IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF ESTROGEN RESPONSE ELEMENTS IN THE HUMAN HERPESVIRUS 8 GENOME

by

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Interaction between viral infection and host genetic susceptibility has increasingly become recognized as an important factor in the etiology of human cancer. Epidemiologic studies suggest that prostate cancer is a complex disease involving host genetic factors and environmental exposures that modify risk. Here we report a novel interaction between infection with human herpesvirus 8 (HHV8) and the human estrogen receptor alpha XbaI polymorphism which is associated with an increased risk of prostate cancer (p=0.032; OR=3.10 95%CI (1.42-6.77)) in an Afrocaribbean population from Tobago. HHV-8 is the causative agent in Kaposi's sarcoma (KS). Despite similar HHV-8 seroprevalences, KS lesions are much less common in females, suggesting that sex hormones influence KS pathology. The estrogen receptor (ER) is a ligand-dependant transcription factor that mediates the genomic effects of hormone signaling. Because of the suggested role of sex hormones in KS development and prostate cancer risk, we hypothesized that some HHV-8 genes might be activated by ER. We computationally scanned the HHV-8 genome for estrogen response elements (EREs). Our analysis identified high scoring EREs in the promoter regions of several genes in the HHV-8 genome, including the regulatory gene K8. Binding of ERs to HHV-8 EREs was confirmed by electrophoretic mobility shift assay and ELISA. To demonstrate that HHV-8 EREs were functional, promoter regions were cloned into reporter plasmids and luciferase expression measured with and without estradiol. Our results demonstrated increased reporter transcription in response to estrogens in cells expressing

ERα. Further, we analyzed other gammaherpesviruses and identified several conserved EREs, including high-scoring EREs in the promoter of the EBV homolog of K8. These results indicate that estrogen may influence transcription from the HHV-8 genome, outside of the normal viral transcription pathway. Our findings may help to explain the differential risk of KS and could represent an important regulatory pathway in other gammaherpesviruses. Prostate cancer and KS present a tremendous burden on public health, not only in the US, but worldwide. Insights into the pathogenesis of these diseases may help us to understand their basic biology and provide potential screening and therapeutic targets

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PREFACE

This dissertation is dedicated to my parents for inspiring me to always be curious about the world around me and to my wife and son for giving me a reason to never give up on my dreams.

1.0 INTRODUCTION

Virtually all organisms on Earth are subject to the constant threat of pathogen infection and have been locked in a figurative arms race with pathogens for millennia. Many of these pathogens spend the majority of their lifecycle inside the host where they are constantly interacting with the host microenvironment, whether in the form of evading host defense mechanisms, pirating genes, or utilizing host cellular processes to accomplish their own goals. As we begin to understand more about the interplay between host and pathogen, it becomes clear that these interactions occur on multiple levels, from simple protein-protein interactions, to the manipulation of gene expression, to the very shaping of the host's genome through selective forces. Genetic variation that influences these integrated biochemical and signaling pathways is likely to contribute to individual differences in disease phenotypes associated with pathogen infection. In order to understand human disease phenotypes resulting from pathogens, we must unravel these complex relationships.

Prostate cancer is a prototypical complex disease, with a variety of interacting factors, including both genetic and environmental causes. Here we describe the identification of a novel interaction between a genetic variant in the human estrogen receptor alpha gene that when present in men from Tobago infected with human herpesvirus 8 (HHV8), significantly increases the risk of prostate cancer. We further

attempt to characterize this novel interaction and in doing so potentially provide insight into the etiology of Kaposi's sarcoma, another disease associated with HHV8 infection.

1.1 TOBAGO POPULATION

Tobago is a small Caribbean island located approximately 11 miles off the coast of Venezuela. Tobago, along with its sister island Trinidad, comprise the nation of Trinidad and Tobago. A national census conducted in 2000 estimated the population of Tobago to be 54,084 (1). Despite their relatively close proximity, Trinidad and Tobago have very distinct population ancestries, largely due to their unique histories as slave trading colonies. Recent estimates of genetic ancestry have found the Tobago population to be of approximately 94.0% African, 4.6% European and 1.4% Native American origin (2). This fairly homogeneous composition makes the Tobago population an ideal population for genetic studies of disease that may otherwise suffer from confounding due to underlying genetic admixture and population substructure. Tobago is also an interesting population for scientific study because it has one of the highest rates of prostate cancer in the world, with a screening-detected prevalence of 14 per 100 men, ages 50-79, screened (3).

1.2 PROSTATE CANCER

1.2.1 Prostate cancer background

Prostate cancer is the most commonly diagnosed non-skin related cancer among men living in the United States and has the second highest mortality rate of all cancers (4). Therefore prostate cancer represents a tremendous burden on public health in the United States as well as worldwide.

Prostate cancer is considered to be a complex disorder with multiple causative factors, including both genetic and environmental influences. Familial aggregation of prostate cancer was first reported by Morganti et al who observed that prostate cancer patients reported a higher family history of prostate cancer than did patients hospitalized with other disorders (5). Additional formal family studies of men diagnosed with prostate cancer confirmed these observations and found an elevated risk among related family members (6,7). Further, studies of twins have shown that identical twins have an increased level of concordance than fraternal twins or other siblings, suggesting that heritable genetic predisposing factors contribute to prostate cancer (8,9). Estimates of heritability indicate that approximately 40% of prostate cancer risk can be attributed to these genes (8,10,11).

Prostate cancer also displays significant variability in incidence depending on racial ancestry, with African Americans at almost twice the risk of Caucasians, while prostate cancer is less common in Asians and Asian Americans (Table 1).

Table 1. SEER Prostate Cancer Statistics 2000-2004

	Incidence	*Mortality*
US Total	*168*	*27.9*
Blacks	*255.5*	*62.3*
Whites	*161.4*	*25.6*
Asians/Pacific Islanders	*96.5*	*11.3*

Incidence and mortality statistics are shown per 100,000 individuals

Differing environmental factors may contribute to risk, as illustrated by the increased risk in prostate cancer in Asians who immigrate to the US. However the incidence of prostate cancer in these immigrants remains similar to that of their ancestral homeland and never reaches levels observed in other groups, suggesting the existence of shared racial genetic predisposition factors (12).

1.2.2 Role of hormones in prostate cancer

The growth and development of the prostate gland has been found to be heavily influenced by steroid hormones. The role of hormones in the prostate was first noted when it was observed that castration of males had a beneficial effect on prostate cancer (13). Androgens have long been known to control proliferation of prostate tissue and androgen deprivation continues to be one of the primary treatments for cancer of the prostate. Androgen deprivation results in a significant atrophy of the prostate gland, primarily affecting the epithelial cells of the prostate lumen (14). Despite this clear

influence on prostate growth, serum levels of androgens does not appear to be associated with prostate cancer risk (15).

More recently, estrogens have been shown to have an important influence on the early developmental morphology of the prostate. In animal models, proper levels of circulating estrogens have been found to be important for gland development. Mice exposed to abnormally high levels of estrogens during prostate gland development were observed to have malformations of the prostate (16) and were predisposed to precancerous lesions in the prostate (17). Prenatal exposure to estrogens has also been reported to result in an effect known as "imprinting" in which the prostate becomes sensitized to estrogens and shows reduced sensitivity to androgens (18). This developmental estrogenization showed a distinct lobe-specific effect on androgen receptor expression (18). Despite these findings, numerous studies have failed to find any correlation between circulating hormone levels in men and risk of prostate cancer (19).

1.2.3 Estrogens

1.2.3.1 Role of estrogens

Despite the general notion that estrogens are a female sex-hormone, both men and women have detectable levels of estrogens in their circulation. Estrogens are biosynthesized from cholesterol in a process known as steroidogenesis. In this process, cholesterol is metabolized into androgenic compounds which then undergo aromatization into estrogens. The three most significant of these estrogenic compounds are estrone, estradiol, and estriol (figure 1).

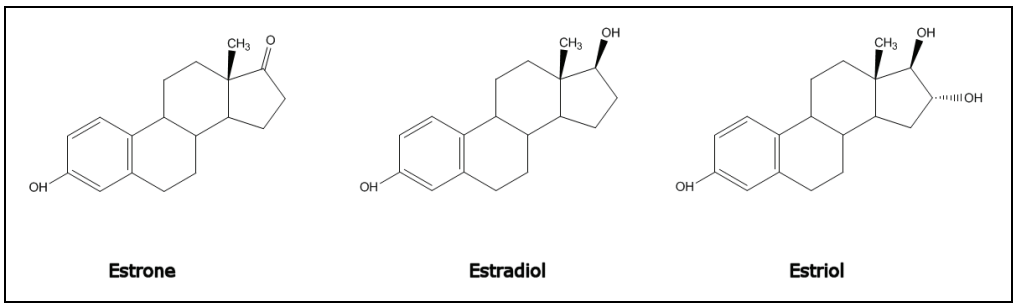

Figure 1. Structure of the Major Biological Estrogens

Derived from Jordan et al (20)

Estradiol can be directly synthesized through the aromatization of testosterone or through an indirect pathway in which androstenedione is first aromatized to estrone which is then converted to estradiol by 17β-hydroxysteroid dehydrogenase. Estriol is synthesized by the direct metabolism of estradiol or by conversion of estrone to hydroxyestrone which is then converted to estriol (21).

Estradiol is the primary estrogenic compound in premenopausal women and is the most potent estrogen of the major estrogens synthesized by the body (22). After menopause the ratio of estrogens changes dramatically and estrone predominates. In men, estrogens are created through aromatization of androgens in peripheral tissue and in the testes (23). Total serum levels of estradiol and estrone increase as men age due to increasing rates of aromatization (24), with a corresponding decline in testosterone level (23). This results in a marked change in the androgen:estrogen ratio. Further, levels of estrogen are known to increase within the prostate gland as men age (25).

1.2.3.2 Actions of estrogens

Estrogens carry out their biological effects through three primary mechanisms. Each of these mechanisms utilizes a protein receptor molecule called the estrogen receptor which specifically recognizes and binds estrogenic compounds. In the first mechanism, estrogens bind to estrogen receptors located in the nucleus which undergo a conformational change which allows them to bind to specific recognition sequences in the genome (26). The bound estrogen receptor then activates nearby proteins involved in RNA transcription or recruits other activator or repressor proteins to the transcription start site. An alternate form of this mechanism, called tethering, involves an indirect binding to DNA wherein the estrogen receptor is bound by protein-protein interaction to another transcription factor that is directly bound to a recognition site. The estrogen receptor has been observed to tether to SP1 and AP1 transcription factors in the promoters of certain estrogen-responsive genes that do not contain estrogen receptor binding sequences (27-29). The third method of estrogen signaling involves a splice variant of the estrogen receptor which localizes to the cell membrane. This method of signaling is much more rapid and involves signaling through the nitric oxide synthase pathway (30).

1.2.3.3 Estrogen receptors

There are two forms of the estrogen receptor transcribed from distinct loci on chromosome 6 and chromosome 14, known as the estrogen receptor α (ESR1) and β (ESR2), respectively. These two receptors share significant homology and appear to have arisen from an ancestral duplication event based on gene synteny in these regions (31). Both receptors have a similar structural organization, including an N-terminal

activation function (AF-1), DNA binding zinc-finger region, hinge region, ligand binding domain, and C-terminal activation function region (AF-2) (32). The AF-2 region is the domain responsible carrying out transcriptional activation in response to estrogens, while the AF-1 region provides a low-level of transcriptional activation that is ligand-independent (32).

1.2.3.4 Ligand binding of estrogen receptors

The estrogen receptors bind a variety of estrogens and estrogen-like molecules with varying affinity. The two receptors can also have differential responses to estrogens as well, with some compounds acting as transcriptional activators when bound to one receptor, but acting as an antagonist when bound to the other. To further complicate matters, local tissue-specific co-regulators, such as members of the SRC family, SMRT, and the TRAP/DRIP protein complex (26), can also modulate the effect of a receptor (33). Binding of estrogens to the receptor occurs at the ligand-binding domain and results in a conformation change in the structure of the estrogen receptor protein (34). This conformational change influences how amino acid residues in the AF-2 region interact with nearby transcription factors and parts of the general transcription machinery (33). Compounds which have an antagonisitic effect of the receptor can also act to inhibit dimerization of the receptor and can influence receptor half-life by causing the receptor to be targeted for ubiquitination and proteasomal degradation (35,36).

1.2.3.5 Estrogen receptor transcription

Transcription of both estrogen receptors is complex process, with each receptor having multiple transcription start sites in the 5'-region. The promoter region of the alpha

receptor has at least 8 different transcription start sites (37). Transcription from these various start sites results in mRNA species with differing 5'-untranslated sequences with over 9 reported untranslated exons (37). Similarly, the ERbeta 5' region includes at least 7 untranslated exons (38) (Figure 2)

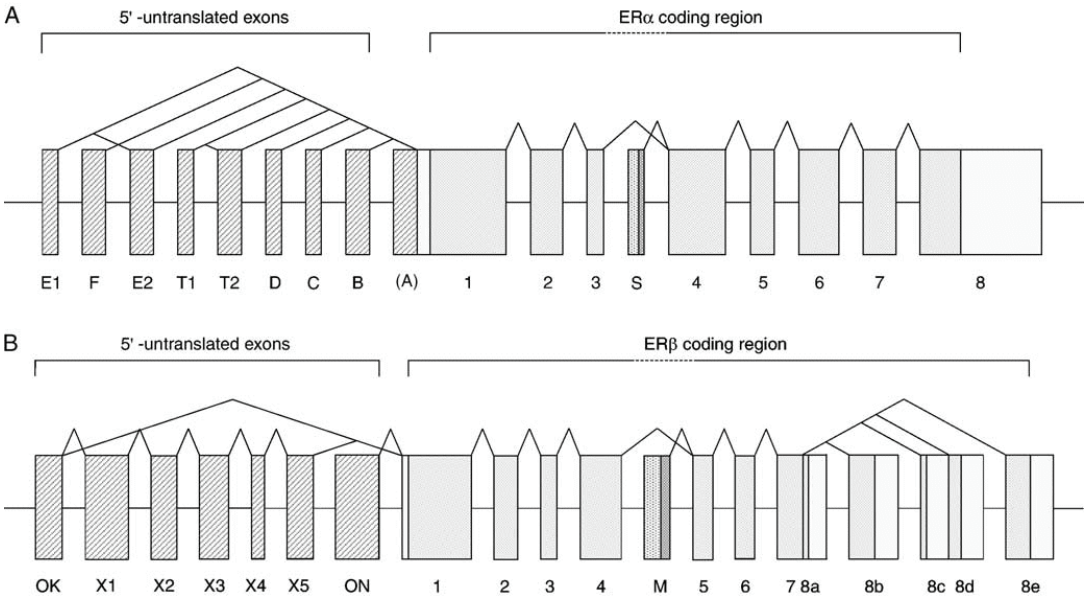

Figure 2. Map of the Estrogen Receptor Coding and Non-Coding Exons

Reprinted with permissions from Hirata et al (38)

The exact function of these untranslated exons is unknown, however they have been hypothesized to be involved in sub-cellular localization and have been reported to play a role in tissue specific expression (39). Although the estrogen receptor mRNA species are exceptionally variable, the translated receptor proteins have a high degree of similarity (37). Several isoforms of each receptor protein have been identified. At least 2 ERalpha proteins have been observed, including the full length 66kDa wildtype protein and a 46kDa receptor protein lacking 173 amino acids from the N-terminus. The 46kDa

protein originates from an alternatively spliced mRNA lacking the first coding exon (1A) with translation occurring from an internal ATG start codon (40). This receptor lacks the AF-1 domain and acts as a competitive inhibitor of AF-1 function of the wildtype 66kDa receptor. However, the truncated receptor is still capable of binding ligands and can activate transcription from an estrogen-responsive promoter (40). In endothelial cells, the 46kDa ERalpha has been shown to localize at the plasma membrane where it plays a role in rapid non-genomic action of estrogens involving the eNOS pathway (30).

The beta estrogen receptor is also expressed as several isoforms. The full length receptor is the most common form, however 2 variants exist, named ERbeta-cx and ERbeta-ins (also known as ERB2). The ERbeta-cx isoform is expressed as a mRNA containing an alternative 3' exon which subsitutes the final 61 amino acids of the receptor, including portions of the ligand binding domain, with 26 unique amino acids (41). The ERbeta-cx variant does not appear to bind estrogen ligands and acts as an inhibitor of wildtype ERalpha and ERbeta (41). The ERbeta-ins isoform is derived from an alternatively spliced ERbeta transcript which contains an 18 amino acid insertion located in the ligand binding domain (42). ERbeta-ins was found to be capable of binding estrogen ligands, however it was shown to have a 35-fold lower affinity for estradiol (42). ERbeta-ins acts similarly as an inhibitor of the wildtype receptor isforms (43).

1.2.3.6 Tissue-specific expression of estrogen receptors

The estrogen receptors have distinct profiles of tissue-specific expression, which can vary greatly even within a single organ. To some degree this variable expression can be traced to the different mode of action of the receptor within tissue where it is expressed. An

example of this is the expression of the membrane-bound form of the alpha estrogen receptor in vascular endothelial cells which is responsible for the rapid signal transduction via the nitric oxide pathway in response to circulating estrogens (30). Another example of the tissue-specific expression of the estrogen receptors can be seen in the prostate gland. The glandular epithelial cells which line the ducts of the prostate primarily express the beta estrogen receptor, while the prostatic stromal cells mainly express the alpha receptor (44,45).

1.2.3.7 DNA binding

It was known for some time that action of estrogens was carried out by binding to estrogen receptors. However, it wasn't recognized for some time that the estrogen receptors acted by binding to specific DNA regulatory elements, until regions of DNA that bound estrogen receptor were discovered upstream of estrogen-responsive genes. Jost et al initially identified a segment of the chicken vitellogenin II gene which was able to bind estrogen receptor *in vitro* (46). After the identification of several other regions capable of binding estrogen receptor and further fine mapping, a general consensus sequence was assembled from conserved regions in each of these sequences (47-49). This region was found to consist of a 13bp palindromic sequence of GGTCA-NNN-TGACC composed of two 5bp half sites separated by a 3bp linker (47). This regulatory element, known as an estrogen response element (ERE), was found to act as an enhancer which had the ability to have an effect on gene expression even when located up to 2.6kb from the transcription start site (TSS) (47). Functional ERE sequences have been shown to be present in front of the TSS as well as inside coding regions and in 3' regions of estrogen-responsive genes (50).

1.2.3.8 Computational modeling of estrogen response elements

The ERE consensus sequence has been shown to be able to tolerate some degree of variability and certain positions in the palindrome have been shown to be more constrained than others. As little as a single change can abolish DNA binding, however up to 2 deviations from the standard ERE consensus have been shown to be able to bind estrogen receptor and increase gene expression (51). Any changes in the ERE are better tolerated if they are present in only one of the two half sites (52). Deviations from the ERE consensus sequence can also be rescued by the presence of a purine immediately flanking the half site (51). Additionally, the presence of AT-rich flanking sequence can increase how potent the response element can modify transcription (52). However, it has been shown that the binding affinity of the receptor to an ERE does not linearly predict how well the bound receptor will modify transcriptional activity (53) and most estrogen responsive genes identified in the human genome do not have perfect ERE consensus sequences (54).

The estrogen receptor protein has been shown to form a stable dimer which binds to the ERE sequences with each receptor monomer interacting with one of the ERE half-sites (55). This DNA-protein interaction occurs in the major groove of the DNA helix with the receptor protein making specific contacts between amino acids in the receptor zinc-finger and the exposed functional groups of the nucleotide bases (53) and both the alpha and beta estrogen receptors appear to interact with the same nucleotides of the ERE (56). The presences of multiple ERE sequences upstream of a gene have been shown to have the ability to act in a synergistic manner and have been called estrogen response units or ERUs (57).

The extensive characterization and limited variability of the ERE sequence makes it well suited for computational modeling which can then be used to predict ERE sites in a novel DNA sequence. Initially, modeling of the ERE sequence was done using IUPAC representation of nucleotide bases and has progressed to more robust computational methods. Currently several computational methods are used to identify ERE sequences with 2 being the most commonly used. The first method involves the use of a position-specific scoring matrix (PSSM) to represent the probability of a particular nucleotide at each position in the ERE. The PSSM is constructed by aligning a group of DNA sequences which are known to act as classical EREs. The number of occurrences of a particular nucleotide base at each position are then totaled and entered into a scoring matrix. These counts are then converted to probability values. Searching for ERE sequences within a novel sequence is then done by scanning along the sequence using a sliding window the size of the ERE sequence. For each iteration, the sequence within the window is then compared to the probability matrix and scored based on its similarity. The second methodology for modeling ERE sequences utilizes a decision tree to separate ERE-like sequences into two categories using two PSSMs. The first PSSM is constructed using sequences present in front of genes shown to respond to estradiol and the anti-estrogen faslodex (50). This list was further refined by experimentally validating that these sequences bound ER protein using a microarray-based chromatin immunoprecipitation assay (ChIP-on-chip) (50). The second PSSM represents ERE-like sequences which were not found to bind ER protein or "non-binding" sequences. one representing sequences experimentally validated to bind ER protein and modify transcription (50). A third method of computationally identifying ERE sequences, utilized in the DragonERE program, incorporates the use of a PSSM matrix and an artificial

neural network trained to differentiate between true ER binding sequences and non-binding ERE-like sequences (58).

1.2.3.9 Estrogen receptor variation

Numerous genetic variants have been identified in the estrogen receptor alpha gene, including a large number of non-coding polymorphisms as well as several missense and non-sense mutations (Summary in Table 2).

Table 2. Reported Estrogen Receptor Alpha Genetic Variation

Location	Change	Rs Number
Promoter	TA repeat	rs3138774
Exon 1	S10S	rs2077647
	S77G	rs9340773
	A87A	rs746432
	S118P	n/a
	Q146P	rs17847065
Exon 2	R157X	n/a
	S168T	rs17847076
	T168T	rs9340802
Exon 3	R243R	rs4986934
Exon 4	P325P	rs1801132
	V364E	n/a
Exon 5	L379L	rs17847067
Exon 6	C447A	n/a
Exon 8	K531R	n/a
	R548H	n/a
	A558A	rs9341068
	T563M	n/a

Table 2 Continued

	A571A	rs9341069
	E587E	rs34840398
	T594T	rs2228480
Intron 1	-1505 A/G	rs48070056
	-1415 T/C	rs9322331
	-397 C/T	rs2234693
	-351 A/G	rs9340799
	GGGA repeat	rs10692281

Of these variants, several have been repeatedly associated with human disease. In particular 2 polymorphisms located 397 and 351 bp upstream of exon 2 have been reported to be associated with a number of disorders including breast cancer (59), bone mineral density (60), bone-fracture (61), osteoarthritis (62), heart-attack (63), cardiovascular disease (63), lung function (64), anxiety-related traits (65), and prostate cancer (66). The PvuII polymorphism (rs2234693), a C->T transition located -397 from exon 2, has been shown to introduce a binding site for the c-myb transcription factor which when inserted into a reporter plasmid was shown to be responsive to c-myb expression (67). While the XbaI polymorphism (rs9340799) has been associated with estrogen-related disease, the A->G transition (Figure 3) has not been shown to have any known effect on function.

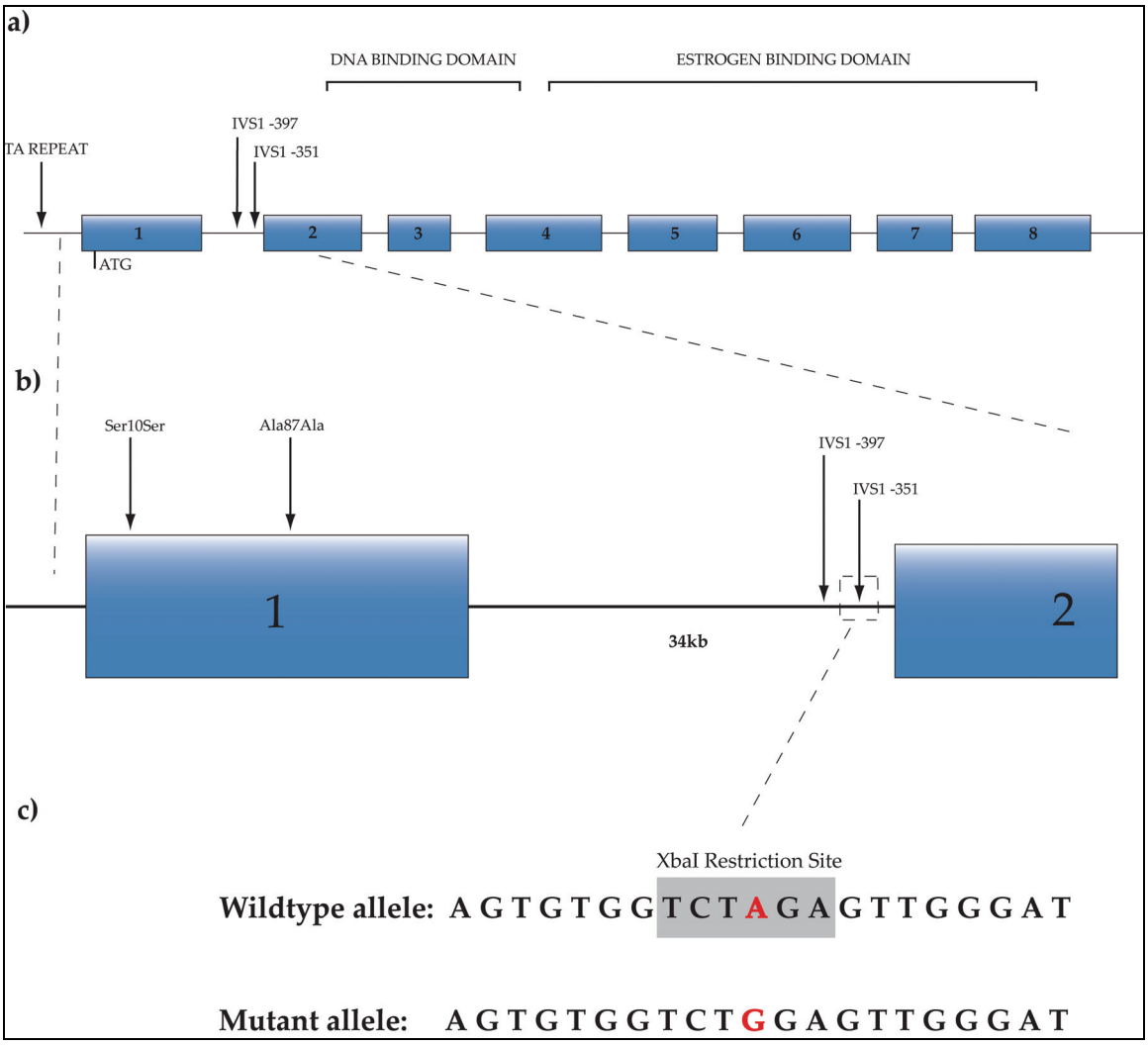

Figure 3. ESR1 Region with XbaI and PvuII Polymorphisms

Adapted from Herrington et al (67)

Variation that influences disease risk has been identified in the estrogen receptor beta as well. A polymorphic CA dinucleotide repeat has been identified in intron 5 of the ESR2 gene and has been associated with several disorders, including systolic blood pressure (68), Alzheimers disease (69), osteoarthritis (70), and risk of hypospadias (71).

1.2.3.10 Linkage disequalibrium in the estrogen receptor alpha gene

The human estrogen receptor alpha gene also displays an extensive amount of linkage disequalibrium (LD) in its 5' region which can be a challenge to attempts to identify causal variation (72). LD is defined as the non-random association of 2 or more alleles in a population (73). LD generally arises when genetic variation occurs in a chromosomal region with limited recombination between the alleles (74). These alleles are then transmitted on the same chromosomal segment until such time has passed as to allow recombination to occur between the alleles. A region of LD has been reported to encompass the region of intron 1 containing the XbaI and PvuII polymorphisms as well the TA dinucleotide repeat in the 5' region (75) In Caucasians, this block of LD extends across a 41kb region of the ESR1 gene while in African populations, this large block of LD is broken down into several smaller fragments (Figure 4). As a result, variants located within this region may be transmitted on a common haplotype background together with the causative allele, result in a spurious association between variants in other parts of the LD block and the disease.

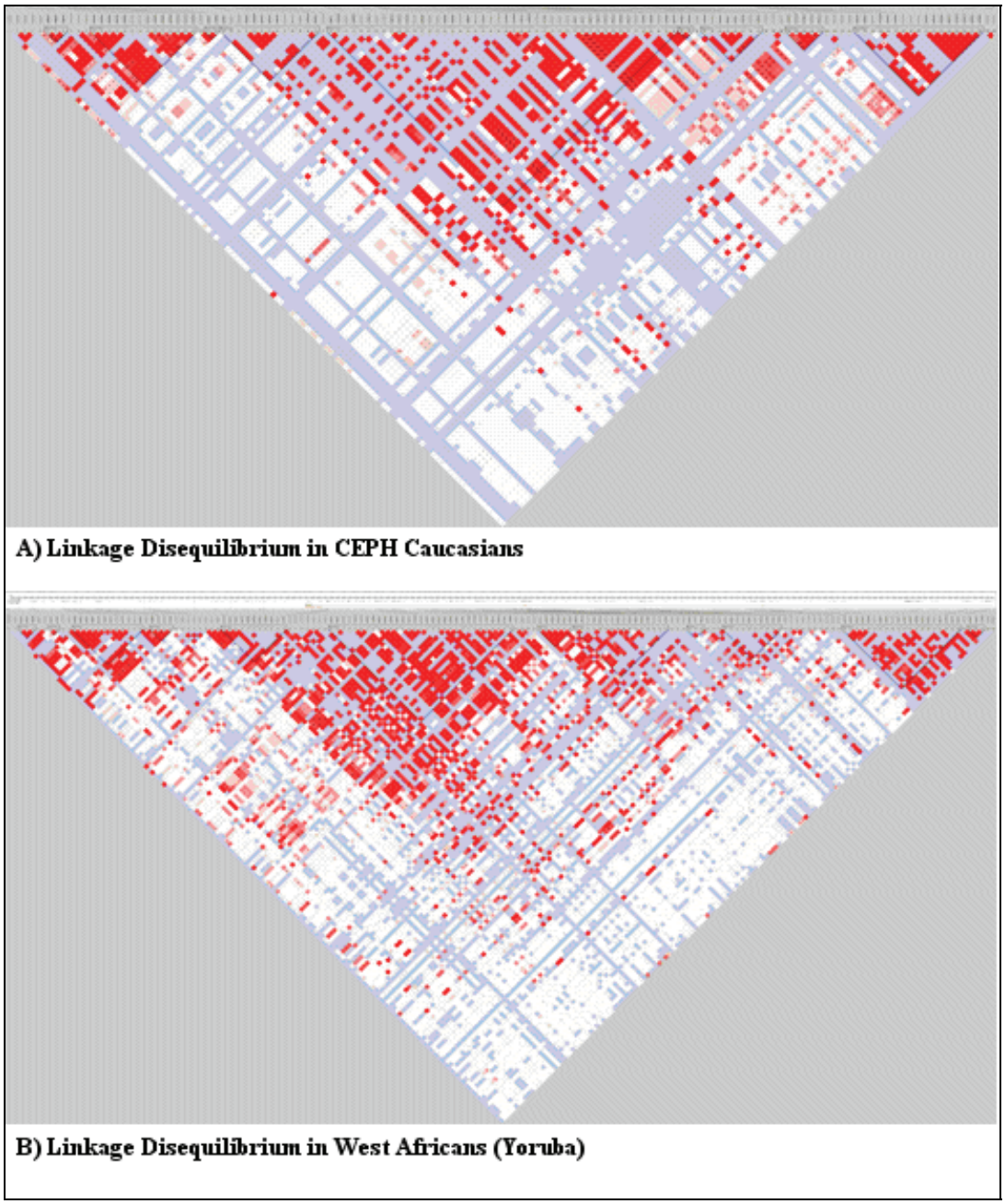

Figure 4. HapMap Plot of Linkage Disequilbrium in ESR1 5'-Region

Linkage disequalibrium for each marker pair is measured using D'. For marker pairs with a log odds of less than 2.0, pairs with D'=1 are indicated in blue, D'>1 in white. For marker pairs with a log odds of 2.0 or greater, pairs with D'=1 are shown in dark red, pairs with D'>1 are indicated in light red. Image generated using the HapMap Project (76) genome browser and HapMap build 36.

1.2.3.11 Viral etiology of prostate cancer

Several epidemiologic studies of prostate cancer have suggested that an infectious agent may contribute to the risk of disease (77-79). Several links between prostate cancer and risky sexual practices have been reported, including associations with several sexually transmitted diseases. Hayes et al reported an increased risk of prostate cancer among men who had a history of gonorrhea or syphilis infection (80). This risk was shown to increase with the lifetime number of infections and was reduced with long-term use of condoms. These results agree with that of Krain which reported that men with prostate cancer have significantly more occurrences of sexually transmitted disease (81). Gonorrhea and syphilis infection are generally regarded as sentinel diseases, therefore associations with these pathogens may simply be a surrogate indicator of other sexually transmitted diseases (80). However, numerous studies have failed to find consistent associations between prostate cancer and other indicators of risky sexual behavior such as lifetime number of sexual partners (80) or HPV infection (82).

Most studies of viral pathogens involved in prostate cancer have failed to consistently identify any particular pathogen associated with an increased risk of disease. However, a recent study of prostate cancer patients who carried two copies of a mutant RNASEL allele found the presence of a novel retrovirus called xenotropic murine leukemia virus (XMRV) in prostate tumor sections (83). While it remains unclear if XMRV contributes directly to prostate cancer risk or is simply present due to reduced viral clearance by the mutant RNASEL enzyme, their finding certainly suggest a relationship between pathogen infection and prostate cancer in genetically susceptible men. Several studies have failed to find any link between herpes simplex infection and prostate cancer (84,85), however, another member of the herpesvirus family has been

implicated in prostate cancer risk. In a study of men from the island of Tobago, Hoffman et al reported that men who showed serological evidence of infection with human herpesvirus 8 (HHV8) had over twice the risk of prostate cancer (86). HHV8 DNA has also been reported to be found in ejaculate of healthy Italian men as well as in urinary tract tissue, including in over 40% of prostate tissue sections (87), suggesting the virus is present in the male urogenital tract. These findings were supported by the PCR identification of HHV8 DNA in the prostate gland of men with Kaposi's sarcoma (KS) (88) Further, Montgomery et al reported finding viral protein expression and increased levels of inflammation in prostate sections of HIV+ men who were seropositive for HHV8 (89). While these studies intriguingly suggest a role for HHV8 in increasing the risk of prostate cancer, several studies have failed to find a serological association between HHV8 and prostate cancer risk (84,90). However, these latter studies were conducted on populations living in diverse areas and the risk of prostate cancer risk may only be associated with HHV8 in genetically susceptible men in areas with high prevalence of HHV8 infection such as Italy, sub-Saharan Africa or Tobago.

1.3 HUMAN HERPESVIRUS 8

1.3.1 Background

Human herpesvirus 8 (HHV8), also known as Kaposi's sarcoma herpesvirus or KSHV, was first identified in 1994 by Moore and Chang using a representational difference analysis technique to extract novel viral DNA from the skin lesion of an HIV+ patient

with Kaposi's sarcoma (91). HHV8 has been identified as the causative agent in all forms of Kaposi's sarcoma (KS) (92) and has been linked to several rare lymphoproliferative disorders (93,94). HHV8 has a double-stranded DNA genome of over 165,000 nucleotides in length which is composed of a 145kb segment of unique sequence situated between G-C rich terminal repeat regions (95). The viral genome is packaged in an icosahedral capsid with a final molecular weight of approximately 300MDa. The HHV8 genome contains approximately 90 predicted open reading frames (ORFs) (96,97). Approximately half of these open reading frames belong to a core of evolutionarily conserved genes present in other herpesviruses (98). Conservation of these core herpesvirus genes includes not only a high degree of protein sequence similarity, but also retained gene synteny (99).

1.3.2 Phylogenetics of the gammaherpesviruses

HHV8 is phylogenetically classified as a member of the gammaherpesvirus family of viruses. The gammaherpesviruses are further classified into 2 subgroups, the lymphocryptovirus genus which includes Epstein-Barr virus and the rhadinovirus genus, of which HHV8 is a member (Figure 5).

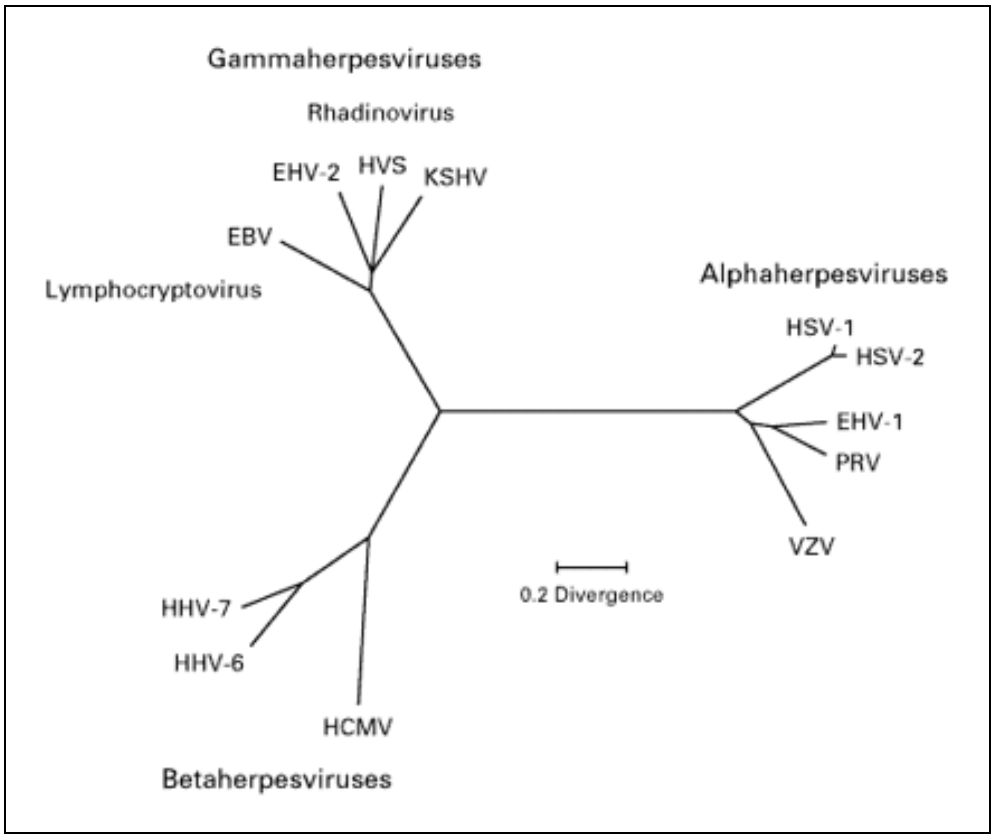

Figure 5. Phylogenetic Tree of the Herpesviridae

Reprinted with permission from Moore et al (99)

The gammaherpesviruses are known to infect a diverse range of mammalian hosts, including sheep, pigs, horses, wildebeests, and a variety of primates species, including both New and Old world monkeys and man (100). The gammaherpesviruses were originally characterized based on their ability to infect lymphocytes, induce lymphoproliferation in immunosuppressed hosts, and by comparison of their DNA polymerase gene sequences. However, the recent availability of full genome sequences has aided their phylogenetic classification based on overall sequence conservation. Currently 12 members of the gammaherpesvirus family have fully annotated reference genome sequences available through the NCBI database (Table 3).

Table 3. List of Gammaherpesviruses with Full Genome Sequences Available at NCBI

Virus Name	Abbreviation	Host	RefSeq Accession
Human herpesvirus 8	HHV8	Human	NC_003409
Human herpesvirus 4	EBV	Human	NC_007605
Cercopithecine herpesvirus 17	RRV	Rhesus monkey	NC_003401
Murid herpesvirus 4	MHV68	Mouse	NC_001826
Alcelaphine herpesvirus 1	AlHV1	Wildebeest	NC_002531
Bovine herpesvirus 4	BHV4	Cow	NC_002665
Callitrichine herpesvirus 3	CalHV3	Common marmoset	NC_004367
Cercopithecine herpesvirus 15	RLV	Rhesus monkey	NC_006146
Ateline herpesvirus 3	HVA	Spider monkey	NC_001987
Ovine herpesvirus 2	OHV2	Sheep	NC_007646
Equid herpesvirus 2	EHV2	Horse	NC_001650
Saimiriine herpesvirus 2	HVS	Squirrel monkey	NC_001350

Of these, 9 belong to the rhadinovirus genus while the remaining 3 members are lymphocryptoviruses. Virtually all members of the gammaherpesvirus family have been shown to induce cancer in their natural or foreign hosts. Most induce a variety of lymphoproliferative disorders of B and T-cells, as well as endothelial lesions similar to KS. For example, infection of rhesus macaques with RRV has been reported to induce lymphoproliferation resembling multicentric Castleman's disease (101), follicular hyperplasia and arteriopathy (102) similar to that observed in MHV68 and AHV1. *Herpesvirus saimiri* has been shown to induce T-cell lymphomas as well (103).

1.3.3 Viral lifecycle

As with most members of the *Herpesviridae*, HHV8 has a biphasic lifecycle, consisting of latent and lytic phases. In the latent or non-productive phase, the virus is not actively producing viral progeny and only a minor subset of genes is expressed (95). These genes play various functional roles, including tethering the viral genome to host chromatin, avoiding immune surveillance, and interacting with the host cell cycle.

In the lytic phase, the viral genome is replicated and packaged into viral capsids, resulting in the production of infectious progeny (95). Genes expressed during the lytic phase are expressed in a temporal manner by function. At the beginning of a lytic phase, an initial set of viral regulatory genes involved in transcriptional activation are expressed. These genes are known as the immediate-early genes (104). The next sets of genes to be transcribed are known as the early genes. These genes are involved in preparing the host cell for DNA replication, large scale protein synthesis and immune evasion (95). Following expression of the early genes, replication of the viral genome takes place. The final set of genes expressed by the virus is known as the late genes and are primarily structural genes that compose the viral capsid (95). Following expression of these genes, the viral capsid is assembled and the viral genome is packaged inside. A group of proteins are attached to the capsid to form what is known as the tegument (95). Finally, the virus capsid buds through the host membrane and is covered in a membrane coat, resulting in a mature viral particle (95).

1.3.4 HHV8 transmission

Transmission of HHV8 is believed to differ significantly depending on geographic location and the level of individuals infected in the population. In the US where levels of HHV8 infection are low, the virus is primarily transmitted as a sexually transmitted disease (105). Homosexual men are particularly at risk (106), however the virus has been observed to be transmitted through heterosexual populations as well (95,107). In contrast, in areas affected by endemic KS where much higher rates of HHV8 infection are present, HHV8 transmission has been observed in young children and generally believed to be passed from parent to offspring and horizontally among infected children (108,109). Several rare cases of vertical transmission have been reported in which young children have viral DNA present less than 24 hours after birth (110).

1.3.5 Rates of HHV8 infection

Globally, the estimates of HHV8 infection vary dramatically by region. In the United States and northern Europe serological studies have estimated the rate of HHV8 infection to be approximately 1-7% (95). In areas of the Mediterranean, such as Italy, the prevalence of infection has been shown to vary from 7-25%, following a gradient increasing from north to south (111) with dramatically higher rates in Sardinia and Sicily of approximately 35% (112). In areas of sub-Saharan Africa where KS in endemic, roughly 30-60% of the population shows serological evidence of infection with HHV8 (95).

1.3.6 HHV8 clades

The HHV8 genome shows some degree of diversity from region to region and has been divided into several distinct subtypes and clades. These subtypes are defined by genetic differences in a hypervariable region of the genome containing the ORF K1 gene (113). The amino acid sequence of the K1 gene varies between 30 and 60% in its N-terminal region and allows the virus isolates to be categorized into 5 groups: A, B, C, D and E (114,115). In North America and Europe, the A and C groups have been found to be the predominant form. In sub-Saharan Africa, the B group predominates, while in Asia the D group is the most common isolate (116). In South America the B and C subtypes are commonly found, while the E subtype has been shown to be present in indigenous populations (115) (Figure 6). HHV8 also is found as 2 distinct allelic variants that have been postulated to arise due to a recombination event with the ancestral herpesvirus (95). These variants, named P (prototype) and M (minority), differ in sequence at the right-hand portion of the genome (95). Despite this diversity, no correlation between clinical symptoms or disease severity and virus clade/subtype has been found (117).

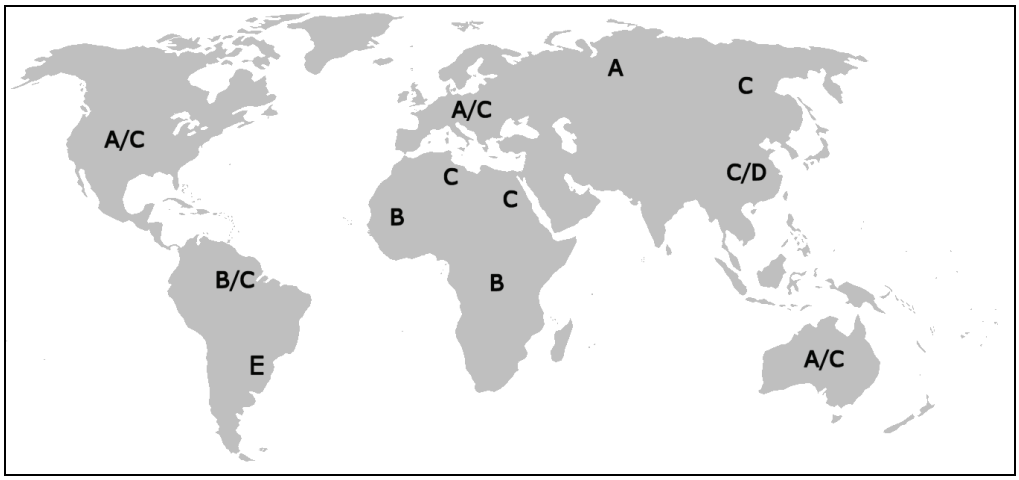

Figure 6. HHV8 Subtype Global Distribution

Derived from Boshoff et al (117)

1.3.7 HHV8 oncogenes

HHV8 genome has been shown to carry a comparatively large number of genes pirated from the host genome, including several which may function as oncogenes. Of these genes, several appear to mimic the regulatory activity of the host gene and often are unresponsive to host feedback mechanisms. The first open reading frame in the HHV8 genome encodes the K1 gene, a transmembrane glycoprotein that has a constitutively active ITAM domain (118,119). Expression of K1 has been shown to be able to transform mouse fibroblasts which are capable of forming tumors in nude mice (120). The K15 gene has also been shown to have oncogenic potential. K15 is spliced to form several unique proteins which contain between 4 and 12 transmembrane-spanning domains with a cytoplasmic tail that contains SH2 and SH3 binding motifs (95). Expression of K15 has been shown to interfere with B-cell receptor (BCR) signaling (121). The Kaposin gene is expressed during both latent and lytic phases and is

expressed as 3 different protein isoforms (122). The smallest of these isoforms, KaposinA has been shown to be able to transform mouse fibroblast cells which can also form tumors when implanted in nude mice (123). Lastly, the ORF 74 gene encodes a viral G-protein coupled receptor with homology to the human IL-8 receptor (124). The vGPCR protein contains a 7-transmembrane spanning region and has been shown to be capable of binding a variety of host signaling molecules, including both CC and CXC chemokines (125). However, unlike the host IL-8 receptor which is response to negative feedback, the vGPCR is constitutively active (125). vGPCR expression has been shown to be capable of inducing morphological changes and tumor formation in rat kidney and NIH 3T3 cells (126), and can immortalize endothelial cells (127). These vGPCR-transformed cells have been shown to express several angiogenic proteins including VEGF, VEGFR-2 and VEGFR-3 (126-128), which have the potential to provide a highly angiogenic environment for nascent prostatic tumors. While these genes have a clear oncogenic potential, they have only been observed to be expressed during the lytic phase of infection. Therefore it is unclear how these genes might contribute to tumor formation and paracrine effects have been proposed as one possible mechanism (126).

1.3.8 Lymphoproliferative disorders associated with HHV8

While HHV8 was originally identified as the pathogen responsible for Kaposi's sarcoma, it has been implicated in several other human lymphoproliferative disorders as well. One of these disorders is a rare primary effusion lymphoma (PEL) that is characterized by a malignant effusion occurring within the visceral cavity (117). The malignant cells in this disorder are derived from preterminally differentiated B-cells (129) and show the

presence of a relatively high copy number of HHV8 DNA (117). HHV8 has also been associated with another lymphoproliferative disorder known as multicentric Castleman's disease (MCD). HHV8 is known to cause a specific subset of MCD affecting HIV positive patients and approximately 50% of MCD in non-HIV patients (130,131). This form of MCD is characterized by the presence of immature B-cell precursors known as plasmablasts. This unique form of MCD has been designated as "plasma-cell variant MCD" (132).

1.3.9 Kaposi's sarcoma

KS is the disorder for which HHV8 is most classically known. The disorder was first identified by the Hungarian dermatologist Moritz Kaposi in 1872 (133). KS presents clinically as a highly vascularized skin lesion. In the initial stage of KS, called the patch stage, the KS lesion develops in proximity to normal blood vessels as groups of irregular spaces lined with endothelial cells with the presence of an inflammatory cell infiltrate (117). KS then progresses to the plaque stage, characterized by the presence of spindle-shaped cells of endothelial origin (95). The KS lesion takes the form of slit-like channels that contain erythrocytes (117). In the final or nodular stage, the KS lesion is composed of sheets of spindle cells (95).

Four epidemiologic forms of KS have been characterized: classic, endemic, iatrogenic, and HIV-associated KS. Classic KS is generally associated with elderly men, particularly of Italian, Greek, and Ashkenazi Jewish ancestry. The classic form of KS has an average age of onset of approximately 60 years of age (134). The endemic form of KS is found in sub-Saharan African, with the highest prevalence occurring in the

equatorial region (134). Endemic KS predates the emergence of HIV in Africa (135) and has a much earlier age of onset than classic KS (136). Infection of children is also observed in endemic KS and transmission from infected parent to child is believed to be the primary mechanism of transmission (108). Iatrogenic or transplant associated KS, occurs post-operatively in organ transplant patients taking immunosuppressant drugs. The recipient of an organ transplant is at a much higher risk of developing KS than is the organ donor. The development of KS has been shown to be linked to the use of immune suppressive drugs that prevent rejection of the transplanted organ (137,138). In most cases of iatrogenic KS, the patient was previously infected with HHV8 rather than contracting the infection through the transplant procedure itself (139). The most recently identified form of KS is HIV-associated KS. This form occurs predominately in homosexual men infected with HIV, but is also observed at lower rates in HIV positive intravenous drug users (140).

One of the most striking observations in the epidemiology of KS is its profound sex bias. While men and women show similar rates of infection with HHV8, men are at significantly higher risk of developing KS. In the US, men develop KS 13 times more frequently than women (4). In areas affected by endemic KS, such as Uganda, estimates of KS prevalence have found it to be 15 times more common in men (134). These differences in KS prevalence cannot be explained by differences in the rate of HIV infection and even in regions with similar rates of HHV8 and HIV infection, men are at a much higher risk of developing AIDS-KS (141). This striking sex bias suggests a possible role for sex hormones in the etiology of KS.

Several studies have linked the onset of KS progression with a marked increase in HHV8 viral load. Whitby et al first observed that the presence of detectable levels of

viral DNA in bloodstream was a predictor of patients with an increased risk of developing KS lesions (142). Further, it was reported that increasing viral load levels are correlated with the onset of KS (143,144).

1.4 SUMMARY

Epidemiologic studies have shown that prostate cancer clearly has a complex etiology, with both genetic and environmental risk factors. The biology of prostate development and regulation has been shown to be strongly regulated by both androgens and estrogens. However, the involvement of the androgen and estrogen receptor in prostate cancer remains unclear. Pathogen infection has similarly been implicated in increasing risk of prostate cancer. In particular, sexually transmitted diseases have frequently been investigated as causative agents. Human herpesvirus 8 has recently been associated with an increased risk of prostate cancer in a population of Afro-Caribbean men from the island of Tobago (3). As previously mentioned, despite similar HHV8 seroprevalences, men are at a much higher risk of developing KS than women, suggesting that hormonal factors may be important in KS pathology. HHV8 has been shown to present in the prostate (89,145) and is known to encode several genes with oncogenic potential. Increased expression of HHV8 oncogenes mediated by the androgen and estrogen receptor may represent a novel causative mechanism in prostate cancer and Kaposi's sarcoma. Here we investigate the role of the androgen and estrogen receptors in HHV8 biology and explore the implications of HHV8-hormone interaction in the etiologies of KS and prostate cancer.

2.0 HYPOTHESIS AND SPECIFIC AIMS

2.1 HYPOTHESIS

We have identified a statistical interaction between estrogen receptor alpha (ESR1) rs2234693 genotype and infection with human herpesvirus 8 (HHV8) that results in an increased risk of prostate cancer in Afrocaribbean men from Tobago. HHV-8 has also been identified as the causative agent in all forms of Kaposi's sarcoma (KS). Despite similar HHV-8 seroprevalences in men and women, men are at a much higher risk of KS, suggesting sex hormones influence KS pathology. The estrogen receptor (ER) is a ligand-dependant transcription factor that mediates the genomic effects of estrogen signaling. Here we propose a direct model of interaction between estrogens and human herpesvirus 8. Specifically, this interaction involves estrogen response elements in the HHV8 genome that modify HHV8 gene transcription in response to estrogens. These estrogen-mediated changes in transcription are responsible for the lower incidence of KS in women and may contribute to the observed increase in prostate cancer risk.

2.2 SPECIFIC AIMS

1) To identify estrogen response element (ERE) sequences in the HHV8 genome by performing:
    a) a computational search of the HHV8 NCBI reference genome sequence for regions resembling the estrogen receptor consensus DNA binding sequence.
    b) Gel-shift assays to show that sequences identified computationally are capable of binding human estrogen receptor proteins.

2) To experimentally validate whether candidate ERE sequences in the HHV8 genome can act as function estrogen response elements, using:
    a) use luciferase reporter assays to show *in vitro* that HHV8 genes with ERE sequences in their promoter region are responsive to estrogen treatment and
    b) show that estrogens are able to modify target gene transcription in the context of the virus.

3) To determine whether candidate ERE sequences found in the HHV8 genome sequence are conserved in the upstream regions of homologous genes found in other members of the gammaherpesvirus family.

3.0 RESEARCH DESIGN AND METHODS

3.1 STUDY POPULATION

Men ages 40-79 were recruited by word of mouth, informed by heath care workers, private physicians, posters, flyers, public service announcements, and public presentations by physicians from Trinidad and the US. Over 60% of the eligible men in Tobago participated in the study. Eligible men were screened by digital rectal exam (DRE) and prostate specific antigen (PSA) testing. A 15ml sample of peripheral blood was collected prior to DRE and frozen at -20°C. PSA levels were analyzed at the University of Pittsburgh Central Pathology Laboratory using automated Microparticle Enzyme Immunoassay (Abbot Ax-SYM PSA assay). Systematic DREs were performed by a physician trained according to a standardized study protocol. Individuals with abnormal DRE results or elevated PSA (≥ 4.0ng/ml) underwent trans-rectal, ultrasound guided biopsies. A total of 405 men were diagnosed with prostate cancer and were designated as cases while 2685 men with normal PSA levels and normal or slightly abnormal DRE results were used as controls. Approval for research of human subjects was obtained from the University of Pittsburgh Institutional Review Board (available in Appendix A) and the Tobago Ministry of Health.

3.2 DNA ISOLATION AND GENOTYPING ANALYSIS

Genomic DNA was obtained from residual blood clots using the QIAgen Blood midi DNA kit according to the manufacturer's instructions and stored at +4°C. The CAG repeat in exon1 of the androgen receptor gene was genotyped by PCR amplification using TET, FAM, or HEX fluorescently labeled primers (designated ARA) in table 4, amplified using 30 cycles of the following conditions: 95°C for 30 seconds, 60.2°C for 30 seconds, 72°C for 30 seconds, followed by a 7 minute extension at 72°C. Amplification products were then run on a 7% polyacrylamide gel using an ABI 377 fluorescence DNA sequencer (Applied Biosystems) along with the GeneScan 500 ROX ladder (Applied Biosystems). Allele sizes were determined by comparison with internal standards with repeat lengths previously determined by direct DNA sequencing.

Table 4. PCR Primers Used For Estrogen and Androgen Receptor Genotyping

Primer Name	Sequence
ARA-F	5'-ACC-GAG-GAG-CTT-TCC-AGA-AT-3'
ARA-R1	5'-AGA-ACC-ATC-CTC-ACC-CTG-CT-3'
ARA-R2	5'-CTG-TGA-AGG-TTG-CTG-TTC-CTC-3'
ARA-R3	5'-CAG-CTG-AGT-CAT-CCT-CGT-CCG-3'
ER-F	5'-ATC-CAG-GGT-TAT-GTG-GCA-ATG-AC -3'
ER-R	5'-ACC-CTG-GCG-TCG-ATT-ATC-TGA-3'

The estrogen receptor alpha PvuII and XbaI polymorphisms (rs9340799 and rs2234693) were assayed by PCR-RFLP analysis. PCR amplification was performed

using the 0.75 ul of 20uM primers ER-F and ER-R in table 4 in a reaction volume of 50ul (28.8ul H_20, 8.0ul of 1.25mM dNTP mix, 5.0ul 10x PCR Buffer (Gibco), 1.5 ul of 50uM $MgCl_2$, 0.3ul of 5U/ul Taq polymerase). Amplification was performed using 30 cycles of 95°C for 30 seconds, 56.8°C for 30 seconds, 72°C for 30 seconds followed by a 10 minute extension at 72°C in a MJ Research thermocycler. Amplification products were then digested overnight at 37°C using XbaI or PvuII restriction endonuclease (NEB). XbaI digestion was performed using 15ul of PCR amplification product, 2.55ul H_20, 2.0ul 10x NEB Buffer #2, 0.2ul bovine serum albumin, and 0.25ul 20U/ul XbaI enzyme. PvuII digestions were performed using 15ul of PCR amplification product, 2.5ul H_20, 2.0ul 10x NEB buffer #2, and 0.5ul of 10U/ul PvuII enzyme. The restriction digest products were then separated by performing gel electrophoresis at 100V on a 2% agarose gel, stained with 5ug/100ml ethidium bromide. Fragment sizes were compared to a 1kbPlus ladder (NEB) and scored as either cut (+) or uncut (-). A total of 260 cases and 541 controls from the Tobago Prostate Cancer study were genotyped for the XbaI and PvuII polymorphisms.

3.3 IMMUNOFLUORESCENCE ASSAY

Serum samples from 154 Tobago prostate cancer and 147 controls were analyzed by enhanced immunofluorescent assay (IFA) (146). A cutoff seropositivity level of 1:100 was used and titer levels were determined by performing IFA on serially diluted samples. Samples were tested in duplicate by the same reader who was blinded to patient genotypes and case/control status (86).

3.4 GENOTYPING DATA ANALYSIS

A sample of 154 cases and 147 controls with overlapping genotyping and serologic results were included in the data analysis. Chi square tests were used to determine significance of serologic and genotyping results. Estimation of the odds ratio (OR) was done by condensing genotype categories into 2 groups (G/G, G/A and A/A) and combining this data with HHV8 seropositivity status, resulting in a total of 4 groups. The reference group for the logistic regression model was comprised of individuals who were seronegative for HHV8 infection and had the low risk genotype (A/A). Two-sided tests of statistical significance were used in the analysis. Androgen receptor repeat sizes were grouped into 2 categories, long (≥20) and short (<20) repeats and analyzed as a dichotomous variable.

3.5 COMPUTATIONAL SEARCH FOR ESTROGEN RESPONSE ELEMENTS

A position specific scoring matrix was constructed by aligning 45 experimentally validated estrogen response element sequences (table 5) published by Vega et al (50). A count matrix was created using the enoLOGOS program (147) (sequence LOGO seen in Figure 7) and was converted to a log transformed weight matrix using the equation: $w_{bi} = -\ln ((c_{bi} + 0.25)/(n+1))$. The weight matrix was then used to score 750bp sequences

upstream of each open reading frame in the HHV8 genome (Accession # NC_003409) using a 13bp sliding window.

The false positive rate of the algorithm was evaluated using 2 methods. First, the algorithm was used to scan all possible reading frames of a 10,000bp sequences that were composed of coding exons (excluding 1st exons) randomly selected from the human genome (NCBI Build 36.1). Transcription factor binding sequences are uncommonly found in protein coding sequences (with the exception of the 1st exon), therefore this sequence should not be enriched for ERE sequences. As a second method, we scanned a total of 100 sequences 10,000bp in length that had been randomly generated based on the dinucleotide content of exons in the human genome. Both methods were found to produce highly similar results. True detection rates were determined by scanning all possible reading frames of an artificial sequence which contained each of the ERE sequences used to construct the weight matrix. Significance cutoff scores were determined by setting sensitivity to 80% and specificity to 99.99% (1 false positive per 10,000bp). Sequences that scored above the significance cutoff and were located within 750bp of the ATG start site of the test gene were designated as candidate sequences for further analysis.

Sequences scoring above the significance cutoff score were then given individual p-values. These values were determined by generating all possible 13-mer sequences and scoring them using our position-specific scoring matrix. Each of the 13-mer sequences was then ranked by score. To determine the probability of finding a sequence at or above a particular score, our experimental sequences were then compared to the ranked list of 13-mers. Therefore our p-values indicate the likelihood of finding a sequence at or above that particular score.

Table 5. List of Experimentally Validated Estrogen Response Element Sequences

Number	Gene	Location	Sequence
1	*PDZK1*	chr1:143,215,756-143,215,768	GGTCAccc**A**G**T**CC
2	*ADORA1*	chr1:199,790,269-199,790,281	GGT**T**AgggTGACC
3	*ADORA1*	chr1:199,790,414-199,790,426	GGT**GT**cttTGACC
4	*AGT*	chr1:227,156,613-227,156,625	GG**G**CAtcgTGACC
5	*GREB1*	chr2:11,603,634-11,603,646	GGTCAaaaTGACC
6	*GREB1*	chr2:11,615,324-11,615,336	GGTCAtcaTGACC
7	*GREB1*	chr2:11,621,861-11,621,873	**A**GTCAgtgT**C**ACC
8	*GREB1*	chr2:11,623,258-11,623,270	GGTCAttcTGACC
9	*CYP1B1*	chr2:38,214,993-38,215,005	GGTC**G**cgcTG**C**CC
10	*CYP1B1*	chr2:38,215,049-38,215,061	GGTCAaag**C**G**G**CC
11	*LTF*	chr3:46,481,739-46,481,751	GGTCAagg**C**GA**T**C
12	*AREG*	chr4:75,676,340-75,676,352	GG**A**CAaggTG**T**CC
13	*ELOVL2*	chr6:11,154,748-11,154,760	GGTCAtctTGA**TG**
14	*VEGF*	chr6:43,844,381-43,844,393	**AA**TCAgacTGAC**T**
15	*LY6E*	chr8:144,170,802-144,170,814	GG**A**CAagaTGACC
16	*PTGES*	chr9:129,597,654-129,597,666	GG**A**CAgccTG**G**CC
17	*CASP7*	chr10:115,428,398-115,428,410	GGTCAgggTGA**A**C
18	*CASP7*	chr10:115,428,492-115,428,504	GGTC**G**gggTGA**A**C
19	*CASP7*	chr10:115,428,572-115,428,584	GGTCAgggTGA**A**C
20	*CASP7*	chr10:115,428,612-115,428,624	GGTCAgggTGA**A**C
21	*CASP7*	chr10:115,428,652-115,428,664	GGTCAgggTGA**A**C
22	*CASP7*	chr10:115,428,689-115,428,701	GGTCAgggTGA**A**C
23	*CASP7*	chr10:115,428,743-115,428,755	GGTCAgggTGA**A**C
24	*CTSD*	chr11:1,741,924-1,741,936	GG**C**C**G**ggcTGACC

Table 5 Continued

25	*PGR*	chr11:100,504,595-100,504,607	GGTCAcca**GCT**C**T**
26	*PGR*	chr11:100,505,180-100,505,192	G**CAGG**agcTGACC
27	*SCNN1A*	chr12:6,355,536-6,355,548	GGTCAgccT**C**ACC
28	*GAPDH*	chr12:6,513,208-6,513,220	GG**A**ACAtcgTGACC
29	*ESR2*	chr14:63,879,248-63,879,260	GGTCAggcTG**GT**C
30	*FLJ30973*	chr15:55,670,850-55,670,862	GG**G**CAgtgTG**G**CC
31	*FLJ30973*	chr15:55,671,545-55,671,557	GGTCAcccTG**CT**C
32	*ABCA3*	chr16:2,319,793-2,319,805	GGTCAcggTG**TT**C
33	*IGFBP4*	chr17:35,849,113-35,849,125	GGTCAttgTGAC**A**
34	*TRIM25*	chr17:52,323,321-52,323,333	GGTCAtggTGACC
35	*BCL2*	chr18:59,136,673-59,136,685	GGTC**G**cca**G**GACC
36	*MGC26694*	chr19:19,035,118-19,035,130	G**T**TCAgagTGACC
37	*GRAMD1A*	chr19:40,182,519-40,182,531	GG**C**C**T**ggcTGACC
38	*ACTN4*	chr19:43,897,093-43,897,105	GGTCActgTGAC**T**
39	*GPR77*	chr19:52,532,131-52,532,143	GGTCActcTGAC**A**
40	*C3*	chr19:6,671,884-6,671,902	GGT**GG**cccTGACC
41	*NRIP1*	chr21:15,359,833-15,359,845	GGTCAaagTGACC
42	*TFF1*	chr21:42,659,626-42,659,638	GGTC**C**tggTG**T**CC
43	*TFF1*	chr21:42,659,906-42,659,918	**A**G**C**CAagaTGACC
44	*TFF1*	chr21:42,660,106-42,660,118	GGTCAcggTG**G**CC
45	*CRKL*	chr22:19,595,695-19,595,707	**A**GTCAatcT**A**ACC

Adapted from Vega et al GenomeBiology (50)

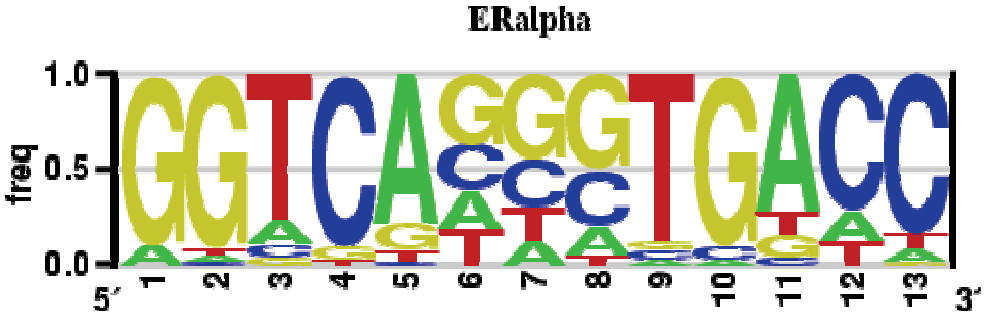

Figure 7. Sequence LOGO Representing the Consensus ERE Sequence Included in the Position-Specific Scoring Matrix

3.6 ELECTROPHORETIC MOBILITY SHIFT ASSAY

Short oligonucleotide probes were synthesized to containing each of the candidate ERE sequences, as well as approximately 20bp of flanking sequence around each ERE (table 6). A sequence containing the ERE from the Xenopus vitellogenin A2 gene was used as a positive control for the analysis. Single stranded probes were hybridized to form a double stranded DNA probes and end labeled with ^{32}P using T4 polynucleotide kinase (NEB). Unincorporated radionucleotides were removed using the Qiagen Nucleotide Removal kit. Nuclear protein extracts were prepared from a T-75 flask of MCF-7 cells using the NucBuster Protein Extraction kit (Novagen) according to the manufacturer's instructions. Protein concentrations were determined using the Bradford assay (BioRad) according to the manufacturer's instructions. Binding reactions were prepared by incubating 10ug of nuclear protein extract with 1ul of labeled DNA probe in the presence of 0.03125ug of polydI:dC to prevent non-specific binding. After a 30 minute incubation at room temperature, the binding reactions were mixed with 6x Maniatis gel loading buffer IV (148) and run on a 6% nondenaturing polyacrylamide gel in 0.1X TBE

at 200V for 2 hours. Gels were then placed in plastic wrap and allowed to expose a phosphorimager plate overnight at room temperature. Plates were then read using a Molecular Dynamics phosphorimager. Cold competition assays were performed as above, but with the inclusion of increasing amounts of unlabelled DNA probe (0, 2, 5, 10, 50, 100, 500, or 1000 fold) in the binding incubation step.

Table 6. Oligonucleotide Probes Used in Electrophoretic Mobility Shift Assays

ERE	Forward Primer	Reverse Primer
Vitellogenin control	5'-CTA-GAA-AGT-CAG-GTC-ACA-GTG-ACC-TGA-TCA-AT-3'	5'-ATT-GAT-CAG-GTC-ACT-GTG-ACC-TGA-CTT-TCT-AG-3'
ORFK8	5'-CTG-CAA-CCG-GTC-AGG-GCG-ACC-TCG-GCG-AC-3'	5'-GTC-GCC-GAG-GTC-GCC-CTG-ACC-GGT-TGC-AG-3'
ORF49	5'-CAA-TCT-ACG-ATC-CCA-GTG-ACC-TAA-ATA-GAG-3'	5'-CTC-TAT-TTA-GGT-CAC-TGG-GAT-CGT-AGA-TTG-3'
ORF74	5'-GAC-AGA-AAG-GTC-ACC-TGG-CCC-AAA-CGG-AG-3'	5'-CTC-CGT-TTG-GGC-CAG-GTG-ACC-TTT-CTG-TC-3'

3.7 NOSHIFT ASSAY OF ESTROGEN RESPONSE ELEMENTS

The NoShift Transcription factor assay was used to confirm the identity of proteins observed to bind ERE sequences in the EMSA analysis. The NoShift Transcription Factor Assay estrogen receptor α kit was obtained from Novagen. Oligonucleotide probes with identical sequence to those used for EMSA experiments (Table 6) were

synthesized with 5' biotin labels (Proligo) and nuclear protein extracts were prepared using the NucBuster kit as per the manufacturer's instructions. Binding reactions were prepared by incubating 5ul of 4x NoShift Bind buffer, 1ul of poly-dI-dC reagent (Novagen), 1ul of salmon sperm (Novagen), 1ul of 10pmol/ul biotinylated oligonucleotide probe, 5ul MCF-7 nuclear extract, and 7ul of nuclease-free H_20. Binding reactions were then incubated on ice for 30 minutes. Wells of a streptavidin-coated microplate (Novagen) were washed 3 times with 200ul of 1x NoShift Wash Buffer (Novagen). 80ul of 1x NoShift Bind buffer was added to each binding reaction and the entire reaction was added to the washed, streptavidin-coated plate. The plate was then sealed and incubated at 37°C for 1 hour. Contents of the plate were then poured off and the plate washed three times with 200ul of 1x NoShift Wash buffer (Novagen). Next, a mouse monoclonal antibody (Novagen) recognizing the human estrogen receptor α protein (clone TE-111) was diluted 1:1000 in NoShift Antibody dilution buffer (Novagen) and 100ul of the diluted antibody solution was added to each well. The plate was sealed and incubated at 37°C. After 1 hour the contents were removed by inverting the plate and washed three times with 200ul NoShift Wash buffer. A 1:100 dilution of HRP-conjugated secondary antibody (Novagen) was prepared and 100ul was added to each well. The plates was again sealed and incubated at 37°C for 30 minutes. Contents of the plate were then removed and the plate was washed five times with 200ul of 1x NoShift Wash buffer. 100ul of tetramethylbenzidine (TMB) substrate was then added to each well and incubated at room temperature until a blue color change was observed. Finally 100ul of 1N HCL was added to stop the reaction and colorimetric readings were taken by measuring absorbance at 450nm using a microplate reader.

3.8 QUANTIFICATION OF ESTROGEN INDUCTION USING LUCIFERASE REPORTER ASSAY

To show that promoters found in the HHV8 genome to contain ERE sequences were responsive to estrogen treatment, we performed a series of reporter assays. The 5'-regions of candidate HHV8 genes found to contain ERE sequences were amplified by PCR using primer sets in table 7. 50ul PCR reactions were prepared using 28ul dH_20, 8.0ul 1.25mM dNTP mix (Invitrogen), 10x PCR buffer (Invitrogen), 2.0ul 50uM $MgCl_2$, 0.75ul of each 20uM primer, 0.2ul 5U/ul Taq polymerase. PCR amplification was performed in an MJ Research thermocycler using 30 cycles of the following conditions: 95°C for 30 seconds, 56.0°C for 30 seconds, 72°C for 30 seconds, followed by a final 10 minute extension at 72°C. The K8 promoter region was found to contain 2 ERE sequences, therefore several truncated portions of the K8 promoter were created (figure 8) in order to determine which of the 2 ERE sequences were necessary for the response to estrogen treatment.

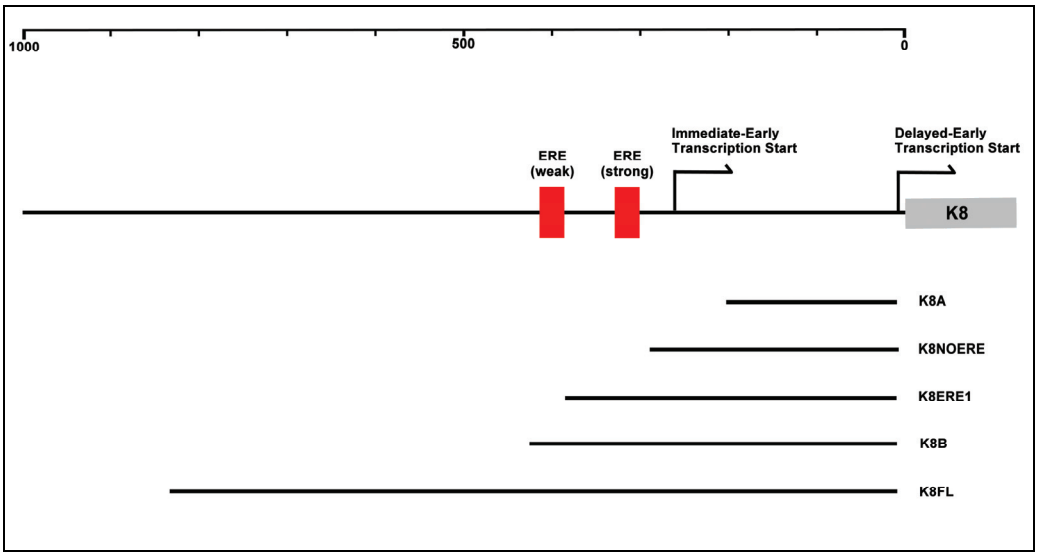

Figure 8. Diagram of HHV8 Promoter Fragments Used in Luciferase Reporter Assays

PCR fragments for the K8FL, K8ERE1, and K8NoERE reporter constructs were cloned into the pGEM-T EASY plasmid (Promega) according to the manufacturer's instructions. PCR fragments for the K8A, K8B, ORF49, and ORF74 constructs were initially cloned in the pCR2.1-TOPO plasmid vector using the Invitrogen™ TA-TOPO kit. Next, pGEM-T EASY plasmids containing the K8FL, K8ERE1, and K8NoERE inserts were digested overnight with KpnI and SpeI (NEB). The pCR2.1 plasmids containing K8A, K8B, ORF49, and ORF74 inserts were digested overnight using KpnI and XbaI (NEB). Restriction digest reactions were then run on a 1% agarose gel in 1X TBE and the digested fragments were excised from the gel and purified using the QIAquick gel extraction kit (QIAgen) according to the manufacturer's instructions. The purified inserts were then ligated using T4 DNA ligase (NEB) into KpnI/NheI digested pGL3-basic luciferase reporter vector from Promega™ (entire procedure illustrated in figure 9). Presence and orientation of inserts was confirmed by restriction digest or direct DNA sequencing. One-Shot competent cells (Invitrogen) were transformed with plasmid constructs using the heatshock method according to manufacturer's instructions. Single colonies were isolated from ampicilin-treated agar plates and used to grow glycerol stocks. Five hundred ml cultures of Luria-Bertani (LB) media treated with ampicilin were inoculated with 25ul of glycerol stock and grown overnight. High purity plasmid DNA was prepared using a cesium chloride centrifugation density gradient protocol followed by phenol-chloroform extraction and resuspended in distilled water. Plasmid DNA concentrations were determined using a Nanodrop spectrophotometer (Thermo).

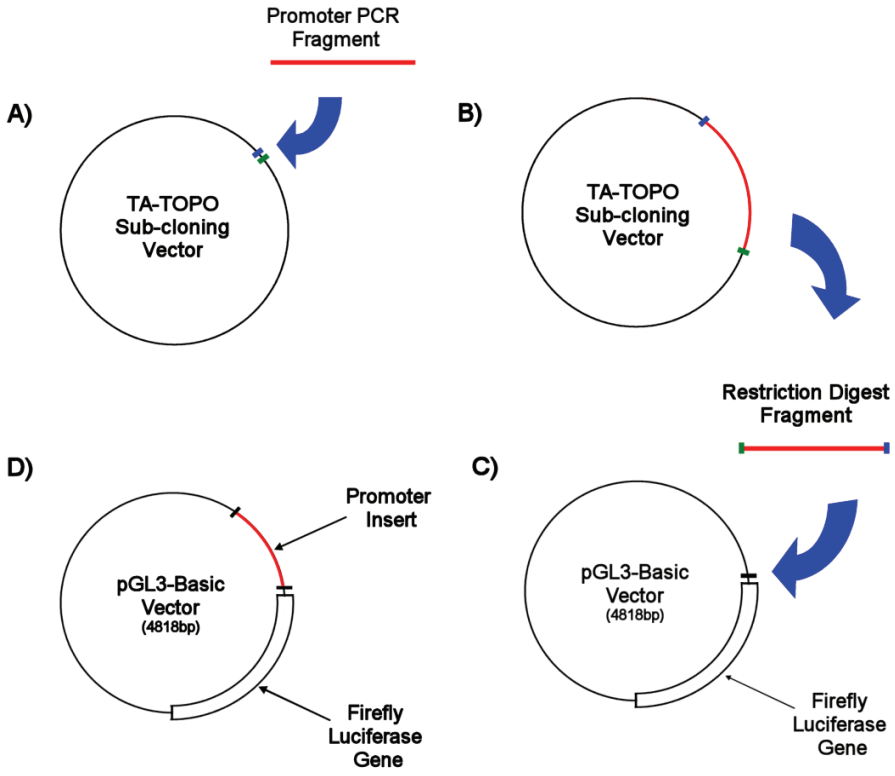

Figure 9. Illustration of Promoter Cloning Procedure

Table 7. PCR Primers Used for Amplifying HHV8 Upstream Regions Containing ERE Sequences for Cloning into pGL3 Luciferase Vectors

ERE	Forward Primer	Reverse Primer
K8FL	5'-GGT-CAT-GAC-GGA-GAC-CAA-GG-3'	5'-GTG-TAA-ACG-TGT-AAC-CCT-GCC-3'
K8B	5'-CGC-GCT-GTT-GTC-CAG-TAT-TC-3'	5'-GTG-TAA-ACG-TGT-AAC-CCT-GCC-3'
K8ERE1	5'-CCT-TCG-GTA-CCC-GGA-GTC-3'	5'-GTG-TAA-ACG-TGT-AAC-CCT-GCC-3'
K8NoERE	5'-CGA-GGT-ACC-AGG-AGT-CCG-3'	5'-GTG-TAA-ACG-TGT-AAC-CCT-GCC-3'
ORF49	5'-AGA-TCT-TGA-CAC-GCC-ACT-CTC-TCC-TTA-G-3'	5'-AGA-TCT-GGT-CAC-TGG-GAT-CGT-AGA-TTG-3'
ORF74	5'-CTA-GAT-GGA-CAC-CCC-GTG-AAC-C-3'	5'-GGC-CTA-CAA-TAA-CAA-GTA-GTA-TAG-G-3'

MCF7 and T47D cell lines were grown to approximately 70% confluency in 6-well cell culture plates in 2ml of Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% fetal bovine serum (FBS) and 1x gentamicin. Media was removed by aspiration and 2ml of fresh OptiMEM (GIBCO) was added to each well. Transfection reactions were set by diluting 4ug of plasmid vectors in a total volume 250ul Optimem and adding 5ul of Lipofectamine reagent (Invitrogen) to 240ul of Optimem. Reagents were then combined and incubated for 30 minutes. The DNA:Lipofectamine complexes were then added to the cell culture plates. Each plate included cells transfected with positive and negative control vectors. The positive control vector, ERE-

Luc, is composed of 2 estrogen response elements inserted into the HSV thymidine kinase promoter. The empty pGL3 vector and pGL3-control vectors were used as the negative controls. After 6 hrs media was aspirated from the wells and replaced with 2ml of phenol red–free RPMI media supplemented with 20% charcoal-stripped FBS. The following morning half the wells were treated with 17β-estradiol (Sigma) at a final concentration of 10^{-8}M. After 18hrs, cell cultures were lysed with Promega GLO-Lysis buffer and cells were detached from plates using cell scrapers and frozen at -80°C. Cell lysates were then thawed and 100ul of lysate was incubated with an equal volume of Promega Bright-Glo Luciferase substrate. Photon emission from each sample was measured using an LJL Catalyst™ plate reader.

The level of induction by estrogen was calculated by taking the ratio of the intensity of estrogen induced divided by uninduced sample. The significance for each vector was determined using a 2-sample paired t-test of the raw values of the induced compared to uninduced samples in each paired sample on the plate.

3.9 ESTROGEN INDUCTION OF HHV8 INFECTED BCBL-1 CELLS

3.9.1 Cell culture and estrogen treatment

BCBL1 cells containing the RTA gene under a doxycyclin-inducible promoter (TREx-BCBL1-Rta) (149) were cultured in a T-125 flask in 300ml of RPMI media supplemented with 10% FBS and 1x Gentamicin for 3 days. Cultures were pelleted by centrifugation and resuspended in 25ml of phenol red-free RPMI. A cell count was

performed by trypan-blue assay. Approximately $1x10^8$ cells were added to three new T-75 flasks in a total volume of 240ml of phenol red-free RPMI media supplemented with 10% charcoal-stripped FBS and placed in a tissue culture incubator at 37°C with 5%CO_2. After 4 hours, the initial T=0 timepoint was harvested and cells were treated with either 10^{-6}M 17β-estradiol, 1X 12-O-tetradecanoylphorbol 13-acetate (TPA), or 20ul ethanol (mock).

3.9.2 RNA preparation

Time points were collected at 0, 2, 4, 8, and 16 hours by removing 40ml of cell suspension (~ $1.8x10^7$ cells total) from each flask and pelleted by centrifugation. The supernatant was removed by aspiration and 2 ml of Trizol reagent (Invitrogen) was added. RNA isolations were performed according to the manufacturer's instructions with the exception that all RNA preps were incubated at -80°C for 30 minutes during the RNA precipitation step. Purified RNA solutions were stored at -80°C.

3.9.3 Preparation of cDNA by reverse transcription

RNA samples were quantified using a NanoDrop spectrophotometer (Thermo). All samples were run in duplicate using 400ng of DNase-treated total RNA per reaction. Reverse transcription was performed by preparing a mastermix solution containing Superscript II (GibcoBRL) and samples were incubated in a thermocycler (MJ Research) at 35°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes. Samples volumes were brought up to 100ul using nuclease-free water (Ambion) and stored at -20°C.

3.9.4 Taqman Realtime PCR quantification of HHV8 K8 mRNA expression

Realtime PCR primers and probes for HHV8 K8 were prepared using Primer Express ver 2.1 (ABI) (table 8). RT-PCR was performed using 1X Taqman Universal PCR Master Mix (ABI), 5uM FAM/TAMRA-labeled probe, 10uM forward and reverse primers, and 20ng cDNA. Control GAPDH expression was measured using 50nM JOE/TAMRA labeled probe. RT-PCR was run using the following conditions: 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. Reactions were run in an ABI Prism 7700 Sequence Detector using SDS ver 1.7 (ABI) to detect fluorescence at 538nm (FAM), 546nm (JOE) and 582nm (TAMRA) in order to determine threshold cycle. Baseline value was calculated by averaging the fluorescent values of cycle 3-15 and then adding 10 times the standard deviation. All reactions were performed in duplicate and values were averaged. Expression of K8 was normalized to GAPDH levels using the comparative Ct method (ABI Prism 7700 Sequence Detection System User Bulletin #2 Dec 11, 1997)

Table 8. PCR Primers and Taqman Probes Used in Quantifying K8 RNA Expression Levels.

Name	Sequence
K8 Forward primer	5'-TCC-CAC-CAT-GTT-GAA-GCT-TG-3'
K8 Reverse primer	5'-TGA-TCC-ACA-CAA-AGT-CTG-GCA-3'
K8 Detection probe	5'-6FAM-TTC-TCC-CGG-ACG-ACG-GCA-CAA-T-TAMRA-3'

3.10 PHYLOGENETIC ANALYSIS

In order to determine if ERE sequences were conserved in other evolutionarily related viruses, we computationally screened all gammaherpesviruses with a genome reference sequence deposited in the NCBI database. Twelve viruses (listed in table 3) were computationally searched for ERE sequences as described in section 3.5. Open reading frames found to have one or more ERE sequences scoring above the significance threshold score of 11.3127 (corresponding to 80% sensitivity and 99.99% specificity) and located within 750bp of the ATG were considered to have an ERE present. Homology of genes was determined based on NCBI genome annotations, conserved gene synteny, and the phylogenetic comparison performed by Nicholas (150). In order to determine the significance of identifying ERE sequences in the upstream region of multiple homologous genes, we performed a simulation analysis to determine the empirical likelihood of the event occurring by chance alone, rather than true evolutionary conservation. To perform the simulation analysis, a new 750bp sequence was randomly generated based on the dinucleotide content of the original homolog sequence. New randomly generated sequences were created for all homologs initially screened for ERE sequences. These new sequences were then searched for ERE sequences and counts of the number of simultaneous occurrences were retained. This was repeated 10,000 times and the total number of repetitions where ERE sequences were found to occur simultaneously in multiple sequences was divided by the total number of repetitions to obtain the frequency of finding EREs in multiple homologs simply due to chance alone.

4.0 RESULTS

4.1 GENOTYPING AND SEROLOGICAL RESULTS

4.1.1 Individual analysis of ESR1, AR, and serology variables

260 cases and 541 controls from the Tobago Prostate Cancer study were genotyped for the XbaI and PvuII polymorphisms located in intron 1 of the estrogen receptor alpha gene by PCR-RFLP analysis. 324 cases and 790 controls were genotyped for the androgen receptor polyglutamine (CAG) repeat. 282 cases and 291 controls from the same cohort underwent serological testing of HHV8 infection by enhanced immunofluoresence assay (IFA). Data was analyzed using chi-square test and odds ratio values were determined by logistic regression. Our analysis identified an association between the ERα (ESR1) XbaI G/G genotype (designated -/-) and an increased risk of prostate cancer (p=0.005; OR=2.48 [95%CI: 1.42-4.34]) (table 9). These results confirm the findings of earlier studies that reported associations between ESR1 polymorphisms and prostate cancer risk (66). Despite the presence of linkage disequilibrium between the XbaI and PvuII sites, we did not observe a significant association between the PvuII polymorphism and prostate cancer risk (data not shown).

Several previous reports have found significant associations between the androgen receptor polyglutamine repeat and prostate cancer risk (151,152), however, our

analysis failed to identify an association between the short form of the androgen receptor polyglutamine repeat (<20 repeats) and prostate cancer risk (p=.63 OR=1.07 [95%CI: 0.82-1.38].) (table 9).

We also observed a statistically significant association between HHV8 seropositivity and an increased risk of prostate cancer (p=0.001; OR=1.80 [95%CI: 1.28-2.52]) (Table 9). Our findings suggest that men from Tobago with serologic evidence of HHV8 infection are at an increased risk of prostate cancer. Interestingly, our serological analysis found an overall high level of HHV8 seroprevalence in the Tobago population, with 23.1% (183 out of 793) individuals found positive for HHV8 antibodies (table 9).

Table 9. Individual Analysis of Association Between Estrogen Receptor XbaI and HHV8 Serology with Prostate Cancer Risk

Parameter	Cases	Non-Cases	P(X^2)	OR(95%CI)	P(OR)
ESR1 +/+	122 (47%)	292 (54%)	----	1.0	----
ESR1 +/-	109 (42%)	221 (41%)	----	1.18 (0.86-1.61)	0.30
ESR1 -/-	29 (11%)	28 (5%)	0.005	2.48 (1.42-4.34)	0.002
AR long	147 (45%)	371 (47%)	----	1.0	----
AR short	177 (55%)	419 (53%)	0.63	1.07 (0.82-1.38)	0.63
HHV8 -	199 (67%)	411 (79%)	----	1.0	----
HHV8 +	96 (33%)	87 (21%)	0.001	1.80 (1.28-2.52)	0.001

4.1.2 Analysis of combined data

When the hormone receptor genotype and serological results were analyzed in combination, we observed several interactions between hormone receptor genotypes and HHV8 serology which were associated with an increased risk of prostate cancer. The first of these interactions was an association between the estrogen receptor alpha and HHV8, in which individuals with at least one XbaI G (-) allele who were seropositive for HHV8, were found to be at an even higher risk of prostate cancer than either of the factors independently (p=0.032; OR=3.10 [95%CI: 1.42-6.77]) (Table 10), indicating the presence of an interaction between these factors and prostate cancer risk. A similar interaction was found between the short form of the androgen receptor repeat and HHV8 seropositivity, that was significantly associated with prostate cancer risk (p= 0.044; O.R=2.46 [95%CI: 1.13-5.35]) (table 10).

Further, when we performed a combined analysis of the ESR1 and AR polymorphisms with HHV8 seropositivity, we found that individuals who were seropositive for HHV8 infection and carried a mutant ESR1 XbaI allele, as well as the short form of the androgen receptor repeat had the highest risk of prostate cancer observed in the entire analysis (p=0.005; OR=9.5 [95%CI: 2.58-35.02]) (table 11).

Table 10. Combined Analysis of Association Between Estrogen Receptor XbaI and HHV8 Serology with Prostate Cancer Risk

Parameter	Cases	Non-Cases	P(X^2)	OR(95%CI)	P(OR)
HHV8 - & ESR1 +/+	56 (36%)	66 (45%)	----	1.0	----
HHV8 - & Any ESR1 -	45 (29%)	47 (32%)	----	1.12 (0.66-1.94)	0.66
HHV8 + & ESR1 +/+	24 (16%)	23 (16%)	----	1.23 (0.66-1.94)	0.54
HHV8 + & Any ESR1 -	29 (19%)	11 (7%)	0.032	3.10 (1.42-6.77)	0.004
HHV8- & long AR	51 (33%)	52 (35%)	----	1.0	----
HHV8- & short AR	50 (32%)	61 (42%)	----	0.84 (0.49-1.43)	0.51
HHV8+ & long AR	24 (16%)	23 (16%)	-----	1.11 (0.55-2.23)	0.76
HHV8+ & short AR	29(19%)	12 (8%)	0.044	2.46 (1.13-5.35)	0.023

Table 11. Analysis of Combined ESR1, AR, and HHV8 Serology Results

Parameter	Cases	Non-Cases	P(X^2)	OR(95%CI)	P(OR)
AR long & ESR1+/+ &HHV8-	28 (15%)	38 (21%)	----	1.0	----
AR short & ESR1- & HHV8+	21 (11%)	3 (1.6%)	0.005	9.5 (2.58-35.02)	0.001

4.2 COMPUTATIONAL ANALYSIS OF ER BINDING SITES IN THE HHV8 GENOME

A computational search of the HHV8 genome reference sequence identified 20 predicted ER binding sites (table 12). These 20 ERE sequences were located in the upstream regions of 18 open reading frames in the HHV8 genome and were distributed relatively randomly (figure 10), with the exception of a cluster of predicted sites located in the region containing several genes involved in transcriptional control and regulating the viral latent/lytic life cycle (figure 11). In this region, the two highest scoring hits in our analysis were located in the promoter region of the K8 gene (EREs 4 and 5 in figure 11). The K8 EREs were located 314bp and 399bp 5' of the ATG start site, in a region previously found to be the K8 promoter (153). The K8 gene encodes a basic leucine zipper (bZIP) protein that is a homolog of the EBV BZLF1 gene (154). However, while BZLF1 is a known transcriptional activator in EBV (155), K8 has been shown to repress

lytic reactivation by inhibiting the major lytic switch gene, ORF50/Rta (156). K8 has also been shown to bind to specific DNA sequences in the lytic origin of replication (157).

Another high-scoring ERE sequence was also located in a non-coding region upstream of the ORF49 gene and the second exon of ORF50 (ERE1 in figure 11). Both of these genes have been shown to function as transcriptional activators. Expression of ORF49 has been reported to act cooperatively with ORF50 to increase the activation of lytic gene expression (158). An expanded analysis of this region identified an additional 2 ERE sequences located inside of the ORF50 reading frame, 666 and 682 bp 5' of the ORF49 ATG (EREs 2 and 3 in figure 11). Therefore a total of 5 high scoring EREs were identified in this cluster of genes.

We selected candidate ERE sequences for further analysis based on 3 criteria: how closely the candidate ERE matched the known ERE consensus, the known functional role of the gene in important transcription regulation and signaling pathways, and finally, a previously demonstrated role in oncogenesis. Based on these criteria, we selected EREs in 3 genes for further functional analysis: K8, ORF49, and ORF74. The K8 and ORF49 genes are interesting candidates because they both have a major role in regulating HHV8 gene expression and control of the lytic/latent switch. Due to its location in the non-coding region shared by ORF49 and ORF 50, the ORF49 ERE could also have had an effect on ORF50 expression. The third ERE selected for our follow up analysis was located upstream of the ORF74 gene. ORF74 encodes a G-protein coupled receptor which has homology to the human IL8 receptor and has been shown to be involved in chemokine signaling (124,125). Expression of ORF74 has been shown to transform mouse fibroblasts and forms tumors in nude mice (126).

Table 12. ERE Sequences Identified in the HHV8 Genome

Rank	ORF	Name	Sequence	Distance	Strand	Score	Sigificance
1	ORF K8	BZLF1 homolog	GGTCAGGGCGACC	314	F	7.177	1.74344E-06
2	ORF K8	BZLF1 homolog	GGGGAGGGTGACC	399	R	9.586	2.68966E-05
3	ORF57	Transcriptional activator	GGTGAGGGGGACC	260	R	9.718	3.08454E-05
4	ORF53	Envelope glycoprotein	GGTCAGGATGAAA	268	F	9.989	4.0248E-05
5	ORF56	DNA replication protein homolog; EBV BSLF1 homolog	GGACAACGTCACC	153	F	9.998	4.06504E-05
6	ORF50	transactivator homolog; EBV BRLF1 homolog	GGTCACTGGGATC	48	R	10.397	5.95003E-05
7	ORF29a	packaging protein homolog; EBV BGRF1 homolog	GGCCTGTGTGACC	428	R	10.403	5.99176E-05
8	ORF31	EBV BDLF4 homolog	GGTCTCGCTGACT	17	F	10.481	6.42985E-05
9	ORF39	glycoprotein M	GGACACGCTGACG	291	R	10.561	6.89328E-05
10	ORF K14	adhesion molecule vAdh homolog	AGTCAGCCGGACC	63	F	10.576	6.98715E-05
11	ORF9	DNA polymerase homolog; EBV BALF5 homolog	GGTGAAGATGATC	376	F	10.617	7.26432E-05

Table 12 Continued

12	ORF53	Envelope glycoprotein	GGGCATGATGAAC	220	F	10.619	7.28667E-05
13	ORF66	EBV BFRF2 homolog	GGGCAGGATGACT	368	F	10.850	8.96603E-05
14	ORF42	EBV BBRF2 homolog	GCTCAGCGTCACC	215	F	10.991	0.0001017
15	ORF K5	BHV4-IE1 homolog	GGACAAGTTGTCC	324	R	11.002	0.000102699
16	ORF74	G protein coupled receptor	GGGCCAGGTGACC	181	R	11.035	0.000105992
17	ORF54	dUTPase homolog; EBV BLLF3 homolog	GGCCACCGTGGAC	22	F	11.109	0.000112846
18	ORF32	EBV BGLF1 homolog; DNA packaging	GGACGGCGTCACC	219	F	11.132	0.000114769
19	ORF60	ribonucleotide reductase, small subunit	GGTCCGGGTAACC	99	R	11.157	0.000116855
20	ORF69	BFLF2 homolog	GGTCGCAGTGGAC	186	F	11.246	0.000126064

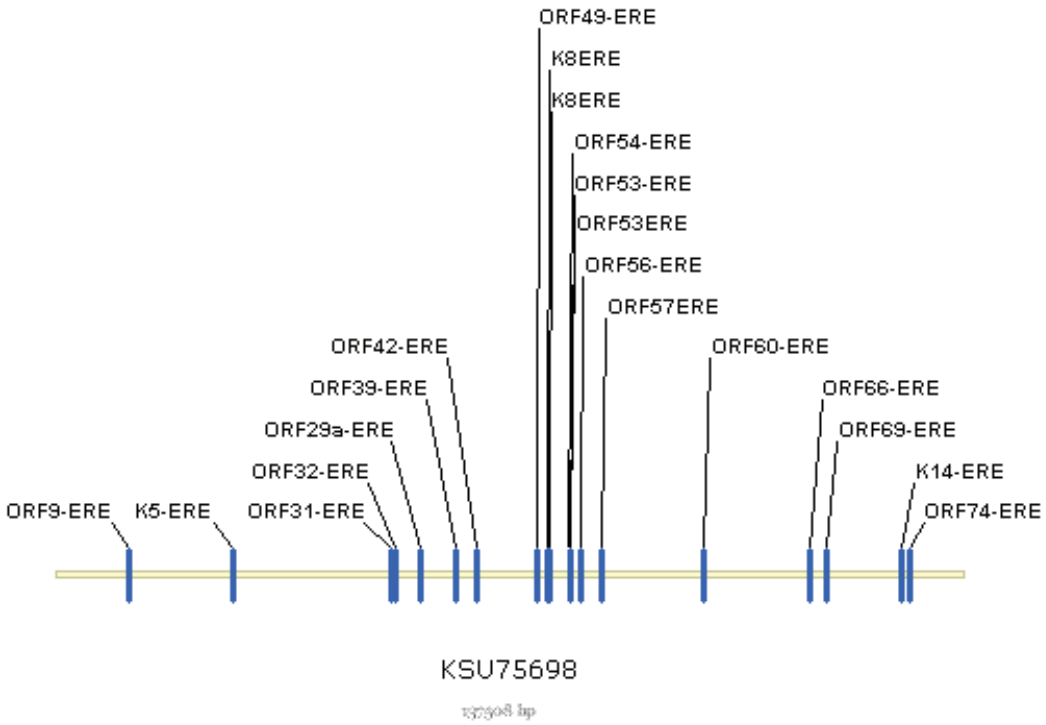

Figure 10. Distribution of Predicted Binding Sites in the HHV8 Genome.

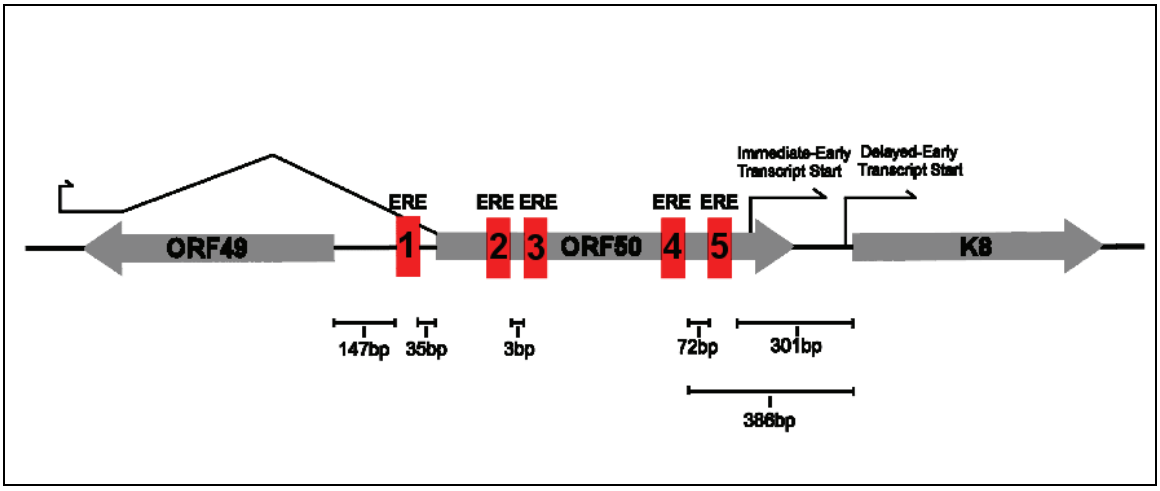

Figure 11. Cluster of Predicted ER Binding Sites in HHV8 Regulatory Region

4.3 DETERMINATION OF ER BINDING TO ERE CANDIDATE SEQUENCES

4.3.1 Electrophoretic mobility shift assay

In order to show that the sequences identified in the HHV8 genome with a high degree of similarity to the ERE consensus sequence were actually able to bind ER proteins, we performed electrophoretic mobility shift assays (EMSA). Radiolabled DNA probes were synthesized to contain candidate ERE sequences identified in the 5'-region of the K8, ORF49, and ORF74 genes. Incubation of these probes with nuclear extracts isolated from MCF7 and HCT116 cells was found to slow band migration resulting in a band shift (Figure 12; HCT116 data not shown). This observation indicates proteins in the nucleus of MCF7 and HCT116 cells were able to bind these candidate sequences in a manner similar to the Xenopus vitellogenin positive control

probe. MCF7 cells are known to express high levels of the alpha ER while HCT116 cells express primarily the beta receptor (159), therefore it is likely that both ERα and β are capable of binding each of our candidate ERE sequences.

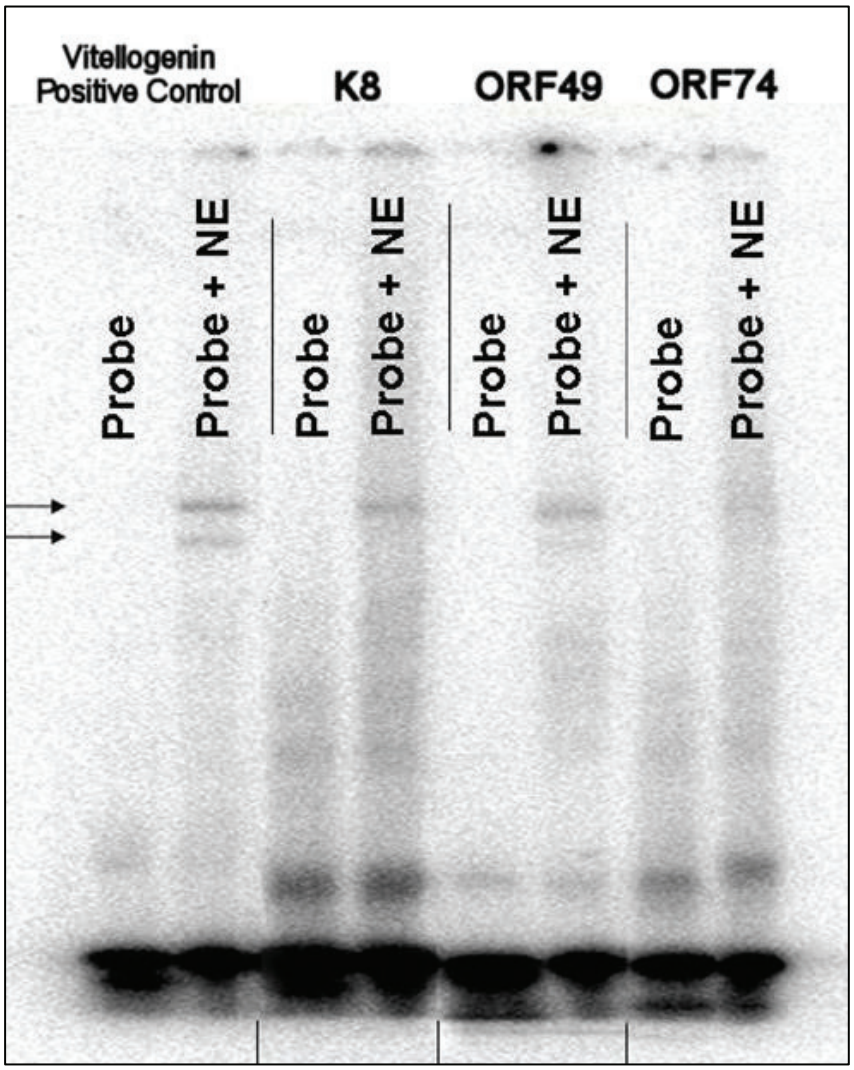

Figure 12. Results of Electrophoretic Mobility Shift Assay of Candidate ERE Sequences

In order to show that binding of proteins to probes containing ERE sequences was occurring in a specific manner and not an artifact resulting from non-specific binding, we

performed a cold competition assay. In this assay, increasing amounts of unlabeled probe were added to the binding reactions to show that the proteins bound to radiolabeled DNA probes could be competed off by the presence of a large excess of unlabeled probe. Results of the cold competition assay (figure 13) indicated that all 3 of the experimental probes and the positive control could be competed off by an excess of probe.

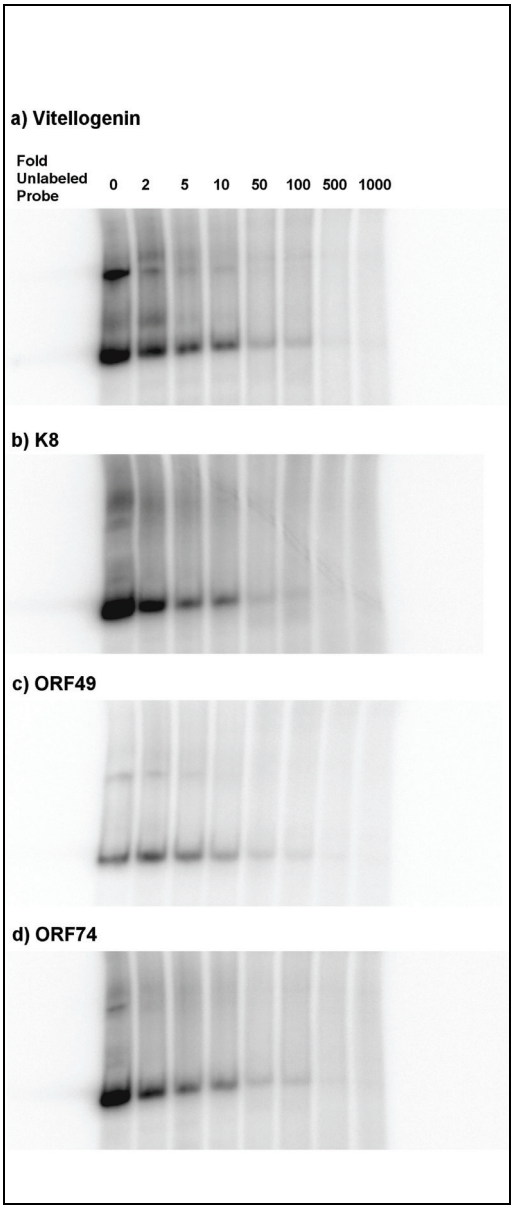

Figure 13. Cold Competition EMSA of HHV8 ERE Sequences

4.3.2 NoShift transcription factor assay

While the results of EMSA experiments strongly indicated that both estrogen receptors were capable of binding to ERE sequences located in front of the K8, ORF49 and ORF74 genes, other DNA binding proteins could have caused the observed band-shift. Therefore to confirm that the estrogen receptor is binding to our DNA probe, we utilized the NoShift transcription factor assay (Novagen), which utilizes a monoclonal antibody to the human estrogen receptor alpha protein. The results of the NoShift analysis confirmed the identity of this protein as the alpha estrogen receptor (figure 14). Our results indicated that all three experimental probes bound the estrogen receptor alpha protein at levels similar to the positive control probe include with the kit, but slightly less than the vitellogenin positive control.

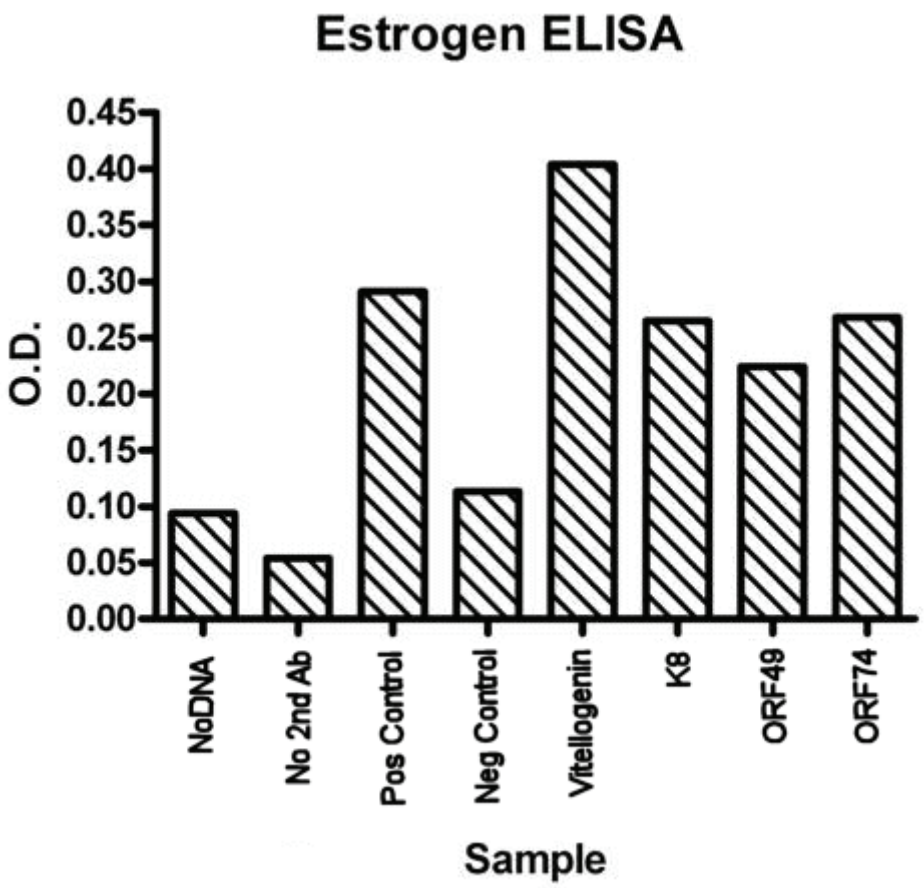

Figure 14. NoShift Transcription Factor Assay of HHV8 ERE Sequences.

4.4 QUANTIFICATION OF HHV8 PROMOTER RESPONSE TO ESTROGEN TREATMENT

4.4.1 Luciferase reporter assay

Binding of ER proteins to sequences with a high similarity to the consensus ERE was expected, however it remained unknown if binding of estrogen receptors to these ERE sequences would

modify target gene transcription in the context of the full promoter. Therefore we used a luciferase reporter assay to determine whether sequences found to contain ERE sequences were responsive to estrogens. Regions upstream of the K8, ORF49, and ORF74 genes were successfully cloned into the pGL3-basic vector. Presence and orientation of the K8 inserts were confirmed by restriction digest mapping, while the ORF49 and ORF74 inserts were confirmed by DNA sequencing. The optimal amount of plasmid for use in transient transfections was optimized by transfecting cells with serial dilutions of the pERE-tk-LUC positive control and selecting the concentration that provided the highest level of response to estrogens in the reporter assay. MCF-7 cells expressing ER-alpha were then transfected and treated with 10^{-8} M 17β-estradiol. Luciferase reporter expression was then measured and levels of cells treated with estrogen were compared to untreated cells. Estrogen treatment was found to induce a 5-fold increase in expression of our positive control and no induction of the empty pGL3-basic vector which contains no EREs (figure 15). We saw a statistically significant 2 fold increase ($p<0.0001$) in expression in the reporter plasmid containing the full length K8 promoter region (figure 15). No significant increases with estrogen treatment were observed in MCF-7 cells transfected with reporter plasmids containing the ORF49 or ORF74 EREs (figure 15). Therefore, of all three experimental constructs, the K8 promoter exclusively appears to be responsive to estrogen treatment in MCF-7 cells expressing the estrogen receptor α.

Because the K8 promoter was found to contain 2 ERE sequences in close proximity, a series of truncated promoter constructs (shown in figure 8) were created to determine which of the EREs contributed to the observed response to estrogen. We also sought to confirm that other DNA elements, such as AP-1 or SP1 sites, in the K8 promoter were not acting in an indirect mechanism to increase reporter transcription in response to estrogens. A truncated promoter

construct in which approximately 400bp of the 5'-flanking sequence was deleted upstream of the 2 identified ERE sequences (construct K8B in figure 8), showed significant response to estrogens (figure 16). This response was similar to levels observed in the full length construct, indicating that DNA elements upstream do not to contribute to the observed estrogen response. Deletion of both ERE sequences (construct K8-NoERE in figure 8) resulted in a substantial decrease in the response to estrogens (Figure 16), indicating that the region containing the 2 ERE sequences was required for estrogen response. A small, but statistically significant increase in expression was seen in this construct lacking both ERE sequences (statistical analysis shown in table 13). This indicates that DNA elements downstream of the EREs have a minor contribution to the response to estrogens as well. This minor response is likely a product of estrogen receptors acting through tethering at an AP-1 site located 27 bp downstream (position 74576-74585 in NC_003409) of the region containing ERE sequences. Deletion of the upstream ERE (construct K8ERE1 in figure 8) resulted in a similar substantial decrease in response to estrogens (Figure 8), suggesting that either the upstream ERE is primarily responsible for the estrogen response or that the presence of both ERE sites is required for the transcriptional response to estrogens. This would be consistent with the hypothesis that both EREs act together as an estrogen response unit to carryout the response to estrogens.

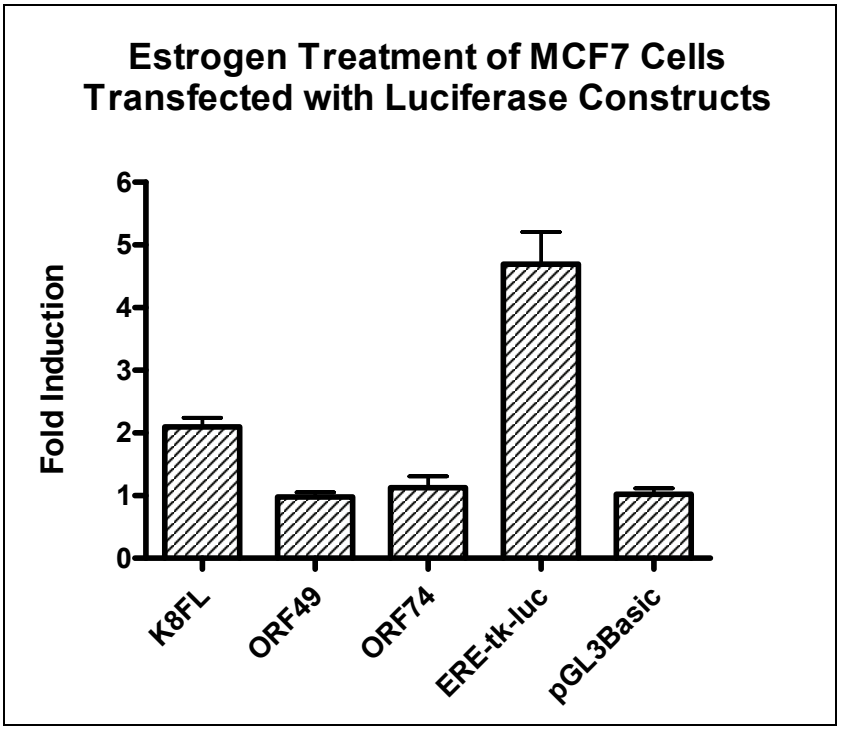

Figure 15. Results of Luciferase Reporter Assay of K8, ORF49 and ORF74 Constructs Treated with Estrogens

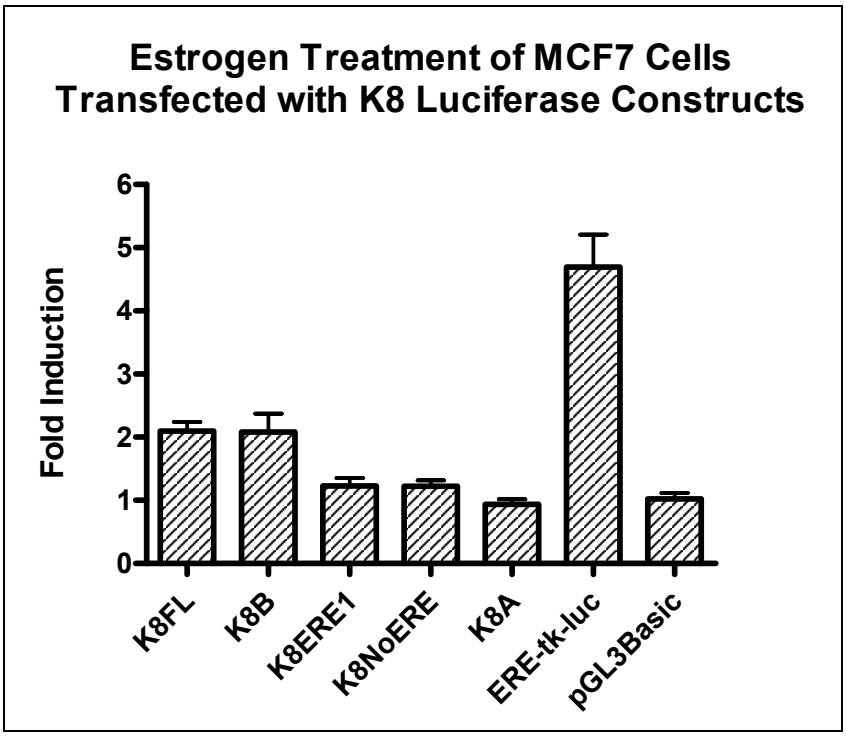

Figure 16. Results of Luciferase Reporter Assay of Truncated K8 Constructs Treated with Estrogens

Table 13. Statistical Analysis of Luciferase Response to Estrogen Treatment

Construct	Mean	Significant	p value
K8FL	2.096 [± 0.06631]	Yes	<0.0001
K8B	2.083 [±0.1184]	Yes	<0.0001
K8ERE1	1.229 [±0.05610]	Yes	0.0083
K8NoERE	1.224 [±0.04074]	Yes	0.0027
K8A	0.9357 [±0.03126]	No	0.1831
ORF49	0.9789 [±0.03229]	No	0.4388
ORF74	1.126 [±0.08346]	No	0.2973
ERE-tk-luc	4.693 [±0.2311]	Yes	<0.0001

4.4.2 Treatment of BCBL1 cells with estrogens

Our analysis using luciferase reporter constructs indicated that the K8 promoter was responsive to estradiol treatment. We therefore sought to determine whether this estrogen response was observed in the natural context of the entire viral genome, rather than a reporter construct. Human BCBL-1 cells (derived from a primary effusion lymphoma) that were latently infected with HHV8, were treated with estrogens. K8 mRNA levels were monitored using Taqman realtime PCR over the course of 16 hours. Results indicated that K8 mRNA levels did not appear to deviate from levels observed in untreated control cells (Figure 17). However, a significant increase in K8 expression was seen in cells treated with TPA, which is a known activator of K8 and HHV8 lytic gene expression. Therefore estrogen treatment does not appear to have a significant effect on HHV8 K8 expression in latently infected BCBL-1 cells.

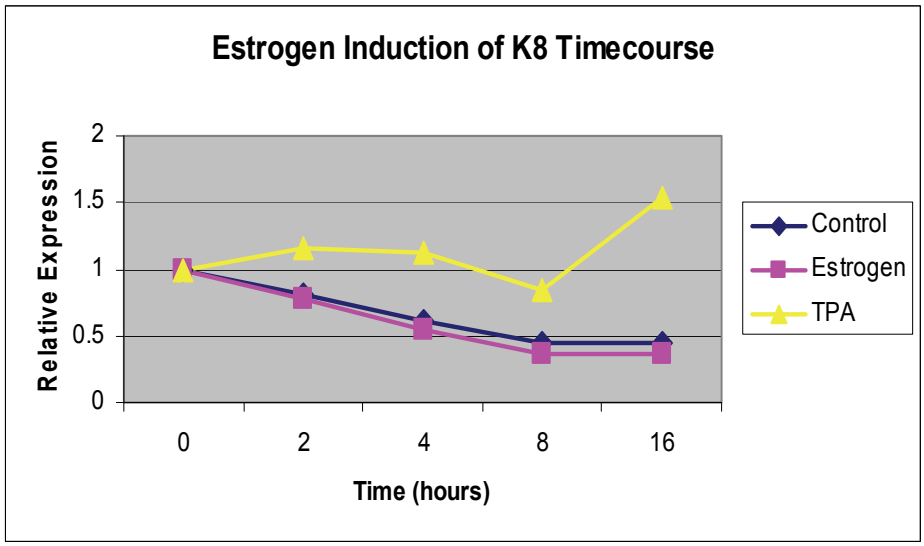

Figure 17. Effect of Estrogen Treatment on K8 mRNA Expression Levels

4.5 COMPUTATIONAL ANALYSIS OF CONSERVATION OF ERE SEQUENCES IN THE GAMMAHERPESVIRUSES

A total of 12 members of the gammaherpesvirus family listed in table 3 were computationally screened for ERE sequences. DNA sequences 750bp in length upstream of each open reading frame were searched using the computational algorithm used in our analysis of the HHV8 genome. We compared these results to the list of HHV8 genes found to have EREs present in their upstream regions. We identified several viral homologs which had ERE sequences present in multiple phylogenetically related viruses. In particular, ERE sequences were identified in 3 out of 7 ($p=0.1035$) gammaherpesviruses with a homolog of the K8 bZIP gene (Table 14 & Figure 18). We also identified ERE sequences in 6 out of 10 viruses ($p=0.0029$) with an ORF74 (IL8R-like, G-protein coupled receptor) homolog (Table 15 & Figure 19). In the case of *herpesvirus saimiri* (HVS), the pirating of a high activity thymidylate synthase gene has resulted in a highly AT-biased genome (160) and only a single ERE sequence was located in the entire genome. Intriguingly, this sequence was located just inside of the HVS ORF 74 coding region, in the promoter region of the ORF73/LANA gene, which has been shown in HVS to repress lytic reactivation by repressing ORF50 expression, similar to the activity of HHV8 K8 (161).

Unexpectedly, we identified a highly conserved ERE in the upstream region of 8/11 ($p<0.0001$) gammaherpesviruses with a homolog of the glycoprotein M (gM) gene (Table 16 & Figure 20). In all cases, these conserved EREs did not appear to be present due to conserved amino acid sequence of an upstream gene.

Table 14. Estrogen Response Elements Upstream of K8 (bZIP) Homologs

Virus	Gene	ERE Found	Sequence
HHV8	K8	Yes	GGTCAGGGCGACC GGGGAGGGTGACC
EBV	BZLF1	Yes	GGACGAACTGACC
RRV	bZIP	No	-----
AhV1	A6	Yes	GGTCACGGGGTTC
CalHV3	ORF43	No	-----
CerHV15	BZLF1	No	-----
OHV2	Ov6	No	-----

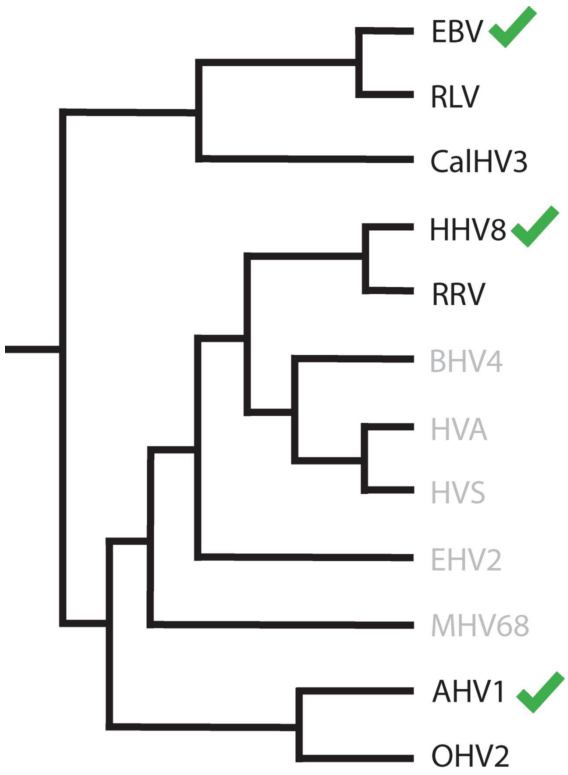

Figure 18. Phylogenetic Tree Illustration of Gammaherpesviruses with Conserved ERE Sequences in Front of K8 Homologs

Table 15. Estrogen Response Elements Upstream of ORF74/GPCR Homologs

Virus	Gene	ERE Found	Sequence
HHV8	ORF74	Yes	GGGCCAGGTGACC
EBV	BILF1	No	-----
RRV	ORF74	Yes	GGTCCAGTTGACC
MHV68	ORF74	Yes	GGCCAAGACAACC
AHV1	A5	No	-----
CalHV3	ORF6/BILF1	Yes	GGTTAAGGTGACA
CerHV15	BILF1	No	-----
HVA	ORF74	No	-----
OHV2	Ov5	Yes	GGGCATCCTGTCC
EHV2	ORF74/E1/E6	Yes/Yes/No	GGGCAGGCTGACC GGTTAGTGTGTCC
HVS	ORF74	No	-----

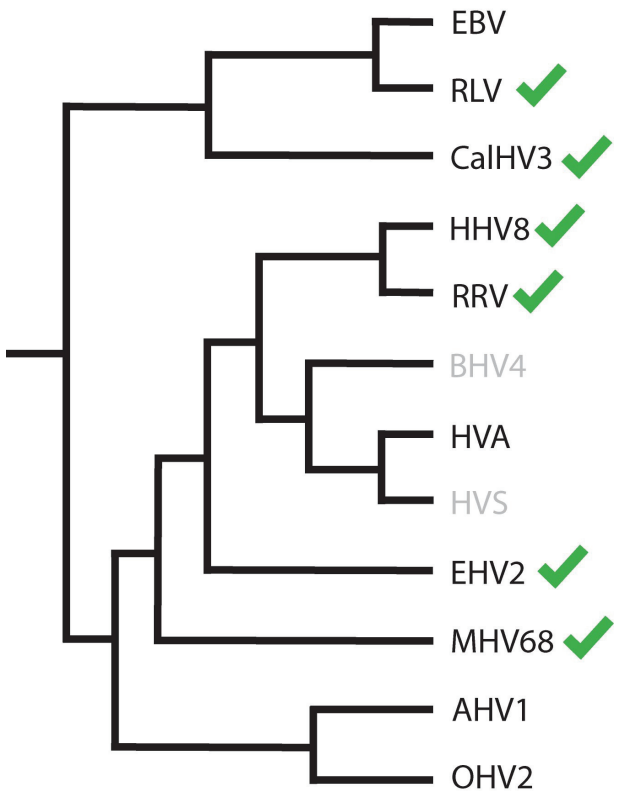

Figure 19. Phylogenetic Tree Illustration of Gammaherpesviruses with Conserved EREs in Front of ORF74 Homologs

Table 16. Estrogen Response Elements Upstream of Glycoprotein M Homologs

Virus	Gene	ERE Found	Sequence
HHV8	ORF39	Yes	GGCGACGGTGACC GGACACGCTGACG GGTCTGGGTGTTC
EBV	BBRF3	Yes	GGGCATCGTGCCC GGTCAACGCGCCC
RRV	ORF39	No	-----
MHV68	ORF39	Yes	GGACAGTCTGACC
AHV1	ORF39	Yes	GGGCAACCTGGCC
BHV4	ORF39	Yes	GGTCATGGTGGAT
CalHV3	ORF39	Yes	GGTCATGCTGTCC
CerHV15	BBRF3	Yes	GGGCATCGTGCCC
HVA	ORF39	No	-----
OHV2	ORF39	No	-----
EHV2	ORF39	Yes	GGTCAGGGTGACT

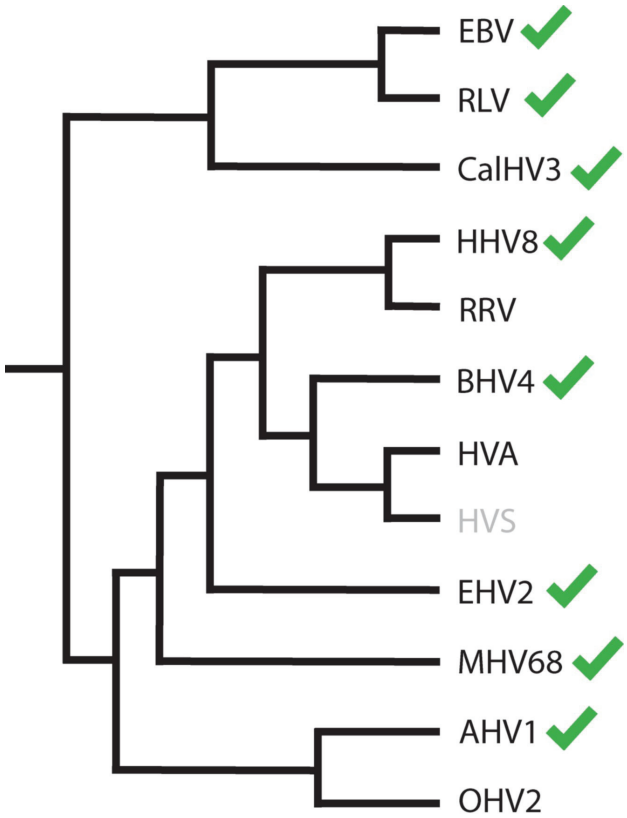

Figure 20. Phylogenetic Tree Illustration of Gammaherpesviruses with Conserved ERE Sequences in front of Glycoprotein M Homologs

5.0 DISCUSSION

5.1 RATIONALE

Prostate cancer is a complex disease with both genetic and environmental factors. The Caribbean nation of Tobago has one of the highest reported screening-detected prevalence of prostate cancer in the world (3). The unique genetic ancestry of Tobago makes it an ideal population for investigating genetic risk factors for prostate cancer. Here we report the discovery of a novel interaction between specific human genetic variation and viral infection that is associated with an increased risk of prostate cancer. To further characterize this interaction, we have performed a detailed functional analysis and characterized a previously unreported biological interaction between these associated factors.

A number of risk factors have been proposed to be important contributors in the etiology of prostate cancer. The growth and development of the prostate gland have been shown to be strongly regulated by the action of steroid hormones (14). Abnormal levels of these hormones have been shown to have profound effects on the prostate and influence on the risk of prostate cancer later in life (16). However, circulating levels of these hormones are unlikely to be responsible for differences in prostate cancer risk (15,19). Pathogens have also been hypothesized to be a risk factor for prostate cancer. Associations between certain types of risky

sexual behavior and increased prostate cancer risk suggest that sexually transmitted diseases (STDs) may play an important role (81).

Several recent reports have found associations between prostate cancer risk and genetic loci involved in immune response to pathogens (162,163). These genes, the macrophage scavenger receptor (MSR1) and ribonuclease L (RNASEL), play various important roles in mediating the immune response to pathogens (164,165). However our recent investigation of the involvement of these loci in prostate cancer risk in the Tobago population failed to find a significant association with prostate cancer (166)(Shea unpublished). Further, the specific mechanism by which these genes contribute to prostate cancer risk has yet to be described. Therefore, while genes involved in immune response appear to be important factors in prostate cancer pathogenesis, variation in the MSR1 and RNASEL genes does not appear to contribute significantly to the increased risk observed in Tobago.

We therefore sought to investigate whether genetic variation in steroid hormone receptors contributed to prostate cancer risk independently or through gene x environment interactions with pathogen infection. Results of our preliminary analysis identified statistically significant associations between the estrogen receptor XbaI polymorphism with a modestly increased risk of prostate cancer (OR=2.48 [95%CI: 1.42-4.34]). Serological evidence of HHV8 infection was also found to be a risk factor for prostate cancer (OR=1.80 [95%CI: 1.28-2.52]). Additionally we identified several previously unreported gene x environment interactions. Individuals who carried at least one mutant estrogen receptor allele and were seropositive for HHV8 infection were found to be at over 3-fold increased risk of prostate cancer (OR= 3.10 [95%CI: 1.42-6.77]. This level of risk was greater than the sum of the individual factors combined, indicating interaction between these factors. A similar interaction was found in HHV8 seropositive men

who carried at least one short AR allele (OR=2.46 [95%CI: 1.13-5.35]). Finally a third significant interaction was observed in men who carried at least one mutant ER allele, one or more short AR alleles, and were seropositive for HHV8 infection (OR= 9.5 [95% CI: 2.58-35.02]). Therefore our finding strongly suggests that an interaction exists between sex hormone receptors and HHV8 infection and that this interaction is an important factor in prostate cancer risk.

While these findings suggest an intriguing link between these factors, the underlying mechanism linking these factors together is unclear. However, several observations regarding the epidemiology of Kaposi's sarcoma provide intriguing evidence for a role of hormones in HHV8 biology. Despite similar levels of HHV8 infection among men and women, men are at a substantially higher risk of developing KS (4). The underlying mechanism explaining this sex bias has yet to be determined, however sex hormones have been hypothesized to play a role. Estrogens are the primary female sex hormone and regulate the expression of a wide number of genes throughout the genome through several distinct mechanisms. One of these mechanisms, called the classical pathway, functions through the binding of estrogens to a hormone receptor protein which translocates to the nucleus and binds to specific DNA regulatory elements located near target genes (26). Binding of the hormone:receptor complex influences mRNA transcription of the target gene by interacting with basal transcription machinery or through the recruitment of other secondary transcription factors (26).

These observations led us to hypothesize that estrogens may act directly on the HHV8 genome to alter gene expression. We therefore sought to identify whether estrogen receptor binding sequences exist in the HHV8 genome. To accomplish this goal, we used a computational approach to search the HHV8 genome for sequences with a high degree of

similarity to the human estrogen receptor consensus binding sequence. We further performed a functional analysis to determine whether ERE sequences identified in the HHV8 genome were able bind estrogen receptor proteins and could modify gene expression in response to treatment with estrogens.

5.2 FINDINGS

5.2.1 Computational analysis

Our computational search of the HHV8 genome identified a total of 20 sequences with a high degree of similarity to the consensus estrogen response elements sequence. The cutoff scores for our algorithm were set to predict an estimate of 1 false positive for every 10,000 base pairs of sequences searched, therefore a total of less than 4.5 false positives was expected. This identification of >4 fold more high scoring sequences than expected indicates that the HHV8 genome is enriched for ERE-like sequences.

Of these 20 sequences with similarity to the ERE consensus sequence, 3 were identified in a cluster of genes with important regulatory roles in controlling latent/lytic gene expression from the HHV8 genome. An expanded search of this region identified an additional 2 sequences inside the ORF50 open reading frame. This cluster contains 2 genes, ORF49 and ORF50/Rta, which are known transcriptional activators of lytic gene expression (158,167). A third gene located within this cluster is the K8 or bZIP gene. K8 has been shown to act as an inhibitor of ORF50 and represses its ability to activate lytic genes (156). The 2 highest scoring ERE sequences identified in our computational analysis were located upstream of the K8 gene, 314bp

and 399bp upstream of the ATG in a region previously described to contain transcription factor binding sites and act as a distal promoter that controls K8 expression levels (153). Each of the 3 genes in this cluster play important regulatory roles in controlling HHV8 gene expression and the switch from latency to the lytic phase, therefore we selected ERE-like sequences found in this region for further functional analysis.

An additional ERE chosen for further analysis was located in front of the ORF74 gene. ORF74 encodes a G-protein coupled receptor with homology to the human interleukin 8 receptor (IL8R) (124). ORF74 has been shown bind several different cytokines and has a constitutive level of signaling that is unaffected by normal IL8R negative feedback pathways (125). ORF74 has also been shown to act as an oncogene that can transform mouse fibroblasts and induce tumors in nude mice (126). In particular endothelial cells expressing ORF74 take on a spindle cell-like conformation that resembles the classical cell type observed in KS lesions (127). ORF74 expression also activates expression of several angiogenic factors such as VEGF (126,168). The clear role of ORF74 in oncogenesis and the morphological changes resulting from its expression make it an interesting candidate for further analysis of whether it is a target of estrogen regulation.

5.2.2 Binding experiments

Electrophoretic mobility shift assay of DNA probes containing the ERE-like sequences found in front of the K8, ORF49, and ORF74 genes indicated that each of these candidate sequences were bound by proteins from MCF7 nuclear extracts. NoShift assay further confirmed the protein binding to these probes was the estrogen receptor alpha. Therefore we can conclude that each of these 3 ERE-like sequences are bound by the human estrogen receptor alpha. A similar band

shift was observed when nuclear extracts from HCT116 cells was used in gel-shift assays, therefore it is likely that both the alpha and beta estrogen receptors bind these sequences. Cold competition assay of our candidate sequences indicated that binding of estrogen receptor to these probes was specific and not a result of artifact.

5.2.3 Reporter assays

Binding of estrogen receptor proteins to sequences with a high degree of similarity to the consensus ERE sequences was largely expected. However, whether these sequences were capable of influencing mRNA expression of a downstream gene was unclear. Therefore we performed a series of reporter assays to further assess whether these sequences were capable of acting as true EREs in the context of the full viral gene promoter.

Results from our luciferase reporter assays indicated that in MCF7 cells expressing the ER-alpha protein, treatment with physiologic levels of estradiol resulted in a significantly increased expression of a luciferase reporter gene under the control of the K8 promoter ($p<0.0001$). We observed a greater than 2 fold increase in reporter expression with the full length K8 promoter. Analysis of reporter constructs containing the ORF49 and ORF74 upstream regions did not show any significant change in reporter expression when treated with estrogens. However, the ORF49 and ORF74 sequences used in our reporter plasmids are 5'-regions upstream of the ATG start site and may not necessarily represent a true gene promoter. In the case of the ORF74 gene, the major mRNA species are transcribed as either a bicistronic mRNA initiated upstream of the K14 locus or as a monocistronic transcript initiated upstream of our ERE sequence. Therefore, the luciferase reporter plasmid used in our experiments may lack the natural promoter elements required for initiating transcription and may be unsuitable for acting

as a promoter by itself. These ERE sequences could still act as an enhancer and could influence transcription at true ORF74 promoter in response to estrogens. Either incorporating a larger portion of the 5'-region into our reporter plasmid or examining ORF49 and ORF74 mRNA expression in the full context of the virus would be necessary to answer this question.

Our computational analysis identified 2 high scoring ERE sequences upstream of the K8 gene, therefore we sought to determine which of these 2 sequences was responsible for the observed response to estrogens. Promoter constructs containing truncated portions of the K8 promoter were created and transfected into MCF-7 cells. We observed a similar response to estrogens in a construct in which most of the region 5' of the ERE sequences was removed indicating that this upstream flanking region does not appear to be responsible for estrogen response. A dramatic decrease in estrogen response was observed when the region containing both ERE sequences was deleted. Therefore the region containing these ERE sequences is essential for response to estrogens. Deletion of the lower scoring upstream ERE sequence also resulted in a loss of estrogen response, indicting that either this ERE or the presence of both ERE sequences is necessary for mediating the response to estrogens.

In order to test whether the ERE sequences identified in front of the K8, ORF49, and ORF74 genes could be induced by cell types expressing the beta estrogen receptor, we repeated our analysis using several cell types expressing ER-beta. Both the colon cancer line HCT116 and CV-1 cells transfected with a ER-beta expression construct were tested, however we did not observe significant reporter expression from any of our reporter constructs, including the positive control plasmid. Because of this we were unable to asses whether ER-beta was able to influence transcription levels of our reporter plasmid.

5.2.4 Estrogen treatment of cells latently infected with HHV8

EMSA analysis using nuclear extracts isolated from HCT116 cells expressing the beta estrogen receptor showed positive band shifts on all of our experimental probes, suggesting binding of ERbeta to these ERE sequences. Therefore we sought to determine if estrogens could influence expression of these genes in cells latently infected with HHV8. For our analysis we used the BCBL-1 cell line which is derived from a primary effusion lymphoma. After treating BCBL-1 cultures with estrogens, we monitored changes in K8 mRNA levels using Taqman Realtime PCR. Our analysis indicated that K8 mRNA levels did not significantly deviate from levels seen in untreated cultures, while TPA strongly induced K8 transcription. Therefore estrogens do not appear to influence K8 levels in BCBL-1 cells. BCBL-1 cells are believed to have originated from a post-germinal center b-cell lineage (169). B-cell populations are known to express high levels of the beta estrogen receptor and comparatively little alpha estrogen receptor (170), therefore our findings indicate that estrogen treatment has little effect on K8 mRNA expression in cell types expressing the beta estrogen receptor. Intriguingly a number of cell types found to support latent infection of HHV8, such as prostate epithelium (89) and b-cells predominantly express the beta estrogen receptor with little or no estrogen receptor alpha expression (170).

5.2.5 Phylogenetic analysis

The presence of estrogen response elements and the use of estrogen signaling pathways by a diverse variety of viral pathogens led us to speculate whether the presence of estrogen response element may be a common to other members of the gammaherpesvirus family. The presence of conserved ERE sequences in other gammaherpesviruses would further confirm that these

sequences are an important part of gammaherpesvirus biology. Therefore we expanded our computational analysis to include all other gammaherpesviruses with fully annotated genome reference sequences. Results of our analysis confirmed the presence of ERE sequences in other gammaherpesviruses, including the discovery of a fully consensus ERE sequence in the Rhesus Rhadinovirus ORF57/Mta promoter. Our analysis also confirmed that ERE sequences were conserved in homologs of the HHV8 K8 and ORF74 genes, although only ORF74 reached statistical significance. However, the presence of 2 ERE sequences in close proximity upstream of the HHV8 and EBV bZIP genes suggests that these sequences may play an important role in human gammaherpesviruses. We further found that ERE sequences were highly conserved in the upstream region of glycoprotein M homologs in the gammaherpesvirus, though the biological significance of this finding is unclear. Glycoprotein M (gM) encodes a structural protein that localizes to the virion (171). The gM protein is known to complex with gN and has been hypothesized to play a role in viral entry and egress (171). The identification of ERE sequences upstream of gM could be confounded by underlying amino acid conservation in the gene located upstream, a helicase/primase conserved among the herpesviruses (UL8 homologs). However the position of these EREs in the gammaherpesvirus UL8 homolog proteins is highly variable, making that unlikely. Overall our computational search successfully identified the widespread presence of ERE sequences in diverse members of the gammaherpesvirus family. Therefore these results provide strong evidence of a biological importance of EREs in the gammaherpesviruses and provide strong corroborating evidence that our initial findings in HHV8 are unlikely to be explained by chance alone.

5.3 MODEL

Our findings indicate that the HHV8 genome is enriched for sequences resembling the estrogen receptor consensus binding sequence. We have shown that these sequences are capable of binding estrogen receptor proteins *in vitro*. Further we have shown that K8 promoter is responsive to treatment with estrogens and that the K8 promoter region containing high scoring ERE sequences is essential for the response to estrogens. Our functional analysis has been limited to cell types expressing the alpha estrogen receptor, however treatment of BCBL-1 cells with estrogens did not appear to have an effect on K8 mRNA levels. Therefore we conclude that estrogens do not appear to directly change K8 mRNA expression in latently infected b-cells, which are the primary cell type harboring HHV8 DNA in non-KS individuals.

Extraneous DNA sequences are unlikely to be maintained in viral genomes due to their comparatively rapid rate of genome evolution (172) and the physical constraints imposed on the viral genome due to limited the space inside the viral capsid. Therefore the presence of estrogen response elements suggests that these sequences are utilized by the virus. This role is unlikely to involve primary infection, as men and women show similar levels of HHV8 seroprevalence and any DNA element that inhibited viral infection would likely be lost rapidly to natural selection.

However the striking sex bias in the incidence of KS in men and women may be a result of the effect of estrogens. The cell type characteristically seen in KS lesions is the spindle cell (95). Analyses of cell surface markers on HHV8-infected spindle cells indicate that they are of an endothelial origin (173), a cell type known to express both the alpha and beta estrogen receptors (30), so in women the presence of higher levels of circulating estrogens may act to increase K8 expression. This increased level of K8 expression may have effect on the ability of HHV8 to establish an initial latent infection in endothelial cells or may act to suppress local

spreading of HHV8 infection to other endothelial cells once an initial endothelial cell is infected (Figure 21). Populations of spindle cells present in early KS lesions are known not to be clonally derived therefore KS lesions likely require the infection of significant numbers of endothelial cells. Reducing the transfer of HHV8 from b-cell populations to endothelial cells may decrease the likelihood of transforming endothelial cells into spindle cells, characteristic of KS. Therefore estrogens may act to reduce the number of endothelial cells infected with HHV8, resulting in the decreased KS incidence in women.

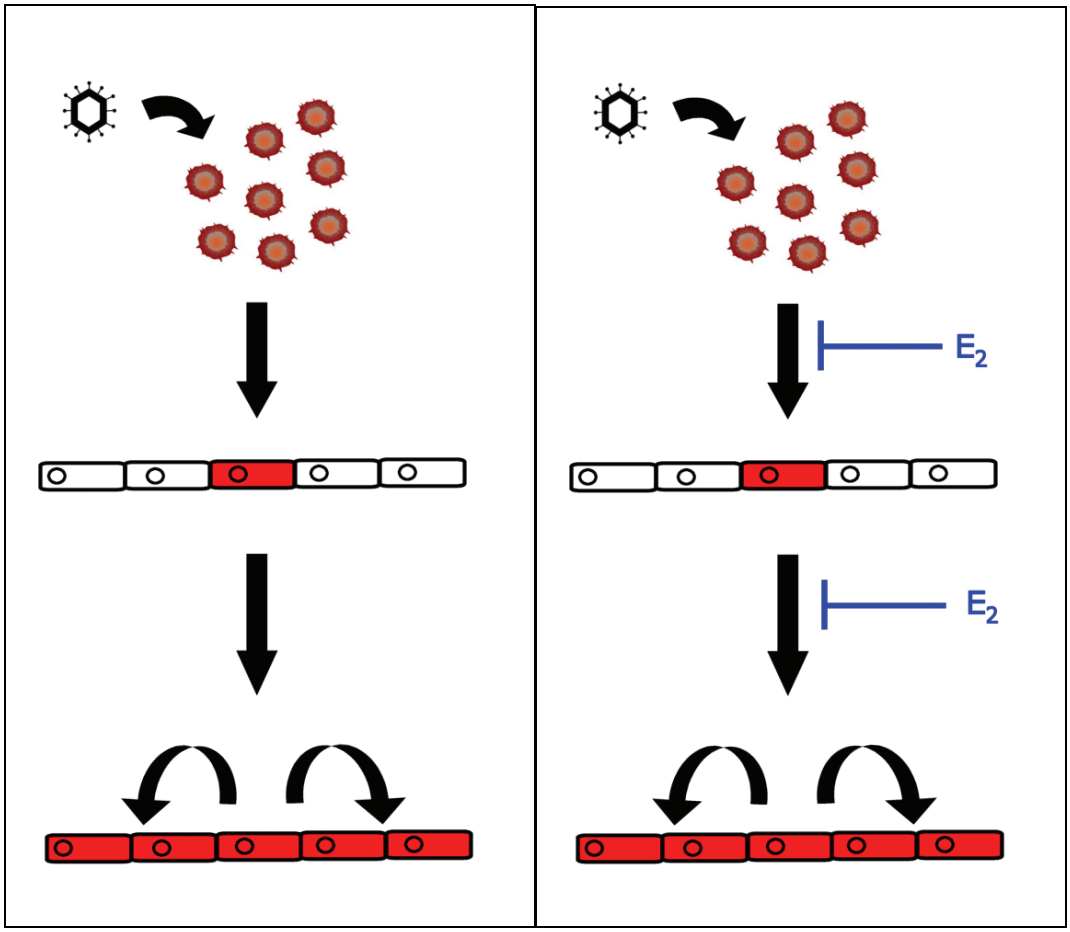

Figure 21. Model of Estrogen Action on HHV8 Pathogenesis

Illustration of proposed model of the effect of estrogens on KS progression. A) HHV8 establishes an initial primary infection and sets up a latent infection in ERα-/ERβ+ b-cells. HHV8 is then transmitted to ERα+/ERβ+ endothelial cells which then experience a local spreading of virus to nearby endothelial cells. B) Blue arrows indicating points where estrogen activation of K8 could repress ORF50 activation and prevent either initial infection of endothelial cells or local spreading.

The role of K8 as an inhibitor of ORF50 also suggests that estrogens may act to limit reactivation of HHV8-infected b-cells. In culture, HHV8-infected b-cells exhibit a "leaky" form

of latency where a small proportion of cells are undergoing spontaneously reactivation (95). The presence of significant levels of circulating estrogens may act to suppress the ability of these cells to reactivate in response to triggers such as TPA or ORF50 expression.

The presence of a DNA element that acted to suppress viral infection of reactivation would generally be selected against by natural selection, as mutant viruses lacking this sequence would replicate and spread more effectively than a wildtype virus containing the suppressive ERE. However, if this element was important part of viral biology that gave it a selective advantage, the element could be retained through balancing selection. Widespread viral infection to all cell types in the host would likely result in either host death or a massive host immune response to viral antigens. Observations of cell types natural infected by herpesviruses include a variety of glandular cells such as prostate (89) and oral epithelia (174). Infection of glandular tissue provides an effective location to allow transmission of virus from an infected host by bodily fluids such as saliva and semen. Intriguingly, these cell types express primarily the beta estrogen receptor (44,175), as do B-cells (170). Therefore the alpha receptor may act to suppress infection in certain cell types in order to limit viral infection to those preferred by the virus for efficient spreading. Therefore limited spreading to preferred cell types would reduce the likelihood of triggering an immune response to HHV8 antigens. Therefore estrogens may play a role in viral trophism.

K8 also has been shown to physically interact with the HHV8 lytic origin of replication (157). Increased levels of K8 expression resulting from estrogens may also influence of the rate of HHV8 viral DNA replication. This proposed role has previously been observed in the estrogen response element in the late leader sequence of the BK virus (176). In the BK virus, estrogens were observed to increase the efficiency of viral particle production by increasing the

expression of a late structural protein involved in capsid formation (176). This observation may also provide a plausible basis for ERE sequences found upstream of structural proteins in the HHV8 genome and may warrant further investigation of these genes.

5.4 ROLE OF HHV8 IN PROSTATE CANCER

The results of our functional analysis failed to identify estrogen regulation of any HHV8 genes with known oncogenic potential, such as ORF74. However, the low levels of estrogens in men would reduce the activation of K8 which would likely ease repression of ORF50. This mechanism may allow local spreading of HHV8 into the prostate. The establishment of a chronic infection in the prostate by HHV8 could trigger inflammatory responses which have been proposed to be a critical part of prostate cancer pathogenesis (177).

Further, our functional analysis has been limited to a small subset of candidate ERE sequences identified in the HHV8 genome. At least 15 other HHV8 genes are potential targets of estrogen regulation which remain untested. Further analysis of these genes as targets of estrogen regulation may reveal the involvement of other HHV8 genes in KS or prostate cancer pathogenesis.

While our findings provide a mechanism linking the estrogen receptor alpha to prostate cancer, the role of the XbaI polymorphism is unclear. Despite its association with a variety of estrogen-related diseases, to date no functional role has been attributed to this variant. Linkage to other variants with true functional roles could explain its effect on prostate cancer. One caveat of our analysis is that use of odds ratios when assessing interaction between common factors

could result in an inflated estimate of the true risk ratios (178). This effect could be reduced by the use of relative risks, however the case-control nature of our study prevents that.

5.5 EXAMPLES OF VIRAL INTERACTION WITH HORMONES IN THE LITERATURE

Various examples of interaction between viruses and steroid hormones have previously been reported and provide evidence that this interaction may be a commonly utilized pathway. One of the earliest characterized hormone response elements was found in the Mouse Mammary Tumor Virus (MMTV). Archer et al reported the presence of a functional glutocorticoid response element (GRE) in the MMTV genome which mediated a strong increase in MMTV RNA transcription when infected cells were treated with glucocorticoids such as dexamethasone and hydrocortisone (179).

More recently, several reports have found interactions between the estrogen receptor and hepatitis viruses. Deng et al reported individuals infected with hepatitis B virus (HBV) who carried the estrogen receptor PvuII polymorphism were at an increased risk of chronic HBV infection (180) and Zhai et al reported a similar interaction associated with risk of hepatocellular carcinoma (181). This association was found to have a plausible biological basis when the human estrogen receptor alpha was shown to physically interact with the hepatitis C virus (HCV) RNA polymerase protein NS5B (182). It was further reported that the estrogen antagonist tamoxifen was able to suppress HCV genome replication (182).

These examples clearly show the widespread utilization of estrogen signaling pathways by evolutionarily unrelated viruses in which each virus has co-opted the pathway for its own

uses. In many cases this interaction is limited to physical binding of viral proteins to the estrogen receptor, however classical estrogen signaling pathways involving the presence of ERE sequences upstream of viral genes have also been described as well. An example of this is the ERE described in the late leader region of the BK virus (176).

While the incidence of KS in women is known to be exceedingly rare, cases of HIV- women diagnosed with KS have been reported. This observation leads us to question what factors contribute to KS progression in this small minority of women. In an intriguing report by Schofer et al, they found that in a small sample of 7 women diagnosed with KS (4 HIV+; 3 HIV-), all 7 had abnormally low serum estradiol levels compared to controls (183). While a small sample size precluded these findings from reaching significance, these results provide tantalizing evidence for a protective effect of estrogens in KS progression.

6.0 CONCLUSION

Our analysis of the HHV8 genome has revealed the presence of several sequences that highly resemble the consensus estrogen receptor binding sequence. We have shown that ERE-like sequences located in front of the K8, ORF49 and ORF74 genes are capable of binding estrogen receptor proteins. Further our analysis indicates that the K8 promoter is responsive to physiologic levels of estrogen and that this response is carried out by a segment of the K8 promoter containing the 2 highest scoring sequences in our analysis. We therefore conclude that in cell types expressing estrogen receptor alpha, that estrogens regulate K8 expression outside of the normal viral program. These findings may provide a mechanism to explain the observed sex-bias in KS incidence and provide a plausible biologic foundation for the interaction of estrogen receptor polymorphisms with HHV8 seropositivity in the risk of prostate cancer. We have also expanded our analysis and discovered the presence of ERE-like sequences in other members of the gammaherpesvirus family, suggesting that this signaling pathway may be utilized by other evolutionarily related viruses.

Here we describe potential insights into the pathobiology of two human neoplasias, prostate cancer and Kaposi's sarcoma. Both of these diseases have significant impacts on public health in the United States and world-wide. In the United States, prostate cancer is the most commonly diagnosed non-skin related cancer and the second leading cause of cancer-related mortality in men. While Kaposi's sarcoma is rarely observed in healthy individuals, it is a

common occurrence in HIV positive men and has a much higher incidence in Mediterranean areas and in parts of sub-Saharan Africa. We believe our findings may provide insights into the underlying biology of Kaposi's sarcoma and may help to explain the increased incidence of prostate cancer observed in Tobago.

APPENDIX

DOCUMENTATION OF INSTITUTIONAL REVIEW BOARD APPROVAL

University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Ground Level
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)

MEMORANDUM

TO: Clareann Bunker, PhD

FROM: Victor Vogel, MD, Vice Chair Victor G. Vogel, MD

DATE: December 11, 2007

SUBJECT: IRB #980722: Molecular Epidemiology of Prostate Cancer in Tobagonians

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Your renewal was reviewed by the Institutional Review Board and approved at the Full Board Meeting (Committee E) held on Wednesday, December 5, 2007.

Please include the following information in the upper right-hand corner of all pages of the consent form:

Approval Date: December 5, 2007
Renewal Date: December 4, 2008
University of Pittsburgh
Institutional Review Board
IRB #980722

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1504.

The protocol and consent forms, along with a brief progress report must be resubmitted at least **one month prior** to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

If this research study is subject to FDA regulation, please forward to the IRB all correspondence from the FDA regarding the conduct of this study.

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

VV:dj

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