QUANTITATION OF HUMAN HERPESVIRUS 8 LOAD AND
FUNCTIONAL ANALYSIS OF THE VIRAL THYMIDINE KINASE

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ABSTRACT

Human herpesvirus 8 (HHV-8) is the latest member of the Herpesviridae to be described, following its discovery in Kaposi’s sarcoma tissue from AIDS patients, in 1994, by Chang et al. using representational difference analysis (RDA).

Qualitative and quantitative-competitive PCR (QCPGR) methods were developed for the detection and quantification of HHV-8 DNA in patient samples. In a study looking at various post-mortem tissues of AIDS patients 32/153 (21%) tissues were found to be positive for HHV-8, whereas 0/47 control tissues were positive. HHV-8 viral loads were shown to vary from <10^1 to 10^5.6 genome copies/µg DNA. There was a significant difference in the viral loads between patients with KS and patients without KS, however there was no significant difference in HHV-8 load in tissue samples when correlated with HIV proviral DNA presence or load.

In order to gain further insight into the molecular mechanisms that govern the antiviral susceptibility of HHV-8, functional studies of the HHV-8 thymidine kinase (TK) homologue were performed. The recombinant HHV-8 TK was shown to be functional for the phosphorylation of deoxythymidine. Sequence homology of the HHV-8 TK with the HSV TK identified three amino acid residues which may be of importance to the TK function, that were targeted for mutagenesis. These mutants were expressed and shown to have limited or no thymidine kinase activity.

The affinity of the HHV-8 TK for known antiviral drugs was examined. Inhibition studies demonstrated that the anti-herpesvirus drugs GCV and ACV were unable to inhibit phosphorylation of dT by HHV-8 TK, whereas the anti-HIV drugs AZT and d4T, and the nucleoside analogue BrdU were competitive inhibitors of dT phosphorylation. In addition, AZT and d4T were shown to be phosphorylated by the HHV-8 TK, however GCV was not. In conclusion, established anti-herpetic agents are extremely poor substrates for the HHV-8 TK, although agents used for the treatment of HIV infection both inhibit the HHV-8 TK and are phosphorylated by its action.
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Ultimately I would like to thank my family for believing in me, and for their continual motivation and support in whatever I have chosen to do throughout my life, including this work.
ABBREVIATIONS

AIDS acquired immunodeficiency syndrome
ACV aciclovir
APS ammonium persulphate
ATP adenosine triphosphate
AZT azidodeoxythymidine
bp base-pairs
BSA bovine serum albumin
B-cell bursa of Fabricius
BrdU bromodeoxyuridine
Cdk2 cyclin dependant kinase-2
CD cell differentiation antigen
CDV Cidofovir
CIP calf intestinal phosphatase
cm centimetres
CNS central nervous system
CPE cytopathic effect
cpm counts per minute
CSF cerebrospinal fluid
DNA deoxyribonucleic acid
DNase deoxyribonuclease
DR direct repeat
ddATP dideoxyadenosine triphosphate
ddCTP dideoxycytosine triphosphate
ddGTP dideoxyguanosine triphosphate
<table>
<thead>
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<tr>
<td>ddTTP</td>
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</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>dT</td>
<td>deoxythymidine</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>d4T</td>
<td>didehydrodeoxythymidine</td>
</tr>
<tr>
<td>EBNA</td>
<td>Epstein-Barr nuclear antigen</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EK</td>
<td>enterokinase</td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii</td>
</tr>
<tr>
<td>EHV</td>
<td>equine herpesvirus</td>
</tr>
<tr>
<td>ES</td>
<td>exanthem subitum</td>
</tr>
<tr>
<td>g</td>
<td>relative gravitational force</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GCV</td>
<td>ganciclovir</td>
</tr>
<tr>
<td>gp</td>
<td>glycoprotein</td>
</tr>
<tr>
<td>hrs</td>
<td>hours</td>
</tr>
<tr>
<td>HCMV</td>
<td>human cytomegalovirus</td>
</tr>
<tr>
<td>HHV-6</td>
<td>human herpesvirus 6</td>
</tr>
<tr>
<td>HHV-7</td>
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</tr>
<tr>
<td>HHV-8</td>
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</tr>
<tr>
<td>HIV</td>
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</tr>
<tr>
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</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
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<td>HSV-1</td>
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<tr>
<td>HVS</td>
<td>herpesvirus saimiri</td>
</tr>
<tr>
<td>IC50</td>
<td>concentration required to inhibit viral replication by 50%</td>
</tr>
<tr>
<td>IE</td>
<td>immediate early</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>IR</td>
<td>internal repeat</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>kbp</td>
<td>kilobase-pairs</td>
</tr>
<tr>
<td>$K_M$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>KS</td>
<td>Kaposi’s sarcoma</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi’s sarcoma-associated herpesvirus</td>
</tr>
<tr>
<td>LATs</td>
<td>latency-associated transcripts</td>
</tr>
<tr>
<td>Lat</td>
<td>latency</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani broth</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>MCP</td>
<td>monocyte chemoattractant proteins</td>
</tr>
<tr>
<td>MCS</td>
<td>multiple cloning site</td>
</tr>
<tr>
<td>MDV</td>
<td>Marek’s disease virus</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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MIP  macrophage inflammatory proteins
mg    milligram
min   minute
ml    millilitre
mM    millimolar
mm    millimeter
mRNA  messenger ribonucleic acid
NF    nuclear transcription factor
ng    nanograms
nm    nanometer
NK    natural killer
OD    optical density
o/n   overnight
ORF   open reading frame
p     protein
PAGE  polyacrylamide gel electrophoresis
PBMC  peripheral blood mononuclear cells
PBS   phosphate-buffered saline
PCR   polymerase chain reaction
PCV   penciclovir
pmol  picomoles
pp    phosphoprotein
RANTES regulated upon activation, normal T expressed and secreted
RFLP  restriction-fragment length polymorphism
RNA   ribonucleic acid
<table>
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<td>ribonuclease</td>
</tr>
<tr>
<td>secs</td>
<td>seconds</td>
</tr>
<tr>
<td>SCID-hu</td>
<td>severe combined immunodeficiency-human</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sdw</td>
<td>sterile distilled water</td>
</tr>
<tr>
<td>Taq</td>
<td>thermus aquaticus DNA polymerase</td>
</tr>
<tr>
<td>tat</td>
<td>transactivating factor</td>
</tr>
<tr>
<td>TBE</td>
<td>90 mM Tris-borate, 2 mM EDTA</td>
</tr>
<tr>
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<td>thymus cell</td>
</tr>
<tr>
<td>TE</td>
<td>10 mM Tris/1 mM EDTA buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N', N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TfbI</td>
<td>transformation buffer I</td>
</tr>
<tr>
<td>TfbII</td>
<td>transformation buffer II</td>
</tr>
<tr>
<td>TIF</td>
<td>transcription-inducing factor</td>
</tr>
<tr>
<td>TK</td>
<td>thymidine kinase</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>U</td>
<td>unique region</td>
</tr>
<tr>
<td>UL</td>
<td>unique long region</td>
</tr>
<tr>
<td>US</td>
<td>unique short region</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet light</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
<tr>
<td>v/v</td>
<td>volume-to-volume ratio</td>
</tr>
<tr>
<td>VZV</td>
<td>varicella-zoster virus</td>
</tr>
<tr>
<td>w/v</td>
<td>weight-to-volume ratio</td>
</tr>
<tr>
<td>wt</td>
<td>wild-type</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
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<td>--------</td>
<td>------------------</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre</td>
</tr>
<tr>
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<td>μM</td>
<td>micromolar</td>
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Chapter One - General Introduction
1.1 Kaposi's sarcoma

In 1872, Moritz Kaposi described five patients with blue-red cutaneous tumours that he named "idiopathic multiple pigmented sarcoma of the skin," later designated Kaposi's sarcoma (KS). Over the years following its description, four different types of this tumour were identified: classic KS, African-endemic KS, iatrogenic or immunosuppressive drug-associated KS, and acquired immunodeficiency syndrome (AIDS)-associated KS (Safai et al., 1980, Friedman-Kien et al., 1990). Before 1981, KS occurred rarely and represented only 0.02-0.06% of all malignancies (Hutt et al., 1981, Martin et al., 1993, Tappero et al., 1993). However, with the advent of the AIDS epidemic, its occurrence increased dramatically.

1.1.1 Epidemiology and clinical features

1.1.1.1 Classic KS

Classic KS primarily affects elderly men of Mediterranean, Eastern European, or Jewish heritage (male:female ratio of 10-15:1), with a peak incidence after the sixth decade of life (Cox et al., 1959, Cottoni et al., 1985). The clinical course of classic KS is usually indolent and is rarely the cause of death. Patients survive an average of 10-15 years before dying of an unrelated illness. Secondary malignancies, such as Hodgkin's disease or non-Hodgkin's lymphoma, develop in more than 35% of cases (Safai et al., 1980, Safai et al., 1981). The lesions of classic KS occur primarily in the lower extremities, especially around the ankles and plantar surfaces (Martin et al., 1993, Tappero et al., 1993). They are usually localised and slow growing. In more than 30% of patients the lesions evolve as macules and progress into
plaques or nodules. As the disease progresses, the nodules coalesce into large, painful tumours that may ulcerate, bleed, and become infected.

1.1.1.2 African-endemic KS

African-endemic KS is primarily seen in eastern African countries, such as Zaire, Uganda, Kenya, Zimbabwe, Tanzania, Gabon, and Chad (Hutt et al., 1981). It occurs in two distinct age groups: young adults (mean age 35 years) with generally benign nodular disease, but sometimes aggressive or florid disease, fatal within 5 to 8 years; and young children (mean age 3 years) with fulminant lymphadenopathic disease, which disseminates rapidly, usually fatal within 1 to 3 years (Olweny, 1984, Pluda et al., 1993). Nodular KS has clinical features similar to those of classic KS, with lesions occurring primarily in the lower extremities (Martin et al., 1993, Tappero et al., 1993). The aggressive and florid forms of KS are more invasive (Martin et al., 1993, Tappero et al., 1993). Florid KS disseminates more rapidly than nodular KS, and aggressive KS infiltrates the subcutaneous soft tissues and bones (Martin et al., 1993, Tappero et al., 1993). Lymphadenopathic KS lesions are usually located in ocular and salivary gland lesions, and rapidly disseminates into lymph nodes and viscera (Martin et al., 1993, Tappero et al., 1993).

1.1.1.3 Iatrogenic KS

Iatrogenic KS has been described primarily in organ transplant recipients, as well as other patient groups, receiving chronic immunosuppressive agents such as corticosteroids, cyclosporin, and azathioprine. KS accounts for 3.4% of all neoplasm's and usually develops
10-22 months post-transplantation (Klepp et al., 1978, Myers et al., 1974, Penn et al., 1983). The incidence of KS after transplantation is 400-500 times greater than in the general population (Harwood et al., 1979), however it varies in different geographic regions. Patients originating from Africa and the Middle East have a higher risk for post-transplantation KS than other populations (Farge et al., 1999). Additional risk factors for developing KS post-transplant are past infection with hepatitis B, and use of antilymphocyte sera (Farge et al., 1999). It generally follows a chronic and indolent course, and rarely disseminates, with spontaneous regression occurring in 24% of patients after discontinuation or reduction of immunosuppressive therapy (Myers et al., 1974, Zisbrod et al., 1980, Tebbe et al., 1991). Cutaneous KS lesions are the most common manifestations, however dissemination to other parts of viscera can occur, presenting in areas such as the lungs, lymph nodes, and gastrointestinal tract (Klepp et al., 1978, Myers et al., 1974, Penn et al., 1983).

1.1.1.4 AIDS-associated KS

KS is the most common AIDS-related neoplasm, seen in approximately 15-20% of cases (McKenzie et al, 1991). The epidemic nature of AIDS first came to light when 26 cases of KS were observed in homosexual men in California and New York City (Centre for Disease Control, 1981, Friedman-Kien, 1981). The proportion of patients with KS has been declining compared with other AIDS-related illnesses, however the overall number of patients with AIDS-KS continues to increase (Haverkos, 1985, Rutherford et al., 1989, Rutherford et al., 1990). Patients who are HIV seropositive are 20,000 times
more likely to develop KS than the general population, and 300 times more likely than other immunosuppressed persons (Beral et al., 1990). In addition, striking differences in acquiring KS exist between the different HIV risk groups. Homosexual and bisexual male AIDS patients are approximately 20 times more likely to present with KS at AIDS diagnosis than haemophiliac patients (DeJarlais et al., 1984, Haverkos, 1985, Beral et al., 1990). Also, there is an increased risk for developing KS in women who have acquired HIV infection by heterosexual contact with bisexual men as opposed to heterosexual intravenous drug users (IDU's) (Biggar et al., 1985, Biggar et al., 1989, Beral et al., 1990).

Unlike classic KS, cutaneous AIDS-KS lesions are generally localised to the upper body such as the head, neck, and back, instead of the lower extremities (Steis et al., 1988, Pluda et al., 1993). They also occur frequently on the palms, soles, and genitalia. AIDS-associated KS displays an extremely aggressive course with multicentric lesions involving the mucous membranes of the respiratory and gastrointestinal tracts. Visceral AIDS-KS progresses rapidly and has a high mortality rate due to lymphatic obstruction, organ infiltration, and pulmonary dysfunction (Sung et al., 1997).

1.1.2 Pathogenesis

KS is a vascular tumour with a complex histology and an uncertain histogenesis. It is characterised microscopically by a proliferation of spindle-shaped cells and irregular slit-like vascular channels (Miles et al., 1990, Ensoli et al., 1990). Most of the spindle cells express endothelial cell markers, such as CD31 and CD34 (Nadji et al., 1981, Rutgers et al., 1986, Sturzl et al.,
However, they also express markers for smooth muscle cells, macrophages and dermal dendrocytes (Nickoloff and Griffiths, 1989, Weich et al., 1991, Wittek, 1991, Huang et al., 1993). Therefore, it is possible that KS cells are derived from either pluripotent mesenchymal precursors or from a heterogeneous population of cells. The survival of KS cells has been shown to rely on the production and secretion of various cytokines, with both autocrine and paracrine function, to control cell proliferation (Salahuddin et al., 1988). These include tumour necrosis factor-alpha (TNF-α), and interleukin-1 (IL-1) and -6 (IL-6), which support spindle cell proliferation (Miles et al., 1990, Corbeil et al., 1991), and proteins that regulate neovascularisation or angiogenesis such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) (Ensoli et al., 1989).

Another intriguing aspect of KS pathogenesis is the fact that more men than women are affected. Studies have suggested that female hormones are protective against the development of KS lesions (Lunardi-Iskander et al., 1995, Harris, 1995). Sera from both humans and mice in the early stages of pregnancy have been demonstrated to have an inhibitory effect on KS Y-1 cells in vitro (Lunardi-Iskander et al., 1995). In addition, mice in the early stages of pregnancy inoculated with KS cell lines did not develop tumours, whereas, those inoculated in the later stages of pregnancy developed small tumours that did not metastasise (Lunardi-Iskander et al., 1995). It has also been reported that β-human chorionic gonadotropin (hCG) can prevent the induction of KS lesions by the KS Y-1 cell line (Harris, 1995).
Although HIV infection does not cause KS, it appears to play an indirect role in its development (Vogel et al., 1988). By co-operating with bFGF, the Tat protein of HIV-1, when released from cells, has been shown to induce the formation of KS-like lesions in nude mice and to enhance the proliferation of KS cell lines and endothelial cells (Ensoli et al., 1994). Also, when antibodies against Tat were added, they were able to block AIDS-KS proliferation (Ensoli et al., 1994). The epidemic nature of HIV-associated KS has suggested clues to its pathogenesis, especially concerning an infectious, possibly sexually transmitted agent. In 1994, Chang et al. identified herpesvirus-like DNA sequences in KS tissue of AIDS patients. The new virus was named Kaposi’s sarcoma-associated virus (KSHV) or human herpesvirus 8 (HHV-8), and is now thought of as the major aetiological factor for KS. The role of HHV-8 in KS is described in Section 1.3.

1.1.3 Treatment

Currently there is no curative therapy for KS; treatment tends to vary with the severity and type of disease. Iatrogenic KS resolves spontaneously in 24% of patients after discontinuation or reduction of the dosage of steroids or immunosuppressive agents (Myers et al., 1974, Zisbrod et al., 1980, Tebbe et al., 1991). Multiple treatment regimens exist for other subtypes of KS. These regimens may be divided into local and systemic types. In general, limited disease responds to local treatment while generalised or aggressive disease requires systemic treatment.
1.1.3.1 Local treatment

Classic KS follows an indolent, benign course and thus requires little or no treatment. When intervention is necessary radiation therapy to the local site is usually sufficient. In patients with limited disease, cutaneous lesions can be managed with a relatively small amount of radiation (Berson et al., 1990). Complete remission may be achieved in 20-70% of cases, however local relapse may occur. Although irradiation is relatively safe, some patients may experience adverse reactions, such as mucositis, pain and post-radiation hyperpigmentation (Cooper et al., 1984, Berson et al., 1990). Other local therapies include surgical excision, liquid nitrogen cryotherapy, argon laser therapy, intralesional chemotherapy, and alitretinoin gel (Tappero et al., 1991, Boudreaux et al., 1993, Hebeda et al., 1995, Webster, 1995).

1.1.3.2 Systemic treatment

Radiation is often adequate even in more aggressive forms such as African-endemic KS, although rarely, systemic chemotherapy is necessary (Pluda et al., 1993, Tappero et al., 1993). In contrast, AIDS-KS is a fulminant disease that usually requires aggressive treatment, especially when it involves visceral organs. Irradiation, systemic chemotherapy, and interferon (IFN)-α are administered primarily for symptomatic relief, and to prevent disease progression (Groopman, 1987, Steis and Longo, 1988, Krown et al., 1992, Lilenbaum and Ratner, 1994).

There are a number of systemic chemotherapy regimens active against the more aggressive forms of KS. Several single cytotoxic agents, including vinca alkaloids (vinblastine, vincristine), paclitaxel, etoposide, liposomal
anthracyclines (doxorubicin, daunorubicin), and bleomycin, have activity against KS (Sung et al., 1997). However, systemic chemotherapy can cause myelosuppression and neutropenia, increasing the risk of opportunistic infections that may further compromise survival of patients with AIDS-KS (Gill et al., 1991, Fischl et al., 1993). When used in low dosages, combination chemotherapy may be as effective as standard dose regimens, whilst decreasing the side effects. These multi-drug regimens are usually reserved for patients with disseminated or rapidly progressive KS. A routinely used combination therapy ABV, which includes adriamycin (doxorubicin), bleomycin, and vincristine, is shown to have minimal toxicity with a good anti-tumour response (Krown, 1991, Gill et al., 1991, Gill et al., 1992).

Another approach is the use of interferon-α in mono- or combination therapy (Groopman et al., 1984, Krown et al., 1986, Volberding et al., 1987, Krown, 1991). In contrast to the cytotoxic agents, IFN-α is considered to be non-immunosuppressive, and has additional anti-retroviral, anti-proliferative and anti-angiogenic effects (Hartshorn et al., 1987, Lane et al., 1988, Fischl, 1991). IFN-α has been used in various dosages and produces response rates, complete and partial, in the range of 30-50%. Anti-tumour response to interferon therapy is seen in patients with CD4 counts greater than 200 cells/mm³, and appears to be best in patients with CD4 counts above 400 cells/mm³ (Mitsuyasu et al., 1986, Evans et al., 1991). High dose IFN treatment, however, is complicated by side effects that result in low tolerance, especially during long-term treatment. In contrast, low dose IFN has been shown to be effective in diseases like hairy cell leukaemia and hepatitis C (Advise et al., 1989, Doane et al., 1990).
1.1.3.3 Antiretroviral therapy

In AIDS-KS patients, the use of zidovudine (ZDV or azidodeoxythymidine, AZT) in combination with low dose IFN-α was tolerated better than either drug alone (Mauss and Jablonowski, 1995). The combination of IFN-α and AZT produces a synergistic inhibition of HIV replication in culture (Hartshorn et al., 1987, Fischl, 1991, Podzamczer et al., 1993). Response rates in the range of 30-60% are seen, and a significant improvement in response rates is seen in patients with CD4 counts less than 100 cells/mm³, in whom IFN alone is rather ineffective (Mauss and Jablonowski, 1995, Fischl et al., 1996, Krown et al., 1990). Furthermore, epidemiologic data show that following the introduction of monotherapeutic and dual therapeutic interventions using AZT the incidence of KS amongst most male homosexuals has declined, despite an increase in the numbers of HIV-infected individuals (Dore et al. 1996, Hermans, 1998, Montaner et al. 1994). The incidence of new cases of KS has declined quite dramatically since 1996, coinciding with the increasing use of highly active antiretroviral therapy (HAART). In patients with ongoing KS a few reports speculate that immune reconstitution following the initiation of HAART therapy may lead to regression of KS.

1.1.3.4 Antitherpetic therapy

With the identification of HHV-8 as the probable causative agent of KS there is new hope for treatment of the disease. Regression of KS lesions was reported in patients treated with foscarnet, which also has activity against HIV
(Morfeldt and Torssander, 1994), while a decreased risk of developing KS has
been observed in a retrospective analysis of HIV-positive patients treated with
foscarnet or ganciclovir (Mocroft et al, 1996). This area is covered in greater
detail in section 1.4.
1.2 Herpesviruses

1.2.1 Herpesvirus structure

The family *Herpesviridae* are a group of around 100 double-stranded DNA viruses, which infect and cause a variety of diseases in most animal species. Eight human herpesviruses have been identified to date. Membership to this family is based on the structure of the virus particle (Figure 1.1), which consists of four distinct morphologic units.

The **core** contains the dsDNA in the form of a torus. This is surrounded by the **nucleocapsid**, composed of 162 hexameric (150) and pentameric (12) capsomeres arranged as a symmetrical icosadeltahedran. Outside the nucleocapsid is an amorphous fibrous material called the **tegument**, which varies in thickness depending on the location of the virions within the cell. Surrounding the tegument is an **envelope**, whose outer surface contains many small spikes consisting of glycoproteins, which appears to be derived from host-cell nuclear membranes. The size of the complete virion varies from 120-300nm, dependent on the thickness of the tegument and the state of the envelope.

1.2.2 Herpesvirus classification

Members of the family *Herpesviridae* are currently classified into 3 subfamilies, the **alpha-**, **beta-**, and **gammaherpesvirinae**. Classification is based, primarily, on their biological properties, such as cell tropism, cell pathology in laboratory culture, and characteristics of latent infection. However, there are exceptions, such as the recently described human herpesvirus 8 (HHV-8 or Kaposi's sarcoma-associated herpesvirus (KSHV)).
Advances in molecular biology have led to the discovery of HHV-8 before it could be propagated in cell culture, and so its classification was based on sequence homology and genome organisation. In the future, as such advances are made, these parameters would need to be taken into account when classifying new viruses.

1.2.2.1 Alphaherpesviruses

The alphaherpesviruses can infect a wide range of cells, which are destroyed rapidly, have a short reproductive cycle, and spread quickly in culture. Their primary site of latent infection is in the neurones of the sensory ganglia, however this is not exclusive. Herpes simplex virus 1 and 2 (HSV-1 and -2), varicella zoster virus (VZV), and pseudorabies virus are members of this subfamily.

1.2.2.2 Betaherpesviruses

In contrast to the alphaherpesviruses, the betaherpesviruses have a restricted host range, with a long reproductive cycle, and a slow progression of infection in culture. Infected cells frequently become enlarged (cytomegalias), and carrier cultures are readily established. The latent virus is often maintained in lymphoid cells or secretory glands, as well as other tissues. Members of this subfamily include human cytomegalovirus (HCMV), and human herpesvirus 6 and 7 (HHV-6 and -7).
Figure 1.1 Schematic diagram showing the major structural components of a herpesvirus particle.
1.2.2.3 Gammaherpesviruses

The gammaherpesviruses have characteristics that are intermediate to those of the alpha- and betaherpesviruses. All members replicate in lymphoid cells in vitro, and some are capable of lytic replication in epithelial cells and fibroblasts. The gammaherpesviruses establish latent infection in lymphocytes and are often associated with cell immortalisation and transformation. These viruses can be further divided into the lymphocryptovirus (γ1) and rhadinovirus (γ2) genera. Epstein-Barr virus (EBV) is the prototype lymphocryptovirus, and HHV-8 and herpesvirus saimiri (HVS) are both rhadinoviruses. Both human gammaherpesviruses, EBV and HHV-8 were first discovered in tumour specimens and both carry one or more genes with potential to cause transformation. Indeed, it is the association of these viruses with cancer that has created most interest.

EBV is the aetiological agent of infectious mononucleosis and is a cofactor in the induction of the neoplastic diseases of Burkitt’s lymphoma (BL), nasopharyngeal carcinoma (NPC), non-Hodgkin’s lymphoma (NHL), post-transplant lymphoproliferative disease (PTLD), and oral hairy leukoplakia. In vitro infection of primary B-lymphocytes with EBV results in the expression of several EBV genes and cell immortalisation. Of those genes which are expressed, five nuclear proteins (EBNAs 1, 2, 3A, 3C and -LP) and an integral membrane protein (LMP1) are critical for cell immortalisation. EBNA3B, LMP2A, and the EBV-encoded small RNAs (EBERs) are also expressed in latently infected B cells, but are not required
for immortalisation. As mentioned previously, HHV-8 is the major aetiological agent of Kaposi’s sarcoma, and is described in greater detail in section 1.4.

1.2.3 Herpesvirus genome

Herpesvirus DNA extracted from virions is linear and double-stranded, however they circularise upon release from the capsids into infected cell nuclei. The molecular sizes of herpesvirus genomes differ from 120 to 300kbp, due to the presence or absence of unique short regions or repeat sequences. The arrangement of these sequences allows the herpesviruses to be classified into six groups, designated by the letters A to F (Figure 1.2).

Both HHV-6 and -7 have group A genome structures, which have large identical repeat sequences at both termini. The genomes of the gammaherpesviruses HHV-8 and HVS, classified as group B, have the terminal sequence directly repeated several times at both termini, whereas EBV has a group C genome, with fewer direct terminal reiterations and other, unrelated sequences, directly repeated internally. Group D genomes, as exemplified by that of VZV, have both a large and a small unique sequence. The latter is flanked by inverted repeats and can invert relative to the large sequence. HSV-1 and -2, and HCMV have group E genomes with both the large and small unique sequences flanked by inverted repeats. Both sequences are able to invert relative to each other, producing four possible isomers. Group F genomes have no terminal or internal reiterations, an example of which is equine herpesvirus 5 (EHV-5).
Figure 1.2 Schematic diagram of the sequence arrangements in the six classes of herpesvirus genomes. Boxes represent sequence repeats.
1.2.4 Herpesvirus life cycle

The mechanisms of herpesvirus replication are very similar throughout all members of the family. Prior to replication, the virus first gains entry into the host cell. To do this the herpesvirus attaches itself to the cell surface via the interaction between its surface glycoproteins and specific cell receptors. The viral envelope and cellular membrane fuse together, releasing the viral capsid into the cytoplasm. The capsid is then transported to the nucleus and the viral DNA is released. After entering the nucleus through the nuclear pores the viral DNA circularises. It is at this point where the herpesvirus either a) establishes latency, or b) replicates to produce progeny virus.

Latency is a characteristic shared by all herpesviruses, to allow their persistence within the host organism without elimination by the immune defences. During latency only a small subset of viral genes are expressed, the number of which varies between viruses. For example, HSV-1 latency involves the expression of only the latency-associated transcripts (LATs), whilst during EBV latency in the immunocompromised up to 11 genes may be expressed. This variation in number is due to the different cell types used for latent infection by the different herpesviruses. EBV establishes latency in dividing cells and so must retain the ability to replicate its genome during latency, however this is not required during latent HSV infection of neurones. Following establishment of latency, various factors may subsequently induce viral reactivation.

Lytic replication of herpesviruses follows a highly co-ordinated cascade of gene expression. It involves the temporal transcription of three classes of genes - the immediate early (IE), early (E) and late (L) genes (or $\alpha$, $\beta$ and $\gamma$
genes). After transcription, the α-mRNAs are transported to the cytoplasm where they are translated into regulatory proteins. This occurs independently of new protein synthesis. These regulatory proteins are then required for the expression of all later genes, in particular the β-genes, the products of which are enzymes involved in viral DNA replication, such as ribonucleotide reductase, thymidine kinase, topoisomerase and DNA polymerase. Viral DNA replication follows, by the rolling circle mechanism, where complete head-to-tail concatamers of viral genomes are formed. This is followed by a final round of transcription and translation to yield the γ-proteins, most of which have structural roles in the assembly of the virion.

In the nucleus, capsid proteins assemble to form empty capsids and genome unit-length viral DNA, cleaved from the concatamers, is packaged to produce nucleocapsids. Viral glycoproteins and tegument proteins accumulate to a ‘patch’ on the nuclear membrane, preceding the attachment of the newly formed nucleocapsids. Budding occurs through the nuclear membrane to release fully enveloped virions. The virions accumulate in the endoplasmic reticulum, prior to release from the host cell by exocytosis.
1.3 Human herpesvirus 8 (HHV-8)

1.3.1 Discovery

Human herpesvirus 8 is the most recently discovered human member of the *Herpesviridae* family. In 1994, Chang *et al.* used a method called representational difference analysis (RDA) to detect unique sequences present in Kaposi’s sarcoma versus normal tissue from AIDS patients. They detected two DNA fragments specific to KS tissue: KS330Bam (330bp) and KS631Bam (631bp). Sequence analysis showed these fragments code for proteins homologous to herpesviral polypeptides. The KS330Bam region had 39% and 51% amino acid homology to the BDLF1 ORF of EBV and the ORF 26 of HVS, respectively. The protein encoded by the KS631Bam region had homology to the tegument protein of EBV (ORF BNRF1, p140) and to the tegument protein of HVS (ORF 75). Following this, Chang *et al.* demonstrated the presence of these herpesviral-like sequences, by PCR amplification of a 233bp fragment of the KS330Bam region (designated KS330\(_{233}\)), in 25 of 27 KS tissues from AIDS patients. In contrast none of 136 non-KS control specimens were positive by PCR for KS330\(_{233}\), suggesting a possible causal role for the newly discovered herpesvirus in KS development.

1.3.2 HHV-8 cell culture and tropism

To date it has been difficult to culture HHV-8 efficiently. Latently infected B-cell lymphoma cell lines have been established from primary effusion lymphoma (PEL) (Cesarman *et al.*, 1995, Arvanitakis *et al.*, 1996, Gao *et al.*, 1996, Renne *et al.*, 1996, Said *et al.*, 1996, Gaidano *et al.*, 1996) and from the peripheral blood of a PEL patient (Boshoff *et al.*, 1998). Like the
lymphoma cells, most PEL cell lines are dually infected with HHV-8 and EBV, but some PEL cell lines infected only with HHV-8 have been established (Gao et al., 1996, Renne et al., 1996, Said et al., 1996, Boshoff et al., 1998). In culture, a small proportion of cells in these PEL-derived cell lines can spontaneously undergo lytic replication and this can be enhanced by treatment with phorbol esters or sodium butyrate (Lennette et al., 1996, Miller et al., 1996, Miller et al., 1997). Such treated cell lines can produce HHV-8 virions and have formed the basis of ultrastructural studies (Renne et al., 1996, Said et al., 1996, Ablashi et al., 1997). HHV-8 virions have morphological features typical of other herpesviruses, with a size of approximately 110 nm. Nucleocapsids with an electron dense core are found in the nucleus of these induced lymphoma cells and enveloped virions are found in the cytoplasm (Renne et al., 1996, Said et al., 1996). Similar particles have also been observed in KS lesions in a few cells with spindle-shaped morphology (Orenstein et al., 1997). However, serial propagation of HHV-8 in tissue culture has so far proved to be inefficient (Foreman et al., 1997). Limited lytic replication and serial transmission have been achieved in 293 cells, but could only be detected by using PCR. Nevertheless it has been possible to culture HHV-8 directly from KS lesions (Foreman et al., 1997) and saliva (Vieira et al., 1997) confirming the presence of HHV-8 virions in tumour samples and saliva.

HHV-8 has been detected in endothelial and spindle cells of KS lesions, in circulating endothelial cells, B-cells, CD8+ T-cells, macrophages and prostatic glandular epithelium (Ambroziak et al., 1995, Boshoff et al., 1995, Corbellino et al., 1996, Harrington et al., 1996, Li et al., 1996, Mesri et
PCR in situ hybridisation experiments first showed that HHV-8 can infect the endothelial cells lining vascular spaces in KS lesions and spindle cells of fully developed, nodular KS lesions, but is not found in normal endothelial cells (Boshoff et al., 1995, Li et al., 1996, Foreman et al., 1997). In situ hybridisation and latency-associated nuclear antigen (LANA) immunohistochemistry have also confirmed HHV-8 gene expression in KS spindle cells within KS tumours (Davis et al., 1997, Rainbow et al., 1997, Staskus et al., 1997, Sturzl et al., 1997).

The presence of circular HHV-8 genomes in PEL cells and KS tissue suggests that the majority of cells in these tissues are latently infected with HHV-8 (Russo et al., 1996, Decker et al., 1996). Only a limited repertoire of HHV-8 genes is expressed in the majority of endothelial tumour cells of KS lesions and/or in PEL cell lines. However, a small population of cells in KS tissue express inducible genes, and these are believed to correspond to lytically infected cells (Staskus et al., 1997, Sturzl et al., 1997). Some of these productively infected cells have the appearance of spindle cells, and HHV-8 virions have been seen by electron microscopy (EM) of KS lesions (Orenstein et al., 1997). The expression of lytic HHV-8 genes in monocytes/macrophages and the presence of linear HHV-8 genomes in PBMC have also been documented (Blasig et al., 1997, Decker et al., 1996), suggesting the presence of productively infected cells that may have a crucial role for the dissemination of the virus into tissues.
1.3.3 Epidemiology and transmission

1.3.3.1 Prevalence in various patient groups

Since its discovery, HHV-8 DNA has been detected by PCR in all forms of KS, as well as two other AIDS-related lymphoproliferative disorders, PEL (or body cavity-based lymphoma) (Cesarman et al., 1995) and multicentric Castleman's disease (MCD) (Soulier et al., 1995). Associations between HHV-8 and sarcoidosis, squamous cell skin carcinoma, angiosarcoma and cutaneous T-cell lymphoma have also been suggested, but have yet to be confirmed (Schulz, 2000). In addition, several groups have linked HHV-8 infection with multiple myeloma (Rettig et al. 1997, Said et al. 1997, Chauhan et al. 1999, and Gao et al. 1998), which again remain unconfirmed by others. Specific criteria must be met to determine whether HHV-8 plays a direct role in disease pathogenesis.

Using PCR, HHV-8 DNA is detectable in more than 90% of lesions from all types of KS (Moore et al., 1995, Huang et al., 1995, Schalling et al., 1995, Dictor et al., 1996, Luppi et al., 1996). However, analysis of peripheral blood mononuclear cells (PBMCs) from AIDS-KS patients demonstrated detectable levels of HHV-8 DNA in only 50% of samples (Whitby et al. 1995). Also, in HIV positive patients without KS around 10% of PBMCs were found to have HHV-8 DNA present. These patients were more likely to develop KS than HIV patients without evidence of HHV-8 infection (Whitby et al. 1995). Moreover, other groups have also reported the detection of HHV-8 DNA in PBMCs of HIV-infected patients prior to the occurrence of KS, further increasing the notion that HHV-8 has a causal role in the development of KS (Moore et al., 1996, Lefrère et al., 1996).
Most of the known human herpesviruses are ubiquitous in the general population, remaining in a latent state throughout the life of the host. However, there is a low detection rate of HHV-8 DNA in healthy blood donors, or other low-risk groups using PCR. Therefore, to get a greater understanding of the prevalence of HHV-8 in the general population and in high-risk groups, more sensitive methods need to be utilised. Serological assays for antiviral antibodies have proved more useful for addressing epidemiological questions. Primary herpesviral infections generally result in antibody responses, which typically remain detectable for the lifetime of the host. During viral latency, whilst viral DNA remains confined to a small population of cells, these antibodies persist. Therefore, the presence of antibodies to HHV-8 will indicate past exposure and thus, ongoing latent viral infection.

KSHV-specific antibodies were first detected using PEL cell lines (Gao et al., 1996, Kedes et al., 1996). These have been used in immunofluorescence assays (IFA) and enzyme-linked immunosorbant assays (ELISA) to measure antibody responses in human sera. Using IFA, antibodies against latency-associated nuclear antigens (LANA) are measured as nuclear fluorescence in uninduced cells, whilst cytoplasmic fluorescence detected in TPA-induced cells measures antibodies against lytic antigens (Lennette et al., 1996). Whereas, ELISAs have been developed to detect antibodies against lytic cycle-associated proteins, recombinant forms of the capsid protein vp19 (encoded by ORF 65) or the gp35/37 envelope protein (encoded by ORF K8.1) (Calabro et al., 1998, Simpson et al., 1996, Chandran et al., 1998). Tests for the latent and the recombinant proteins are highly
specific, but sensitivity ranges from 70-90% for AIDS-KS patients and 95-100% for classic KS patients. However, the lytic IFAs have sensitivities approaching 100% for all KS types, although they also have lower specificity (Schulz, 2000).

Despite these differences, there is general agreement on the overall geographical distribution of HHV-8 seroprevalence between studies using the various assays (Table 1.1), which correlates well with KS prevalence prior to the onset of the AIDS epidemic. There is low prevalence of HHV-8 in the UK and USA compared to Mediterranean countries, which have a slightly higher prevalence in accordance with the incidence of classic KS seen in these countries. In addition, HHV-8 prevalence is highest in African countries corresponding to the high incidence of African-endemic KS.

In HIV-infected individuals there are significant differences in HHV-8 seroprevalence between the different risk groups. Antibodies to HHV-8 have been detected at a high incidence in homosexual or bisexual men, 20-40% of individuals with the latent and recombinant assays (Gao et al., 1996, Kedes et al., 1996, Simpson et al., 1996, Martin et al., 1998, Renwick et al., 1998), and 90% of individuals with the lytic IFA (Lennette et al., 1996). However, in HIV-positive haemophiliacs, intravenous drug users (IVDUs), and women the prevalence of anti-HHV-8 antibodies is closer to that of the general population (Gao et al., 1996, Kedes et al., 1996, Simpson et al., 1996, Lennette et al., 1996, Martin et al., 1998 and Renwick et al., 1998). This data parallels the reports of higher incidence of KS among homosexuals and women with bisexual partners than haemophiliacs and IVDUs (Beral et al., 1990), and
would suggest a major route of transmission for HHV-8 is through sexual contact, especially among homosexual men.
Table 1.1 Geographical distribution of HHV-8 prevalence in the general population (KS negative/HIV negative), determined by the various serological assays that detect antibodies to HHV-8 LANA, vp19 and lytic antigens.

<table>
<thead>
<tr>
<th>COUNTRY</th>
<th>% POSITIVE FOR HHV-8 ANTIBODIES</th>
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<tbody>
<tr>
<td></td>
<td>LANA/ORF 73 ( ^{a,g} )</td>
</tr>
<tr>
<td>UK/US</td>
<td>0-10</td>
</tr>
<tr>
<td>ITALY/GREECE</td>
<td>4-35</td>
</tr>
<tr>
<td>AFRICA</td>
<td>11-53</td>
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</tbody>
</table>

\( ^{a} \) Gao et al., 1996; \( ^{b} \) Kedes et al., 1996; \( ^{c} \) Simpson et al., 1996; \( ^{d} \) Lennette et al., 1996; \( ^{e} \) Rabkin et al., 1998; \( ^{f} \) Calabro et al., 1998; \( ^{g} \) Zhu et al., 1999; \( ^{h} \) Rezza et al., 1998
1.3.3.2 Routes of transmission

HHV-8 DNA has been detected in semen of individuals with and without KS, however the frequency and consistency of such findings have varied greatly (Lin et al., 1995, Monini et al., 1996, Howard et al., 1997, Viviano et al., 1997, Diamond et al., 1997, LaDuca et al., 1998). Although this could account for sexual transmission of the virus, detection rates appear to be lower than in PBMCs and so quantities of HHV-8 in semen may be insufficient for transmission. Recent studies into the sexual behaviour of homosexual men have demonstrated that HHV-8 infection and seroconversion are significantly associated with number of sexual partners, HIV-1 seropositivity, and history of sexually transmitted diseases (STDs), as well as specific sexual practices, including insertive and receptive anal intercourse, insertive anilingus, and insertive fisting (Martin et al., 1998, Gambus et al., 1999, O’Brien et al., 1999). Grulich et al. (1999) demonstrated a significant association between HHV-8 seropositivity and hepatitis A virus (HAV) seropositivity, and a non-significant association with oral-anal contact. HAV is mainly transmitted by the faeco-oral route in homosexual men (Corey and Holmes, 1980). In addition, several studies have found an association between history of oral-anal contact and KS risk (Beral et al., 1992, Grulich et al., 1997, Darrow et al., 1992, Jacobson et al., 1990), and so Grulich et al. suggested this may also be a route of transmission for HHV-8. Although, this has not been seen by other groups looking at sexual risk factors for HHV-8 infection (Melbye et al., 1998, Renwick et al., 1998). In addition, the presence of HHV-8 DNA in faeces of HIV-positive individuals with KS could not be demonstrated (Whitby et al., 1995, LaDuca et al., 1998), although HHV-8
DNA has been detected in gastrointestinal tissues of HIV-positive individuals (Alero Thomas et al. 1996).

Transmission of HHV-8 by heterosexual contact is less evident. Two studies of female commercial sex workers, in Honduras and Cameroon, demonstrated a significant association between HHV-8 infection and both HIV-1 infection and commercial sex work (Sosa et al., 1998, Bestetti et al., 1998). Also, a recent retrospective study demonstrated the transmission of HHV-8 between two married, HIV-1 infected, heterosexual partners (Masquelier et al., 2000). Both partners developed AIDS-associated KS at approximately the same time, however the husband was HHV-8 seropositive at least one year prior to his wife's seroconversion. DNA sequence comparison of the HHV-8 minor capsid protein, and ORF 75, showed complete identity between the patients. Thus, strongly suggesting the transmission of HHV-8 from the man to his wife.

HHV-8 infection is found in elderly men of Mediterranean descent with KS, in persons with African-endemic KS of all ages, and in transplant patients with KS. These are, for the most part, populations at low risk for sexually transmitted diseases, and would suggest that HHV-8 may have some other route of transmission. Presence of HHV-8 DNA in saliva appears to be greater than in semen, suggesting some form of oral contact might carry a higher risk for transmission than intercourse (Boldogh et al., 1996, Koelle et al., 1997, Blackbourn et al., 1998 and LaDuca et al., 1998). This idea is supported by Vieira et al. (1997) who have demonstrated that HHV-8 isolated from cell-free salivary fluid of KS-positive subjects, persists in inoculated 293 cell cultures. They have also described the induction of viral replication by
TPA, and the expression of virus-specific RNAs in 293 cells inoculated with cell-free saliva fluid. Presence of HHV-8 in saliva appears to be restricted to HIV-positive and HIV-negative/KS-positive individuals and remains undetectable in HIV-negative/KS-negative persons, although at present not all population groups at risk of developing KS have been investigated.

In populations at risk of African-endemic KS, HHV-8 infection is prevalent in children, whereas childhood infection is rare in the USA. Bourboulia et al. (1998) investigated mother to child transmission of HHV-8 in South Africa in 107 women and 112 of their children, aged between 6 months and 14 years. The results showed that 42% (8/19) of the children whose mothers were HHV-8 seropositive were themselves seropositive, whereas only 1% (1/93) of the children whose mothers were seronegative for HHV-8 were seropositive. In a subsequent report the same authors observed the proportion of HHV-8 seropositive children increased in relation to maternal titre of HHV-8 antibody suggesting a high viral load of HHV-8 may increase the likelihood of transmission of the virus from mother to child (Sitas et al., 1999).

Gessain et al. (1999) investigated HHV-8 infection in Cameroon children ranging from 0 to 20 years old and pregnant women. They found a high prevalence (46%) of HHV-8 antibodies in newborn children (0-6 months). However, children between 6 months and 4 years had a much lower HHV-8 seroprevalence, but around 4 years of age a linear increase in HHV-8 seropositivity was observed, with 39% of 12-14 year olds and 48% of children above 15 years being seropositive, similar to the rate (54%) observed in pregnant women. In a second study, the same group looked at intrafamilial
transmission of HHV-8, in a village in French Guyana (Plancoulaine et al., 1999). The results showed a strong familial aggregation in HHV-8 seroprevalence characterised by high mother-child and sibling-sibling correlations (with odds ratios (OR) of 2.8 and 3.8, respectively), whereas there was no correlation in HHV-8 infection between spouses (OR=0.6), or father and child (OR=0.8). These data, along with others, suggest that HHV-8 transmission in the endemic population would occur by more casual routes, such as close contact, especially between mother and siblings (Mayama et al., 1998, Lyall et al., 1999). The high prevalence of HHV-8 initially observed in new-borns more likely reflects the passive transmission of maternal IgG.

Post-transplantation KS is associated with HHV-8 infection of the transplant recipient. The presence of HHV-8 antibodies before and after kidney transplantation has been demonstrated to be highly predictive of the emergence of post-transplantation KS and conferred a 28-fold increased risk of KS (Farge et al., 1999). It has been suggested that in endemic countries this is primarily due to reactivation of the virus as a result of immunosuppressive therapy (Parravicini et al. 1997), whereas, in non-endemic countries KS development can occur after primary HHV-8 infection, transmitted from the donor through the transplanted organ (Regamey et al. 1998).

1.3.4 The HHV-8 genome structure and organisation

The long unique region (LUR) of all known rhadinoviruses spans some 110 to 130kb and contains at least 75 reading frames. The first size estimation for the HHV-8 genome from two BCBL-derived cell lines suggested
an approximate size of 270kb (Moore et al., 1996, Mesri et al., 1996). In contrast, size measurements by pulsed-field gel electrophoresis (PFGE), using lytically infected cells and purified virus particles, suggested that HHV-8 DNA is in the genomic size range of other rhadinoviruses at 165kb (Renne et al., 1996). The almost complete nucleotide sequence of HHV-8 DNA has been determined from a BCBL cell line and from a KS biopsy specimen (Russo et al., 1996, Neipel et al., 1997). This data showed that the genome structure was characteristic of rhadinoviruses, with a 140.5kb LUR flanked by two terminal repeats consisting of several 801bp repeat subunits of high (84.5%) G+C content.

The LUR contains at least 81 open reading frames, including 66 with homology to herpesvirus saimiri ORFs and 15 genes unique to HHV-8. Homologous genes are classified according to the corresponding HVS ORFs starting from the left-hand end of the genome. HHV-8 is missing the first three genes found in HVS, and so the first homologous gene begins at ORF4 and extends throughout the genome to ORF-75. Genes unique to HHV-8 are designated with a 'K' prefix and numbered sequentially (ORFs K1-15).

Like other herpesvirus genomes, that of HHV-8 contains genes encoding proteins that are required for replication and assembly of progeny virus. These conserved genes are arranged into four or five blocks, as previously described by Chee et al. (1990), and in the case of HHV-8 are numbered gene blocks I-IV", and IV". They include i) major structural proteins such as the major capsid protein (ORF-25), ii) DNA synthetic proteins, including the thymidine kinase (ORF-21) and the DNA polymerase (ORF-9), iii) glycoproteins, e.g. gB (ORF-8), gH (ORF-22), and gM (ORF-38), and iv) a
viral proteinase and assembly protein (ORF-17). In between the conserved

gene blocks are regions containing genes unique to HHV-8 or conserved in

other rhadinoviruses, referred to as divergent loci (DL) A, B, C, D, and E. It

appears that many of these non-conserved genes are homologues of cellular
genomes, encoding proteins involved in cell cycle regulation or cell signalling,

and some DNA synthetic enzymes (Table 1.2).

<table>
<thead>
<tr>
<th>Cellular homologue*</th>
<th>HHV-8</th>
<th>HVS</th>
<th>EBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCPH</td>
<td>ORF-4</td>
<td>ORF-4</td>
<td>-</td>
</tr>
<tr>
<td>vIL6</td>
<td>ORF-K2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DHFR</td>
<td>ORF-2</td>
<td>ORF-2</td>
<td>-</td>
</tr>
<tr>
<td>TS</td>
<td>ORF70</td>
<td>ORF-70</td>
<td>-</td>
</tr>
<tr>
<td>CC-chemokines</td>
<td>ORF-K4, 4.1 &amp; 6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>ORF-16</td>
<td>ORF-16</td>
<td>BHRF1</td>
</tr>
<tr>
<td>vIRF</td>
<td>ORF-K9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>vFLIP</td>
<td>ORF-71 (K13)</td>
<td>ORF-71</td>
<td>-</td>
</tr>
<tr>
<td>v-Cyclin</td>
<td>ORF-72</td>
<td>ORF-72</td>
<td>-</td>
</tr>
<tr>
<td>N-CAM family</td>
<td>ORF-K14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-8R/GCR</td>
<td>ORF-74</td>
<td>ORF-74</td>
<td>-</td>
</tr>
<tr>
<td>FGAM</td>
<td>ORF-75</td>
<td>ORF-3 &amp; 75</td>
<td>BNRF1</td>
</tr>
</tbody>
</table>

* CCPH = complement control protein; vIL-6 = viral interleukin 6; DHFR = dihydrofolate

reductase; TS = thymidylate synthase; vIRF = viral interferon response factor homologue;
vFLIP = viral FLC inhibit protein; N-CAM = neuronal cell-adhesive membrane protein

family; IL-8R/GCR = interleukin 8 receptor or G-protein-coupled receptor homologue; FGAM =

phosphoribosylformylglycinamidine synthase.
Figure 1.3 Schematic representation of the HHV-8 genome showing open reading frames (ORF) common to all herpesviruses and unique to HHV-8 and other primate rhadinoviruses.
Figure 1.4 A phylogenetic tree for the DNA polymerase of HHV-8 and other primate rhadinoviruses. ChRV-1,2, Chlorocebus rhadinovirus 1 and 2; RFHVMm, Mn, retroperitoneal fibromatosis herpesvirus of rhesus and pigtailed macaques; RRV, MneRV-2, rhesus and pigtailed macaque rhadinoviruses; HVA, herpesvirus ateles; HVS, herpesvirus saimiri; PtRV-1, Pan troglodytes rhadinovirus-1 (With kind permission from Tom Schulz).
Also, two putative origins of replication, ori-L and ori-R, have been identified within the HHV-8 genome. Ori-L is a 1050bp domain in DL-B with typical features of a herpesvirus lytic cycle DNA replication origin. These include clustered consensus binding motifs for AP-1 (Jun/Fos) and CTF, multiple short repetitive motifs that resemble features of EBV ori-Lyt, two motifs matching sites found within CMV ori-Lyt, and two long AT-rich palindromes which resemble the core loop structures of HSV ori-L and ori-S. Ori-R is situated in DL-E and is an almost identical inverted copy of ori-L. The positions of the two domains within the HHV-8 genome closely match those of EBV and, as in EBV, also lie directly adjacent to high G+C content tandem repeats. In addition, there are several internal repeat regions present in the LUR, i) waka/jwka – a 143bp sequence repeated twice, ii) fnrk – a sequence of 20 and 30bp repeats, iii) vnct – a sequence of 13bp repeats, iv) zppa – a sequence of 23bp repeats, v) moi – 15 different 11 to 16bp repeats, and vi) mdk – a complex A+G-rich repeat sequence.

1.3.4.1 Strain variation and geographic distribution

At the left-hand side (LHS) of the HHV-8 genome the viral gene ORF-K1 is possibly the most divergent reading frame and has evolved into four subtypes, A, B, C, and D. The B and D subtypes differ from the A and C subtypes by 30% and 24%, respectively, whereas A and C differ from each other by 15%. KS patients from Africa or of African heritage were found to be almost exclusively subtype B, whereas the rare D subtypes were found only in KS patients of Pacific Island heritage. In contrast, subtypes A and C predominate in all parts of Europe and the US, however C subtypes also
occur in the Middle East and Asia. The pattern of variability in K1 is consistent with the idea that the four major HHV-8 subgroups evolved with different populations (Zong et al., 1999, Cook et al., 1999).

Analysis of sequence variability at internal constant-region loci (ORF26, T0.7/K12, and ORF75) revealed consistent subtype A, B, C, and D patterns matching those in ORF-K1 (Poole et al., 1999). However, a very different pattern of strain subgrouping occurs at the extreme right-hand side (RHS) of the HHV-8 genome, whereby two alternative highly diverged predominant (P) and minor (M) subtypes of ORF-K15 protein are essentially unlinked to the LHS (Glenn et al. 1999). The P and M forms are structurally related integral membrane proteins that have only 33% overall amino acid identity to one another. It may be that this relatively low homology between the two K15 subgroups represents a level of evolutionary divergence that is consistent with an origin for one of them (the M allele) by recombination with a closely related \( \gamma \)-2-herpesvirus (Poole et al., 1999). Whether this virus was of human or animal origin is not known.

1.3.5 Molecular pathogenesis and transformation potential

Epidemiological studies provide strong evidence for the role of HHV-8 in the pathogenesis of KS, PEL and MCD. More recently, sequence analysis and molecular biological studies have identified a number of potential viral oncogenes that may contribute to HHV-8-related neoplasia. The presence, expression, and functional integrity of the many captured cellular homologues identified (Table 1.2) enhance the possibility that HHV-8, like EBV and HVS, probably has the potential to directly influence the proliferation state of its host
cells. EBV is the closest known relative of HHV-8 in humans. The EBV nuclear antigens (EBNAs) and latent membrane proteins (LMPs) of EBV are known to be crucial for the maintenance of viral latency and for growth transformation of the host cell by the virus. Genes homologous to the EBNAs and LMPs of EBV are not present in the rhadinoviruses, including HHV-8. However, there is a striking correlation between the cell-homologous genes encoded by HHV-8 and cellular genes induced by EBV through the EBNA and LMP proteins. EBV induces huIL-6, cyclin-D, an IL-8 receptor, cellular Bcl-2, and the complement-controlling protein CR-2. This suggests that HHV-8 may modify the same cell regulatory and signalling pathways as EBV, but use different strategies to achieve these effects. The sole purpose of these genes appears to be as a defence against stereotypical cellular responses to viral infection, i.e., cell cycle shutdown, induction of apoptosis, and in vivo enhancement of cell-mediated immunity.

1.3.5.1 Genes involved in cell cycle control

**Nucleotide metabolites**

Sarisky et al. have reported that both viral dihydrofolate reductase (vDHFR) and thymidylate synthase (vTS) retain the normal functional activities of their cellular homologues. As enzymes involved in nucleotide metabolism, including the viral phosphoribosylformylglycinamidine synthase (vFGAM), these could be utilised by HHV-8 to cause replication of DNA to occur outside of the S phase of the cell cycle, which in turn would also facilitate the proliferation of transformed cells.
Cyclin-D homologue

HHV-8 encodes a cyclin-D homologue (ORF-72) which is expressed during latency and promotes phosphorylation of the retinoblastoma protein (pRB), like that of HVS through the activation of cyclin-dependent kinase 6 (CDK-6), and can thereby overcome a cell cycle checkpoint imposed by this tumour suppressor protein (Chang et al., 1996, Godden-Kent et al., 1997). In addition, the substrate specificity of CDK-6 is altered by v-cyclin to allow phosphorylation of histone H1, an ability also exhibited by HVS v-cyclin but not by cellular cyclin-Ds (Godden-Kent et al., 1997). The broader substrate specificity of the HHV-8 cyclin-CDK complex opens the possibility that it is able to fulfil functions other than promoting G1 progression, e.g. to mediate progression through further checkpoints imposed later in the cycle. Furthermore, the v-cyclin, unlike cellular D-type cyclins, is resistant to p16, p21, and p27 CDK inhibitors (Swanton et al., 1997), suggesting that expression of the HHV-8 cyclin may re-establish cell cycle homeostasis in the presence of active cellular antiviral responses that would otherwise induce cell cycle arrest.

1.3.5.2 Genes involved in control of cell apoptosis

Bcl-2 homologue

As well as preventing pRB-mediated cell cycle arrest, over-expression of v-cyclin can rapidly induce apoptosis in cells with wild-type p53. Therefore, for infection to persist it may be expected that HHV-8 would encode genes that can prevent apoptosis. Like EBV and HVS, HHV-8 also carries a gene
(ORF-16) with homology to the anti-apoptotic, Bcl-2 gene. Transcripts of the HHV-8 vBcl-2 have been detected in HHV-8-associated malignancies, including classical and AIDS-KS lesions, suggesting a possible pathogenic role analogous to Bcl-2 (Sarid et al., 1997). The same group backed this up by demonstrating vBcl-2 has the ability to suppress Bax-induced cell death in yeast and human cells. Another group reported that overexpression of the vBcl-2 blocks Sindbis virus-induced apoptosis at levels comparable to those displayed by cellular Bcl-2, Bcl-xL, and the EBV Bcl-2 homologue BHRF1 (Cheng et al., 1997). Although there is disagreement as to whether or not vBcl-2 heterodimerises with cellular members of the Bax-Bcl-2 family, which appears to be crucial for cellular Bcl-2 to prevent Bax-mediated apoptosis. Like other viral Bcl-2 homologues, HHV-8 vBcl-2 appears to be expressed during lytic replication, during which time the cell may be particularly prone to apoptosis, indicating its prime function is to prolong the survival of lytically infected cells.

**FLICE-inhibitory protein**

In 1997, Thome et al. identified a new family of anti-apoptotic agents, FLICE (FADD [Fas-associated death domain]-like IL-1β-converting enzyme)-inhibitory proteins (FLIPs), present in several γ-herpesviruses which interfere with apoptosis signalled through death receptors. The vFLIPs, including the HHV-8 vFLIP encoded by ORF-71/K13, contain two domains with significant homology to mammalian death-effector domains (DED). Like the other viral FLIPs, the HHV-8 encoded FLIP protects cells from Fas-mediated apoptosis by inhibiting caspase activation, via DED-DED interactions with the adaptor
protein FADD, and permits clonal growth in the presence of death stimuli in vitro (Djerbi et al., 1999). In addition, HHV-8 vFLIP transduced murine B lymphoma cells (A20) injected into immunocompetent recipient mice rapidly develop into aggressive tumours showing a high rate of survival and growth. Transcription studies have shown that, unlike vBcl-2, the HHV-8 vFLIP is expressed in latently infected PEL cell lines and KS spindle cells suggesting a role in HHV-8 pathogenesis and tumourigenesis (Sarid et al., 1998, Talbot et al., 1999).

**Interleukin-6 homologue**

Cellular IL-6 has been implicated in the pathogenesis of several malignancies such as KS, multiple myeloma and Castleman’s disease. Both murine and human IL-6 have been shown to inhibit apoptosis in myeloma cell lines (Lichtenstein et al., 1995), and HHV-8 ORF-K2 encodes an interleukin 6-like cytokine (vIL-6) that may play a similar role in infected cells.

AIDS-KS cell lines have been reported to secrete large amounts of biologically active IL-6 and IL-6 receptor, which have also been shown to be present at higher levels in KS lesions than in surrounding normal dermis in vivo. Furthermore, addition of recombinant human IL-6 to cultured KS spindle cells gives rise to increased proliferation rates (Miles et al., 1990). However, only 1-2% of cells in KS lesions have been reported to express HHV-8-encoded vIL-6 (Moore et al., 1996), thus it is unlikely that vIL-6 significantly contributes to KS pathogenesis. In contrast, high level vIL-6 expression is observed in the PEL cell lines BCP-1 and BC-1 (Moore et al., 1996), and in HHV-8 positive Castleman’s disease tissue (Parravicini et al., 1997).
addition, recombinant vIL-6 has been reported to support the growth and survival of the IL-6-dependent mouse hybridoma cell line B9 (Moore et al., 1996, Nicholas et al., 1997) and the human myeloma cell line INA-6 (Burger et al., 1998). Thus, vIL-6 is proposed to play a role in the pathogenesis of PEL and MCD.

More recently, nude mice inoculated with NIH3T3 cells expressing vIL-6 were observed to develop tumours more rapidly than control cells, and tumours positive for vIL-6 appeared more vascular (Aoki et al., 1999). The same study detected high levels of vascular endothelial growth factor (VEGF), an angiogenesis and Kaposi's-spindle-cell growth factor, in cells expressing vIL-6, and using immunohistochemical staining detected VEGF in spleen, lymph nodes, and tumour tissues from mice injected with vIL-6-producing cells. A recent study has also reported that vIL-6 stimulates expression, and induces secretion of endogenous human IL-6 from cells derived from MCD patients and various other cell lines (Mori et al., 2000). However, the two cytokines are produced by different cells within the affected tissue. vIL-6 positive cells were arrayed around the periphery of the germinal centres of plasma cells of a lymph node of MCD, whereas the hIL-6 was detected predominantly in the germinal centres and in rare surrounding cells (Parravicini et al., 1997). Thus, vIL-6 plays an important paracrine role in the pathogenesis of HHV-8 associated diseases.
1.3.5.3 Genes involved in cell-mediated immunity

Interferon-response factor homologue

In addition to cell cycle shutdown and apoptosis, enhanced immune recognition is another important cellular defence against virus infection. Many of these antiviral effects are co-ordinated at the cellular level through interferon (IFN) signal regulation. Moore et al., (1996) identified the protein encoded by HHV-8 ORF-K9 as an interferon regulatory factor (vIRF) homologue. Members of the IRF family positively or negatively regulate IFN signal transduction through binding to IFN-stimulated response elements (ISRE) in the promoters of genes under IFN induction control. HHV-8 vIRF inhibits IFN-β signal transduction by interfering with IRF-1, a positive regulator of IFN-β mediated gene expression (Gao et al., 1997, Zimring et al., 1998 Li et al., 1998). IRF-1 also plays a role in cellular responses mediated by other cytokines involved in antiviral and inflammatory immune responses, including IFN-α, IFN-γ, TNF-α, IL-1β, and IL-6. Thus, by inhibiting IRF-1, HHV-8 can potentially interfere with antiviral immunology at numerous points. Moreover, vIRF prevents IFN-β-induced transcription of the CDK inhibitor p21, suggesting a possible effect on cell cycle regulation (Gao et al., 1997) (See above). In addition, full cellular transformation of NIH3T3 cells was induced upon expression of vIRF, and injection of these vIRF-expressing NIH3T3 cells into nude mice led to the formation of tumours (Gao et al., 1997, Li et al., 1998). Preliminary studies demonstrated that vIRF was not significantly expressed in KS tumours, but was expressed in PEL cell lines at low levels during latency and at markedly enhanced levels post induction of the lytic cycle. This suggests that vIRF is a transforming oncogene active in B-cell
neoplasia’s that may provide a unique immune escape mechanism for infected cells.

**G-protein coupled receptor homologue**

The ORF-74 genes of both HHV-8 and HVS encode a G-protein coupled receptor (GCR) with high sequence homology to the IL-8 receptor. The GCR homologue encoded by HHV-8 is a potent, constitutively active molecule which when expressed in rat fibroblasts leads to enhanced proliferation and increased expression of VEGF (Arvanitakis et al., 1997, Bais et al., 1998). When these cells are transplanted into nude mice, the formation of highly angiogenic tumours is observed, supporting a role for this receptor in tumourigenesis. Bais et al. also found that vGCR can activate the JNK/SAPK and p38MAPK pathways, by triggering signalling cascades like those induced by inflammatory cytokines that are angiogenesis activators and mitogens for Kaposi’s sarcoma cells and B-cells. In virus-infected lymphoid cells and in KS tumours HHV-8 GCR is expressed primarily during the early phases of lytic replication (Kirshner et al., 1999), and so it is unlikely that cells expressing detectable GCR transcripts in KS will go on to proliferate. In fact, the role of vGCR in KS pathogenesis may rely more on the paracrine release of potent angiogenic factors to trigger neovascularisation, a hallmark of KS histology.

**Chemokine homologues**

Unique among known human herpesviruses, HHV-8 encodes two genes, vMIP-1 (ORF-K6) and vMIP-2 (ORF-K4), whose products show sequence similarity to the MIP/RANTES CC chemokine family. Chemokines
and their receptors play a fundamental role in leukocyte migration and activation and haematopoiesis. Like other members of the MIP/RANTES family of chemokines, HHV-8 vMIP-1 can prevent nonsyncytia-inducing (NSI) HIV-1 entry and replication by binding to the CCR5 chemokine receptor, which is also the HIV-1 co-receptor (Moore et al., 1996). vMIP-2 also causes potent inhibition of HIV entry, but binds predominantly to the CCR3 chemokine receptor (Boshoff et al., 1997). Binding of vMIP-2 to CCR3 also has an agonistic role in the activation and chemotaxis of eosinophils, suggesting it may function to recruit cells to enhance HHV-8 pathogenesis (Boshoff et al., 1997). These data contrast to a report of the antagonistic action of vMIP-2 as an inhibitor of the chemotactic response to MIP-1α, -β, and RANTES in monocytes, suggesting vMIP-2 functions to block recruitment of cells as part of the viral defence mechanism (Kledal et al., 1997). Nevertheless, both vMIP-1 and vMIP-2 induce angiogenesis in chick embryos, which could suggest a role in the neoangiogenesis characteristic of KS lesions (Moore et al., 1996, Boshoff et al., 1997).

1.3.5.4 Non-homologous genes

**ORF-K1**

For both EBV and HVS, genes located close to the left end of the genome, LMP-1 and STP/TIP respectively, have been shown to be relevant for malignant transformation. Expression of STP in transgenic mice results in the development of epithelial and lymphoid tumours (Murphy et al., 1994, Kretschmer et al., 1996). The N-terminus of STP has been shown to associate with the cellular ras proto-oncogene to activate the ras signal
induction pathway (Jung et al., 1995), whereas, the carboxyl terminal cytoplasmic region of LMP-1 interacts with cellular tumour necrosis factor receptor associated factors (TRAFs), which have been shown to be essential for B-cell transformation by EBV (Mosialos et al., 1995, Izumi et al., 1997). However, sequence analysis has revealed that no obvious homologue of these genes is present in HHV-8. Instead, the K1 ORF is present at the same position, with the same orientation as LMP-1 but inverted relative to STP (and TIP).

ORF-K1 encodes a membrane glycoprotein, expressed during the lytic replication cycle (Sarid et al., 1998), which, although it has no sequence homology to STP or LMP-1, has both a transmembrane region and a short intracytoplasmic tail similar to the transforming genes of other rhadinoviruses encoded at equivalent genomic positions. Thus, K1 could be a credible candidate for a transforming gene. The transforming potential of K1 was demonstrated by Lee et al. (1998) using Rat 1 fibroblasts, who showed that expression of the K1 gene produced morphological changes and focus formation in rodent fibroblasts indicative of transformation. In addition, a recombinant herpesvirus in which the STP oncogene of HVS was replaced with K1 could immortalise primary T-lymphocytes to IL-2 independent growth and also induce lymphoma in common marmosets. However, as cells undergoing lytic replication would not be expected to survive it is unclear whether the in vitro transforming properties of K1 contributes to pathogenesis in vivo.
Latency-associated nuclear antigen

The latency-associated nuclear antigen (LANA/LNA-1), encoded by ORF-73 of the HHV-8 genome, is a highly immunogenic protein that is expressed predominantly during viral latency, in most KS spindle cells and in cell lines established from BCBLs (Rainbow et al., 1997, Kedes et al., 1997). LANA is found in intranuclear bodies that are associated with the heterochromatin and also contain the viral episomal genome (Ballestas et al., 1999, Szekely et al., 1999). It may thus play a role in the maintenance of HHV-8 episomal DNA in persistently infected cells and assist in the segregation of viral genome upon cellular division (Ballestas et al., 1999). More recently, LANA has also been shown to associate with histone H1 in BCBL cells, and a region at the left end of the HHV-8 genome which colocalises to the host chromosomes with LANA (Cotter et al., 1999). It was proposed that this chromosomal association of the HHV-8 genome is mediated by LANA and involves a tethering mechanism by which viral episomes are linked to host chromatin through simultaneous interaction with host chromosomal proteins including histone H1 and cis-acting KSHV DNA elements. LANA has also been shown to interact with p53 to repress its transcriptional activity and its ability to induce apoptotic cell death in vivo (Friborg et al., 1999). Indicating overall that LANA may prolong cell survival and, in so doing, sustain HHV-8 persistence.

Kaposin

One of the most abundant transcripts expressed in KS spindle cells and PEL cell lines is the latency-associated 0.7kb transcript, T0.7 (Staskus et
al., 1997, Sturzl et al., 1997). It contains three ORFs, one of which, ORF-K12 encodes a small 60 aa hydrophobic protein, kaposin. Functional studies on kaposin are limited, however one study reported that kaposin can induce tumourigenic transformation (Muralidhar et al., 1998). Constructs with kaposin expressed either from its endogenous promoter or from a heterologous promoter induced focal transformation upon transfection into Rat-3 cells. All transformed Rat-3 cell lines containing kaposin sequences produced high-grade, highly vascular, undifferentiated sarcomas upon subcutaneous injection into athymic nu/nu mice. Furthermore, kaposin protein was localised to the cytoplasm and detected in both transformed and tumour-derived cells by immunofluorescence.
1.4 Antiviral chemotherapy

Although HHV-8 is in a highly controlled latent state in the majority of infected cells, it appears that many of the lytic cycle gene products may be involved in the pathogenesis of HHV-8-associated diseases (as described above). Therefore, prophylactic antiviral therapy to limit in vivo lytic HHV-8 replication may decrease the incidence of KS and other associated diseases. In fact, in vitro studies have shown that HHV-8 is susceptible to ganciclovir (GCV), foscarnet, cidofovir, penciclovir (PCV) and (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) but relatively insensitive to acyclovir (ACV) (Kedes et al., 1997, Medveczky et al. 1997, Neyts et al., 1997). In addition, retrospective studies of HIV infected patient groups at risk of KS have suggested that the incidence of KS was lower in patients receiving GCV or foscarnet, but not ACV (Glesby et al. 1996, Mocroft et al. 1996). These antiviral drugs act by inhibiting viral DNA polymerase, thus preventing lytic replication. However, the nucleoside analogues GCV, PCV, BVDU, and ACV require phosphorylation in order to have antiviral activity. Two protein families, viral thymidine kinase (TK) and phosphotransferase (PT), have been identified in herpesviruses with the ability to activate these drugs. Only the alpha- and gamma- human herpesviruses encode a TK, whereas all human herpesviruses encode a PT (Table 1.3). The HSV UL-23 and the CMV UL-97 are the respective prototypes of the TK and PT families.
Figure 1.5 Diagrams showing the chemical structures of the antiherpesvirus drugs ganciclovir (GCV), aciclovir (ACV), foscarinet and cidofovir (CDV), and the antiretroviral drugs azidodeoxythymidine (AZT) and didehydrodeoxythymidine (d4T).
### Table 1.3 Herpesvirus kinases implicated in the activation of antiviral drugs.

<table>
<thead>
<tr>
<th>Herpesvirus</th>
<th>Thymidine Kinase</th>
<th>Phosphotransferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1</td>
<td>UL-23</td>
<td>UL-13</td>
</tr>
<tr>
<td>HSV-2</td>
<td>UL-23</td>
<td>UL-13</td>
</tr>
<tr>
<td>VZV</td>
<td>ORF-36</td>
<td>ORF-47</td>
</tr>
<tr>
<td>EBV</td>
<td>BXLFL1</td>
<td>BGLF4</td>
</tr>
<tr>
<td>CMV</td>
<td>-</td>
<td>UL-97</td>
</tr>
<tr>
<td>HHV-6</td>
<td>-</td>
<td>U-69</td>
</tr>
<tr>
<td>HHV-7</td>
<td>-</td>
<td>U-69</td>
</tr>
<tr>
<td>HHV-8</td>
<td>ORF-21</td>
<td>ORF-36</td>
</tr>
</tbody>
</table>

### 1.4.1 Thymidine kinase

Like most other human herpesviruses, the HHV-8 genome encodes a viral thymidine kinase (ORF-21). Cellular thymidine kinase (TK) is used in the salvage pathway of nucleotide biosynthesis for the phosphorylation of thymidine (dT) to thymidine monophosphate (TMP). Ordinarily, thymidine is not available to cells in sufficient quantity to support DNA replication. Instead, cells depend on synthesis of TMP from deoxyuridine monophosphate (dUMP). Thymidine is only produced from the breakdown of TMP when DNA is degraded. For this reason TK is called a "salvage" enzyme because it allows thymidine, produced by degradation processes, to be salvaged.

In herpesviruses, the TK is required for the phosphorylation of nucleosides to their monophosphates, which are then phosphorylated by cellular phosphotransferases to nucleoside triphosphates as eventual substrates for viral DNA synthesis. Herpesviral TK differs from cellular TK in
many ways. For example, mammalian cellular TK is substrate specific, using Mg\(^{2+}\)ATP to monophosphorylate only one substrate, thymidine. In comparison, the TKs of both HSV and EBV are able to phosphorylate a range of nucleoside analogues, to carry out multiple phosphorylation on thymidine, and to use nucleotides other than ATP as a phosphate donor.

### 1.4.2 Nucleoside analogues

The thymidine kinases of the \(\alpha\)-Herpesvirinae have been exploited for many years as activators of anti-herpesvirus drugs (Cheng et al. 1981, De Clercq, 1993). The majority of antiviral compounds that have been studied for herpesviruses are nucleoside analogues. The best studied and therefore most widely used is acyclovir (ACV). The structure of this compound is based on the nucleoside guanosine, one of the building blocks of DNA. Guanosine consists of the purine, guanine, covalently linked to the 1'-carbon on the sugar molecule, ribose. The difference in ACV is the ribose ring, which is acyclic with the 2'- and 3'-carbons missing.

During the synthesis of DNA a condensation reaction occurs between the DNA chain 3'-hydroxyl group and the 5'-phosphate of an incoming nucleoside triphosphate resulting in the formation of a phosphodiester bond and elongation of the DNA chain by one base. However, if acyclovir is present the drug inhibits viral DNA polymerase in carrying out further condensation reactions (because acyclovir does not possess a 3'-hydroxyl) resulting in the premature termination of DNA synthesis. Prior to the incorporation of acyclovir into DNA, the drug is initially activated by phosphorylation at the 5' hydroxyl position. In herpes simplex virus infected
cells the drug diffuses across the cell membrane and phosphorylation is carried out by the TK. Once phosphorylated, the drug is subsequently fully activated to the triphosphate form by cellular phosphotransferases and can now act as a DNA polymerase inhibitor resulting in the inhibition of viral replication. Acyclovir can be administered orally, intravenously or topically and is well tolerated with very few reported side effects. This is predominantly due to the drug being a good substrate for viral TK but not cellular TK so in uninfected bystander cells the drug remains in an unphosphorylated inactive form.

Acyclovir is most effective for the treatment of HSV-1 and VZV (Wagstaff et al., 1994) while its efficacy against HCMV is lower. However, ganciclovir (GCV), another nucleoside analogue has been shown to be more potent at inhibiting HCMV replication and is usually the first-choice antiviral used to treat HCMV infections. The structure of GCV is very similar to ACV the difference being GCV possesses a 3’-hydroxyl group. Therefore GCV, unlike ACV, is not an obligate chain terminator because the 3’-hydroxyl provides a suitable substrate for DNA polymerase to continue adding nucleoside triphosphates. This seemingly slight difference improves the antiviral activity against a broad range of the herpesviruses, however it increases cytotoxicity presumably because GCV can be incorporated into nascent cellular DNA.

The mechanism of action of GCV, like ACV, is reliant on the sequential phosphorylation at the 5’-hydroxyl position. However, HCMV does not encode a TK homologue although the UL-97 gene has been shown to catalyse the initial phosphorylation of GCV (Littler et al., 1992, Sullivan et al.,
1992). The increased efficacy of GCV in HCMV infected cells can be attributed to the favourable activation of GCV over ACV by the UL97 protein and also, HCMV is a slower growing virus than HSV and so GCV, with its increased intracellular half-life may be expected to be more potent.

Penciclovir (PCV), a nucleoside analogue is related to GCV in that it also has a 3’-hydroxyl and is not an obligate chain terminator. The difference occurs in the ribose ring where in PCV there is a carbon atom replacing the oxygen. This drug is predominantly administered for alphaherpesvirus infections.

1.4.3 Other antiviral compounds

1.4.3.1 Foscarnet

As described in the previous section (1.4.2) the synthesis of viral DNA, catalysed by viral DNA polymerase, is a condensation polymerisation. The end products of the reaction are the elongation of the DNA chain by one base and a pyrophosphate group. Foscarnet is a structural analogue of pyrophosphate and functions by occupying the pyrophosphate binding site on the virally encoded DNA polymerase, thus inhibiting efficient exchange of pyrophosphate during DNA synthesis resulting in a non-competitive inhibition of DNA polymerisation. Foscarnet has broad-spectrum anti-herpesvirus activity and is usually administered for HCMV infections that have failed GCV therapy due to resistance. Foscarnet is generally well tolerated with the main side effect being nephrotoxicity. The oral bioavailability is poor and so the drug is usually administered intravenously.
1.4.3.2 Cidofovir

Cidofovir is an acyclic nucleotide phosphonate analogue of cytosine. Like foscarnet, nucleotide analogues are not dependent on being initially phosphorylated by virally encoded TK or PT. Cidofovir is taken up by cells and di- and tri-phosphorylated by cellular enzymes, and its mechanism of action is by preferentially inhibiting viral DNA polymerase over cellular DNA polymerase. Cidofovir is indicated in CMV patients that have acquired resistance to either GCV or foscarnet. A distinguishing feature of cidofovir is that its plasma half-life is relatively long and so requires infrequent intravenous administration compared to GCV or foscarnet therapy.
1.5 Human immunodeficiency virus (HIV)

Human immunodeficiency virus (HIV) is a member of the lentivirus subfamily of retroviruses. Other members include simian immunodeficiency virus, visna virus and equine infectious anaemia virus. Retroviruses are RNA-containing viruses that encode the reverse transcriptase (RT) enzyme. Upon infection, RT transcribes the viral RNA into proviral DNA. This subsequently becomes integrated into host chromosomal DNA and serves as a template for viral genomic and messenger RNA transcription by the host cell's RNA synthetic and processing systems. HIV was first isolated in 1983 (Barré-Sinoussi et al., 1983) just two years after the identification of AIDS. Further, independent HIV isolates were reported in 1984 (Gallo et al., 1984, Levy et al., 1984), which, together with serology, made a convincing case for HIV as the cause of AIDS.

AIDS was initially observed in 1981, in the United States, when a handful of homosexual men presented with Pneumocystis carinii pneumonia (PCP) and KS. By the end of 1982 the disease was being detected in drug abusers, haemophiliacs, blood transfusion patients, sexual partners of high-risk group members, and children born to mothers at risk. These observations indicated that AIDS was caused by an infectious agent spreading by sexual intercourse, contaminated blood and blood products, and vertical transmission, i.e. in utero, intrapartum, or perinatally, and finally led to the discovery of HIV.

HIV and AIDS are now seen worldwide, with an estimated 33.4 million of the world's population (adults and children) living with the disease. Two immunodeficiency associated human lentiviruses have now been identified.
HIV-1, which is the prototype AIDS virus and is prevalent in Europe, the US and Central Africa, and HIV-2, which is largely confined to regions of West Africa and appears to show less virulence and penetrance than HIV-1.

1.5.1 HIV-1 pathogenesis

Primary HIV infection entails symptomatic fever and lymphadenopathy in about 50-70 % of infections. The symptoms include acute influenza-like or mononucleosis-like syndrome, often associated with a rash. Viraemia is seen at this time, which is detectable between one week and three months post-infection. After seroconversion there follows an asymptomatic phase of infection lasting 2-15 years. Virologically, however, this does not represent latent infection but rather a high turnover of HIV production and of infected CD4+ lymphocytes with equally active replenishment (Ho et al., 1995, Wei et al., 1995). A large number of CD4+ cells in the lymph nodes also appear to become infected during this period (Pantaleo et al., 1993, Haase et al., 1996), leading to the death of follicular dendritic cells and the collapse of the architecture of the lymph nodes.

Ultimately, immune destruction results in end-stage disease, i.e. AIDS, associated with opportunistic infections, malignancies and neurological disorders. PCP is one such infection and is the most common cause of mortality in patients with AIDS. Other opportunistic infections include Cryptococcus neoformans, Candida albicans, Mycobacterium tuberculosis and Toxoplasma gondii. In addition, Cytomegalovirus, Herpes simplex and Hepatitis B and C viruses are often reactivated in AIDS patients. The commonest AIDS-defining malignancy exhibited by patients is KS, however
non-Hodgkin's lymphoma and anogenital squamous carcinoma are also observed in high frequency. These events correlate with various virological and immunological changes such as a decrease in CD4 levels to below 200 x 10^6/l and a rapid rise in blood HIV RNA levels, which are used as predictors for AIDS diagnosis. Other markers include β_2 microglobulin, a plasma measure of immune activation, and p24 antigen.

1.5.2 HIV-1 life cycle

The first step in the viral life cycle is the interaction of the viral envelope with specific molecules or receptors on the surface of cells. The main target for HIV infection is the CD4+ receptor on T lymphocytes (Dalgleish et al., 1984). However, monocytes, macrophages, Langerhans cells, follicular dendritic cells, and glial cells are also shown to express this receptor and are known to be susceptible to HIV infection. Mediated by the gp41 glycoprotein, the virus binds to the CD4 molecule via the gp120 envelope protein. Whilst this is necessary it is not sufficient for HIV infection. The chemokine receptors CCR5 and CXCR4 have been identified to act as co-receptors to CD4 (Feng et al., 1996, Weiss and Clapham, 1996).

Following fusion with the cell membrane the viral nucleocapsid is then internalised and partially uncoated. The single-stranded viral RNA is transcribed into linear double-stranded DNA (provirus), by the virion-associated reverse transcriptase, which then migrates to the nucleus and is randomly integrated into the host genome. Mediated by the viral enzyme integrase, the termini of the long terminal repeat (LTR) regions of the viral DNA are covalently joined to the host cell DNA. Following integration, the cell
may either remain latently infected and integrated provirus passed on to progeny cells during mitosis (with little or no expression of virus), or may be productively infected, which is controlled by cellular activation.

Transcription involving cis- and trans-acting regulatory elements in the LTR produces viral mRNAs. The viral transcriptional transactivator Tat acts through a signal at the 5’ end of newly initiated viral transcripts to augment initiation and/or elongation by the host cell RNA polymerase II complex. The unspliced, singly spliced and multiply spliced viral transcripts are transported to the cytoplasm and translated into new viral proteins, which assemble at the cell membrane. Viral RNA associates with the nucleocapsid protein and then buds from the cell, with the cell membrane incorporating gp120 and gp41 forming the viral envelope. During and after budding, the p55 (gag) polyprotein precursor is cleaved by viral proteases, generating the mature gag proteins, p17, p24, p7 and p6. The mature HIV virion is now free to infect other cells.

1.5.3 Treatment of HIV infection

There are many sites throughout the HIV life cycle with the potential to be targets for antiretroviral drugs, including virus-cell attachment, fusion and entry, the viral integrase, and nucleocapsid assembly. In reality, however, only the reverse transcriptase and protease activities have been utilised in routine clinical use.

The first antiretroviral drug to be used was zidovudine or azidodeoxythymidine (ZDV or AZT), a nucleoside reverse transcriptase inhibitor (NRTI) (Mitsuya et al., 1985). AZT is a pyrimidine analogue with an
azido group replacing the 3' hydroxyl group on the ribose ring. AZT is phosphorylated by cellular thymidine and thymidylate kinases to the mono- and diphosphate forms, respectively. The formation of AZT-diphosphate appears to be a rate-limiting step, reflected by high levels of monophosphate and its ability to inhibit thymidylate kinase. A cellular nucleoside diphosphate kinase completes the final phosphorylation to its active form of AZT-triphosphate (AZT-TP), a competitive substrate for RT with deoxythymidine-TP (dTTP) which is incorporated into the elongating 3' end of the yet-unintegrated proviral DNA. This results in the premature termination of chain elongation due to the inability of AZT-TP to form a normal phosphodiester linkage. The first clinical use of AZT was in 1986, when it was shown to benefit patients with symptomatic disease (Fischl et al., 1987). Later, AZT was administered to both asymptomatic and symptomatic patients, however, in 1994 a large placebo-controlled study showed that early treatment with AZT was no better than deferring therapy until patients were symptomatic (Concorde Coordinating Committee, 1994).

Stavudine (d4T) was the second dT analogue to be approved for HIV chemotherapy and has a similar mechanism of action as AZT. D4T inhibits HIV replication in vitro with similar potency to AZT, and in patients with at least 6 months of prior AZT experience altering therapy to d4T has been shown to be superior to continuing with AZT. Further nucleoside and non-nucleoside RTIs have been developed, such as didanosine (ddl), zalcitabine (ddC), lamivudine (3TC) and nevirapine, but have had limited clinical efficacy as monotherapies. The use of some of these drugs as part of a dual combination therapy with AZT or d4T, however, has proven more successful
than monotherapy in delaying disease progression, reducing viral load and improving survival rates. Nevertheless, these two drug combination regimens ultimately fail, primarily due to the development of resistant mutations in HIV RT.

The introduction of protease inhibitors, such as saquinivir, indinivir and ritonivir, in 1995 provided a class of drugs with a new site of action that had a marked potency against HIV. Moreover, it was the use of these drugs in combination with two RT inhibitors, known as highly active antiretroviral therapy (HAART) that had exceptional effects on HIV-infected patients. Plasma virus fell to undetectable levels (<50 copies/ml) within 2 to 4 months in patients treated with HAART and it became difficult to culture the virus from blood. This also coincided with increasing CD4 cell counts and control of opportunistic infection and neoplasia (Carpenter et al., 1997, Perelson et al., 1997, Gulick et al., 1997). In addition, time to AIDS diagnosis and survival expectations appeared to be substantially better in patients treated with HAART compared to those treated in the pre-HAART era (The CASCADE Collaboration, 2000).
Aims of the thesis

The objectives of the work carried out for this higher degree were:

1. The development of qualitative and quantitative-competitive PCR methods for the detection and quantification of HHV-8 DNA.

2. The use of these PCR assays to look at the presence of HHV-8 DNA in various post-mortem tissues of AIDS patients, to investigate how widely disseminated HHV-8 is throughout the body, and to correlate the results with the pathological presence of KS and with HIV proviral loads.

3. To clone and express the HHV-8 thymidine kinase homologue, ORF 21 in an E. coli expression system.

4. To examine the activity of the recombinant HHV-8 TK with respect to phosphorylation of its natural substrate deoxythymidine, and nucleoside analogues used in the treatment of herpesvirus and HIV infections.
Chapter 2 - Materials and Methods
2.0 Development of a qualitative PCR for HHV-8

To detect the presence of HHV-8 DNA in patient samples a nested qualitative PCR was developed, which amplifies a 233bp region of the KS330BamHI DNA sequence previously described by Chang et al. (1994).

2.0.1 Patient samples

All the studies in this thesis received ethical approval from the Royal Free Hospital ethical committee.

Blood samples were obtained from a renal transplant recipient who had developed KS 56 months post-transplant with consent, as part of established surveillance protocols. The patient received immunosuppressive therapy post-transplantation, consisting of azathioprine, cyclosporin A, and prednisolone, until transplant failure when immunosuppressive treatment was stopped. Post-mortem tissue samples were obtained from 12 AIDS patients and 3 control patients (Two were in road traffic accidents and the third died from a subdural haematoma).

2.0.2 Extraction of DNA from patient tissue and blood samples

Two different methods were utilised for the extraction of total DNA from tissue and blood samples:

DNA was extracted from patient tissue samples using the Promega genomic DNA extraction kit. Six hundred microlitres of nuclei lysis solution were aliquoted into 1.5ml eppendorfs and chilled on ice. 10-20mg of fresh or thawed tissue was added to the chilled nuclei lysis solution, with 10μl proteinase K (20μg/μl), and incubated overnight, at 55°C. To remove RNA
from the nuclear lysate, 3μl of RNase solution was added. Samples were initially mixed by inverting the tubes 25 times, and then incubated at 37°C, for 15-30 mins. Samples were allowed to cool to room temperature, for 5 mins, before the addition of 200μl of protein precipitation solution.

The RNase-treated lysates were vortexed vigorously for 20 seconds, and centrifuged for 3 mins, at 13000rpm, to pellet the precipitated protein. Supernatants were carefully removed from the tubes and transferred to clean 1.5ml tubes containing 600μl of isopropanol. The solutions were gently mixed by inversion until the strands of DNA had formed a visible mass, at which point the DNA was pelleted by centrifugation at 13000rpm, for 1 min. The supernatants were decanted and 600μl of 70% ethanol was used to wash the pellet, followed by centrifugation as before. The ethanol was carefully aspirated, using a fine-tip Pasteur pipette, without disturbing the pellet. Tubes containing the DNA pellet were inverted onto absorbent paper and air-dried for 10-15 mins. The DNA pellets were finally rehydrated in 100μl sterile distilled water, by incubating at 65°C for 1hr, and then stored at -70°C until required for analysis.

The QIAamp DNA blood mini kit was used to extract DNA from citrate treated whole blood. Two hundred microlitres of whole blood were aliquoted into a 1.5ml eppendorf tube containing 20μl of QIAGEN protease. Two hundred microlitres of Buffer AL was added to the sample, vortexed for 15 secs and incubated at 56°C for 10 mins. Samples were briefly centrifuged in a microfuge before the addition of 200μl of ethanol (96-100%), followed by
vortexing for 15 secs to mix. After a brief spin in a microfuge, the mixture was applied to a QIAamp spin column, placed in a 2ml collection tube, and centrifuged at 8000rpm for 1 min. The 2ml collection tube was replaced with a fresh tube and 500µl of Buffer AW2 was added to the column, followed by centrifugation at 13000rpm for 3 mins. To prevent any carryover of Buffer AW2, the collection tube was replaced and the column centrifuged at 13000rpm for 1 min. The 2ml collection tube was discarded and the spin column was placed in a clean 1.5ml eppendorf. Two hundred microlitres of distilled water was added to the spin column and incubated at room temperature for 5 mins, before eluting by centrifuging at 8000rpm for 1 min. Samples were stored at -70°C until required for analysis.

2.0.3 Optimisation of the HHV-8 PCR

The first round oligonucleotide primers, 8EF and 8ER (Table 2.1), were designed to produce a 300bp fragment of the KS330 region. This product was then used as a template for a second round of PCR using nested primers, 8IF and 8IR (Table 2.1), which amplified the 233bp fragment KS330233.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>8EF</td>
<td>5' - CCA GCT AGC AGT GCT ACC CCC ATT - 3'</td>
</tr>
<tr>
<td>8ER</td>
<td>5' - ATG GAC AGA TCG TCA AGC ACT CGC - 3'</td>
</tr>
<tr>
<td>8IF</td>
<td>5' - AGC CGA AAG GAT TCC ACC ATT GT - 3'</td>
</tr>
<tr>
<td>8IR</td>
<td>5' - TCC GTG TTG TCT ACG TCC AGA CG - 3'</td>
</tr>
</tbody>
</table>

Table 2.1 Sequences of primers for nested HHV-8 PCR.
Optimisation of each round of the PCR reaction was performed with primer concentrations ranging from 50ng to 200ng. Each reaction was performed in triplicate, in 1 x KCl buffer containing 1.5mM MgCl₂, with 187.5μM of each dNTP and 1 unit of BioTaq polymerase. Target DNA, extracted from a KS biopsy from an HIV +ve patient, was added to a final reaction volume of 50μl. The cycling parameters used were identical for both rounds:

- **Hot start**: 95°C for 6 mins → 1 cycle
- **Denaturation**: 94°C for 30 secs
- **Annealing**: 60°C for 30 secs → 39 cycles
- **Extension**: 72°C for 30 secs
- **Final extension**: 72°C for 5 mins → 1 cycle

PCR amplicons were detected by electrophoresing 10μl of the amplified reaction against an appropriate molecular weight marker on a 3% (w/v) agarose gel.

When the optimum primer concentration had been determined, the concentration of MgCl₂ was optimised. MgCl₂ concentrations of 1mM, 1.5mM and 2mM were used in duplicate reactions, with the KCl buffer being replaced with 1 x NH₄Cl₂ buffer, without MgCl₂. The concentrations of the other reagents, and the cycling conditions used previously remained the same. The sensitivity of the nested PCR assay was determined by amplifying known quantities of an HHV-8 positive control plasmid (see section 2.0.5), ranging from $10^6$ to $10^0$ copies.
2.0.4 Analysis of PCR amplification by agarose gel electrophoresis

One hundred millilitre agarose gels were made to the appropriate concentration (w/v) in 1 x TBE buffer (Tris-HCl, boric acid, EDTA), melted in a microwave oven and ethidium bromide was added to a final concentration of 0.1μg/ml. The gel mix was poured into a gel casting tray, with the gel comb, and allowed to set. Once set, the gel was placed in an electrophoresis tank containing 1 x TBE buffer. Five microlitres of DNA loading buffer (0.02% w/v bromophenol blue in 15% w/v Ficoll) was added to 10μl of each reaction and loaded into separate wells, alongside the φX174/HaeIII cut DNA marker to estimate the molecular size of amplified DNA. DNA was separated by electrophoresis at 120V for 30 mins and visualised under ultra-violet (UV) light.

2.0.5 Construction of an HHV-8 positive control

To construct a positive control for HHV-8 PCR amplification the 300bp fragment, described in 2.0.3, was ligated into the cloning vector pUC18. The pUC18 was supplied pre-cut with SmaI and dephosphorylated. Therefore, the insert required phosphates on its 5' end to form phosphodiester bonds with the vector. To achieve this the fragment was amplified with phosphorylated primers.

2.0.5.1 Phosphorylation of primers

5μg of primers 8EF and 8ER were phosphorylated in 1 x forward reaction buffer, using 100pmoles ATP and 20U T4 polynucleotide kinase, in a final volume of 50μl. The reaction was carried out at 37°C for 30 mins and
stopped by increasing the temperature to 68°C for 10mins, which inactivated the enzyme. The 300bp fragment was amplified using 100ng of each phosphorylated primer, under optimised PCR conditions, and purified before being cloned.

2.0.5.2 Purification of amplified DNA from agarose gels

Amplified DNA was separated by electrophoresis in a TAE (Tris-HCl, acetic acid, EDTA) low-gelling point agarose gel. DNA bands were visualised under UV light and excised from the gel using a sterile scalpel blade. The excised DNA band was then purified from the gel using the Wizard PCR preps DNA purification system. The 300μl agarose slice was transferred to a 1.5ml eppendorf tube and incubated at 70°C, until the agarose had melted. One millilitre of purification resin was added to the melted agarose and mixed thoroughly for 20 seconds. A Wizard Minicolumn was placed into a fresh 1.5ml eppendorf and a syringe barrel attached, without the plunger. The DNA/resin mix was pipetted into the syringe barrel and slowly pushed through with the syringe plunger. The barrel was detached from the Minicolumn and the barrel removed. The syringe barrel was then reattached and 2ml of 80% isopropanol syringed through the Minicolumn. The syringe was discarded and the Minicolumn transferred to a fresh eppendorf, before centrifugation at 10000g, for 2 mins, to dry the resin. The eppendorf was replaced and 50μl of sterile distilled water applied to the column. The column was left for no more than 30 mins before centrifuging at 10000g for 20 seconds, to elute the DNA fragment. The purified DNA was stored at 4°C or -20°C, for future use.
2.0.5.3 Ligation of HHV-8 DNA into the cloning vector pUC18

Equimolar ratios of the insert DNA and vector were determined using the following equation:

\[ X \text{ ng of insert DNA} = \frac{Y \text{ ng of vector DNA} \times \text{No. of insert base pairs}}{\text{No. of vector base pairs}} \]

Therefore, for a 1:1 ratio, 11.2ng of the HHV-8 300bp fragment were blunt-end ligated into 100ng of pUC18 (SmaI digested and dephosphorylated). Ligation reactions with vector:insert ratios of 1:2 and 1:3 were also performed. Each reaction, set up in a 20μl volume, contained 2 Weiss Units of T4 DNA ligase and 1mM hexaminecobalt chloride, in 1x T4 ligase buffer (including ATP), and were incubated at 16°C, overnight. Ligation reactions were transformed into competent JM109 *Escherichia coli* cells.

2.0.5.4 Preparation of competent *E.coli* cells

A single colony of JM109 *E.coli* cells was selected and used to inoculate 5ml of LB (Luria-Bertani) media. The cells were grown at 37°C to an OD$_{550}$ of 0.3 absorbance units (AU) and subcultured 1:20 into 100ml LB. The culture was then grown to an OD of 0.48 AU, chilled on ice for 5 mins and centrifuged at 6000rpm for 5 mins at 4°C. The supernatant was carefully decanted and the cell pellet resuspended in 2/5 volume (40ml) of ice-cold transformation buffer I (Tfbl), consisting of 30mM potassium acetate, 100mM RbCl$_2$, 10mM CaCl$_2$, 50mM MnCl$_2$ in 15% (v/v) glycerol, adjusted to pH5.8 with acetic acid. The resuspended cells were incubated on ice for 20 mins and, pelleted by centrifugation at 5000rpm for 5 mins, at 4°C. The cells were resuspended in 4ml of ice-cold Tfbl I (10mM MOPS, 10mM RbCl$_2$, CaCl$_2$ in
15% (v/v) glycerol, adjusted to pH 5.8 with KOH) and left on ice for a further 20 mins. The cell suspension was aliquoted into 200µl volumes in 1.5ml eppendorf tubes and immediately snap frozen in a methanol/dry ice bath. The competent cells were stored at -70°C until required.

2.0.5.5 Transformation of E.coli cells with plasmid DNA

Frozen competent JM109 cells were thawed and incubated for 10 mins on ice. 10µl of plasmid DNA (one-tenth the volume of cells) were added to 100µl of cells and incubated on ice for a further 30 mins. The cell/plasmid mix was heat-shocked at 42°C for 90-120 secs, with constant agitation, and then cooled on ice for 1 min. 390µl of pre-warmed LB broth was added to the mix and incubated for 1 hr at 37°C. Aliquots of 100µl and 200µl were plated onto LB/agar plates containing 50µg/ml ampicillin, plus 0.5mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 40µg/ml 5-bromo, 4-chloro, 3-indolyl-β-galactopyranoside (X-gal) if blue/white colony screening was required.

2.0.6 Small-scale preparations of plasmid DNA

Small-scale plasmid DNA preparations from transformed E. coli was performed using the QIAprep-8 miniprep kit. This method utilises the alkaline lysis of bacteria, and a silica gel membrane to bind the plasmid DNA on filter strips, under vacuum. One to 5ml cultures, initiated from a single colony, were harvested by centrifugation in a microfuge and the cell pellet was resuspended in 250µl of P1 buffer (50mM Tris-HCl, pH8.0, 10mM EDTA, 100µg/ml RNase A). The bacteria were lysed with the addition of 250µl of buffer P2 (200mM NaOH, 1% SDS). Five hundred microlitres of buffer N3
(3.0M potassium acetate, pH5.5) was added to precipitate protein and chromosomal DNA, and the suspension was clarified by centrifugation at 13000rpm, for 10 mins in a microfuge. The supernatant was applied to the well of a QIAprep-8 strip and the vacuum applied. Under vacuum, the well was washed with 1ml of buffer PB (buffer composition not available), to remove carbohydrate contamination. The buffer was allowed to flow through and each well was further washed with two 1ml aliquots of buffer PE (buffer composition not available). Once the liquid had passed through, the vacuum was applied for a further 3 mins to allow the membrane to dry to enhance elution. The bound DNA was eluted from each well under vacuum by the addition of 100µl of SDW.

2.0.7 Large-scale preparations of plasmid DNA

Large quantities of plasmid DNA were extracted from transformed E. coli using the Qiagen Maxi plasmid purification protocol, which is based on the alkaline lysis of bacteria, followed by binding of the plasmid DNA to Qiagen anion exchange resin. The column, under the appropriate conditions, binds only DNA, and impurities are washed away. A 150ml culture of E. coli was harvested by centrifugation at 5000g in an IEC PR-7000, and resuspended in 10ml of buffer P1 (50mM Tris-HCl, pH8.0, 10mM EDTA, 100µg/ml RNase A).

To lyse the cells, 10ml of buffer P2 (200mM NaOH, 1% SDS) were added to the suspension, mixed gently and incubated at room temperature for 5 mins. Proteins and chromosomal DNA were precipitated by the addition of chilled P3 buffer (3.0M potassium acetate, pH 5.5) and incubation on ice for 20 mins. The suspension was filtered using a Qiagen maxi-prep filter and the
supernatant containing the plasmid DNA was loaded onto a Qiagen-tip 500, pre-equilibrated with 10ml of QBT buffer (750mM NaCl, 50mM MOPS, pH 7.0, 15% ethanol, 0.15% Triton X-100). The supernatant was allowed to enter the column by gravity flow, after which the column was washed twice with 30ml of buffer QC (1.0M NaCl, 50mM MOPS, pH 7.0, 15% ethanol). The bound DNA was eluted with a high salt buffer, QF (1.25M NaCl, 50mM MOPS, pH 8.5, 15% ethanol). The DNA solution was transferred to a 50ml conical tube and the DNA was precipitated with the addition of 10.5ml of isopropanol, followed by centrifugation at 8000g in an IEC PR-7000 centrifuge. The supernatant was aspirated and the DNA pellet was washed with 70% ethanol. The DNA was air dried and resuspended in an appropriate volume of SDW (200-500μl), and either, used immediately, or stored at -20°C.

2.0.8 Restriction endonuclease digestion

To determine whether cloning was successful, the plasmid DNA was digested with restriction endonucleases, at cleavage sites found within the multiple cloning site (MCS) of the vector. A double digest was performed, with 10 units of both EcoRI and HindIII, and 10μl of HHV-8 plasmid DNA (pUC18-8300), in 1 x buffer B to a final volume of 20μl. Digestion reactions were carried out at 37°C for 1hr, and then separated by agarose gel electrophoresis, as in section 2.0.3.

2.0.9 DNA sequencing

Clones were sequenced using the Sanger dideoxy-mediated chain termination method. Ten micrograms of plasmid DNA in 20μl of SDW was
denatured by the addition of 5µl 1M NaOH/1mM EDTA and incubated at room temperature for a minimum of 5 minutes. Spin purification columns were prepared by making a small hole in the bottom of both a 0.5ml and 1.5ml eppendorf tube. A 20µl solution of glass beads was added to the 0.5ml eppendorf tube, below a mixture of 2 parts Sepharose CL-6B and 1 part TE. The 0.5ml eppendorf tube was placed in the 1.5ml eppendorf, and both were seated in a 15ml tube for centrifugation at 1400rpm for 5 mins, to compact the Sepharose. The pierced 1.5ml eppendorf was replaced with a fresh tube and the denatured plasmid DNA was spun through the column by centrifugation, as above.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>M13 Universal</td>
<td>5' – GTA AAA CGA CGG CCA GT – 3'</td>
</tr>
<tr>
<td>M13 Reverse</td>
<td>5' – GTT TTC CCA GTC ACG AC - 3'</td>
</tr>
</tbody>
</table>

Table 2.2 Sequence of M13 primers used for sequencing.

One microlitre of either the M13 universal or reverse oligonucleotides (10ng/µl), see Table 2.2, was annealed to 5µg of denatured DNA (8µl), in 1µl 10 x TM buffer (100mM Tris/100mM MgCl₂), and incubated at 37°C for 15 mins. The hybridised DNA and oligonucleotide were allowed to cool to room temperature before sequencing using the United States Biochemical T7 Sequenase™ version 2.0 sequencing kit. To the annealed DNA/oligonucleotide, 1µl dithiothreitol (0.1M), 2µl dGTP labelling mix (diluted 1/5 in sterile distilled water), 0.5µl [α-35S] dATP (ICN) and 2µl Sequenase
version 2.0 (diluted 1/8 in Sequenase dilution buffer) were added, which was then incubated at room temperature for 3 mins.

Into 4 appropriately labelled tubes, 2.5μl of ddGTP, ddATP, ddTTP and ddCTP were aliquoted, separately. Sequencing was terminated by adding 3.5μl of the sequencing reaction to each tube, with a 5 minute incubation at 37°C. Each reaction was then stopped by the addition of 4μl of stop solution and stored at -20°C until required, or heated to 95°C for 3 minutes before separation by polyacrylamide gel electrophoresis (PAGE).

2.0.10 Polyacrylamide gel electrophoresis of sequenced DNA

An 8% denaturing polyacrylamide gel was prepared by mixing 75g of Urea, 30ml of 40% acrylamide/2% bis-acrylamide, 15ml of 10 x TBE, and 45ml of SDW on a heated stirrer. The two sequencing plates required to make the gel were cleaned thoroughly with 70% ethanol, and the inside of one plate was coated in dimethyl-dichlorosilane, to prevent the gel from adhering to both plates upon separation. Spacers, 0.5mm in depth, were placed on either side of one plate. To create a wedge gel, two smaller spacers about 2cm in length were placed on the bottom of these, followed by the second plate. The plates were then sealed together with tape. When the acrylamide mix had dissolved, it was allowed to cool to room temperature before the addition of 900μl of 10% ammonium persulphate and 80μl of N,N,N',N'-tetramethylethylenediamine (TEMED). The gel was then poured carefully between the plates, and shark-tooth combs inserted upside down, to create a straight line along the top. Once the gel had set, the combs and the
tape along the bottom of the plates were removed. The gel was clamped into a sequencing tank and 1 x TBE poured into the top and bottom reservoirs. The combs were placed in between the plates, with the teeth resting along the top of the gel to create the wells.

Five microlitres of each sequence reaction were loaded into the wells, in the order G, A, T and C, to ease reading, and were electrophoresed at 65 Watts. When the gel had run, the plates were separated and the gel fixed in 10% glacial acetic acid, for 20 mins, after which the gel was transferred to 3MM Whatman chromatography paper, covered with cling film, and dried under vacuum at 80°C for 2hrs. The dried gel was placed in an autoradiography cassette, exposed to Hyperfilm for 1-4 days, and developed in the X-ray processor of the Royal Free Hospital X-ray department.

2.1 Development of a quantitative-competitive PCR for HHV-8

To quantify the amount of HHV-8 DNA present in patient samples a quantitative-competitive PCR assay was developed, which uses a competitive internal control sequence that differs from the wild-type HHV-8 target sequence by the presence of an EcoRI restriction endonuclease site.

2.1.1 Construction of an HHV-8 control sequence

The strategy used to introduce an EcoRI restriction site into the wild-type HHV-8 sequence was based on a site-directed mutagenesis method developed by Chen and Przybyla (1994), and is summarised in Figure 2.1. A first round PCR amplification of the pUC18-8300 plasmid was performed using
the M13 reverse primer (see Table 2.2) and the mutagenic primer, 8MUT2 (Table 2.3).

8MUT2 5’ - GCT GCT GCA GAA TTC CGT GCC CCA GTT G - 3’

Table 2.3 Mutagenic primer used to incorporate an EcoRI site into the HHV-8 sequence (Mutated bases in bold).

A proof-reading enzyme, BIO-X-ACT polymerase was used with the following cycling conditions: 25 cycles of 95°C for 30 secs, 45°C for 30 secs, and 72°C for 45 secs. The product from this reaction was then used as a primer, along with the M13 universal primer, to amplify a second round of PCR on the pUC18-8300 plasmid. The same conditions as the first round were used, except for an initial hot start cycle of 95°C for 5 mins. Digestion with HindIII and Sacl restriction endonucleases was performed on the second round product and the pUC18 vector. The two were then ligated together in equimolar quantities and transformed into competent JM109 E. coli cells. The mutant construct was characterised by digestion with EcoRI and DNA sequence analysis.
Figure 2.1 Schematic diagram of site directed mutagenesis used for introducing an EcoRI restriction site into the wt HHV-8 sequence (represented as %). M represents the target mutation site, GAATAG, which is converted to M*, GAATTC.
2.1.2 Standard curve production and calibration of the quantitative PCR

To assess the capacity of the control sequence to provide accurate quantification of HHV-8 DNA, known amounts of both the pUC18-8300 plasmid and the HHV-8 control sequence, ranging from $10^1$ to $10^6$ copies, were mixed together and amplified. Conditions were as described above, except that a single round of PCR was performed when using $10^4$ – $10^6$ copies, whilst a nested PCR using 17 cycles was used for $10^1$ – $10^3$ copies. In either single round or nested PCR procedures, 2ng of one primer was phosphorylated with 15 kBq of $[\gamma-32P]$ ATP at its 5’ end, as described in section 2.0.5.1. After amplification, the PCR amplicons were digested with EcoRI, which only digests the HHV-8 control sequence, and separated by electrophoresis on 10% polyacrylamide gels run in TBE. The separated fragments were visualised by autoradiography, and the resulting bands scanned by densitometry, on a Shimadzu CS-9001PC dual-wavelength flying spot scanner. By comparing the intensities of the bands for the target and control amplicons, the amount of template present was calculated. The mean of three experiments was plotted against the known input amounts to produce the standard curve.
2.2 Production of recombinant HHV-8 thymidine kinase

2.2.1 Cloning of the HHV-8 TK into pThioHisB

The 1.8kb HHV-8 TK fragment was amplified by PCR from DNA extracted from lung tissue of an AIDS-KS patient who had had no previous exposure to anti-herpetic drugs. Amplification was performed using the 5'-phosphorylated primers, HHV8TK1 and HHV8TK2 (Table 2.4), to incorporate an EcoRI restriction site at its 3' end. The proof-reading enzyme BIO-X-ACT polymerase was used, with the following conditions: 94°C for 2 mins, then 25 cycles of 94°C for 10 secs, 65°C for 30 secs, and 68°C for 5 mins.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHV8TK1</td>
<td>5’ - AGA TCT GCT CAG GGA TTT CTT AAC CTC G - 3’</td>
</tr>
<tr>
<td>HHV8TK2</td>
<td>5’ - GAA TTC AAG GGC CGC CAA GAA GGC TAG AC - 3’</td>
</tr>
</tbody>
</table>

Table 2.4 Sequence of primers used to amplify the 1.8kb HHV-8 TK (EcoRI restriction site in bold).

The amplicon was purified (section 2.0.5.2) and initially cloned into pUC18. Large-scale preparations of the pUC18-8TK clone, and the expression vector pThioHisB (Figure 2.2) were performed. Twenty micrograms of both were restricted with EcoRI, for 4 hrs at 37°C, and purified as previously.
Figure 2.2 Schematic diagram of the expression vector pThioHisB (Invitrogen).
The digested pThioHisB was then dephosphorylated to prevent its ends from re-ligating together: - 30μg pThioHisB (EcoRI digested), 10U Calf intestinal phosphatase (CIP), 10 x reaction buffer. The reaction was incubated at 37°C for 1 hr, after which the enzyme was inactivated, by incubating to 75°C for 10 mins.

The HHV-8 TK fragment was ligated into pThioHisB, downstream of a thioredoxin fusion, to produce the clone pThio8TK. Clones in the correct orientation were detected by digestion with Psfl, and separation on a 1% agarose gel. DNA sequence analysis was then utilised to confirm the presence of the entire HHV-8 TK ORF. A selected clone was then used to transform SY211, TK-deficient *E.coli* cells for expression. The cells were also transformed with pThioHisB alone, as a positive control.

### 2.2.2 Expression of the HHV-8 TK homologue

Optimal expression of the HHV-8 TK was determined by performing time course experiments. The conditions for expression were as follows: - 5ml of LB media, including 100μg/ml ampicillin, was inoculated with a single colony and incubated overnight at 37°C, in a shaking incubator. 2ml of the overnight culture was then used to inoculate 50ml of fresh LB (containing ampicillin), and grown at 37°C to an OD$_{550}$ of 0.5AU (mid-log phase). This was referred to as time, t=0. A 1ml sample was taken and the cells pelleted in a microcentrifuge at maximum speed. The supernatant was decanted and the pellet frozen at -20°C until required. Expression was induced by adding IPTG to a final concentration of 1mM, and incubating the culture at room
temperature with shaking. 1ml samples were taken regularly over a 24 hour period, and pelleted and stored as above.

Pellets were quickly thawed at 37°C and resuspended in 1/20 culture volumes of lysis buffer (20mM Tris-HCl, pH8.0, 2.5mM EDTA, 5mM imidazole, including 10μg/ml of aprotinin and leupeptin, and 250μg/ml of Pefabloc), then stored on ice. Cell suspensions were sonicated using a Soniprep150, with three 10-second bursts at full power, then frozen in a methanol/dry ice bath and thawed quickly at 37°C. After repeating the freeze/thaw sonication cycle 3 times, the lysates were centrifuged at 6000rpm for 15 minutes at 4°C. The supernatants (soluble fraction) were decanted and pellets (insoluble fraction) resuspended in the same volume of lysis buffer. Expression of the HHV-8 TK was determined by SDS-PAGE and western blot analysis using an Anti-Thio™ mouse monoclonal antibody. Optimal expression of the HHV-8 TK for protein was determined to be after 20 hrs induction at room temperature. Pellets were frozen and stored at −70°C until required.

2.2.3 Protein separation by SDS-PAGE

Expressed protein was separated using the NOVEX™ NuPAGE electrophoresis system. Briefly, samples (50μl) were prepared by the addition of 25μl 4 x NuPAGE sample buffer, 10μl 0.5M DTT and 15μl SDW, and denatured by incubation at 95°C for 5 mins. Twenty five microlitres of each sample were loaded into the wells of a 4-12% NuPAGE Bis-Tris gel, and electrophoresed for 50 mins, at 200V, in a XCELL II™ Mini-Cell electrophoresis tank, using MOPS SDS running buffer. Samples were run
alongside 5μl of Rainbow coloured protein molecular weight Marker to estimate the molecular size of the expressed proteins.

2.2.4 Protein detection by coomassie brilliant blue staining

Staining of NuPAGE gels was carried out by soaking in coomassie brilliant blue staining solution (40% methanol, 10% acetic acid, 50% SDW, and 0.25% coomassie brilliant blue R250, v/v/v/w) for approximately 1 hr. The gel was then destained in 40% methanol, 10% acetic acid, and 50% SDW (v/v/v) until protein bands were visible (approx. 2 hr).

2.2.5 Protein detection by western blot analysis

After protein separation, the NuPAGE gel was placed on a piece of filter paper, pre-soaked in transfer buffer, and a pre-soaked polyvinylidene difluoride (PVDF) membrane positioned on the gel. A second pre-soaked filter paper was put on top, to create a sandwich. The gel/membrane sandwich was placed into an XCELL II blot module, between blotting pads pre-soaked in transfer buffer, with the membrane proximal to the anode. The blot module was then inserted into the Mini-Cell, and protein was transferred electrophoretically to the PVDF membrane, at 25 Volts for 1.5 hrs.

The PVDF membrane was removed and incubated overnight in 10ml blocking solution (3% BSA, 0.01% sodium azide in TBS buffer (10mM Tris-HCl, pH7.5, 150mM NaCl)), at room temperature. The membrane was then washed twice in 20ml TBS-Tween/Triton buffer (20mM Tris-HCl, pH7.5, 500mM NaCl, 0.05% Tween 20, 0.2% Triton X-100), for 5 mins. This was
followed by a 1 hr incubation in 15ml Anti-Thio™ mouse IgG antibody (diluted 1:5000 in TBS-Tween/Triton and 1% BSA). The membrane was washed twice in TBS-Tween/Triton buffer, and transferred to 15ml goat anti-mouse IgG conjugated to alkaline phosphatase for 1 hr. After which the membrane was washed twice in TBS-Tween/Triton buffer, followed by a single wash in TBS buffer to remove detergent. To visualise immunoreactive proteins, the PVDF membrane was incubated in 10ml Sigma Fast BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium). The membrane was washed in distilled water to stop colour development, and allowed to dry on blotting paper.

2.2.6 Production of HHV-8 TK mutants

Using multiple sequence alignment and homology modelling of the core region of the HHV-8 TK (amino acid residues 223 - 580) on the 3-D structure of the HSV-1 TK complexed with dT and GCV, three residues in the HHV-8 TK sequence likely to be important for binding ATP (Gly 265), Mg²⁺ (Asp 362), and deoxythymidine (Phe 372) were identified. These residues were mutated using the Promega GeneEditor in vitro site-directed mutagenesis kit, summarised in Figure 2.3, with slight modifications to the manufacturers instructions, as described below.

Spin purification columns were prepared as for DNA sequencing (see section 2.0.9). Two micrograms of pThio8TK DNA was denatured by the addition of 5µl 1M NaOH/1mM EDTA and incubated at room temperature for a minimum of 5 minutes. A control reaction using pGEM-11ZF(+) vector DNA
was included. The denatured DNA was then spun through the purification columns at 1400rpm, for 5 mins. 200ng of denatured DNA was annealed to 1.25pmol of phosphorylated mutagenic primer, represented in Table 2.5, and 0.25pmol of Top strand selection oligonucleotide, 1 x annealing buffer to a final volume of 20μl. The control DNA was annealed to the lacZ control knockout oligonucleotide and the bottom strand selection oligonucleotide. The reactions were incubated at 37°C for 15 mins and then allowed to cool slowly to room temperature.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>TK1.dT</td>
<td>5' TCC CCA GCA GTG GTG TGC CCT CTC ATG CAC CTG 3'</td>
</tr>
<tr>
<td>TK2.ATP</td>
<td>5' GGG GTA ATG GGT GTG GCC AAA TCA ACG CTG GTC 3'</td>
</tr>
<tr>
<td>TK3.Mg</td>
<td>5' CAC TGG TGC GTC TTT GCC AGG CAT CTC CTC TCC 3'</td>
</tr>
</tbody>
</table>

Table 2.5 Sequence of primers used for site-directed mutagenesis of HHV-8 TK (mutated bases in bold).

Mutagenesis was then performed according to manufacturers instructions. The three HHV-8 TK mutants were expressed using the same conditions optimised for the wild-type HHV-8 TK (Section 2.2.2).
1. Alkaline denature dsDNA template, anneal mutagenic oligonucleotide and Selection Oligonucleotide

2. Synthesize mutant strand with T4 Polymerase and T4 DNA Ligase

3. Transform BMH71-18mutS cells with mutagenesis reaction. Grow O/N with the GeneEditor Antibiotic Selection Mix

4. Isolate plasmid DNA and transform JM109. Select mutants on ampicillin plates containing the GeneEditor Antibiotic Selection Mix

Figure 2.3 Schematic diagram of the GeneEditor *in vitro* mutagenesis procedure
2.3 Functional analysis of the HHV-8 thymidine kinase

2.3.1 Thymidine kinase assay

Kinase reactions were performed in a buffer containing 160mM Tris-HCl (pH 7.5), 7.5mM NaF, 1.6mM DTT, 5µM ATP, 5µM MgCl₂, 1% BSA, and 5µM [³H-methyl] deoxythymidine (dT) (5.05Ci/mmol specific activity), to a total volume of 75µl, plus 10µl of expressed protein. Samples were incubated at 37°C and aliquots (50µl) taken every 30 mins, over a 3 hr period, and spotted onto 20mm positively charged DE81 chromatography discs to separate the monophosphate from the unphosphorylated dT. The discs were washed twice in 2mM ammonium acetate, followed by 95% ethanol, allowed to dry at room temperature, and placed in vials containing 5ml scintillant (0.4% PPO, 0.01% POPOP, in toluene). Radioactivity was measured using a LKB Wallac 1217 rackbeta liquid scintillation counter. For enzyme kinetic analysis, reactions were performed as above, except with increasing concentrations of [³H-methyl] dT (2-20µM), and ATP (5-20µM), together with MgCl₂, at 37°C for 3 hrs. For the phosphorylation of nucleoside analogues the [³H-methyl] dT was replaced by [³H-methyl] azidodeoxythymidine (AZT) (15Ci/mmol specific activity), [³H-methyl] ganciclovir (GCV) (13.5Ci/mmol specific activity), or [³H-methyl] didehydrodeoxythymidine (d4T) (34.3Ci/mmol specific activity).

2.3.2 Nucleoside analogue inhibition

Inhibition assays were performed as above, first with 5µM [³H-methyl] dT and increasing concentration of unlabelled AZT, d4T, GCV and
bromodeoxyuridine (BrdU) (10^0-10^4\mu M), and second, over a range of both dT and nucleoside (0-10\mu M) concentrations.

2.3.3 Calculation of enzymatic activity

In order to assess the enzymatic activity of the recombinant thymidine kinase the data obtained was represented in the form of double reciprocal plots of 1/V vs 1/[S] (Lineweaver-Burk plots), using Microcal Origin. Line of best fit was plotted through the points and the Michaelis constant, $K_m$, for the substrate was determined using the equation:-

$$\frac{-1}{K_m} = \text{intercept on the x-axis.}$$

For inhibition studies, data was also represented as Lineweaver-Burk plots. As before line of best fit was plotted, and then the inhibition constant, $K_i$, for each inhibitor determined using the following equations:-

$$\frac{-1}{\alpha K_m} = \text{intercept on the x-axis}$$

and

$$\alpha = \frac{1+[I]}{K_i}.$$
Chapter 3 - Development of qualitative and quantitative-competitive PCR for the detection and measurement of HHV-8 DNA
3.1 Introduction

Human herpesvirus-8 is the latest member of the *Herpesviridae* to be described, following its discovery in Kaposi’s sarcoma tissue from AIDS patients, in 1994, by Chang *et al.* using representational difference analysis (RDA). Kaposi’s sarcoma is the most frequent malignancy observed in HIV positive individuals (McKenzie *et al.*, 1991), and has been documented in other immunocompromised patients, such as renal transplant recipients (Myers *et al.*, 1974). The use of PCR has led to the detection of HHV-8 DNA in all forms of KS, as well as two other AIDS-related lymphoproliferative disorders, primary effusion lymphoma (PEL) (Cesarman *et al.*, 1995) and multicentric Castleman’s disease (MCD) (Soulier *et al.*, 1995). Evidence compatible with a causal role for HHV-8 in KS comes from two reports of the clinical effects of anti-herpes drugs. Regression of KS lesions was reported in patients treated with foscarnet, which also has activity against HIV (Morfeldt and Torssander, 1994), while a decreased risk of developing KS has been observed in a retrospective analysis of HIV-positive patients treated with foscarnet or ganciclovir (Mocroft *et al.*, 1996).

Viral load measurements have been central to understanding the pathogenic mechanism of many viruses (Hagiwara *et al.*, 1993; Ho *et al.*, 1995; Mellors *et al.*, 1996; Stagno *et al.*, 1975; Wei *et al.*, 1995;). At the time of performing the experiments in this thesis there were only semiquantitative methods available for the measurement of HHV-8 (Dupin *et al.*, 1995). However, several strategic approaches could be adapted. HHV-8 can be cultured in latently infected B cell lines (Renne *et al.*, 1996), however not
easily, and so would not be suitable for development as a quantitative method. The development of these cell lines has allowed the seroepidemiology of HHV-8 to be partially elucidated (Miller et al., 1996, Moore et al., 1996). Anti-HHV-8 antibodies were used in the generation of serological assays for detecting HHV-8 infection (Kedes et al., 1996, Gao et al., 1996). Using established formats, for example, enzyme immunoassays (EIA), these tests could be adapted for quantitative purposes. However, such systems are indirect, because they do not give a true reflection of how much latent virus is present, but of how much virus can be artificially stimulated.

Since the majority of data on HHV-8 has been generated using PCR methods I developed a quantitative-competitive PCR system to allow the accurate quantification of HHV-8 in clinical specimens. Quantitative-competitive PCR assays for cytomegalovirus, HHV-6 and -7, and HIV have previously been developed in our laboratories (Fox et al., 1992; Kidd et al., 1996; Clark et al., 1996), and shown to be highly reproducible and to allow accurate quantification of target sequence, even in the presence of inhibitory substances. Quantification of HHV-8 would allow analysis of the potency of antiviral drugs against HHV-8 replication and any associated effects on the development of KS. Also, in HIV-infected patients, it would allow the correlation of HHV-8 viral load with the viral load of HIV to help understand its relationship with KS.

In this chapter I describe the development of the nested PCR method for the detection and quantification of HHV-8 DNA, and show its utilisation by
analysing blood DNA extracts from a renal transplant patient, who developed KS 56 months post-transplantation.
3.2 Results

3.2.1 Optimisation of qualitative HHV-8 PCR

The optimum primer and magnesium ion concentrations, for both rounds of the nested PCR, were determined by visually comparing the intensities of amplicon bands for different concentrations of the reagent, after electrophoresis on a 3% agarose gel. Figure 3.1.A shows a titration of the first round primers, 8EF and 8ER. The intensity of the bands increased slightly from 50ng to 100ng. There was no visible difference in band intensity between 100ng, 150ng and 200ng of primers, however, there was an increase in the presence of primer dimers. Therefore, the optimum primer concentration was determined to be 100ng per reaction.

The concentration of magnesium ions at which optimal amplification was observed was 2mM, as shown in Figure 3.1.B by an increase in band intensity with increasing concentration. The second round of the nested PCR was optimised as described above, and conditions were determined to be the same as the first round. Using the optimised conditions, the sensitivity of the assay was then measured by amplifying cloned HHV-8 DNA, for this region, over a range of copy numbers. Figure 3.2 shows the assay to have an ultimate sensitivity of 10 copies of target HHV-8 DNA sequence.
Figure 3.1 Optimisation of qualitative PCR for the HHV-8 sequence KS330233.

A) An agarose gel showing the optimisation of first round primer concentration. Optimum amplification of the HHV-8 target was observed using 100ng of both 8EF and 8ER primers. B) An agarose gel demonstrating optimal amplification of the HHV-8 target DNA using 2mM MgCl₂. (On both gels M refers to a PCR size marker)
Figure 3.2 Determination of PCR sensitivity. Known quantities (in triplicate) of HHV-8 positive control plasmid, ranging from $10^6$ to $10^0$ copies, were amplified using the optimised nested PCR conditions (M is the HaeIII digested φX174 DNA marker).
3.2.2 Generation of the HHV-8 control plasmid

The quantitative-competitive PCR assay developed uses a competitive internal control sequence that differs from the wild-type HHV-8 target sequence by the presence of a restriction endonuclease site. A site-directed mutagenic approach was used to produce the HHV-8 control sequence (see Figure 2.1), which was identical to the wild-type sequence except for the mutation of 2 nucleotides at positions 180 and 181 of the 300 bp target sequence, GAATAG, to yield a recognition site for EcoRI, GAATTC. The presence of the mutation was confirmed by DNA sequence analysis, as shown in Figure 3.3.

3.2.3 Calibration of the quantitative-competitive PCR

To determine the accuracy of the quantitative-competitive PCR for quantifying HHV-8 DNA a standard curve was produced. Known quantities of wild-type cloned HHV-8 target sequence and control sequence DNA were mixed and co-amplified, with each copy number performed in triplicate. A representative autoradiograph of a quantitative analysis is shown in Figure 3.4. By performing a series of PCRs with known copy numbers, and plotting the mean calculated wild-type copy number as a function of the input wild-type copy number, the standard curve shown in Figure 3.5 was generated. The calculated and actual copy numbers of HHV-8 were highly correlated (R=0.998; P=6x10^{-11}; for a linear fit curve), indicating that the quantitative method was highly reproducible and could allow accurate quantitation of HHV-8 viral load.
Figure 3.3 Autoradiograph showing the DNA sequence of both the wt HHV-8 and the control sequences, to demonstrate the introduction of an EcoRI restriction site (GAATTTC), by site-directed mutagenesis.
Figure 3.4 Autoradiograph of quantitative HHV-8 PCR for the production of a standard curve. $10^3$ genome copies of control sequence mixed, in triplicate, with $5 \times 10^2$, $10^3$, and $2.5 \times 10^3$ genome copies of plasmid containing the *wt* HHV-8 sequence. The first lane represents control sequence only.
Figure 3.5 Calibration of the HHV-8 quantitative-competitive PCR. Known copy numbers of wild-type sequence were co-amplified with constant control sequence copy numbers. Calculated wt numbers were obtained and plotted against actual input copy numbers. Each point represents the mean of triplicate determinations, with standard deviation shown as error bars. Line of best fit was plotted through the points by linear regression.
3.2.4 Quantification of HHV-8 load without the use of radioactivity

One drawback with the assay is the use of a radiolabelled primer for determining viral loads by scanning densitometry. This was rectified by a minor modification. Pictures of polyacrylamide gels showing separation of the wt and control sequences (Figure 3.6) were scanned directly using a Umax 600S flatbed scanner, allowing the removal of the autoradiography step. The resultant digitised images were then analysed using the public domain program, NIH image. Using this modification, pictures of the gels used previously for autoradiography were scanned, and these results correlated with those determined using autoradiography. As represented in Figure 3.7, this alternative method is as accurate for quantifying HHV-8 load (R=0.995, P=2.5x10⁻⁹; for a linear fit curve).

3.2.5 Quantification of HHV-8 DNA in the blood of a renal transplant patient with KS.

Nine blood samples, taken over a period of 31 months, from a renal transplant recipient who developed KS (56 months post-transplant), were subjected to qualitative and quantitative nested PCR for HHV-8. Two blood samples were positive for HHV-8, and these correlated to the clinical appearance of Kaposi's sarcoma on the groin and inner thigh. The HHV-8 PCR positive samples were then co-amplified with 1, 5, 10 and 50 genome copies of control standard, respectively. Quantitation showed that at diagnosis of KS there was 28,320 HHV-8 genomes/ml blood, while 2 days later the viral load had reduced to 13,680 genomes/ml. This reduction correlated with removal of the immunosuppressive drugs, azathioprine and
cyclosporin A. HHV-8 DNA was undetectable after 4 months, despite the continued presence of KS lesions. The viral load profile, with relation to the clinical management of this renal patient is shown in Figure 3.8.
Figure 3.6 Photograph of a polyacrylamide gel of quantitative HHV-8 PCR, used previously for autoradiography (Figure 3.4), for the production of a standard curve using NIH image. $10^3$ genome copies of control sequence mixed, in triplicate, with $5 \times 10^2$, $10^3$, and $2.5 \times 10^3$ genome copies of plasmid containing the *wt* HHV-8 sequence. The first lane represents control sequence only.
Figure 3.7 Curve showing the correlation of calculated HHV-8 copy number by quantitative PCR using autoradiography or NIH imager. Line of best fit was plotted through the points by linear regression.
Figure 3.8 Use of the QC-PCR for HHV-8 to determine the HHV-8 load in the blood of a renal transplant patient who developed KS (Day 0). The effects of withdrawal of immunosuppressive therapy on HHV-8 DNA load should be noted.
3.3 Discussion

This chapter describes the development and application of an assay for the quantification of HHV-8 viral DNA. The method used had been previously employed in for the quantification of HHV-6, HHV-7, CMV and HIV, and uses the same primers to co-amplify target DNA with a competitive internal standard, differing from the wild-type sequence by the presence of a unique restriction site (Fox et al, 1992; Kidd et al, 1996; Clark et al, 1996; Atkins et al, 1996). In this case an EcoRI site was introduced into the wild-type HHV-8 sequence, using a PCR mutagenic approach. The assay could accurately quantify HHV-8 within the range of $5 \times 10^4$ to $10^8$ molecules of target DNA, in a highly reproducible fashion. Because of the risks involved with using radioactivity the assay was modified, by removing the need for this. Linear regression analysis showed this approach to be highly correlated with the autoradiography method.

The utility of the assay to provide insight into modulations of HHV-8 load in clinical samples was assessed by analysing blood DNA extracts from a renal transplant patient diagnosed with Kaposi's sarcoma, prior to rejection of the transplant. Peak HHV-8 viral load was observed one week after KS was first observed, and had decreased to 13,680 copies/ml two days later. This reduction correlated with removal of the immunosuppressive drugs, azathioprine and cyclosporin A. Analysis of blood samples taken 4 and 5 months later showed that HHV-8 DNA was below the limit of detection. Despite the abrupt virologic response following reduction in immunosuppressive therapy, KS lesions persisted for 3 months and the individual eventually suffered graft loss due to renal rejection. It is well
recognised that cessation of immunosuppression can allow such patients immune systems to regenerate sufficiently to allow the remission of KS lesions (Brooks et al, 1986). It could be that the immune response also controlled HHV-8 infection either directly or indirectly by destroying the KS cells. It is probable that these effects are also a paradigm for the activity of highly active anti-retroviral therapy (HAART) on the incidence of KS in HIV infected individuals (Bower et al, 1999). More extensive long-term follow-up studies are required to give a better idea of the correlation between HHV-8 infection and immunosuppression, and provide insight into the role of HHV-8 in the pathogenesis of KS.

More recently the development of Taqman and related PCR technologies which detects amplification of target DNA in real-time, by the target specific release of a fluorescent reporter molecule during the PCR reaction, has occurred. The technique has been adapted for detecting HHV-8 DNA in human tissue samples (Kennedy et al, 1997), and White and Campbell (1999) have recently reported the use of this technique for quantitation of HHV-8 DNA, with similar levels of accuracy to the assay developed in this chapter. However, it will be interesting to compare the internal calculated QC-PCR results to the real-time PCR results, especially in clinical samples where inhibitory substances may be present.

In summary, the results described in this chapter demonstrate that the competitive PCR method is highly satisfactory for the purpose of quantifying HHV-8 load. Furthermore, use of this assay should enable a greater understanding of HHV-8 pathogenesis, and the effects of drugs, such as immunosuppressants and antivirals on HHV-8 replication.
Chapter 4 – Analysis of HHV-8 DNA levels in post-mortem tissues of AIDS patients
4.1 Introduction

Kaposi’s sarcoma is the most frequently observed malignancy in HIV seropositive individuals, and is one of the first signs for the onset of AIDS. It is now the consensus that HHV-8 is the major aetiologic agent for KS development. However, this does not take into account the varying nature of KS in the different patient groups. The clinical features of AIDS related-KS differ from the other types of KS. Cutaneous lesions tend to present on the upper body instead of the lower extremities, and also occur frequently on the palms, soles, and genitalia (Pluda et al., 1993, Steis and Longo, 1988). AIDS-associated KS displays an extremely aggressive course with dissemination of multicentric lesions to the mucous membranes of the respiratory and gastrointestinal tracts, as well as other major organs. Visceral AIDS-KS progresses rapidly and has a high mortality rate. For example, 15-50% of patients present with KS in the pulmonary tract (Tappero et al., 1993), a condition which is estimated to contribute to 25% of deaths in AIDS-KS patients (Steis and Longo, 1988) in the pre-HAART era.

The more aggressive nature of AIDS-KS is most likely due to the involvement of HIV. How this occurs is still unknown. Formation of KS-like lesions in nude mice, and stimulation of spindle cell proliferation, has been induced by the co-operation of the HIV-1 Tat protein with basic fibroblast growth factor (bFGF). bFGF mRNA is expressed in vivo in both classical and AIDS KS cell lines, suggesting a key role in pathogenesis. However, epidemiological evidence shows that the risk of KS is not uniform among HIV-positive persons. The prevalence of KS in HIV-positive gay men is 10-20 times that observed in equally immunodeficient HIV-positive haemophiliacs.
and children. Also, KS is common in HIV-positive female partners of bisexual men, but uncommon in HIV-positive intravenous drug users.

The aim of this chapter was to look at the presence of HHV-8 DNA in various post-mortem tissues of AIDS patients, to investigate how widely disseminated HHV-8 is throughout the body, and to correlate the results with the pathological presence of KS, and with HIV proviral loads.
4.2 Results

4.2.1 PCR analysis of post-mortem tissue samples from AIDS patients

DNA was extracted from post-mortem tissue samples from 12 AIDS patients and 3 control patients (two were in road traffic accidents and the third died from a subdural haematoma), using the Promega extraction kit, as in section 2.0.2. Thirty-two out of 151 (21%) tissues from the AIDS patients were positive for HHV-8 DNA, however none of the 47 control tissues were HHV-8 positive (Table 4.1). Of the positive samples, 4/68 (6%) were from males without KS, 25/55 (45%) were from males with KS, and 3/28 (10%) were from women without KS. The tissues most frequently positive for HHV-8 DNA were the heart, stomach and duodenum.

4.2.2 Quantitation of HHV-8 DNA in positive samples

HHV-8 viral loads were measured in the HHV-8 positive tissue samples and ranged from $10^1$ to $10^{5.6}$ genome copies per $\mu$g of DNA (Table 4.2). Patients diagnosed with KS at post-mortem had significantly higher viral loads than those without KS, represented in Figure 4.1 (KS median $10^{2.47}$ genome copies/$\mu$g DNA versus without KS median $10^1$ genome copies/$\mu$g DNA; $P=0.02$ by Mann Whitney ‘U’ test).
<table>
<thead>
<tr>
<th>Patient Sex KS</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>Controls A</th>
<th>B</th>
<th>C</th>
</tr>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>X</td>
<td>X</td>
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<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| TISSUE       |   |   |   |   |    |    |    |    |    |    |    |    |      |   |   |
| Lymph Node   | + | + | - | + | -  | -  | -  | -  | -  | -  | -  | -  |       |   |   |
| Spleen       |   |   |   |   |    |    |    |    |    |    |    |    |       |   |   |
| Brain        |   |   |   | + | -  | n/a| -  | -  | -  | -  | -  | -  |       |   |   |
| Lung         | + | + | - | + | n/a| -  | -  | -  | -  | -  | -  | -  |       |   |   |
| Heart        |   |   |   | + | +  | +  | -  | -  | -  | -  | -  | -  |       |   |   |
| Kidney       | + | - | - | - | -  | -  | -  | -  | -  | -  | -  | -  |       |   |   |
| Adrenal      | + | - | - | + | -  | -  | -  | -  | -  | -  | +  | -  |       |   |   |
| Oesophagus   | - | + | - | + | n/a| -  | -  | -  | -  | n/a| -  | -  |       |   |   |
| Duodenum     | - | + | + | + | n/a| -  | -  | -  | -  | n/a| +  | -  |       |   |   |
| Colon        | - | - | - | + | n/a| n/a| +  | -  | n/a| n/a| -  | -  |       |   |   |
| Pancreas     | - | - | - | n/a| -  | -  | +  | n/a| n/a| -  | -  | -  |       |   |   |
| Liver        | + | + | - | - | -  | -  | -  | -  | -  | -  | -  | -  |       |   |   |
| Stomach      | + | + | + | - | n/a| -  | -  | -  | +  | n/a| -  | -  |       |   |   |
| Sal. Gland   | + | n/a| - | - | -  | -  | -  | -  | -  | -  | -  | +  | n/a   |   |   |

Table 4.1 HHV-8 qualitative PCR results of post-mortem tissues from AIDS patients and controls (n/a = no available sample).
<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>7</th>
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<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TISSUE</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph Node</td>
<td>3.04</td>
<td>4.06</td>
<td>-</td>
<td>2.47</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
<td>1.50</td>
<td>-</td>
<td>n/a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td>5.60</td>
<td>+n/a</td>
<td>-</td>
<td>1.02</td>
<td>n/a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td>2.55</td>
<td>&lt;1</td>
<td>2.59</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adrenal</td>
<td>2.76</td>
<td>-</td>
<td>-</td>
<td>&gt;4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>-</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>-</td>
<td>2.30</td>
<td>-</td>
<td>3.14</td>
<td>n/a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Duodenum</td>
<td>-</td>
<td>2.91</td>
<td>+n/a</td>
<td>3.47</td>
<td>n/a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>-</td>
</tr>
<tr>
<td>Colon</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>n/a</td>
<td>&lt;1</td>
<td>-</td>
<td>n/a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pancreas</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>n/a</td>
<td>-</td>
<td>3.25</td>
<td>n/a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stomach</td>
<td>2.07</td>
<td>3.69</td>
<td>1.66</td>
<td>-</td>
<td>n/a</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sal. Gland</td>
<td>1.77</td>
<td>n/a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Table 4.2 HHV-8 viral loads (log_{10} genomes/μg cellular DNA) in post-mortem tissues of AIDS patients (n/a = no available sample, + = positive for HHV-8 by qualitative PCR).
Figure 4.1 Scatter diagram comparing the HHV-8 viral loads in samples of patients with and without Kaposi’s sarcoma at the time of post-mortem. Statistical analysis using the Mann-Whitney ‘U’ test showed a significant difference (P=0.02) in the median HHV-8 viral load in samples from patients with KS compared to those without KS.
4.2.3 Correlation of HHV-8 infection with HIV infection

HIV qualitative and quantitative PCR analysis had been performed on all post-mortem samples previous to this study. HIV proviral DNA had been detected in 22 of the 32 HHV-8 positive tissues, with proviral loads ranging from $10^{0.7} - 10^{5.1}$ genome copies/µg DNA. Samples in which HIV proviral DNA was detectable had elevated HHV-8 loads (median load of $10^{2.185}$ genomes/µg DNA) compared to those where HIV proviral load was below detectable levels (median load of $10^{1.25}$ genomes/µg DNA), but this difference did not reach statistical significance ($P=0.18$, Mann Whitney ‘U’ test) (Figure 4.2). No correlation was observed between HIV proviral load and HHV-8 viral load ($R=0.23$, $P=0.20$ by linear regression), represented in Figure 4.3.
Figure 4.2 Scatter diagram comparing the HHV-8 viral loads in samples of patients positive or negative for HIV at the time of post-mortem. Statistical analysis using the Mann-Whitney 'U' test showed no significant difference (P=0.18) in HHV-8 viral load in samples from patients with HIV to those without HIV.
Figure 4.3 Correlation of HHV-8 viral load with HIV proviral load in the post-mortem tissue samples (R=0.23, P=0.20, by linear regression).
4.3 Discussion

In this chapter I have studied HHV-8 infection in various organs of AIDS patients, post-mortem. HHV-8 is widely disseminated at death, being detected in organs such as the heart, lungs, kidneys and brain. Forty-five percent of tissues from patients with KS were positive for HHV-8 compared to only 8% of tissues from patients without KS. Of these positive samples, there was a significant difference in the viral loads between patients with KS and patients without KS. Prior to this study, all previous investigations of HHV-8 load have used semiquantitative techniques.

The tissues where HHV-8 DNA was most frequently detected in this study were the heart, stomach and duodenum. A high prevalence of HHV-8 infection in the heart is consistent with HHV-8 presence in the blood, since it has been shown that 52% of PBMCs from AIDS-KS patients are positive for HHV-8 by PCR (Whitby et al. 1995), although this may be the result of contaminating virus from blood. When patients have visceral KS the gastrointestinal tract is one of the sites primarily affected and the high levels of HHV-8 infection in the stomach and duodenum is consistent with this pathology.

The high incidence of KS among homosexuals and women with bisexual partners suggests HHV-8 might be transmitted sexually. A recent case study into the sexual behaviour of homosexual men has demonstrated that HHV-8 infection and KS development are significantly associated with anal intercourse (Gambus et al., 1999). Presence of HHV-8 DNA in gastrointestinal tissues could be linked with transmission via the faeco-oral route, as suggested in various studies. Alero Thomas et al. (1996) detected
HHV-8 DNA in 50% of duodenal and rectal biopsies, as well as duodenal aspirates, and Grulich et al. (1999) demonstrated an association between HHV-8 orf65 serology and hepatitis A, which is also believed to be transmitted by the faeco-oral route in homosexual men (Corey and Holmes, 1980). However, HHV-8 DNA has not been detected in faeces of HIV-positive individuals (Whitby et al., 1995, LaDuca et al., 1998).

In this chapter, the results also suggest there to be no direct interaction between HHV-8 and HIV, with no significant difference in HHV-8 load in tissue samples when correlated with HIV proviral DNA presence or load. Similar data has also been reported in two recent studies investigating HHV-8 load in PBMCs of HIV positive KS patients, using Taqman PCR technology (Campbell et al., 1999, and Pellet et al., 1999). The involvement of HIV infection in the pathogenesis of KS is still unresolved.

In summary, this chapter represents the first data with fully quantified HHV-8 loads in AIDS patients, identifying various organs that are possible sites of viral replication.
Chapter 5 – Production and expression of a recombinant HHV-8 thymidine kinase
5.1 Introduction

DNA sequence analysis of the HHV-8 genome revealed close homology to the $\gamma_2$-herpesviruses (Moore et al. 1996), with the products of many open reading frames (ORFs) potentially able to manipulate the cellular environment (reviewed by Schulz, 1998). The virus encodes both a thymidine kinase (TK) and a herpesvirus protein kinase (PK) homologue (Moore et al. 1996, Russo et al. 1996). Sequence comparisons show the HHV-8 TK to have 24.0% and 26.8% homology to the $\gamma$-herpesviruses EBV and HVS, respectively, and 12.0% homology to the $\alpha$-herpesvirus HSV. The five conserved sites or domains observed in all herpesvirus TKs are also present in the HHV-8 encoded TK.

In the $\alpha$-Herpesvirinae, the viral TK performs the initial phosphorylation of nucleosides to their monophosphate en route to the triphosphate, as eventual substrates for DNA synthesis. The $\alpha$-herpesvirus TK can monophosphorylate a broad range of substrates including the anti-herpesvirus drugs, aciclovir (ACV) and ganciclovir (GCV) (Cheng et al. 1981, De Clercq, 1993). In vitro studies have shown that HHV-8 is susceptible to GCV and cidofovir but relatively insensitive to ACV (Medveczky et al. 1997). Retrospective studies of HIV infected patient groups at risk of KS have suggested that the incidence of KS was lower in patients receiving GCV or foscarnet, but not ACV (Glesby et al. 1996, Mocroft et al. 1996).

In this chapter I describe the cloning and expression of the HHV-8 TK homologue, ORF 21, for eventual analysis as a potential target for antiviral drugs.
5.2 Results

5.2.1 PCR amplification of the HHV-8 thymidine kinase

The HHV-8 thymidine kinase homologue was amplified by PCR, initially from HHV-8 DNA positive lymph node of an AIDS patient, using the BIO-X-ACT proof-reading DNA polymerase (as in section 2.2.1) and a 3’ primer which possessed an EcoRI restriction site to facilitate cloning. However, the patient had received GCV therapy for a year as treatment for CMV, and so if HHV-8 TK has GCV kinase activity there is a possibility that GCV resistant TK mutants may have developed within this patient. As a precautionary measure the HHV-8 thymidine kinase was subsequently amplified from HHV-8 DNA positive lung tissue of an AIDS patient that had not received any antitherpetic drugs. The products were separated by agarose gel electrophoresis and revealed a single band of 1.8kb (Figure 5.1).

5.2.2 Cloning of HHV-8 TK into the expression vector pThioHisB

The HHV-8 TK PCR product was purified and blunt-end ligated into the Smal restriction site of pUC18. Clones with EcoRI restriction sites at both the 5’ (within the poly linker) and 3’ ends were selected for ligation into the EcoRI site of the pThioHisB expression vector, downstream and in-frame with the thioredoxin gene, to produce the clones pThio8TK\textsubscript{a} and pThio8TK\textsubscript{b}, from the first and second patient samples, respectively. There is a single PstI restriction site within the HHV-8 TK (see figure 5.3) and one in the poly linker region of pThioHisB. PstI digestion of pThio8TK clones revealed bands of 1317bp or 570bp after agarose gel separation (Figure 5.2). Clones in the correct orientation for expression were 1317bp in size.
Figure 5.1 Agarose gel showing the amplification of the HHV-8 TK homologue, from HHV-8 PCR positive lung tissue of an AIDS patient, represented in lanes 1-6 by bands of 1.8 kilobases. Lanes labelled -ve and M represent the negative control and the HaeIII digested φX174 DNA marker, respectively.
Figure 5.2 Agarose gel showing the separation of pThio8TK clones digested with the *Pst*I restriction enzyme. Clones in the correct orientation for expression are represented by bands of 1317 base pairs in size.
5.2.3 Sequencing of the pThio8TK clones

The HHV-8 TK open-reading frame was confirmed to be present within the pThio8TK clones by DNA sequence analysis, as described in section 2.0.8, and aligned with the HHV-8 TK sequence obtained from Genbank (accession number gi|1136820). Four base differences were observed between the published HHV-8 TK sequence and that of the pThio8TK\_a clone, all of which resulted in the presence of different amino acids, aa80 H→L, aa398 E→D, aa399 G→D and aa543 E→Q (Figures 5.3 and 5.4). Eleven base differences were observed in the pThio8TK\_b clone (Figure 5.3). However, only three resulted in the presence of a different amino acid, aa156 M→T, aa167 L→R, and aa307 M→L (Figure 5.4), the remaining eight were non-coding mutations.

5.2.4 Expression of the recombinant HHV-8 TK

Expression of the HHV-8 TK homologue as an 80kD thioredoxin-fusion protein was initially performed at 37°C. Optimal expression was determined by time course experiments to be at 16hr post-induction with IPTG (data not shown). However, following sonication, the majority of the protein was insoluble (Figure 5.5). The HHV-8 TK homologue was then expressed at room temperature, and was maximal at 20 hours post-induction (Figure 5.6). Under these conditions the HHV-8 TK was detected predominantly in the soluble fraction (supernatant) post-sonication, with a small amount detectable in the pellet (Figure 5.7). No corresponding protein bands were observed in the control expression. The soluble fractions were used in all further enzymatic studies.
<table>
<thead>
<tr>
<th>HHV-8 TK</th>
<th>ATGGCAGAAG  GCGGTTTGG  AGCGGACTCG  GTCGGGCGCG  GCGGAGAAAA  50</th>
</tr>
</thead>
<tbody>
<tr>
<td>pThio8TKₐ</td>
<td>-------- -------- -------- -------- -------- T --------</td>
</tr>
<tr>
<td>pThio8TKₐ</td>
<td>-------- -------- -------- -------- -------- T --------</td>
</tr>
<tr>
<td>HHV-8 TK</td>
<td>GGCCTCTGTG  ACTAGGGGAG  GACAGTGGGA  CTTGGGGAGC  TCAGAGCACG  100</td>
</tr>
<tr>
<td>pThio8TKₐ</td>
<td>-------- -------- -------- -------- -------- T --------</td>
</tr>
<tr>
<td>HHV-8 TK</td>
<td>AATCAAGCAC  CTCACCAACC  AGCACGGATA  TGGACGGACCT  CCCTGAGGAG  150</td>
</tr>
<tr>
<td>pThio8TKₐ</td>
<td>-------- -------- -------- -------- -------- T --------</td>
</tr>
<tr>
<td>HHV-8 TK</td>
<td>AGGAAACCAC  TAACGGGAAA  GTCTGTAAAC  ACCTGCAAGT  TATAGGACGT  200</td>
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<tr>
<td>pThio8TKₐ</td>
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<tr>
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<td>GCCACCAGTC  CCGACACGA  AGCCGTTGGG  TTTATGCAC  GAAACTCCC  250</td>
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<td>HHV-8 TK</td>
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<tr>
<td>HHV-8 TK</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>HHV-8 TK</td>
<td>AACGCTGGTC  AAGCAGGATGT  GCGGACATTC  GCCCCAGGAG  AGAGTGCACAA  800</td>
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Figure 5.3 DNA sequence alignment of the pThio8TK clones with the HHV-8 TK sequence obtained from Genbank (accession number gi|1136820). The PstI restriction site at 490bp was used to select clones in the correct orientation for expression.
Figure 5.4 Alignment of the published HHV-8 TK amino acid sequence with the pThio8TK clones, showing the differences resulting from DNA polymorphisms. The grey boxes numbered I-V are the five domains conserved amongst the herpesvirus TKs.
Figure 5.5 Coomassie blue stained polyacrylamide gel showing the expression of the HHV-8 TK homologue, as a thioredoxin-fusion (80kD), at 37°C. The protein is present in the pellet, post-sonication, suggesting it is insoluble. There are no equivalent protein bands in the vector only expression control (pThioHisB).
Figure 5.6 SDS-polyacrylamide gels showing the protein expression time-course (0 to 24 hrs) for A) pThio8TK and B) pThioHisB (control). Optimal expression for HHV-8TK was observed at 20 hrs post-induction, as a band of 80kD. Equivalent protein bands were not observed with expression of the pThioHisB vector alone.
Figure 5.7 Western blot showing the expression of HHV-8 TK (80kD), at room temperature. The protein is present in both the pellet and soluble fractions of pThio8TK, post-sonication, but not in those using vector alone (pThioHisB).
5.3 Discussion

As the eventual aim of this study was to express the HHV-8 TK gene product for biochemical analysis it was necessary to minimise the risk of Taq induced errors. For this reason, the proof-reading enzyme Bio-X-Act polymerase (Bioline) was used to amplify the HHV-8 TK gene. The HHV-8 thymidine kinase was originally amplified from a patient that had received GCV therapy for a year as treatment for CMV. If a functional HHV-8 TK has GCV kinase activity there is a possibility that GCV resistant TK mutants may have developed within this patient. Four base pair differences from the published sequence were observed, all of which resulted in amino acid substitutions. Whilst it is possible these substitutions may be resistant mutations, none fall within the five conserved domains of herpesvirus TKs, although the residues D398 and D399, as well as being adjacent to one another, are close to the nucleotide binding site (see Figure 6.3).

To rule out the possibility of using a resistant TK for functional studies the gene was subsequently amplified from the tissue of an AIDS patient known not to have received any antiviral drugs. However, eleven base pair differences from the published sequence were observed with this clone, pThio8TKb, of which only three resulted in amino acid substitutions. As with the pThio8TKa clone, these substitutions did not occur in any of the five conserved domains, and although may be a result of Taq error they are more likely to be strain variation rather than resistant mutations induced by the use of antiviral drugs. In this instance, the function of the expressed protein would hopefully, not be compromised.
Expression was initially performed at 37°C, where optimal expression was observed at 16 hours. However, after cell lysis the majority of the recombinant TK was present in the pelleted fraction, suggesting the protein was insoluble. For proteins, temperature is an important factor during folding, in order to achieve the correct conformation. If a protein is not in the correct conformation, its solubility can be affected. Decreasing the temperature at which expression occurs has been shown to aid both protein folding and solubility (LaVallie et al., 1993). It is also true that cells grow more slowly at lower temperatures. When repeated at room temperature, the expression of the recombinant HHV-8 TK was optimal at 20 hours post-induction. I found that by reducing the temperature for expression the solubility of the recombinant TK was greatly improved. In conclusion, in this chapter I have described the cloning and expression of a soluble recombinant form of the HHV-8 thymidine kinase.
Chapter 6 – Functional analysis of the HHV-8 thymidine kinase
6.1 Introduction

The α-herpesvirus TK can monophosphorylate a broad range of substrates including the anti-herpesvirus drugs, aciclovir (ACV) and ganciclovir (GCV) (Cheng et al. 1981, De Clercq, 1993). However, the TK encoded by HHV-8 has greater homology to the EBV TK (Moore et al. 1996), which appears to be inefficient at catalysing the phosphorylation of ACV and GCV, although it can phosphorylate thymidine analogues such as azidodeoxythymidine (AZT) (Gustafson et al. 1998, Littler and Arrand, 1988, Tung and Summers, 1994). Using transfected 293 cells, the HHV-8 TK has been shown to phosphorylate GCV inefficiently (Cannon et al. 1999), while in vitro studies have shown that HHV-8 is susceptible to GCV and cidofovir (CDV) but relatively insensitive to ACV (Medveczky et al. 1997). Retrospective studies of HIV infected patient groups at risk of KS have suggested that the incidence of KS was lower in patients receiving GCV or foscarnet, but not ACV (Glesby et al. 1996, Mocroft et al. 1996).

At the beginning of the AIDS epidemic, KS was one of the most common AIDS-defining illnesses. However, over the ensuing years the frequency of KS has decreased in parallel with the introduction of specific anti-HIV drugs, either as monotherapy or, more recently, as multiple combination therapy (Dore et al. 1996, Hermans, 1998, Montaner et al. 1994). Since the EBV TK is able to phosphorylate AZT (Gustafson et al. 1998, Tung and Summers, 1994), I have investigated whether the HHV-8 TK could phosphorylate thymidine analogues used in the treatment of HIV infection, and also if these compounds could serve as inhibitors of the HHV-8 TK.
6.2 Results

6.2.1 Phosphorylation of deoxythymidine by the pThio8TK

The kinetics of deoxythymidine phosphorylation by the HHV-8 TK, and determination of Michaelis constants ($K_m$) for the reaction, were performed using $[^3H]$-methyl deoxythymidine as the substrate. Both pThio8TK proteins catalysed the phosphorylation of deoxythymidine in an ATP-dependent fashion which obeyed classical enzyme kinetics (Figure 6.1). However, pThio8TKb showed a greater level of dT phosphorylation with time compared to pThio8TKa. All subsequent reactions were performed using the pThio8TKb expressed protein. The $K_m$ values for both deoxythymidine and ATP as substrates of the HHV-8 TK, calculated from double reciprocal plots of $1/V$ and $1/[S]$, were $18.5(\pm 5.9)\mu M$ and $6.6(\pm 1.7)\mu M$, respectively (Figure 6.2).

6.2.2 Identification of catalytic residues of the HHV-8 thymidine kinase

Three residues in the HHV-8 TK sequence likely to be important for binding ATP (Gly 265), Mg$^{2+}$ (Asp 362), and deoxythymidine (Phe 372) were identified following alignment of the HHV-8 TK sequence, not including the N-terminus (aa223 - aa580), with the HSV-1 TK sequence (aa34 - aa376) (Figure 6.3). After site-directed mutagenesis of these residues to Ala, Ala and Cys respectively, each mutant was expressed to the same level as the wt HHV-8 TK (Figure 6.4) and could be solubilised accordingly (see Chapter 2). Enzyme kinetic analysis (Figure 6.5) of the mutants showed that each was severely compromised functionally, with the $\Delta$dT and $\Delta$Mg mutants
showing no phosphorylation of deoxycytidinone and the 8TKΔATP showing very low levels of phosphorylation (0.4% of wild-type levels).
Figure 6.1 Graphs showing the phosphorylation of deoxythymidine by the HHV-8 thymidine kinase homologue. A shows an increase in dT phosphorylation with time for both pThio8TK$_a$ and pThio8TK$_b$, but no increase for the control (pThioHisB); B shows an increase in phosphorylation with both increasing dT and ATP concentration.
Figure 6.2 Double reciprocal plots (Lineweaver-Burk) for both A) dT and B) ATP. The Michaelis constants $K_{dT}$ and $K_{ATP}$ are determined from the points at which the lines converge on the X-axis. Linear regression analysis was used to compute the line of best fit through the data points.
Figure 6.3  Sequence alignment of the HHV-8 TK (aa223 - aa580) with the HSV-1 TK (aa34 - aa376), showing the amino acid residues selected for mutation Gly 265, Asp 362, and Phe 372, represented by the dark grey boxes. The light grey boxes represent the ATP (aa48 – aa69) and nucleotide (aa161 – aa192) binding sites of HSV-1 TK, determined by Brown et al. 1995. The vertical lines identify residues conserved in both sequences and the colon identifies closely related residues.
Figure 6.4 Western blot showing the expression of the HHV-8 TK mutants 8TKΔdT, 8TKΔATP, and 8TKΔMg. Protein is expressed at similar levels as wild type HHV-8 TK and is present in both the pellet and soluble fractions, post-sonication.
Figure 6.5 Comparison of the enzymatic activity of wt HHV-8 TK and mutants 8TKΔdT, 8TKΔATP and 8TKΔMg. All mutants were substantially reduced in their ability to phosphorylate dT.
6.2.3 Inhibition of the HHV-8 TK by various nucleoside analogues

Using a fixed concentration of [$^3$H-methyl] deoxythymidine, the effects of increasing amounts of unlabelled-nucleoside analogue concentration on deoxythymidine phosphorylation were investigated. AZT was the most potent inhibitor of deoxythymidine phosphorylation, with 50% inhibition occurring at a concentration of 13μM, versus 70μM and 100μM for bromodeoxyuridine (BrdU) and didehydrodeoxythymidine (d4T), respectively. In contrast, high concentrations (5mM) of GCV and ACV produced either a minimal reduction (6%) or no reduction in the phosphorylation of deoxythymidine, respectively (Figure 6.6). Lineweaver-Burk plots for AZT, BrdU and d4T showed that these compounds acted as competitive inhibitors of deoxythymidine phosphorylation by HHV-8 TK, with $K_I$ values of 2.3μM, 25.2μM and 37.3μM, respectively (Figure 6.7 A, B and C).

6.2.4 Phosphorylation of nucleoside analogues by the HHV-8 TK

To determine whether AZT, d4T or GCV could be phosphorylated by the HHV-8 TK, enzyme kinetics were performed with the tritiated analogues replacing deoxythymidine. In the case of AZT, efficient phosphorylation was observed (Figure 6.8A) with a $K_M$ of 2.1μM, whilst d4T was a less efficient substrate (data not shown). No phosphorylation of GCV was detected (Figure 6.8B), consistent with the inhibition data shown in Figure 6.6.
Figure 6.6 Inhibition of dT phosphorylation by HHV-8 TK by various nucleoside analogues. dT phosphorylation decreases with increasing concentration of AZT, BrdU, d4T, but not GCV or ACV.
Figure 6.7 Lineweaver-Burk plots showing increasing inhibition of dT phosphorylation with increasing concentrations of A) AZT, B) BrdU, and C) d4T, respectively. All the lines intersect on the Y-axis showing that the compounds act as competitive inhibitors of dT phosphorylation.
Figure 6.8 Phosphorylation of AZT but not GCV or ACV by the HHV-8 TK.  

A  Time-course of AZT phosphorylation;  
B  Time-course of GCV and ACV phosphorylation.
6.3 Discussion

The thymidine kinases of the \textit{\(\alpha\)-Herpesvirinae} have been exploited for many years as activators of anti-herpesvirus drugs (Cheng \textit{et al.} 1981, De Clercq, 1993). However, the HHV-8 TK is more closely related to the EBV TK which has been shown in some studies to be functionally impotent in the phosphorylation of drugs such as ganciclovir (Gustafson \textit{et al.} 1998, Littler and Arrand, 1988, Tung and Summers, 1994). In order to characterise the HHV-8 TK we used a prokaryotic expression-based approach, which yielded high quantities of functional protein. Enzyme kinetic analysis showed that the HHV-8 TK was an efficient deoxythymidine kinase, with a \(K_m\) for deoxythymidine and ATP of 18.5\(\mu\)M and 6.6\(\mu\)M, respectively. The EBV TK has a comparable \(K_m\) for deoxythymidine (22\(\mu\)M) whereas the \(K_m\) for ATP is higher at 25\(\mu\)M (Tung and Summers, 1994). However, similar analysis by Gustafson \textit{et al.} (2000) suggest HHV-8 TK is less active than EBV TK, with a \(K_m\) for dT of 33.2\(\mu\)M.

The N-terminal region of the HHV-8 TK, like other gammaherpesviruses, is extended by around 200 amino acid residues compared to other herpesvirus TKs. Therefore, sequence alignment of a truncated version of the HHV-8 TK (amino acids 223-580) with the HSV-1 TK was used to identify conserved amino acids previously shown for the HSV-1 TK to be involved in substrate recognition (Brown \textit{et al.} 1995, Evans \textit{et al.}, 1998). The mutations introduced into the HHV-8 TK were amino acid residues 265 Gly→Ala, 362 Asp→Ala and 372 Phe→Cys. Gly265 of HHV-8 TK was selected because it is highly conserved in all herpetic TKs, and is homologous to the HSV-1 TK residue Gly61 present in the ATP binding
domain. Also, Asp362 was chosen because it is the only Asp residue conserved in all herpetic TKs, and the equivalent residue in HSV-1 TK, Asp162, is responsible for co-ordinating Mg$^{2+}$ binding to ATP. Although the amino acid residue Phe 372 is not conserved in all herpesvirus TKs it was selected because it aligns with a HSV-1 TK residue shown to be important for dT binding Tyr162 which can only be functionally replaced by a Phe residue (Munir et al., 1992). Site-directed mutagenesis of these residues produced modified HHV-8 TK proteins which were either non-functional (the Mg$^{2+}$ and deoxythymidine binding site mutants) or had significantly reduced enzymatic activity (in the case of the ATP-binding mutant, D362).

The HHV-8 TK could be competitively inhibited by thymidine analogues such as BrdU, AZT and d4T but not by the guanosine analogues, ganciclovir or aciclovir. These data are similar to competition assays using HHV-8 TK expressed as a glutathione S-transferase (GST) fusion protein (Gustafson et al., 2000), and consistent with the inhibition of deoxythymidine phosphorylation reported for the EBV TK (Gustafson et al. 1998, Tung and Summers, 1994). In the present study, $K_i$ values for AZT and d4T were 2.3$\mu$M and 37.3$\mu$M, respectively, indicating AZT to be a potent inhibitor of deoxythymidine phosphorylation by the HHV-8 TK.

In addition to demonstrating competitive inhibition of HHV-8 TK by AZT, I have shown that AZT is also a substrate for the enzyme. In contrast, phosphorylation of GCV could not be detected, despite multiple experiments with many different enzyme/substrate concentrations. These data are consistent with similar studies using heterologously expressed EBV TK (Gustafson et al. 1998, Littler and Arrand, 1988, Tung and Summers, 1994),
but contrast with some studies using mammalian cell transfection approaches (Moore et al. 1999). Recent data show that the HHV-8 protein kinase homologue is more efficient than the HHV-8 TK at phosphorylation of GCV (Cannon et al. 1999).

Since the $K_m$ for AZT was 2.1$\mu$M it is a ten-fold more efficient substrate for the HHV-8 TK than its natural substrate, deoxythymidine. *In vivo*, this effect would serve to increase intracellular levels of AZT monophosphate and hence the levels of active triphosphate. However, build-up of AZT-MP is associated with cell toxicity, due to inhibition of cellular thymidylate kinase. Gustafson et al. (2000) report that the HHV-8 TK has thymidylate kinase activity, in addition to thymidine kinase activity, thus bypassing this inhibition by diphosphorylating AZT. As the seropositivity of HHV-8 amongst HIV positive individuals is relatively high (for example, in male homosexuals, HHV-8 seroprevalence rates are between 30-50%) (Kedes et al. 1996) the relevance of a viral-encoded enzyme being able to specifically activate AZT should not be underestimated. Indeed, HHV-8 latency has been identified in cells of monocytic origin, which are also important sites for HIV replication (Blasig et al. 1997).

In conclusion, I have shown that established anti-herpetic agents are extremely poor substrates for the HHV-8 TK, although agents currently used for the treatment of HIV infection both inhibit the HHV-8 TK and are phosphorylated by its action. Further studies are warranted to determine whether other anti-retroviral nucleoside analogues are substrates for the HHV-8 TK and to identify inhibitors of this enzyme for the therapy of KS.
Chapter 7 – General Discussion
7.1 General discussion

Kaposi's sarcoma is a neoplasm that was initially documented in elderly Mediterranean men (Kaposi, 1872). Over the years other types of KS have emerged: African-endemic, iatrogenic and AIDS-associated KS (Safai et al., 1980, Friedman-Kein et al., 1990). With the onset of the AIDS epidemic several theories were put forward as to the cause of KS, from various viruses such as hepatitis and CMV to the nitrite inhalants popular amongst gay men. However, epidemiological studies of KS in HIV-infected individuals and immunosuppressed transplant recipients have suggested clues to its pathogenesis, especially with respect to an infectious, possibly sexually transmitted agent. It was in 1994 that Chang et al., using a molecular biological technique called representational difference analysis (RDA), identified novel herpesvirus-like sequences present in AIDS-KS tissue but absent from normal tissue. Using PCR the new herpesvirus, HHV-8, was subsequently detected in all KS types (Moore et al., 1995, Huang et al., 1995, Schalling et al., 1995, Dictor et al., 1996, Luppi et al., 1996) and two AIDS-associated lymphomas, MCD and PEL (Cesarman et al, 1995, Soulier et al, 1995), and is now seen as the major aetiological factor for KS.

The polymerase chain reaction has been used to increase our knowledge of HHV-8 epidemiology and pathogenesis (Section 1.3). However, our understanding of the pathogenic mechanisms of many other viruses, including HIV, CMV and hepatitis C virus, has been improved using viral load measurements (Hagiwara et al, 1993; Ho et al, 1995; Mellors et al, 1996; Stagno et al, 1975; Wei et al, 1995). At the time of performing the studies in
this thesis no method for the determination of HHV-8 viral load had been described. Such an assay would be beneficial to HHV-8 research, and so in Chapter 3 of this thesis I describe the development of a quantitative-competitive PCR (QCPGR) method for the measurement of HHV-8 viral load. Calibration of the assay demonstrated that HHV-8 load could be accurately and reliably quantified within the range of 50 to $10^6$ genome copies.

Using the QCPGR, I was able to examine the extent of HHV-8 infection in the organs of AIDS patients at post-mortem (Chapter 4). Comparable to the aggressive nature of KS in AIDS patients at the time of death, HHV-8 infection was widely disseminated throughout the bodies of those examined. In addition, patients with KS at time of death had a greater number of HHV-8 positive organs and significantly higher viral loads ($P=0.02$) than AIDS patients without KS. This is the first evidence that HHV-8 DNA is widely distributed in internal organs of AIDS-KS patients without obvious histopathological evidence of KS in the body. Presence of HHV-8 DNA may represent reservoirs of persistent HHV-8 infection, and transmission of the virus between different cell types, with the higher viral loads potentially identifying sites of lytic replication.

Identification of HHV-8 infected organs could give clues to how the virus may be spread. For example, HHV-8 was detected at fairly high viral loads in the lymph nodes of some patients (described in Chapter 4), and one study has suggested that, analogous to EBV, the lymphoid system is a potential reservoir of cells latently infected with HHV-8 (Bigoni et al., 1996).
Upon immunosuppression, as in the case of HIV-infected individuals and transplant recipients, the virus may be reactivated from these cells represented by the higher viral loads observed in my data. Also, the heart was one of the tissues most frequently positive for HHV-8. Although this maybe blood contamination, it correlates with HHV-8 detection in blood of AIDS-KS patients (Whitby et al., 1995), which could suggest the presence of infectious virus in blood. Thus, transmission of HHV-8 via blood would be a possibility, and consistent with this suggestion there has been one report of parenteral transmission by blood transfusion (Blackbourn et al., 1997). However, the contribution made by parenteral transmission to the spread of HHV-8 is probably not very significant as the prevalence of HHV-8 antibodies is the same among both blood donors and intravenous drug users, in endemic and non-endemic countries (Simpson et al., 1996, Calabro et al., 1998).

The major route of transmission of HHV-8 among HIV-infected individuals appears to be through sexual contact, observed by the high numbers of HHV-8 infected homosexual and bisexual men. Seroepidemiology studies have indicated that transmission of HHV-8 has similar risk factors involved as those known to increase the risk of AIDS-KS. More specifically certain sexual practices, including receptive and insertive anal intercourse, are risk factors for both infection with HHV-8 and development of KS (Martin et al., 1998, Gambus et al., 1999). Grulich et al. (1999) showed an association between HHV-8 orf65 serology and hepatitis A, which is believed to be transmitted by the faeco-oral route in homosexual men (Corey and Holmes, 1980). The presence of HHV-8 DNA in 50% of duodenal
and rectal biopsies, as well as duodenal aspirates, has been reported previously by one group (Alero Thomas et al., 1996). In this study I have also detected HHV-8 DNA in various gastrointestinal tissues and, as suggested above, this would be consistent with transmission of HHV-8 via the faeco-oral route.

At the beginning of the AIDS epidemic, KS was one of the most common AIDS-defining illnesses. However, over the ensuing years the frequency of KS has decreased with the introduction of new anti-HIV drug regimens, more specifically, highly active anti-retroviral therapy (HAART). The underlying reasons for the resolution of KS following HAART may be attributed to either the decrease in HIV viral load or immune reconstitution, although HAART may impart its therapeutic effects via inhibition of HHV-8 replication. Direct involvement of HIV infection in the pathogenesis of KS would suggest some interaction between HIV and HHV-8. However, in this study both HIV proviral DNA presence and load appeared to have no significant association with HHV-8 load.

The *in vivo* efficacy of antiviral compounds for the treatment of HHV-8 infection has yet to be determined. To date, most reports of the effects of antiviral therapy on HHV-8 have been based on patients being treated for other infections, i.e. anti-HIV and CMV therapies. Longitudinal analyses using QCPGR could examine the effects of therapies on HHV-8 load and disease progression, and provide information on HHV-8 replication dynamics as has been demonstrated for CMV, HIV, HBV and HCV in patients treated with
specific antiviral regimens (Ho et al., 1995, Wei et al., 1995, Neumann et al., 1998, Emery and Griffiths, 2000). By monitoring patients, such as transplant recipients and HIV-positive patients, for HHV-8 infection it should be possible to identify those who are at risk of developing KS (Whitby et al., 1995). Those patients considered to have a high risk of developing the disease could potentially benefit from antiviral prophylaxis, especially during periods of immunosuppression, whether chemically or virally induced. Antiviral intervention is likely to be more useful in the suppression of HHV-8 replication in patients at risk of KS, i.e. HHV-8 seropositive individuals, rather than for the treatment of KS once established.

Retrospective studies of HIV infected patient groups at risk of KS have suggested that the incidence of KS was lower in patients receiving ganciclovir (GCV) or foscarnet, but not acyclovir (ACV) (Glesby et al. 1996, Mocroft et al. 1996). In addition, in vitro studies have shown that HHV-8 is susceptible to GCV, foscarnet, cidofovir, penciclovir (PCV) and (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) but relatively insensitive to ACV (Kedes et al., 1997, Medveczky et al. 1997, Neyts et al., 1997). It would be valuable to gain further insight into the molecular mechanisms that govern the antiviral susceptibility of HHV-8 in order to identify not only new anti-HHV-8 drugs but also compounds that may be active against the other human herpesviruses. For example, the treatment of HSV infection exploits the viral thymidine kinase to initially phosphorylate the anti-herpetic drugs ACV and GCV prior to phosphorylation by cellular kinases to their active triphosphate forms (Cheng et al. 1981, De Clercq, 1993). HHV-8 ORF 21 encodes a thymidine kinase
homologue that could be similarly exploited for the activation of antiviral
drugs, following detailed biochemical characterisation of the HHV-8 TK.

Chapter 5 describes the cloning and expression of the HHV-8 TK using
an *E.coli* expression system. Heterologous expression systems are designed
to express large amounts of the desired protein, although this often results in
expression of the protein in an insoluble form. The latter problem was initially
encountered for the recombinant HHV-8 TK when expressed in *E.coli* at 37°C,
however by decreasing the expression temperature to room temperature
solubility of the expressed HHV-8 TK was greatly improved. Time limitations
meant that purification of the protein could not be achieved, and so
subsequent biochemical analyses were performed using a crude lysate of the
expressed recombinant HHV-8 TK. In addition, to eliminate the possibility that
any enzymatic activity observed was not a result of impurities or
contaminating *E.coli* proteins, all reactions were carried out concurrently with
a crude lysate of the expression system without the recombinant HHV-8 TK.
Enzyme kinetic analysis was performed by using a nucleoside kinase assay
and demonstrated the recombinant HHV-8 TK was able to phosphorylate its
natural substrate deoxythymidine (dT) with high affinity (\(K_m=18.5\mu M\)) (Chapter
6). In addition, site-directed mutagenesis of the HHV-8 thymidine kinase
identified amino acid residues important to the activity of the enzyme in
conserved catalytic domains known to be involved in substrate binding and
recognition in the HSV-1 TK.
Gly61 of the HSV-1 TK is found within the nucleotide (ATP) binding domain and is highly conserved in all herpesvirus TKs (Evans et al., 1998). The homologous residue in HHV-8 TK, Gly265, was substituted by an alanine residue. Although both amino acid residues have small, non-polar side chains, the substitution resulted in a significant reduction in enzyme activity. The substitution of alanine for glycine is effectively the addition of a methyl group onto the amino acid side chain, which may extend into the ATP binding pocket, thus interfering with ATP binding. Asp362 of HHV-8 TK is homologous to the Asp162 residue of HSV-1 TK and was also substituted by an alanine residue, producing a non-functional enzyme. In HSV-1 TK Asp162 is responsible for Mg$^{2+}$ co-ordination (Brown et al., 1995), which may require the Mg$^{2+}$ molecule to interact with the charged carboxyl group of the aspartic acid side chain. By substituting the aspartic acid, at position 362 of the HHV-8 TK, with an alanine the charged carboxyl group is consequently removed thus preventing any possible interaction with Mg$^{2+}$. Phe372 of the HHV-8 TK is not conserved in all herpesvirus TKs, however it aligns with the Tyr172 residue of HSV-1 TK which has been shown to be important for nucleoside binding and stacks against the thymine base of the substrate (Brown et al., 1995), and which can only be functionally replaced by a phenylalanine residue (Munir et al., 1992). Phe372 was substituted with a cysteine residue and resulted in a non-functional HHV-8 TK, possibly as a consequence of the replacement of the ‘bulky’ phenyl group of Phe372 with the smaller thiol group of cysteine causing a local conformational change, or maybe introducing a new disulphide bond.
There are several other amino acid positions which may be important in the overall catalytic activity of the HHV-8 TK that could be targeted for site-directed mutagenesis. These include Arg363, homologous to Arg163 of HSV-1 TK shown to be of primary importance in the phosphorylation of substrates (Black and Loeb, 1990, Brown et al., 1995), and other residues of the nucleoside binding pockets highly conserved in other herpesvirus TKs, i.e. Glu287, Tyr300, and Pro373 (Brown et al., 1995). In addition, like other gammaherpesviruses, the N-terminus of HHV-8 TK has an extension of approximately 200 amino acid residues compared to other herpesvirus TKs, and is especially proline, glycine and serine rich. The purpose of this elongated N-terminal region has yet to be determined, but may have some distinct function such as interacting with other cellular or viral proteins, or may be involved in the limited substrate specificity of the gammaherpesvirus TKs. The creation of N-terminal deletion mutants and site-directed mutagenesis of specific residues could help decipher its role in TK function.

After determining the recombinant HHV-8 TK homologue was functional I then investigated its affinity for known antiviral drugs. Inhibition studies demonstrated that the anti-herpesvirus drugs GCV and ACV were unable to inhibit phosphorylation of dT by HHV-8 TK, whereas the anti-HIV drugs AZT and d4T, and the nucleoside analogue BrdU were competitive inhibitors of dT phosphorylation, with $K_I$ values of $2.3 \mu M$, $37.3 \mu M$ and $25.2 \mu M$, respectively. Moreover, subsequent analyses investigating these compounds as substrates for the HHV-8 TK found that AZT ($K_M=2.1 \mu M$) and d4T were phosphorylated but GCV and ACV were not. This implies that HHV-8 TK has
a limited substrate range as both AZT, d4T and BrdU are analogues of the natural substrate of TK deoxythymidine, whereas GCV and ACV are acyclic guanine analogues. This is consistent with reports of other gammaherpesviruses, EBV and HVS, and a more recent report on HHV-8 TK substrate specificity (Honess et al., 1982, Tung and Summers, 1994, Gustafson et al., 1998, Gustafson et al., 2000). In addition, the implication that GCV is not a substrate for the HHV-8 TK would suggest that worries about the pThio8TKα protein being resistant to GCV need not have been a concern.

Both AZT and d4T have been used in monotherapeutic and multiple drug regimens for the treatment of HIV infection and it is interesting to note that plasma levels which exceed the Ki for HHV-8 TK can be achieved during AZT therapy (Borvak et al. 1992, Morse et al. 1990, Wintergerst et al. 1995). Potentially, therefore, HHV-8 may be susceptible to inhibition by AZT and, to a lesser extent d4T. In fact, recent data using in vitro analysis of TPA-induced BCBL-1 cells have shown that AZT is a more potent inhibitor of HHV-8 lytic replication than either GCV or CDV (IC50 values of 0.91 μM for AZT versus 6.7 μM and 3.1 μM for GCV and CDV, respectively) (Fletcher et al. 1999). It is therefore possible that HHV-8 TK phosphorylates AZT, which is then incorporated into the elongating HHV-8 DNA, and acts as a chain terminator thus inhibiting the HHV-8 DNA polymerase. A more provocative possibility is that in patients with active HHV-8 replication levels of AZT-TP are increased by phosphorylation by the HHV-8 TK, thus facilitating in the inhibition of HIV replication. Indeed, cells of monocytic origin that are important sites for HIV
replication have also been identified as sites of HHV-8 latency (Blasig et al. 1997).

An important issue to address is whether the enzymatic studies performed here have clinical relevance. In this context, two observations are of note. Firstly, epidemiologic data show that the incidence of KS amongst most male homosexuals has declined following the introduction of mono, dual and triple therapeutic interventions using AZT, despite an increase in the numbers of HIV-infected individuals (Dore et al. 1996, Hermans, 1998, Montaner et al. 1994). Secondly, two studies have reported improved response rates of Kaposi's sarcoma when AZT was added to standard interferon alpha treatment (Fischl et al. 1996, Krown et al. 1990). As stated previously, these effects were initially ascribed to a reduction in HIV load or immunosuppression, but the data presented here, along with other studies (Fletcher et al. 1999, Gustafson et al., 2000), suggests the anti-retroviral drug AZT, and maybe d4T, has a more direct effect on HHV-8 replication.

In conclusion, the data presented in this thesis has helped to further characterise the pathogenesis and identify mechanisms for the control of HHV-8 infection. Viral load measurements using QCPCR would aid the analysis of the in vivo effects of new and old antiviral drugs in controlling HHV-8 infection. In addition, the potential for the HHV-8 TK homologue to phosphorylate dT analogues may be exploited in the search for new antiviral compounds as HHV-8 therapies. Compounds found to be efficiently phosphorylated by the HHV-8 thymidine kinase could be further characterised
by analysing their effects on the distinct cell populations productively infected or immortalised by HHV-8. This could be used to identify less toxic anti-HHV-8 drugs and would help to optimise future therapeutic strategies involved in the control of infection and disease. More recently the development of Taqman and related PCR technologies adapted for detecting HHV-8 DNA in human tissue samples has occurred (Kennedy et al, 1997). Also, White and Campbell (1999) have recently reported the use of this technique for quantitation of HHV-8 DNA, with similar levels of accuracy to the assay developed in this thesis.
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APPENDIX

Reagents and sources

**Amersham**

**BDH**
Isopropanol, Potassium acetate, Glacial acetic acid, Methanol, N,N,N',N'-tetramethylethylenediamine (TEMED).

**Bioline**
BioTaq polymerase, KCl buffer, MgCl₂, NH₄Cl₂ buffer, Bio-X-Act DNA polymerase, Optiperm buffer.

**Boehringer Mannheim**
T4 polynucleotide kinase, Forward reaction buffer, EcoRI, HindIII, Sacl, PstI, Buffer B, Pefabloc, calf intestinal phosphatase.

**Gibco BRL**
pUC18 BAP (SmaI cut).

**ICN**
[α-35S]dATP

**Invitrogen**
pThioHisB, Anti-Thio™ mouse monoclonal antibody.

**Moravek**
**Novex**

NuPAGE Electrophoresis system, Lithium dodecyl sulphate (LDS) sample buffer, Antioxidant.

**Promega**

Genomic DNA extraction kit, dNTP's, 8X174HaeIII DNA marker, Wizard PCR preps DNA purification system, GeneEditor *in vitro* site-directed mutagenesis kit, pGEM-11ZF(+).

**Qiagen**

Qiamp DNA blood mini kit, Qiaprep-8 miniprep kit, Qiagen EndoFree Maxi-plasmid purification kit.

**Sigma**

Tris-HCl, boric acid, (EDTA), ethidium bromide, bromophenol blue, Ficoll, hexaminecobalt chloride, RbCl2, CaCl2, MnCl2, (MOPS), KOH, glycerol, Luria-Bertani (LB) broth, ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal), low-gelling point agarose, NaOH, Sepharose CL-6B, dithiothreitol (DTT), urea, dimethyl-dichlorosilane, ammonium persulphate, imidazole, aprotinin, leupeptin, sodium dodecyl sulphate (SDS), coomassie brilliant blue R250, sodium azide, bovine serum albumin (BSA), Tween 20, Triton X-100, Sigma Fast BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium), sodium fluoride, ammonium acetate, PPO, POPOP, toluene, dT, AZT, d4T, bromodeoxyuridine (BrdU), GCV.

**Whatman**

3MM chromatography paper, DE81 chromatography paper.
PUBLICATIONS


Grzywacz, M., E. Lennette, M. J. Lock, D. J. Blackbourn, and V. C. Emery. Quantitation of HHV-8 DNAemia following renal transplantation: correlation with HHV-8 serology (Submitted for publication).

Lock, M.J., N. Thorley, J. Teo, and V.C. Emery. Azidodeoxythymidine (AZT) and Didehydrodeoxythymidine (d4T) as Inhibitors and Substrates of the Human Herpesvirus-8 Thymidine Kinase (Submitted for publication).