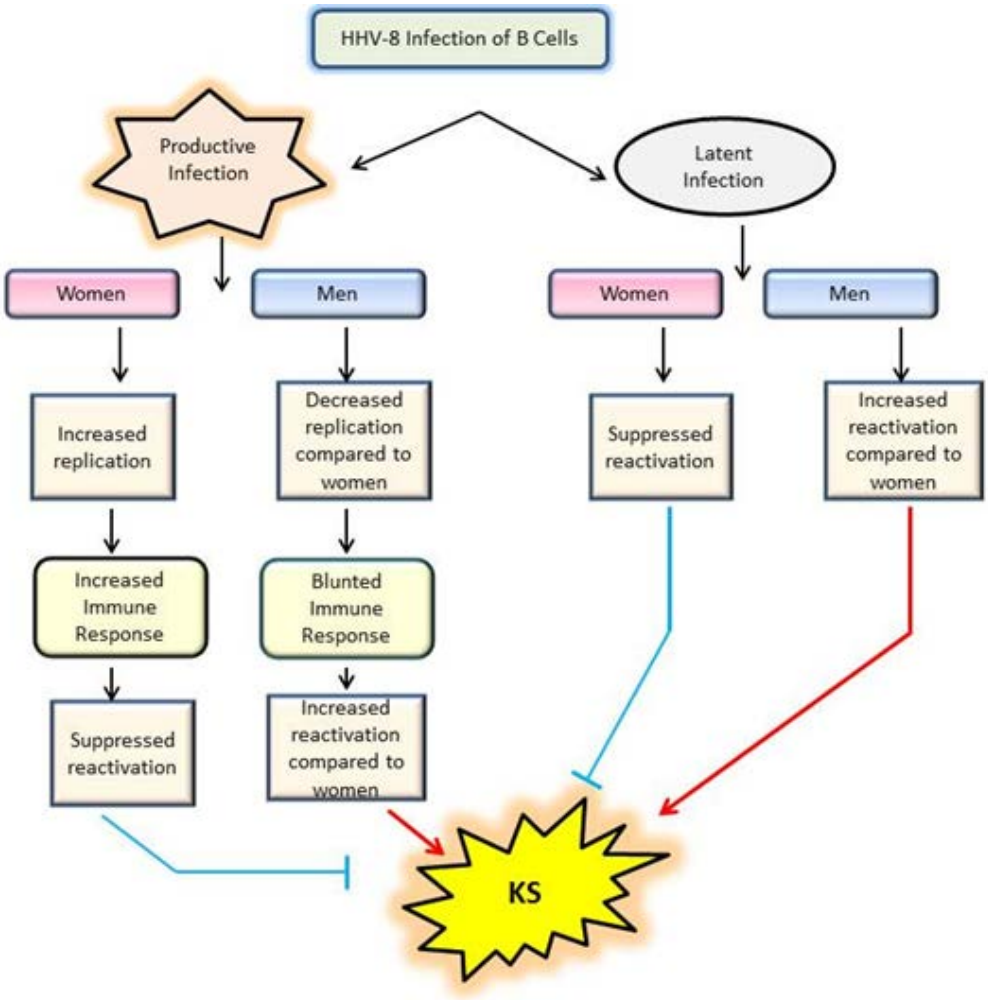


Figure 22. Diagram of the Proposed Model of Estrogen's Effect on Kaposi's Sarcoma Development.

5.2 PUBLIC HEALTH SIGNIFICANCE

The role of sex hormones in development of KS has not been investigated in the context of controlling cellular or viral transcription. The results of the present study are important as they help explain how estrogen could modulate viral and cellular transcription and protect women from developing KS. This study will hopefully pave the way for future studies on how

estrogen modulates the full viral replication program and also yield therapeutic targets to help understand the pathology and prevent KS development.

6.0 FUTURE DIRECTIONS

Upcoming studies should include the screening of the whole HHV-8 genome for estrogen receptor binding sites through chromatin immunoprecipitation assays. This screening would help map out all the EREs possible in the genome with proof of estrogen receptor binding and confirm the computational analysis results.

The infection of activated B cells with HHV-8 in presence or absence of estrogen should be performed, to measure the production of infectious virus consecutive to the infection using the TCID50 assay method. RT-PCR using RNA from the infected activated B cells, on a subset of viral genes, to see how they compare to the BCBL-1 naturally reactivating treated with estrogen. Additionally, a study matched cases of men and women comparing their humoral and cytotoxic-T lymphocytes responses regarding HHV-8 infection should be performed.

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