Persistent Infection with Human Herpesvirus-6 in Patients with an Inherited Form of the Virus: A Newly Described Disease

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Persistent Human Herpesvirus-6 Infection in Patients with an Inherited Form of the Virus: a Newly Described Disease

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Ed Seto, Ph.D.

Date of Approval
November 5, 2013

Keywords: HHV-6, Chronic Fatigue Syndrome, Inherited Herpesvirus Syndrome, Virus Integration, Telomere

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DEDICATION

I dedicate this dissertation to my grandparents, Ladric and Retinella Lewis, my parents, Beverley and Everard Pantry, and to my brothers, Marlondale and Lamonte Pantry. I would also like to dedicate this dissertation, to my niece and nephew, Chaniah and Marlondale Pantry. Thank you for always believing in me, and for always supporting me. This degree is yours, as much as it is mine.
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First, I would like to thank Dr. Peter Medveczky for the time and effort that he has put into helping me throughout my years in the lab. I admire you as a scientist, and a person, and I hope to one day follow in your footsteps. Had you not pushed me to beyond my limits, I would not be the person I am today. I would also like to thank Maria Medveczky for being an excellent teacher. Over the years, the three of us have gone from being colleagues to being family. I appreciate you both very much.

I would also like to acknowledge Drs. Gloria Ferreira, George Blanck, Ed Seto, and David Knipe for serving on my committee and guiding me through the dissertation process.

Finally, I would like to acknowledge the professors, staff, and students in the Department of Molecular Medicine.
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<th>Description</th>
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<tbody>
<tr>
<td>AAV2</td>
<td>Adeno-associated virus type 2</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>CFS</td>
<td>Chronic fatigue syndrome</td>
</tr>
<tr>
<td>CIHHV-6</td>
<td>Chromosomally integrated HHV-6</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>Cre</td>
<td>Cyclization recombinase</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DIHS</td>
<td>Drug-induced hypersensitivity</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle's medium</td>
</tr>
<tr>
<td>DR</td>
<td>Direct repeat</td>
</tr>
<tr>
<td>DRESS</td>
<td>Drug rash with eosinophilia and systemic symptoms</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent in situ hybridization</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>gB</td>
<td>Glycoprotein B</td>
</tr>
<tr>
<td>gH</td>
<td>Glycoprotein H</td>
</tr>
<tr>
<td>HBLV</td>
<td>Human B-lymphotropic virus</td>
</tr>
<tr>
<td>HEK293</td>
<td>Humane embryonic kidney 293</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HHV-6</td>
<td>Human herpesvirus-6</td>
</tr>
<tr>
<td>IE</td>
<td>Immediate early</td>
</tr>
<tr>
<td>IHHV-6</td>
<td>Inherited HHV-6</td>
</tr>
<tr>
<td>IHS</td>
<td>Inherited herpesvirus syndrome</td>
</tr>
<tr>
<td>Jjhan</td>
<td>T cell lymphoma line obtained from the HHV-6 Foundation</td>
</tr>
<tr>
<td>loxP</td>
<td>locus of X-over P1</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>Molt3</td>
<td>T cell lymphoma line obtained from the HHV-6 Foundation</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>TERRA</td>
<td>Telomere repeat-containing RNA</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
</tbody>
</table>
ABSTRACT

Human Herpesvirus 6A (HHV-6A) and 6B (HHV-6B) are ubiquitous betaherpesviruses. Both viruses are associated with a variety of adult disorders including neurological disorder, such as multiple sclerosis and chronic fatigue syndrome. HHV-6 viruses are capable of establishing latency by integration into the telomeres of the host chromosome and are transmitted in a Mendelian manner in approximately one percent of the population. To date little is known about the immunological and neurological consequences of HHV-6 inheritance. This study focused on a unique population of individuals that inherited HHV-6 and present with chronic fatigue-like symptoms, including hypersomnia, generalized fatigue, headache, and short term and long term memory impairment. The central hypothesis of this study was that active replication of HHV-6 correlates with patient symptoms. To address this aim we first looked at the reactivation of integrated HHV-6 in vitro by inducing viral replication with epigenetic modifiers trichostatin A (TSA), valproic acid, sodium butyrate, and carbamazepine, and found TSA to be an effective method of inducing reactivation of HHV-6 from its integrated form. Additionally, a reactivated HHV-6A virus isolated from a patient with inherited HHV-6 was fully sequenced and the nucleotide and amino acid sequence was compared to that of fully sequenced HHV-6 laboratory strains, as well as the inherited virus. The reactivated virus was found to be very similar to the HHV-6A GS strain; however, there was some divergence at the right end of the viral genome and
regions of the genome that do not contain herpesvirus core genes. Interestingly, the sequenced reactivated virus was found to differ from the HHV-6 virus which was inherited. Finally, HHV-6 replication was assessed by performing reverse transcriptase PCR assay for the viral glycoprotein U100 in patients receiving antiviral treatment. Results indicated that short term antiviral treatment was insufficient to abrogate viral replication, while treatment of six weeks or longer eliminated viral mRNA in patient blood samples. Furthermore, sequencing of the viral mRNA and inherited viral DNA indicate that the source of the mRNA detected in patient blood samples was an exogenously acquired HHV-6 virus, as the U100 glycoprotein sequences were not identical. Together these studies indicate that although HHV-6 can be reactivated from its integrated form, individuals in this unique population harbored an exogenous HHV-6 virus, in addition to the inherited virus; we termed this condition inherited herpesvirus syndrome. The fact that these individuals are able to acquire exogenous HHV-6 viruses suggest that there may be some level of immune tolerance or immune dysfunction; we suggest that further studies focus on uncovering the immune response to HHV-6 in individuals with an inherited form of the virus.
**INTRODUCTION**

**Herpesviridae Structure**

The *Herpesviridae* family of viruses is comprised of greater than 200 herpesviruses that infect mammals, birds, and reptiles. Irrespective of the natural host, all herpesvirus virions range in size from 120-260nm and have a similar structure (1). At the core of the virus particle, is the double stranded DNA genome that is tightly packaged into an icosahedral nucleocapsid comprised of 161 hexameric and pentameric capsomeres. Surrounding and tightly associated with the nucleocapsid is an amorphous, fibrous structure made of a compilation of viral and cellular proteins known as the tegument. Viral tegument proteins are important for propagating infection and often interfere with the host innate response to the virus. Examples of tegument proteins include HSV UL41 protein that targets cellular mRNA for degradation, and US3 that inhibits toll like receptor 2 (2,3). The most outer layer of the virion is a lipid bilayer envelope with glycoprotein protrusions. Glycoproteins often serve as ligands for cellular receptors or some are essential for cell fusion (1,4).

**Classification of Human Herpesviruses**

The nine human herpesviruses may be classified into three subfamilies and six genera based on DNA sequence, similarity of encoded proteins, site of latency, and other biological properties *(Table 1)*. The human members of the Alphaherpesvirinae
subfamily include herpes simplex viruses 1 & 2, as well as Varicella-zoster virus; the
Alphaherpesviruses infect and establish latency in sensory ganglia. Cytomegalovirus
(CMV), human herpesvirus 6A, 6B, and 7, are members of the Betaherpesvirinae
subfamily. CMV establishes latency in monocytic cells and the bone marrow; however,
there is no conclusive evidence indicating the natural site of latency for the HHV-6
viruses or for HHV-7. The viruses belonging to the Gammaherpesvirinae subfamily are
known for their oncogenic potential; gammaherpesviruses establish latency in lymphoid
cells. The two human gammaherpesviruses, Epstein-Barr virus and Kaposi’s sarcoma-
associated herpesvirus, both establish latency in B-lymphocytes.

Figure 1: The Structure of Herpesviruses. Each herpesvirus particle is comprised of
a double stranded DNA genome contained within a capsid, tegument, and envelope.
Protruding from the surface of the envelope are glycoproteins (1).

Genome Organization of Human Herpesviruses

The genomes of the human herpesviruses range in size from 145 to 230 kbp (1).
Despite some conservation in the encoded genes, the genomic arrangement varies
from one virus to another (Figure 2). The majority of the genome length is attributed to
the unique length region, which contains the majority of the protein-coding genes (1). The unique length segment may occur as a single unit or may be separated via one or more blocks of intervening repeated sequences, creating a unique long or two or more unique short regions (1).

Table 1: Classification of Human Herpesviruses (1)

<table>
<thead>
<tr>
<th>Designation</th>
<th>Common Name /Abbreviation</th>
<th>Subfamily</th>
<th>Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHV-1</td>
<td>Herpes simplex virus 1 (HSV-1)</td>
<td>Alpha</td>
<td>Simplexvirus</td>
</tr>
<tr>
<td>HHV-2</td>
<td>Herpes simplex virus 2 (HSV-2)</td>
<td>Alpha</td>
<td>Simplexvirus</td>
</tr>
<tr>
<td>HHV-3</td>
<td>Varicella-zoster virus (VZV)</td>
<td>Alpha</td>
<td>Varicellovirus</td>
</tr>
<tr>
<td>HHV-4</td>
<td>Epstein Barr virus (EBV)</td>
<td>Gamma</td>
<td>Lymphocryptovirus</td>
</tr>
<tr>
<td>HHV-5</td>
<td>Cytomegalovirus(CMV)</td>
<td>Beta</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>HHV-6A</td>
<td>Human herpesvirus 6A (HHV-6A)</td>
<td>Beta</td>
<td>Roseolovirus</td>
</tr>
<tr>
<td>HHV-6B</td>
<td>Human herpesvirus 6B (HHV-6B)</td>
<td>Beta</td>
<td>Roseolovirus</td>
</tr>
<tr>
<td>HHV-7</td>
<td>Human herpesvirus 7 (HHV-7)</td>
<td>Beta</td>
<td>Roseolovirus</td>
</tr>
<tr>
<td>HHV-8</td>
<td>Kaposi’s sarcoma-associated herpesvirus (KSHV)</td>
<td>Gamma</td>
<td>Rhadinovirus</td>
</tr>
</tbody>
</table>

Additionally, the genomes of the human herpesviruses characteristically contain a variable number of repetitive elements at the ends and/or within the genome. When internal repeats are inversions of terminal or internal reiterations (inverted repeats), isomers may arise due to recombination of complementary sequences during viral
replication (1). Of the human herpesviruses, only HSV-1, HSV-2, VZV, and CMV have inverted repeats(1).

The genome of HHV-6A and HHV-6B are 159kbp and 162kbp, respectively. An 8-13kb segment, designated direct repeat left (DR\textsubscript{L}) or right (DR\textsubscript{R})(5–7), is duplicated in the same orientation at both ends of the unique length. At the outer regions of the direct repeat are a variable number of telomere repeats that are either perfectly reiterated (perfect repeats) or repeated with other interspersed hexameric sequences (imperfect repeats) (7). The telomere repeats of HHV-6 are identical to that of the human telomere repeats and the perfect repeats at the right end of the viral genome are in the same orientation of the human telomeres, facilitating recombination between viral and host repeats. In addition to the telomere repeats, the genomes of HHV-6A and HHV-6B have major repetitive elements located at near the right end of the unique length region: R1, R2, and R3; HHV-6B has an additional repetitive element R0 located at the junction of the DR\textsubscript{L} and the unique segment (1,5,6).

**Herpesvirus Replication: Lytic Replication, Latency, and Reactivation**

Three replication properties shared by all herpesviruses are (1) replication of the viral DNA within the nucleus (2) a replication cycle includes primary infection, and a period of lytic replication during which new virus production occurs or the establishment of latency, and (3) after a variable period of latency, reactivation may occur, leading to the production of infectious viruses.
Figure 2. The Genome Organization of Human Herpesviruses. Solid lines represent the unique length (U) regions of the genome: The unique (U), unique Long (UL), unique short (US), and long unique regions (LUR) are represented by solid lines. Box regions represent repetitive elements of the genome: TR (terminal repeats), TRL (long terminal repeat), or TRS (short terminal repeat). For HHV-6A, 6B, and 7 the direct repeats (DRL or DRR), the black boxes represent the perfect telomere repeats, while the gray boxes represent the imperfect telomere repeats (1,5,8,9).

**Lytic Replication**

Infection of herpesviruses begins with receptor binding, fusion of the viral and cellular membrane, and the release of the viral genome into the nucleus of the cell (1). Prior to the start of lytic replication, the DNA genome of the virus circularizes, and viral gene products are synthesized in a lytic cascade. At the earliest time points of infection,
tegument proteins are expressed. Next, the immediate early (IE) genes that function as transcriptional regulators are expressed. IE gene expression occurs independent of protein synthesis, and is therefore produced in the presence of cycloheximide in vitro. Next, the early genes, which are expressed independently of viral DNA synthesis, are synthesized; these genes encode proteins such as the viral primase/helicase, and DNA polymerase, which are required for viral DNA replication. In the final phase of the lytic cascade, the late genes that encode structural proteins for new virion production, such as capsid proteins and glycoproteins, are synthesized.

During DNA replication, the circularized viral genome is duplicated by a rolling-circle mechanism (10,11). At the start of replication, a short RNA primer is made by the viral primase. Replication begins at an origin of replication and proceeds around the circle, resulting in the formation of catenated genomes. For new viral particles to be made, cleavage of the concatemers must occur and single length genomes need to be packaged into empty nucleocapsids. During lytic replication, the production and release of new viral particles results in the lysis of the host cell.

**Latent Infection**

As an alternate to lytic replication, herpesvirus may enter a period of latent infection following infection of new target cell cells (1). In contrast to lytic replication, gene expression is highly restricted during latency. Genes that are essential for the production of new viruses are repressed, and only genes required for maintenance of the viral genome and latency associated genes are expressed. In most cases, during
herpesvirus latency, multiple circularized copies of the viral genome found within the nucleus are tethered to the chromosome.

**Latency of HHV-6**

HHV-6A infects cells via the CD46 receptor, while HHV-6B infects cells via the CD134 receptor (12,13). It has been suggested that the natural site of latency for HHV-6 viruses is the monocyte/macrophage cell population (14). Additionally, there is *in vitro* evidence of establishment of latency in other cell types including astrocytes, T-lymphocytes (15,16). There is no conclusive evidence on the natural site of latency during HHV-6 infection.

In contrast to other human herpesviruses, HHV-6 viruses do not form circular episomes during latency. Initial reports of HHV-6 latency in patient PBMCs indicated a covalent linkage between HHV-6 DNA and high molecular weight cellular DNA (17). However, the mechanism of latency and the orientation of the genome during latency were not elucidated for another decade. More recently, Arbuckle *et al.* demonstrated that latency of HHV-6 viruses occurs by homologous recombination between telomere-like repeats at the telomeres at the end of the host chromosomes (15). This method of integration was observed *in vitro* as well as in patient PBMC samples (15). Southern blot hybridization and cloning and sequencing of the integration site, showed that during integration, the HHV-6 genome is oriented so that the perfect telomere repeats at the right end of the viral genome is adjacent to the subtelomere of the human chromosome (15). Additional experiments also demonstrate that during integration both direct repeats
are present and the imperfect telomere repeats at the left end of the viral genome adjoins the telomere of the host chromosome (18). **(Figure 3)**

*Figure 3: The Model of HHV-6 Integration.* During homologous recombination the presence of perfect telomere repeats (black box) and imperfect telomere repeats (grey box) at the end of the direct repeats allow integration into the host telomeres via homologous recombination (18).

**Reactivation**

Herpesvirus latency is not abortive; the quiescent virus may produce infectious virus particles at a later point. A variety of factors may lead to the reactivation of herpesviruses in their natural hosts, such as immune suppression, UV exposure, and in some cases hypoxia (19,20). In tissue culture models, some latent herpesviruses are inducible by chemical compounds which serve as epigenetic modulators which alter chromatin structure or mediate RNA polymerase to access DNA. To name a few, DNA methyltransferase inhibitor, 5-azacytidine, and HDAC inhibitors, trichostatin A (TSA) and sodium butyrate, are all capable of inducing viral gene transcription (21–25).
HHV-6 History

In 1986, HHV-6 was first isolated from the PBMCs of six immunodeficient individuals who presented with a variety of lymphoproliferative disorders, including HTLV, B-cell lymphoma, and acute lymphocytic leukemia (26). Preliminary reports identified the virus as a novel herpesvirus that primarily infects B-lymphocytes; therefore, the virus was designated human B-lymphotropic virus or HBLV (26). Shortly after initial identification, studies demonstrated that in culture HBLV/HHV-6 selectively infects and efficiently replicates in T-lymphocytes with a preference towards CD4+ T-cells (27–29). Although HHV-6 viruses are primarily lymphotropic, additional sites of replication for the viruses include monocytes/macrophages, and astrocytes (14,16).

HHV-6 Transmission

After primary infection, HHV-6 is detectable in saliva, nasal cavity, cervix and female genital tract (30–32). The main route of HHV-6 transmission is oral transmission via saliva; most children are infected by the age of two (33).

Additionally, HHV-6 viruses may be transmitted congenitally by one of two mechanisms: transplacental/perinatal infection or inheritance (15,34–38). Integration of HHV-6 in the telomeres of germline cells, a condition known as chromosomally integrated HHV-6 (CIHHV-6), results in vertical transmission of the virus in a Mendelian manner. All CIHHV-6 positive individuals harbor one copy of the viral genome in every nucleated cell; there has been a single report of an individual who inherited one copy of the viral genome from each parent (37). Over the past twenty years, several independent groups reported HHV-6 integration/inheritance; evidence was primarily
obtained by fluorescent in situ hybridization studies to determine integration site (38–41). More recently, Arbuckle et al. cloned and sequenced the integration site, while also performing partial sequence analysis to confirm that the integrated virus is the same in all CIHHV-6 family members (15). The rate of inheritance in the general population is approximately 1% in the United States and the United Kingdom, with an increased prevalence seen in hospitalized patients (40,42–49). To date eight unique HHV-6 sites of integration have been reported in the literature: 1q, 9q, 10q, 11p, 17p, 18q, 19q, 22q (15,17,37–39,50–53). Congenital HHV-6 infection may also result from intrauterine or transplacental infection. HHV-6 has successfully been detected in cervical swabs of pregnant women (30,54), as well as in placenta samples obtain during birth (55), and recent reports confirm that transplacentally acquired HHV-6 may occur due to reactivation of a maternal chromosomally integrated HHV-6 (CIHHV-6) strain (30,36). Although inheritance of HHV-6 is relatively low in the general healthy population, it is considered the major mode of congenital infection (34).

Transplant related HHV-6 transmission may also occur by either donor transmission or reactivation of the virus. Transmission of integrated HHV-6 via transplant has been noted in hematopoietic stem cell recipients, as well as liver, kidney, and small bowel transplant patients (40,45,46,56). The implications of CIHHV-6 transmission is not fully understood; however, a common problem is misdiagnosis of active replication and treatment with antivirals in the absence of HHV-6 related clinical symptoms (41,51,57,58). Additionally, transmission via solid organ transplantation resulting in antibody responses characteristic of primary HHV-6 infection has been documented (59).
HHV-6 Epidemiology

**Prevalence**

Seroprevalence of HHV-6 ranges up to 100% in most regions of the world (60). However, there is a disproportionate number of childhood infections with HHV-6B in the United States, Japan, and some regions of Europe, rather than HHV-6A (61,62). In these regions, infections with HHV-6A are generally seen in adult populations who are experiencing immunological or neurological disorders. An exception to this trend is seen in regions of Sub-Saharan Africa, where the prevalence of HHV-6A is as high as 85% in healthy children (63).

The rate of inheritance of HHV-6 is approximately 1% in the United States and the United Kingdom. However, and increased prevalence has been noted in some populations of transplant patients and pediatric encephalitis patients (34,42,46,56,64–67). Inheritance of both HHV-6A and HHV-6B occurs. However, there are insufficient data at this time to determine which variant is most commonly inherited.

**HHV-6 Disease Associations and Complications**

**Exanthema subitum**

In most regions of the world, primary HHV-6B infection occurs by the age of two and the virus is considered ubiquitous in the healthy population (1,68). HHV-6B is one of the causes of exanthema subitum, also known as roseola infantum or sixth disease (69). Exanthema subitum is characterized by a sudden onset fever, followed by a rash on the trunk, extremities, and face. Although rare, complications associated with
exanthema subitum include convulsions and encephalitis (1). Primary infection with HHV-6 in adults is rare, but may result in a mononucleosis-like disease (70).

**AIDS Progression**

While HHV-6A has not conclusively been implicated as the etiological agent for any diseases, it may play a role in AIDS progression. HHV-6 viruses predominantly replicate in CD4+ T-cells, the target cells of the HIV virus. In vitro, HIV and HHV-6 can together productively infect CD4+ T cells, and dual infection has been noted in infants and adult (62,63). HHV-6 infection leads to trans-activation of HIV LTR and increased HIV gene expression (71,72). Furthermore, in a pig-tail macaque model, animals infected with both HHV-6A and simian immunodeficiency virus (SIV) progression to AIDS and depletion of CD4+ and CD8+ cells occurred faster than in animals infected SIV alone.(73). Accelerated progression to AIDS was also noted when animals were infected with HHV-6 prior to SIV infection.

**Multiple Sclerosis**

The cellular receptor for HHV-6A viruses is the ubiquitously expressed CD46 receptor; in contrast, CD134 is used by HHV-6B viruses for infection. In vitro studies have demonstrated experimental infection of a variety of cell types including neuronal cells (12,16,74) with both viruses. The nasal cavity is a reservoir for HHV-6 viruses; and from there HHV-6 gains access to the central nervous system via the olfactory pathway (31). Both HHV-6A and HHV-6B are able to infect cells of neuronal origin. However, only HHV-6A is able to establish productive infection in astrocytes and oligodendrocytes; HHV-6B infection in these cell types is abortive (16,31,74,75).
It has been suggested that HHV-6 plays a role in the progression of multiple sclerosis (MS); however, this relationship remains controversial. Staining of neurons and oligodendrocytes on brain specimens in the United States detected using antibodies against HHV-6 virion protein and DNA binding protein in both control and MS brains. However, HHV-6 antigen detection in oligodendrocyte and neuronal plaques was significantly higher in MS brains than control brains (76). Other studies describe significance higher prevalence of IgG titers directed at the HHV-6 U94 rep homologue protein and elevated IgM antibody directed against p41/38 HHV-6 early antigen in MS patients during relapse (77–79). HHV-6 DNA and infectious virus could also be detected and isolated from the cerebral spinal fluid of MS patients (79,80). Despite these data, contradicting reports have found no significant differences in DNA and antibody levels between individuals with MS and controls (81).

**Chronic Fatigue Syndrome**

Chronic fatigue syndrome (CFS) is a chronic multi-systemic disease characterized by long-standing fatigue, impaired memory and concentration, hypersomnia, headache, and musculoskeletal pain (82). Additionally, individuals suffering from CFS also present with hypothalamic dysfunction and quantitative encephalography consistent with encephalopathy (83,84). To date, the etiological agent of CFS has not been established. However, many have suggested that a viral agent may be a trigger because patients often report having flu-like symptoms prior to CFS onset (84,85).
HHV-6 exhibits immunomodulatory effects and has the ability to productively infect cells of neuronal origin; thus, many have suggested these viruses to be a trigger for CFS (84). In the literature, there are reports supporting and refuting these claims. Studies supporting a role for HHV-6 in CFS, indicate a significant increase in antibody titers to HHV-6 early antigen p41 and late antigen gp110 in affected individuals (86). Elevated titers suggest viral reactivation; improved cognitive function was also described with the use of valganciclovir treatment in individuals with elevated HHV-6 titers (83,87). However, other studies found no difference in HHV-6 IgG levels in individuals with CFS (88).

Research on the role of HHV-6 in CFS pathology is far from complete. For example, the prevalence of CIHHV-6 in the CFS population has not been fully explored. CIHHV-6 adds a level of complexity to the question of how HHV-6 affects CFS pathology because inheritance of the virus may potentially alter an individual’s immune response and may contribute to the disease pathology.

**Drug-Induced Hypersensitivity**

Drug-induced hypersensitivity (DIHS)/drug rash with eosinophilia and systemic symptoms (DRESS) is a rare but severe systemic drug allergic reaction, characterized by rashes, fever, and lymphadenopathy. Triggers for DIHS/DRESS include anticonvulsants, some classes of antibiotics, and anti-inflammatory drugs (50,89,90). Many anticonvulsants, such as valproic acid and carbamazepine, are HDAC inhibitors; thus, these epigenetic modifiers often lead to the reactivation of latent herpesviruses. Multiple studies have linked HHV-6 to DIHS; HHV-6 replication has been detected in
DIHS skin lesions and anti-HHV-6 IgG and IgM has also been detected in patient serum. The presence of both HHV-6 specific IgM and IgG during DIHS, suggests that primary HHV-6 infection and HHV-6 reactivation may be contributing factors to the syndrome.

**HHV-6 Detection in Clinical Samples**

The most common method for detecting primary HHV-6 infection is indirect immunofluorescence to detect anti-HHV-6 IgM and IgG antibodies (91,92). Additionally, active replication may be detected by PCR or RT-PCR for the detection of viral DNA or RNA in plasma or serum or whole blood (92–95). Following primary infection, IgG antibodies often persist and IgM antibody diminish and do not always reappear with reactivation. Therefore, quantitative PCR is most often used for detecting viral reactivation or reinfection. Different methodologies, however, must be used to detect active HHV-6 replication in individuals with CIHHV-6.

In contrast to transient infections with HHV-6, CIHHV-6 positive individuals exhibit a persistent high viral load (1x 10^6-1 x 10^7 copies/ml) in PBMCS, and hair follicles, leukocytes, and other clinical samples are also positive (34,96,97). The antibody response to HHV-6 in CIHHV-6 harboring individuals is variable with regards to the presence of IgG antibodies in the serum of these individuals (37,38). In vitro studies and clinical reports indicate that HHV-6 may be reactivated from its integrated form (15,36); however detection of reactivation in samples from individuals with CIHHV-6 is difficult using PCR based methodologies. HHV-6 antibody response during reactivation is also variable and may yield inconclusive results. Detection of mRNA, however, seems
to be a promising marker for viral reactivation; thus, several research groups successfully detected active HHV-6 replication using reverse-transcriptase PCR based assays (30,35,93–95,98).

**Conserved and Unique Genes of HHV-6**

The genomes of HHV-6A and HHV-6B are colinear to human cytomegalovirus and to date there are greater than sixty open reading frames with known or implied function (99). Protein-encoding open reading frames occur within the unique length region as well as the direct repeats of the virus, and proteins are encoded on both strands of the viral genome. Similar to other herpesviruses, the majority of genes encoded by HHV-6A and HHV-6B are conserved across all herpesvirus subfamilies. Conserved herpesvirus genes include tegument and capsid proteins, and proteins essential for the helicase/primase and DNA polymerase complex. Additionally, one block of genes at the left end of the virus (U2 to U19) is specific to betaherpesviruses; and another seven other genes are unique to roseoloviruses (U20, U21, U23, U24, U24A, U26, U100). There are only five genes that are unique to HHV-6A/6B. (Table 2)

**HHV-6 Interstrain and Intrastrain Variation**

To date, four HHV-6 genomes have been completely sequenced (Table 3) (5,6,9,100). Whole genome sequence comparison has been performed comparing the two B viruses, HST and Z29, to HHV-6A U1102 (5,6). The sequencing for HHA-6A GS has been completed and made available recently; however, the genetic relationships
Figure 4: A Diagram of the HHV-6 Genome. The HHV-6 genome includes 7 gene blocks with are conserved across all herpesvirus subfamilies (numbered open arrows), as well as one gene block specific to betaherpesviruses. The black boxes indicate the perfect telomere repeats, while the gray box represents the imperfect telomere repeats. (5,99)

Table 2: HHV-6 Specific Genes

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR3</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>U6</td>
<td>Unknown</td>
</tr>
<tr>
<td>U22</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>U83</td>
<td>Chemokine/Cytokine</td>
</tr>
<tr>
<td>U94</td>
<td>Parvovirus rep homologue</td>
</tr>
</tbody>
</table>

between the GS and other sequenced HHV-6A viruses have yet to be elucidated. Full genome studies focusing on intrastrain variation are also limited. However, studies have looked at the nucleotide and amino acid sequence variation of several genes in clinical samples, and there are preliminary data on partial differences between HHV-6B Z29 and HHV-6B HST (5,6).

The amino acid identity between HHV-6A and B isolates averages approximately 90% (5,6). The center of the genome (U32-U77) is highly conserved, and the most conserved open reading frames include the genes encoding the helicase/primase complex (U77), capsid assembly proteins (U40), and the putative terminase (U60/66). The region of greatest divergence is the right end of the genome, which encodes open
reading frames U86 to U100 as well as the three major repetitive elements, R1, R2, and R3. The amino acid identity in this region is 72% (5). Other variable genes, such as U47 (glycoprotein) and the U83 (chemokine) are scattered throughout the genome and are generally located between conserved herpesvirus gene blocks (Figure 4). In addition to differences in protein encoding genes, HHV-6B viruses have an expanded DR region, when compared to HHV-6A U1102 (5,6).

A few studies have focused on the nucleotide and amino acid sequence divergence in clinical samples. Sequence analysis from clinical strains isolated from the United States, Japan, Germany, Uganda and Zaire highlights key differences demonstrating inter and intrastrain variation (101). For the U38 chemokine, the amino acid identity between HHV-6A U1102 and HHV-6BZ29 is 85.7% (5). Key interstrain differences are as follows: (1) none of the HHV-6A viruses encode a mature chemokine with a functional signal peptide (2); the majority of U83A isolates have a 2 amino acid truncation at the C-terminus., with U1102 being the exception (101). Upon further study, HHV-6B viruses could further be divided into groups, one group with a shortened signal peptide and the other group, which includes HHV-6BHST and HHV-6BZ29 with a full length signal peptide. Intrastrain amino acid divergence for the B isolates ranged from 0 to 3.5% (101).

Glycoproteins B and H are highly conserved in the herpesvirus family; however, distinct differences in nucleotide and amino acid sequences between variants clearly segregate HHV-6A viruses from HHV-6B. Intrastrain variation in the amino acid sequences of 31 HHV-6B clinical samples was 0.9% and 0.3% for gB and gH, respectively (102). Despite the small magnitude of difference within strains, the B
isolates could be further segregated into two groups based on gB and gH sequences. The segregation, however, differed depending on which gene was analyzed, suggesting that intrastrain recombination between HHV-6B viruses may occur (102).

Other studies on inter and intrastrain variation in clinical samples focused on envelope glycoprotein O (U47), and the polymerase processivity factor (U27). Sequencing of the hypervariable gene U47 gene is useful for genotyping HHV-6 viruses in clinical samples (63,103). Amino acid sequence identity between U1102 and GS is 97.5%, while the identity to HHV-6B Z29 is only 89%. Sequencing results of a portion of the U47 reports up to 6% variation within HHV-6 variants for this region and up to 8% variation between groups (103). In other studies by Bonnafous et al., the interstrain variation for the U27 DNA polymerase processivity factor gene is 7.6% when comparing HHV-6A and HHV-6B clinical isolates, while intrastrain differences were 1.2% and 0.3%, for A and B isolates, respectively (104).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genbank Accession</th>
<th>Geographic Location of Isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHV-6A U1102</td>
<td>NC_001664</td>
<td>Uganda</td>
</tr>
<tr>
<td>HHV-6A GS</td>
<td>KC465951</td>
<td>North America</td>
</tr>
<tr>
<td>HHV-6B Z29</td>
<td>NC_000898</td>
<td>Zambia</td>
</tr>
<tr>
<td>HHV-6B HST</td>
<td>AB021506</td>
<td>Japan</td>
</tr>
</tbody>
</table>

Table 3: Fully Sequenced HHV-6 Strains
Adaptive Immune Response to HHV-6 Infection

Cellular Response to HHV-6

Both CD4+ and CD8+ HHV-6 specific T-cells have been isolated from the PBMCs of healthy donors. In the case of both CD4+ and CD8+ the frequency of HHV-6 specific T-cells is low, but the responding population could be expanded in vitro (105–107). Expanded CD4+ and CD8+ populations characteristically secrete IFNγ and TNFα, while also performing cytolytic functions mediated by the secretion of perforin (CD4+) or granzyme B (CD8+) (105,106,108). Additionally, CD8+ clones were found to also secrete granulocyte macrophage stimulating factor. Studies on T-cell response to HHV-6 have identified 8 HLA-DR restricted CD4+ epitopes and 5 HLA-A restricted CD8+ epitopes (105–107).

Table 4: HHV-6 T-cell Epitope Specificity (105–107)

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene Product</th>
<th>CD4+</th>
<th>CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>U11</td>
<td>Tegument/Antigenic virion protein</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>U14</td>
<td>Phosphoprotein 85</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>U38</td>
<td>DNA Polymerase</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>U48</td>
<td>Glycoprotein H</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>U54</td>
<td>Virion transactivator</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>U57</td>
<td>Capsid Protein</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>U71</td>
<td>Myristolated virion protein</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>U90</td>
<td>Immediate early transactivator</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Antibody Response to HHV-6

During primary infection, anti-IgG and anti-IgM antibodies are produced, with IgM antibodies being the first to be detected (67,91). IgG titer begins to increase about one week post infection and peaks a week later. Additionally, there is an increase in IgG avidity over the course of infection (60,67).

The antibody response to HHV-6 in CIHHV-6 harboring individuals has not been clearly defined; thus, studies in the future should focus on this topic. However, one study by Tanaka-Taya et al. measured antibody titer to two HHV-6 antigens (IE-A and glycoprotein gB) in the PBMCs of CIHHV-6 individuals and compared the response to that of healthy non-CIHHV-6 individuals (38). Interestingly, 57% of individuals with CIHHV-6 had antibodies to the IE-A antigen present in the serum, while IE-A antibodies were undetectable in non-CIHHV-6 individuals. On the other hand, 14% of CIHHV-6 versus 60% of the healthy individuals had an antibody response to gB (38). Glycoprotein B is considered one of the major neutralizing epitopes and variant specific gB antibodies have been detected (109). The absence of gB antibodies or reduced anti-gB titers may reduce the ability of CIHHV-6 individuals to mount an immune response to an exogenous HHV6 infection.

Treatment of HHV-6 Associated Diseases

Primary HHV-6 infections are usually self-limiting and do not require antiviral therapy. However, in the case of complications due to viral reactivation antiviral treatment may be necessary until symptoms subside. HHV-6 viruses do not respond to the nucleoside inhibitors acyclovir and penciclovir, in vitro or in vivo (110). Patients
experiencing HHV-6 related post-transplant complications, such as encephalitis, respond best to valganciclovir and foscarnet, or the pyrophosphate analogue, cidofovir (53,110,111). Additionally, a preliminary study by Montoya et al. recently described the successful treatment with valganciclovir of two related patients with chromosomally inherited HHV-6 who were exhibiting neurological symptoms in conjunction with active HHV-6 replication (83). However, to date no drugs have been approved by the FDA specifically for the treatment of HHV-6.

**Coinfection and Superinfection with Herpesviruses**

Reinfection with HHV-6 has been noted, suggesting that despite the development of neutralizing antibodies and T-cell immune responses the virus is able to subvert the immune system. Indirect evidence supporting the possibility of co-infection and superinfection in children comes from a study by Bates et al (63). In this study, febrile and non-febrile children in Zambia were tested for HHV-6 prevalence and the detected viruses were genotyped by determining the amino acid sequence of the U47 envelope glycoprotein O. In this study, co-infections were reported at a rate of 21% to 50%, in non-febrile and febrile children, respectively. Thirteen percent of the non-febrile children had co-infection with both HHV-6A and HHV-6B at 18 months of age; co-infection with both variants was associated with an elevated viral DNA load in the serum of nearly four times the average for a single infection. HHV-6 reinfection was also noted in a study of children in the United States (112). More recently, Burbelo et al. designed a novel HHV-6 variant specific serological test for the detection of HHV-6A and HHV-6B U11 antigen (88). In a screening of 25 African serum samples, 50% of the samples HHV-6A seropositive and 80% were HHV-6B seropositive. This implies that a fraction of
the individuals in this study had either been reinfected by another HHV-6 virus or superinfected with both HHV-6 variants prior to seroconversion. The information on reinfection and superinfection in CIHHV-6 positive individuals is limited; thus, no reports of this nature have been reported prior to the current study.

Human cytomegalovirus, another betaherpesvirus, is associated with encephalitis, retinitis, and other diseases in the immunocompromised. In individuals with a functional immune system, CMV establishes persistent infection in salivary gland tissue and myeloid cells and viral shedding may occur for years after primary infection (113). Despite robust antibody production in response to CMV and the high frequency of CMV-specific T-cells in the peripheral blood, asymptomatic CMV reactivation is frequent in healthy individuals and constant immune surveillance is needed to control the virus (114–116). Additionally, mixed infections frequently occur and individuals with persistent infection may be superinfected with additional strains of the virus (117). The key to CMV persistent infection and superinfection is the subversion of the host immune system by viral proteins which inhibit MHC function (118). Furthermore, a functional consequence of persistent CMV infection is immunosenescence: excessive expansion of CMV-specific T-cell clones lead to premature senescence and reduced T-cell responsiveness (119).

Development of Immune Tolerance

During development, multiple mechanisms are used to ensure self-tolerance. In the thymus, T-cells that are specific to self-antigens are deleted by negative selection (120). In the periphery, presentation of self-antigens in the absence of co-stimulatory
molecules leads to T-cell anergy or inactivation (121). For B-cells, self-tolerance occurs by one of three mechanisms: receptor editing to replace self-reactive receptors, deletion of self-reactive clones, and anergy (122).

Inheritance of HHV-6 presents the possibility for immune tolerance to viral antigens. CIHHV-6 reactive T-cells may be deleted by negative selection during development or T-cell anergy may be induced. Humoral responses may also be lower, if viral genes are expressed during development and are recognized as self. Alternately, in the event that reactivation of the inherited virus occurs after birth and CIHHV-6 specific antibodies or T-cells are developed. These individuals may still be susceptible to superinfection if the exogenous virus differs significantly from the inherited virus.
OBJECTIVES AND HYPOTHESIS OF THE CURRENT STUDY

This study focuses on a unique patient population that displays chronic fatigue syndrome-like symptoms, such as CNS dysfunction (impaired concentration and short term memory, and mild dementia), headache musculoskeletal pain, headache, and long-standing fatigue and has inherited HHV-6. The central hypothesis of this study is that these CIHHV-6 harboring patients suffer from CFS-like symptoms because of reactivation/active replication of HHV-6. Investigation of this hypothesis will be accomplished by three specific aims.

1: Test the ability of various compounds to cause reactivation of CIHHV-6 in vitro. The rationale for this is aim was that quantitative PCR is unable to differentiate between actively replicating HHV-6 virus in patients with CIHHV-6; therefore, it is essential to develop an assay to detect replicating virus.

2: Test in vivo reactivation of latent HHV-6 in patient samples. In this part epigenetic modifiers will be used to induce replication of latent HHV-6 in patient samples, and the induced virus will be characterized and compared to prototype HHV-6 strains and to the inherited virus.

3: Test the efficacy of antiviral treatment in suppression of HHV-6 reactivation/replication in patients with CFS-like symptoms. The purpose of the final aim is to correlate HHV-6 replication status with neurological symptoms and evaluate the
effect of short term and long term antiviral treatment. Additionally, the prevalence of CIHHV-6 will be assessed in a population of individuals displaying CFS-like symptoms.
MATERIALS & METHODS

Note to Reader

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Study Subjects, Treatment, Statistical Analysis

Four patients diagnosed with inherited HHV-6 provided written informed consent prior to the start of this study, and the study was approved by the Institutional Review Board. All subjects presented with more than 0.5 million DNA copy numbers of HHV-6 in whole blood, with concomitant positive results of HHV-6 in hair follicles as detected by Viracor-IBT laboratories, Inc. Patients presented with fatigue and neurological symptoms including, but not limited to, depression, hypersomnia, memory and cognitive impairment. In this study, patients received therapeutic antiviral therapy consisting of either twice daily valganciclovir (patients 1, 2, and 3) or 60 mg/kg foscarnet (patient 4) per day. Patients receiving short term treatment received 900 mg valganciclovir twice daily; patients receiving long term treatment received 900mg valganciclovir twice daily for three weeks and 450mg twice daily for three weeks or longer. Blood samples were collected in Quiagen PAXgene DNA, RNA, and heparin tubes before and during treatment.
A two-tailed Fisher exact test was used to determine the significance of CIHHV-6 prevalence among a cohort of 337 US adult patients suffering from neurological symptoms.

**Isolation of RNA & DNA from Blood or PBMCs**

For RNA isolation, patient whole blood was collected in Quiagen PAXgene blood RNA tubes and incubated at room temperature for at least 2 hours prior to processing. Cells from whole blood were pelleted by centrifugation for 10 minutes at 3,780 rpm (3,000xg) in a Fisher Scientific accuSpin 1R (rotor #3450). Alternately, PBMCs or immortalized T-cells were pelleted at 1,500 rpm in a Fisher Scientific accuSpin 1R (rotor #3450). After collection by centrifugation, cells were homogenized with TRIzol Reagent (Invitrogen), separated with chloroform, precipitated with isopropanol, and washed with 75% isopropanol. The pelleted RNA was resuspended in diethyl pyrocarbonate (DEPC)-treated water.

For DNA isolation, patient whole blood was collected in Paxgene blood DNA tubes, and DNA was isolated using Wizard Genomic DNA Purification Kit (Promega). Alternately, for DNA isolation from cells, cells were pelleted at 1,500 rpm in a Fisher Scientific accuSpin 1R (rotor #3450) and DNA isolation was also performed using Wizard Genomic DNA Purification Kit (Promega), according to the manufacturers protocol.

**Isolation of DNA from Agarose Gel and Cloning**

Amplified PCR products were separated on a TBE agarose gel and visualized using ultraviolet light. Bands corresponding to the PCR-amplified products were excised
and DNA was isolated from the gel fraction using the Wizard SV Gel and PCR Clean-Up System (Promega). The DNA was then TA cloned using the PCR4 TOPO (Invitrogen) or pGEMT-Easy (Promega) cloning system. For DNA generated using the Primestar High Fidelity Polymerase, an A-tailing reaction was performed prior to TA cloning. Briefly, the purified PCR fragment was incubated with 0.2mM dATP and 5 units of RedTaq Polymerase (Sigma) for 30 minutes at 70 °C. The A-tailed product was then ligated with the TA vector.

For PCR4 TOPO cloning system, 1.5µl of the ligated DNA was mixed with 50µl of competent E. coli ElectroMAXDH10B strain (Invitrogen) and incubated on ice for 30 minutes. The chilled DNA/bacteria mixture was then put into a 1mm gap cuvette and electroporated at 1500V. The transformation mixture was then incubated in SOC broth for 1 hr at 37º C with rotation, and the incubated mixture was plated on Luria Burtani (LB) plates containing 50 mg/ml kanamycin and 40mg/ml X-gal (5-bromo-4-chloro-3-indoly-β-D-galactopyranoside). The composition of LB plates is as follows: 10g tryptone and sodium chloride, 5g yeast extract per 1 liter of water, pH 7.5). The plates were incubated for 14-16 hours at 37 C.

For the pGEMT-Easy cloning system, 2µl of ligated DNA was mixed with 40µl of competent JM109 (Promega) and incubated on ice for 20 minutes. The DNA/bacteria mixture was then incubated at 42ºC for 45 to 50 seconds. The transformation mixture was then incubated in SOC broth for 1.5 hr at 37º C with rotation. The incubated mixture was plated on LB plates containing 100 mg/ml ampicillin with 80µg/ml X-gal and 0.5mM IPTG (Isopropyl-β-D-thiogalactopyranoside). The plates were then incubated for 14-16 hours at 37º C.
The following day, white colonies were picked and incubated in 4 ml of LB containing 50mg/ml kanamycin or 100mg/ml ampicillin and incubated with shaking for 16 hours. Three milliliters of liquid cultures were used for plasmid isolation using the Fermentas Gene Jet Plasmid Miniprep Kit. Restriction enzyme digestions with EcoRV were performed on isolated plasmids to confirm the presence of the correct sized inserts.

HEK293 Single Cell Clones & Fluorescent In Situ Hybridization

HEK293 cells were infected with HHV-6A U1102, and single cell clones were obtained by limited dilution and seeding in a 96 well plate. Individual clones were expanded and maintained in DMEM (Dulbecco’s Modified Eagles Medium) with 10% FBS (Fetal Bovine Serum), supplemented with 50µg/ml gentamicin. Genomic DNA was isolated from each clone using the Wizard Genomic DNA purification kit. To confirm HHV-6 infection, PCR assay was performed to detect HHV-6 ORF U94 and subsequent agarose gel electrophoresis.

To confirm integration of the HHV-6A genome, single cell HEK293 cells were treated with colcemid and harvested according to standard cytogenetic protocol (123). Metaphase chromosomes were by hybridized with fluorescein labeled HHV-6A U1102 cosmid probes pMF335-631 and pMF311-12 (124). Five or more metaphase chromosomes were examined for each cell line. FISH experiments were performed at the University of Minnesota Cytogenetics Core Laboratory.
In vitro Reactivation of Integrated HHV-6 and Virion DNA Isolation: Patient Samples

Patient PBMCs were isolated by density gradient centrifugation (Lymphoprep) and maintained in RPMI-1640 medium with 10% FBS and 50µg/ml gentamicin and treated with 10 ng/ml TPA (12-O-tetradecanoylphorbol-13-acetate) and 10ng hydrocortisone as previously described (15). Patient cells were co-cultured with Molt3 T-cell line for two weeks until the formation of syncytia was observed. Virion particles were isolated by centrifugation of supernatant from infected cells at 27,000rpm in a Beckman L7-65 ultracentrifuge (SW41 rotor). Following centrifugation, DNA was extracted using Wizard Genomic DNA Purification Kit (Promega) or phenol chloroform extraction.

Reactivation of Integrated HHV-6: HEK293 Clones and Assay to Detect Catenated Genomes

HEK293 cell clones harboring integrated HHV-6A were maintained in DMEM with 10% FBS and with 50µg/ml gentamicin and treated varying concentrations (160 ng/ml, 80 ng/ml, 40 ng/ml, 20 ng/ml or 10 ng/ml) of TSA for three days. Alternately, single cells were treated with the same concentrations of sodium butyrate, carbamazepine, or valproic acid. Untreated cells were used as a negative control in all experiments.

After treatment, DNA was isolated from the cells using Promega’s Wizard Genomic isolation, and PCR amplification was performed to detect catenated genomes (Figure 5). Amplified DNA was separated by agarose gel electrophoresis, blotted onto nitrocellulose, and probed with α32-dATP DR probes or with α32-dATP HHV-6A BAC vector probe.
Figure 5: Schematic of PCR Assay for Detecting Catenated Viral Genomes. After induction with various epigenetic modifiers, PCR will be performed with primers specific to the unique length of the HHV-6 viral genome. A PCR product will only be generated if the viral genome is joined in a head-to-tail manner.

cDNA Synthesis and Nested Reverse Transcriptase PCR Assay

Blood samples were collected in PAXgene blood RNA tubes and RNA was isolated using Trizol reagent. Briefly, 30 ng of total RNA and random primers were used for first strand cDNA synthesis using the GoScript Reverse Transcription System (Promega). Alternately, RNA isolated from uninfected or infected Molt3 T lymphocytes was used as a negative or positive control, respectively. An equivalent of 5ng of total RNA was used for nested PCR reaction.

Two rounds of PCR were performed to using RedTaq Polymerase (Sigma) and primers specific to the U100 glycoprotein. For a cellular control, beta actin was amplified with 30 cycles of PCR. Additionally, a reverse transcriptase null reaction was performed to ensure the absence of DNA in RNA samples. Amplified products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized using ultraviolet light in the Gel Doc Molecular Imaging System (Bio-Rad).
Table 5: Primers Used for Nested RT-PCR and Beta Actin

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>U100 Outside F (93)</td>
<td>5’-CTAAATTTCCTACCTCCGAAATGT-3’</td>
</tr>
<tr>
<td>U100 Outside R (93)</td>
<td>5’-GAGTCCATGAGTTAGAAGATT-3’</td>
</tr>
<tr>
<td>U100 Inside F (93)</td>
<td>5’-ACTACTACCTTAGAAGATATAG-3’</td>
</tr>
<tr>
<td>U100 Inside R (93)</td>
<td>5’-AAGCGCGTGCGAGTTCCCCAA-3’</td>
</tr>
<tr>
<td>Beta actin F</td>
<td>5’-CTGGAACGGTGAAAGGTGACA-3’</td>
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<tr>
<td>Beta actin R</td>
<td>5’-AAGGGACTTCCTGTAACAATGCA-3’</td>
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Table 6: PCR Cycling Conditions: U100 Nested RT-PCR

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (minutes)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94</td>
<td>2</td>
<td>1x</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>1</td>
<td>30x</td>
</tr>
<tr>
<td>Annealing</td>
<td>43 (outside)/53 (inside)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>5</td>
<td>1x</td>
</tr>
</tbody>
</table>

Table 7: PCR Cycling Conditions: Beta Actin

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (Celsius)</th>
<th>Time (minutes)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94</td>
<td>2</td>
<td>1x</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>1</td>
<td>30x</td>
</tr>
<tr>
<td>Annealing</td>
<td>52</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>5</td>
<td>1x</td>
</tr>
</tbody>
</table>

PCR Amplification for Sequence Comparison or Detection of Viral DNA in Infected Cells

Selected HHV-6 open reading frames were amplified by PCR amplification using DNA isolated from whole blood or PBMCs as a template. Primestar High Fidelity
Polymerase (Takara) was used to perform each reaction. Two step or three step amplification was performed, according to the manufacturer's protocol. To confirm viral infection, ORF U94 was amplified by PCR with RedTaq polymerase and using DNA isolated from HHV-6A infected HEK293 single cell clones as a template.

**Sequencing**

For cDNA sequencing and sequencing for genome comparison clones were sequenced at the Moffitt Cancer Center Molecular Genomics Core Facility using the ABI3130XL capillary sequencing instrument. Alternatively, pyrosequencing of virion DNA was performed at the Recombinant Herpesvirus and HTS sequencing Core Laboratory at the University of Florida using the Roche 454 GS-FLX Assembly of the consensus sequence obtained from pyrosequencing was assembled using HHV-6A U1102 as a reference.

**Restriction Digest and Southern Blot for Comparison of Inherited and Reactivated Viral Genomes**

For evaluating the heterogeneity in inherited HHV-6 strains, 5 µg of cellular DNA from patient PBMCs or 1ng of virion DNA was digested with Sacl. For comparison of the reactivated HHV-6 strain to the U1102 prototype strain, 45 ng of virion DNA was digested with BamHI and Sacl. Digested DNA was separated agarose gel run in 1x TBE buffer, stained with Sybr Green (Manufacturer) or ethidium bromide and visualized using the GE Typhoon Imager or Bio-Rad Geldock, respectively.
Southern Blot

Preparation of Gel for Transfer

After separation of DNA on an agarose gel run in 1xTBE Buffer, the gel was incubated under the following conditions with gentle rocking to prepare the gel for DNA transfer.

Table 8: Southern Blot Gel Preparation

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution</th>
<th>Incubation time (minutes)</th>
<th>Number of washes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depurination</td>
<td>0.25M HCl</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>DNA denaturation</td>
<td>1M NaCl, 0.5M NaOH</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>Neutralization</td>
<td>1.5M NaCl, 0.5M TrisHCl</td>
<td>15</td>
<td>2</td>
</tr>
</tbody>
</table>

Transfer

After washing was completed, the gel was transferred to Hybond C mixed ester nitrocellulose by vacuum blotting for 30 minutes (125). A 10x saline sodium citrate (SSC) solution was used as a transfer agent to prevent gel from drying during transfer. After transfer, the DNA was immobilized by baking the membrane at 80ºC for one hour.

Preparation of $\alpha^{32}$-dATP Probe

An HHV-6A BAC vector was used to generate an $\alpha^{32}$P-dATP probe (4). Initially, a combination of 20-100ng BAC vector DNA, 1.5µg random primers (Promega) was heated at 100 ºC for 3 minutes. Following denaturation, 50 µM dNTP-dATP, 5U DNA Polymerase I Klenow, DNA polymerase buffer (to a final concentration of 1x), and 50
µCi $\alpha^{32}$P dATP (Perkin Elmer) was added. The complete probe mixture was incubated for 4 hours at room temperature (22-25°C) and then the reaction was stopped with 45 microliters stop buffer (20mM NaCl, 50mM EDTA, 20mM Tris, 1% SDS)

Table 9: Primers Used for Sequence comparison

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>U11F</td>
<td>5’-TCACGACGCGATCGCTGACTCGC-3’</td>
</tr>
<tr>
<td>U11R</td>
<td>5’-ATGGATCTGCAAGACATCCGATTCCG-3’</td>
</tr>
<tr>
<td>U15F</td>
<td>5’-GCAGAAACGACGCTGACTCGC-3’</td>
</tr>
<tr>
<td>U15R</td>
<td>5’-ACGGGAGAGCTGCTGCTGAGCCG-3’</td>
</tr>
<tr>
<td>U18F</td>
<td>5’-CTAAGGCGACTGGGATGAAGACGCTA-3’</td>
</tr>
<tr>
<td>U18R</td>
<td>5’-CTCTTGATGACATGTGGCAATTTGCTGG-3’</td>
</tr>
<tr>
<td>U20F</td>
<td>5’-CTGCAACATGCGCAGCCAGTTGCTACCCGG-3’</td>
</tr>
<tr>
<td>U20R</td>
<td>5’-GGCCGTCACCTTTCTTCGACGTG-3’</td>
</tr>
<tr>
<td>U31F</td>
<td>5’-CAAGAAACGACGCTGACTCGC-3’</td>
</tr>
<tr>
<td>U31R</td>
<td>5’-CGTCAAGAAGTTGTTACGGGTTG-3’</td>
</tr>
<tr>
<td>U38F</td>
<td>5’-TCACATTACCTGCAATAGCAG-3’</td>
</tr>
<tr>
<td>U38R</td>
<td>5’-CTCTCTCTCTCCGAGCTCATGC-3’</td>
</tr>
<tr>
<td>U47F</td>
<td>5’-CCACGTAGTAGTGTACTACCCAGCTG-3’</td>
</tr>
<tr>
<td>U47R</td>
<td>5’-CATTTGTGCTCAACTCACTCCGCAGATG-3’</td>
</tr>
<tr>
<td>U79F</td>
<td>5’-CTCTCCTGCTGATAAACGGC-3’</td>
</tr>
<tr>
<td>U79R</td>
<td>5’-CCAATTTGGAAATTTCTGAC-3’</td>
</tr>
<tr>
<td>U83F</td>
<td>5’-GAGAAGGTTGTAATATGAGTG-3’</td>
</tr>
<tr>
<td>U83R</td>
<td>5’-CTCTCCTGCTGATAAACGGC-3’</td>
</tr>
<tr>
<td>U86F</td>
<td>5’-CCACCGCCAGTGTGCTGAC-3’</td>
</tr>
<tr>
<td>U86R</td>
<td>5’-GACACCAAGGCAGTGTGAC-3’</td>
</tr>
<tr>
<td>U90F</td>
<td>5’-CTGAAACTTTGACATCTGATAAGG-3’</td>
</tr>
<tr>
<td>U90R</td>
<td>5’-CGCGGTGTCTCAATTTGAC-3’</td>
</tr>
<tr>
<td>U91F</td>
<td>5’-CTCTGAGAGTTGCTCTGCAC-3’</td>
</tr>
<tr>
<td>U91R</td>
<td>5’-GTCACATGCTTGCATCCCATC-3’</td>
</tr>
<tr>
<td>U95F</td>
<td>5’-CCTATCGGTGGGATCCATCCACC-3’</td>
</tr>
<tr>
<td>U95R</td>
<td>5’-CTCTTGGTTGGATCCATTCC-3’</td>
</tr>
<tr>
<td>U100F</td>
<td>5’-CCAGAAAGGCCTGCTGACATCCATC-3’</td>
</tr>
<tr>
<td>U100R</td>
<td>5’-CCGTCGTCATAGGATCCTCTGGCGC-3’</td>
</tr>
</tbody>
</table>
Prehybridization & Hybridization

The nitrocellulose membrane with the immobilized DNA was incubated in 10ml prehybridization buffer at 42º C with rocking for 10-30 minutes. While the membrane was being incubated, 10ml of the hybridization buffer was boiled with the α\textsuperscript{32}P labeled probe. The membrane was then incubated with the hybridization/probe mixture for 16 hours at 42ºC with gentle rocking.

Table 10: Prehybridization and Hybridization Buffers for Southern Blot

<table>
<thead>
<tr>
<th></th>
<th>Prehybridization</th>
<th>Hybridization</th>
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</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>25ml</td>
<td>25ml</td>
</tr>
<tr>
<td>20x SSC</td>
<td>12.5ml</td>
<td>12.5ml</td>
</tr>
<tr>
<td>50x Deinhardt’s Solution</td>
<td>5ml</td>
<td>1ml</td>
</tr>
<tr>
<td>Sonicated salmon sperm DNA</td>
<td>2.5ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td>1M NaPO4</td>
<td>2.5ml</td>
<td>0.75ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>0.25ml</td>
<td>0.25ml</td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td>N/A</td>
<td>10ml</td>
</tr>
<tr>
<td>Water</td>
<td>2.5ml</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Following incubation, the membrane was washed three times in a heated wash buffer (55-60ºC) containing 0.1x SSC, 0.1% SDS solution. The washed membrane was wrapped in plastic wrap and exposed to a phosphoimager screen for 4 hours- 5 days. The image was developed using a GE Typhoon Imager.

Alignment of nucleotide sequences

Complete alignment of nucleotide sequences was performed using ClustalX 2.0.10. Parameters were as follows for pairwise alignments: Gap opening 10, Gap extend 0.1, Protein weight matrix Gonnet 250, DNA weight matrix: IUB. For multiple
alignments: Gap opening g10, Gap extend 018.2, Delay divergent sequence 30%, DNA transition 0.5, Gonnet series, DNA weight matrix: IUB.
RESULTS

Note to Reader

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AIM 1: Test the Ability of Various Compounds to Cause Reactivation of Integrated HHV-6 In Vitro

**Generation of HEK293 Single Cell Clones**

To develop an *in vitro* model of HHV-6 latency, HEK293 cells were infected with HHV-6A U1102. By limited dilution, single cell clones were isolated in individual wells of a 96-well plate and expanded. Although multiple single-cell clones were generated, some clones stopped dividing for an unknown reason. Originally 50 viable single HEK293 cells were isolated, and ten of these were initially positive for HHV-6. However, seven of the clones lost the viral genome after prolonged subculturing. Three clones stably maintained the HHV-6A genome, as indicated by PCR amplification of ORF U94, as reported previously (15). The viable clones were designated C8, D2, and G6. As an additional means of confirming HHV-6 integration, fluorescent *in situ* hybridization (FISH) was performed using cosmid probes specific to large regions of the HHV-6 genome. FISH for all three clones revealed hybridization of HHV-6 at the ends of 4',6-
diamidino-2-phenylindole (DAPI) stained metaphase chromosomes, confirming viral integration into the telomere ends of the chromosomes (Figure 6). Two clones, D2 and G6 had one integration site; the final clone C8 had two integration sites (Figure 6).

**Figure 6: Fluorescent In Situ Hybridization of HEK293 Clones Infected with HHV-6A.** Metaphase chromosomes of three HEK293 single cell clones with integrated HHV-6 were stained with DAPI (blue). In this figure, hybridization was performed with HHV-6 cosmid probes pMF311-12 and pMF335-631 which were labeled with fluorescein (green).

**Detection of Circularized Viral Genome During Reactivation**

Both HHV-6A and HHV-6B preferentially integrate into the telomeres as a means of establishing latency (15,18). The ability to reactivate from latency is essential to the spread of viral infection. To investigate the effect of epigenetic modifiers on the replication states of latent HHV-6 virus, HEK293 single cell clones were treated with varying concentrations of trichostatin A, sodium butyrate, valproic acid, or carbamazepine for three days. Once cytopathic effect was observed, DNA was isolated from the treated cells. To confirm the reactivation of integrated virus, a PCR-based assay was developed to detect the circularized HHV-6 genome. The designed primers
were specific to the unique length of the viral genome and oriented in opposite directions. Therefore, a PCR product would only be generated in the event that circularization of the viral genome occurs. Southern blotting was then performed on the PCR generated products using either and probed with an α\textsuperscript{32}P labeled probe surrounding the viral DRs or with an α\textsuperscript{32}P HHV-6A BAC vector probe.

Initially, all three single cell clones were treated for three days with varying concentrations (160 ng/ml, 80 ng/ml, 40 ng/ml, 20 ng/ml or 10 ng/ml) of TSA or the other epigenetic modifiers, sodium butyrate, valproic acid, or carbamazepine to determine their ability to reactivate from the integrated form. Southern blotting and hybridization with radiolabeled BAC vector probe revealed that of the three clones, only the D2 clone was capable of reactivation, as indicated by the variation in banding patterns after development of the Southern Blot (Figure 7). Furthermore, of the epigenetic modifiers tested, only TSA induced the reactivation of latent HHV-6A virus (Figure 8). These results indicate that in some cases integration of HHV-6 may be a dead end phenomenon. Possible explanations for this will be presented in the discussion.

To qualitatively assess the variation in size of the direct repeat during HHV-6 reactivation, Southern blotting was performed on TSA-treated D2 clone as described above; however, a probe specific to the unique regions of the viral genome surrounding the DRs was used. Upon induction, with variable concentrations of TSA, multiple distinct bands were detected after hybridization. Differences in DR size at each TSA concentration demonstrate that upon circularization there is variation in the DR length (Figure 9). Possible reasons for this will be discussed at a later point.
Figure 7: Trichostatin A Effectively Induces the Reactivation of HEK293 Clone D2. HEK293 single cell clones (C8, D2, or G6) latently infected with HHV-6A were treated with varying concentrations of TSA (Lane 1: 160 ng/ml, Lane 2: 80 ng/ml, Lane 3: 40 ng/ml, Lane 4: 20 ng/ml, Lane 5: 10 ng/ml Lane 6: untreated). Following induction, PCR was performed to detect the formation of viral concatemers. PCR products were separated by electrophoresis, blotted onto nitrocellulose and probed with a α\(^{32}\text{P}\) HHV-6A BAC vector probe.
Figure 8: TSA is Most Effective at Inducing the Reactivation of Latent HHV-6 Virus. HEK293 cells latently infected with HHV-6A were treated with varying concentrations of four epigenetic modifiers: sodium butyrate, carbamazepine, Tricostatin A, and valproic acid. (Lane 1: 160 ng/ml, Lane 2: 80 ng/ml, Lane 3: 40 ng/ml, Lane 4: 20 ng/ml, Lane 5: 10 ng/ml Lane 6: untreated). Following induction, PCR was performed to detect the formation of viral concatemers. PCR products were separated by electrophoresis, blotted onto nitrocellulose and probed with α32-dATP HHV-6A BAC vector probe.
Figure 9: During Reactivation DR length is Variable. Uninfected HEK293 or HEK293 cells latently infected with HHV-6A were treated with varying concentrations of TSA (160 ng/ml, 80 ng/ml, 40 ng/ml, 20 ng/ml or 10 ng/ml) or left untreated. Following induction, PCR was performed to detect the formation of viral concatemers. Additionally, the concatemer-formation assay was also performed on lytic infected or uninfected Jhan T-cells. PCR products were separated by electrophoresis, blotted onto nitrocellulose and probed with a α32-dATP DR probe.

AIM 2: Test if Inherited HHV-6 in Patient Samples can be Reactivated In Vitro

Reactivated virus is infectious

In vitro experiments with HEK293 clearly demonstrated that HHV-6 can be reactivated from its latent form in HHV-6A containing cell lines. In the second aim of this project, the goal was to test if inherited HHV-6 in patient samples could be reactivated in vitro. Here, we used T-cells isolated from a patient with inherited HHV-6 who resides in
the United States. In a previous publication, confirmed HHV-6 integration at 18q23 and vertical transmission of the virus from parent to this patient, who was previously designated Family 1/Sibling 2 (15). In attempts to induce the inherited virus, PBMCs isolated from whole blood obtained from Family 1/Sibling 2 were treated with TPA and hydrocortisone, a combination previously shown to reactivate integrated HHV-6, while having limited amounts of toxicity (15). During induction, the treated PBMCs were co-cultured with Jihan or Molt3 T cell lines until primary cells reached their replication limits and died. Co-cultured cells were observed for visible cytopathic effect. As a control, PBMCs latently infected with U1102 were also induced with TPA and hydrocortisone. Visible cytopathic effect indicates that the induced virus is infectious (Figure 10).

*Reactivated virus is distinct from HHV-6A U1102*

The reactivated virus from Family 1/Sibling 1 was confirmed to be an HHV-6A strain by PCR analysis. In primary molecular analysis, the reactivated viral genome was compared to the HHV-6A U1102 laboratory stain. Restriction enzyme digestion and Southern blot analyses indicated that the viral genome was similar, but distinct from the U1102 strain. When probed with a radiolabeled BAC vector, a unique restriction pattern was observed, although the majority of visualized bands were colinear to the U1102 fragments (Figure 11).

*Nucleotide and amino acid characterization of the reactivated virus*

To fully characterize the reactivated viral genome, we performed pyrosequencing on the isolated virion DNA. The generated consensus sequence was assembled using the HHV-6A U1102 genome as a reference. However, there were gaps in the nucleotide
Figure 10: TPA/hydrocortisone Induced Virus from Patient PBMCs is Infectious. PBMCs latently infected with HHV-6 were treated with 10ng TPA /Hydrocortisone and co-cultured with uninfected T-cells. Visible cytopathic effect indicates that the induced virus is infectious.

sequence, most notable in the regions surrounding the repetitive elements in the viral genome. As needed, select open reading frames were cloned and sequenced to complete the nucleotide sequence. Amino acid sequences were deduced using the Expasy translate tool, and both nucleotide and amino acid sequenced were aligned using Clustal Omega. In this study, the reactivated viral genome was compared to HHV-6A U1102, HHV-6A GS, and HHV-6B Z29.
Figure 11: Reactivated Virus Isolated from Family 1/Sibling 2 is Distinct from HHV-6A U1102. Virion DNA isolated from the PBMCs of Family 1/Sibling 2 and HHV-6A U1102 were digested using Sacl and BamHI. Fragments were separated by electrophoresis, blotted onto nitrocellulose and probed with a radiolabeled HHV-6A U1102 BAC clone.

The nucleotide sequence generated by pyrosequencing was 156.6 kbp in length. This length was comparable to the HHV-6AGS which is 156.9 kbp in length. Both HHV-6A GS virus and this reactivated virus were shorter than the HHV-6A U1102 genome.
and the HHV-6B Z29 genome, that are 159.3kbp and 162kbp, respectively. Similar to
reports comparing HHV-6A U1102 to HHV-6B Z29, there was noted divergence in the
length of the repetitive regions within the viral genome. The reactivated virus was found
to have a shortened DR_L and DR_R when compared to U1102, GS, and Z29. However
when compared to HHV-6A GS, the R3 region, located between open reading frame
U91 and U94, was approximately 300bp longer. On the other hand, the repetitive R1/
R2 and R3 of HHV-6AU1102 were approximately 250bp and 420bp longer than the
reactivated virus, respectively.

Previous publications have thoroughly covered the difference in nucleotide and
amino acid sequence between the HHV-6A U1102 and HHV-6B Z29 strain (5,6). To
evaluate the level of interstrain variation, we focused on analyzing the differences
between the reactivated virus and the two HHV-6A strains, GS and U1102. The
reactivated was more closely related to the GS (>99% nucleotide identity) isolate than
U1102 (97% nucleotide identity). At the nucleotide level, all open reading frames were
greater than 99% identical to the HHV-6A GS. Of the 83 open reading frames analyzed,
only eleven genes had predicted amino acid sequences that were identical for the
reactivated virus and both HHV-6A laboratory strains (Table 11). Interestingly, three of
these genes are neither located within the herpesvirus core gene blocks, the
betaherpesvirus gene block, nor conserved between Roseoloviruses. These genes
were U47A, U60, and U63. The lack of divergence suggests they may be important to
the biological properties of the HHV-6A viruses. These genes will be further discussed
at a later point. The nucleotide identity between GS and the reactivated virus ranges
from 99 to 100%, while the amino acid identity ranges from 97 to 100%. The nucleotide
identity between the U1102 and the reactivated virus ranges from 97% to 100%, while the nucleotide identity ranges from 92% to 100% (Table 12). Interestingly, the predicted U18 envelope glycoprotein and the predicted U20 membrane protein nucleotide sequences had early stop codons. The predicted U18 amino acid sequence was 66 amino acid shorter than HHV-6A GS and predicted for HHV-6A GS and U1102, while the U20 amino acid sequence was 188 shorter than the HHV-6 GS and U1102 strains (Figure 12 & 13).

### Table 11: Open Reading Frames with 100% Identity Between Reactivated Virus, HHV-6A GS and HHV-6A U1102

<table>
<thead>
<tr>
<th>Open Reading Frames with 100% Amino Acid Identity</th>
<th>Conserved Gene?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene ID</td>
<td>Gene Product</td>
</tr>
<tr>
<td>U24A Protein U24A</td>
<td>Yes</td>
</tr>
<tr>
<td>U25 Tegument protein UL43</td>
<td>Yes</td>
</tr>
<tr>
<td>U29 Capsid triplex subunit 1</td>
<td>Yes</td>
</tr>
<tr>
<td>U32 Small capsid protein</td>
<td>Yes</td>
</tr>
<tr>
<td>U35 DNA packaging protein UL33</td>
<td>Yes</td>
</tr>
<tr>
<td>U47A Envelope glycoprotein 24</td>
<td>No</td>
</tr>
<tr>
<td>U48 Envelope glycoprotein H</td>
<td>Yes</td>
</tr>
<tr>
<td>U56 Capsid triplex subunit 2</td>
<td>Yes</td>
</tr>
<tr>
<td>U60 DNA packaging terminase subunit 1</td>
<td>No</td>
</tr>
<tr>
<td>U63 Protein UL92</td>
<td>No</td>
</tr>
<tr>
<td>U75 Tegument protein UL7</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The Virus Reactivated from Patient PBMCs Differs from the Inherited Virus.

Previous reports indicate that HHV-6 may be reactivated from its inherited form (15,36). In this study, five open reading frames (U11, U18, U20, U47, and U91) were cloned and sequenced using genomic DNA isolated from whole blood obtained from Family
1/Sibling 2 as template. To determine if the inherited virus was induced by TPA/hydrocortisone treatment, we compared the reactivated virion DNA sequence and the genomic DNA sequences. In all cases, the reactivated sequences differed from the inherited sequences (Table 13). Of the genes studied, the immunodominant U11 tegument protein and the U91 membrane protein had the greatest difference when compared to the inherited virus. Variation in nucleotide sequence indicates that this individual presenting with CFS-like symptoms harbored an HHV-6 strain distinct from the virus which was inherited by germline transmission.

Table 12: Most Divergent Open Reading Frames When Comparing the Reactivated Virus to HHV-6A GS and HHV-6A U1102

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Putative Function</th>
<th>Nucleotide Identity</th>
<th>Amino Acid Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GS</td>
<td>U1102</td>
</tr>
<tr>
<td>U11</td>
<td>Tegument protein pp150</td>
<td>99.7</td>
<td>97.8</td>
</tr>
<tr>
<td>U17</td>
<td>Tegument protein vlCA</td>
<td>100.0</td>
<td>99.0</td>
</tr>
<tr>
<td>U18</td>
<td>Envelope glycoprotein UL37</td>
<td>99.6</td>
<td>97.6</td>
</tr>
<tr>
<td>U19</td>
<td>Protein UL38-anti-apoptotic</td>
<td>99.6</td>
<td>98.1</td>
</tr>
<tr>
<td>U20</td>
<td>Membrane protein U20</td>
<td>99.9</td>
<td>98.8</td>
</tr>
<tr>
<td>U24</td>
<td>Protein U24</td>
<td>100.0</td>
<td>97.7</td>
</tr>
<tr>
<td>u31</td>
<td>Large tegument protein</td>
<td>99.97</td>
<td>99.49</td>
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<tr>
<td>u47</td>
<td>Envelope glycoprotein O</td>
<td>99.95</td>
<td>98.44</td>
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<td>U46</td>
<td>Envelope glycoprotein N</td>
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<td>U90</td>
<td>Regulatory protein IE1</td>
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<td>97.7</td>
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<tr>
<td>U91</td>
<td>Membrane protein UL124</td>
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<td>97.2</td>
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Figure 12: Nucleotide Alignment of U18 Envelope Glycoprotein Sequences. PCR-amplified HHV-6 glycoprotein U18 was TA cloned and sequenced. Obtained nucleotide sequences were translated using Expasy Translate and aligned using Clustal Omega.

<table>
<thead>
<tr>
<th>U18 Envelope Glycoprotein</th>
<th>AA Length</th>
<th>% Identity</th>
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</thead>
<tbody>
<tr>
<td>Reactivated</td>
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</tr>
<tr>
<td>HHV-6A GS</td>
<td>293</td>
<td>95.2</td>
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<tr>
<td>HHV-6A</td>
<td>293</td>
<td></td>
</tr>
<tr>
<td>U1102</td>
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</tr>
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</table>

AIM 3: Test the Efficacy of Antiviral Treatment in Suppression of HHV-6

Reactivation/Replication

Prevalence of CIHHV-6 in Cohort of Patients with Neurological Symptoms

To reveal the prevalence of CIHHV-6 in 337 US patients suffering from a wide range of CFS-related neurological symptoms, including long term fatigue, peripheral blood samples were tested by quantitative PCR by Viracor-IBT laboratories, Inc. Seven
patients had higher than 0.5 million HHV-6 copies per ml of blood. A two-tailed Fisher exact test was used to determine the significance of CIHHV-6 prevalence among these patients. The CIHHV-6 rate among this cohort of US adult patients suffering from CFS patients suffering from neurological symptoms was 2.1% (7/337). This is significantly greater (p=.03) than the expected value of 0.8% reported in the general US population (34,64).

**CFS Patients with CIHHV-6 Express Viral mRNAs, and Long Term Antiviral Treatment Abrogates Viral mRNA Production**

The four patients enrolled in this study harbored over 0.5 million copies of HHV-6 DNA copy numbers per ml of peripheral blood. Patients presented with a variety of neurological symptoms including but not limited to headache, blurred vision, and memory impairment, as well as other symptoms such as generalized pain and long term fatigue.

To determine if the symptoms correlated with replicating HHV-6, nested RT-PCR was employed to detect the late envelope glycoprotein U100 mRNA in whole blood samples from patients with CIHHV-6 (93). Initial RT-PCR assays were performed on blood samples isolated from symptomatic patients prior to the administration of antiviral medications, and follow-up samples were taken during or after treatment. For each RT-PCR reaction, 5ng of total RNA isolated from peripheral blood was used. In all cases, U100 mRNA was detected in blood samples, in the absence of antiviral treatment (Figure 14, 15, 16). On the other hand, ten control single donor samples obtained from
**Figure 13: Nucleotide Alignment of U20 Membrane Protein Sequences.** PCR-amplified HHV-6 glycoprotein U20 was TA cloned and sequenced. Obtained nucleotide sequences were translated using Expasy Translate and aligned using Clustal Omega.

<table>
<thead>
<tr>
<th>U20 REACTIVATED_AA</th>
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</thead>
<tbody>
<tr>
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<tr>
<td>U20_GS_AA</td>
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<tr>
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<tr>
<td>U20_UL102_AA</td>
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</tr>
<tr>
<td>U20_GS_AA</td>
<td>VVAELLPDL17CMLGECHSTVRPL6HVIFREFLRHACLQTVSLQIFSSELVCYFVMNK</td>
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<th>U20 REACTIVATED_AA</th>
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</tr>
<tr>
<td>U20_GS_AA</td>
<td>TGLS95YFPLANHTTQV1DLNEKNGFVDLIVLSFLQRSLHV15SYAESFCEVELIL</td>
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<tr>
<th>U20 REACTIVATED_AA</th>
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<tr>
<td>U20_GS_AA</td>
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<tbody>
<tr>
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<tr>
<td>U20_GS_AA</td>
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### U20 Membrane Protein

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</tr>
<tr>
<td>HHV-6A GS</td>
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<tr>
<td>HHV-6A U1102</td>
<td>422</td>
<td>94.44</td>
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Table 13: Nucleotide Comparison: Inherited vs. Reactivated Virus

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</tr>
<tr>
<td>U18</td>
<td>16/882</td>
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</tr>
<tr>
<td>U20</td>
<td>16/1268</td>
<td>1.34</td>
</tr>
<tr>
<td>U47</td>
<td>31/1858</td>
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</tr>
<tr>
<td>U91</td>
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<td>2.84</td>
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</table>

the local blood bank were negative for U100 mRNA (data not shown). In the absence of reverse transcriptase, PCR products were not detected. Long term (≥six weeks) administration of foscarnet (patient 4) or valganciclovir (patient 3) resulted in the abrogation of U100 mRNA expression (Figure 15 and 16), while short term (three weeks) administration of valganciclovir was not sufficient to eliminate viral gene expression in two unrelated patients (patients 1 and 2) (Figure 14). Five weeks after the cessation of intravenous foscarnet treatment (patient 4), U100 mRNA was detected in the whole blood of patient 4. In all cases, resolution of symptoms was concurrent with the reduction of mRNA expression. This suggested that U100 mRNA detection is a reliable method of detecting HHV-6 persistence. However, treatment efficacy was variable and appears to be dependent on the length of treatment, with treatment length of greater than or equal to six weeks being optimal for valganciclovir and foscarnet.
The Late U100 viral mRNA may Originate from an Exogenous HHV-6 Strain

Amplified U100 cDNA was sequenced to confirm that the RT-PCR products were derived from mature mRNA that lacks introns rather than the inherited viral DNA.

Sequencing was also performed to determine if the detected mRNA originated from the

Figure 14: Amplification of Envelope Glycoprotein U100 mRNA after Three Week Valganciclovir Treatment. Nested reverse transcriptase PCR was used to amplify HHV-6 glycoprotein U100 mRNA from whole blood. Total RNA was converted to cDNA and two rounds of PCR were performed. Second round PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized using UV light. Top U100 RT-PCR (Lane 1: 100bp Ladder, Lane 2: Patient 1, before treatment, Lane 3: Patient 1, 3 weeks post treatment, Lane 4: Patient 2, before, Lane 5: Patient 2, 3 weeks post treatment, Lane 6: negative control (Molt3 T-cells), Lane 7: positive control Molt3 T-cells infected with HHV-6A U1102. Bottom: Beta-actin RT-PCR)
Figure 15: Amplification of Envelope Glycoprotein U100 mRNA after Long Term Valganciclovir Treatment. Nested reverse transcriptase PCR was used to amplify HHV-6 glycoprotein U100 mRNA from whole blood. Total RNA was converted to cDNA and two rounds of PCR were performed. Second round PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized using UV light. Top U100 RT-PCR (Lane 1: 100bp Ladder, Lane2: Patient 3, before treatment, Lane 3: Patient 3, 6 weeks post treatment, Lane 4: Patient 3, 12 weeks post treatment, Lane 5: negative control (Molt3 T-cells), Lane 6: positive control (Molt3 T-cells infected with HHV-6A U1102). Bottom: Beta-actin RT-PCR.
Figure 16: Amplification of Envelope Glycoprotein U100 mRNA after Long Term Foscarnet Treatment. Nested reverse transcriptase PCR was used to amplify HHV-6 glycoprotein U100 mRNA from whole blood. Total RNA was converted to cDNA and two rounds of PCR were performed. Second round PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized using UV light. Top U100 RT-PCR (Lane 1: 1kb Ladder, Lane 2: Patient 4, before treatment, Lane 3: Patient 4, 6 weeks post treatment, Lane 4: Patient 4, 5 weeks after cessation of treatment, Lane 5: negative control (Molt3 T-cells), Lane 6: positive control (Molt3 T-cells infected with HHV-6A U1102). Bottom: Beta-actin RT-PCR.

inherited virus, or from an exogenous virus. The U100 gene of the inherited viral genome was also amplified and both cDNA and inherited HHV-6 DNA sequences of U100 were cloned and sequenced. For sequences from the inherited viral DNA
fragments, introns were removed using previously accepted splice sites prior to alignment using ClustalW2. Isolated cDNA sequences were also compared to the U100 sequences for HHV-6A U1102 and GS, or HHV-6B Z29. All patient cDNA sequences were more similar to HHV-6A U1102 than HHV-6A GS and differed from HHV-6A U1102 by only a CG inversion at bases 193/194 (Figure 17). On the other hand, the inherited genomic sequences were more similar to HHV-6A GS. The inherited HHV-6A sequences were greater than 99% identical to each other for three patients, as were all the reactivated viral sequences.

The cDNA and inherited genomic sequences differed from each other in all cases. U100 cDNA shared only an average of 96.3% nucleotide sequence identity with the inherited HHV-6A viral genome. U100 cDNA from patient 1 was more similar to HHV-6A U1102, while the inherited HHV-6 DNA sequence was 98% identical to HHV-6B Z29 (Figure 17). In addition, the U100 mRNA sequence and inherited viral DNA sequences shared only a 90% nucleotide sequence identity (Figure 18). Differences in the genomic DNA and cDNA sequences demonstrate that these symptomatic patients with inherited HHV-6 harbor more than one HHV-6 virus, and the U100 mRNA detected by RT-PCR originates from an exogenous virus.

**Inherited HHV-6 Viral Genome Sequences are Heterogeneous**

Our results clearly indicated that the reactivated virus from these patient samples did not emanate from the major inherited virus. In all cases the reactivated viruses were more similar to HHV-6A U1102 than HHV-6A GS. Additionally, there was a high level of
similarity between the U100 DNA sequences of the inherited viruses. To compare the genome of the inherited HHV-6 viruses to the HHV-6A GS virus, we performed
Figure 17. Nucleotide Alignment of cDNA and Genomic Sequences Compared to HHV-6 Laboratory Strains. PCR amplified HHV-6 glycoprotein cDNA or corresponding genomic DNA fragments were TA cloned and sequenced. Obtained sequences were aligned using the Mauve algorithm and visualized in JalView in the ViPr database.
Figure 18. Nucleotide Alignment of cDNA and Genomic Sequences for Patient 1.
PCR amplified HHV-6 glycoprotein cDNA or corresponding genomic DNA fragments were TA cloned and sequenced. Obtained sequences were aligned using Clustal Omega.

restriction fragment length analysis on DNA from four patient’s PBMCs or GS virion DNA.

Briefly, DNA was subjected to restriction enzyme digestion using SaeI; fragments were separated by electrophoresis, blotted onto nitrocellulose and probed with a radiolabeled HHV-6A U1102 BAC clone (Figure 19). Despite similarities among the inherited HHV-6 sequences, Southern blot hybridization showed distinct banding patterns for each inherited virus and a significantly diverged banding pattern from HHV-
6A GS, indicating that all inherited viruses evaluated in this experiment markedly differ from HHV-6A GS and are also uniquely distinct from each other.

**Figure 19: Restriction Fragment Analysis of Inherited HHV-6 Viruses.** Cellular DNA from patient PBMCs or virion DNA from HHV-6 GS was digested using Sacl. Fragments were separated by electrophoresis, blotted onto nitrocellulose and probed with a radiolabeled HHV-6A U1102 BAC clone. Lane 1: HHV-6A GS virion DNA, Lane 2: patient 5, Lane 3: patient 3, Lane 4: patient 4, Lane 5: patient 6; patient 6 is from Family 1, Sibling 2 (15,18).
DISCUSSION

Note to Reader

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Latency of HHV-6

In the first part of this study, single cell HEK293/HHV-6A clones were established in vitro. Interestingly, HHV-6A infected HEK293 cells could be segregated into three groups: (1) cells that stopped replicating after HHV-6 infection, (2) cells that continued replicating after HHV-6 infection that were initially PCR positive for HHV-6A and later lost the viral genome and (3) cells that were infected with HHV-6A and maintained the viral genome in the telomeres, as evident by FISH analysis. These results were unexpected and indicate areas that need to be addressed in the future.

HEK293 cells are positive for telomerase, a reverse transcriptase that extends the 3’ ends of chromosomes (126); however, replicative senescence followed HHV-6 infection in some HEK293 infected single cell clones. This suggests that HHV-6 integration may abrogate telomerase activity in specific chromosomes. Alternatively, HHV-6 integration may lead to telomere instability and lead to senescence if integration
of the viral genome occurs in a region of the human genome essential for cell division, or for other unknown reasons.

Subtelomeres are transcribed by RNA polymerase II into non-coding RNA known as TERRA (telomeric repeat-containing RNA) (127). TERRA, exclusively located in the nucleus, plays a role in heterochromatin formation, but also induces telomere (128,129). TERRA molecules are similar in sequence to telomeric DNA and competes with telomeres for telomerase binding (128). Furthermore, TERRA induces telomere shortening by stimulating the expression of exonuclease 1 expression at chromosome ends (128,129). Insertion and transcription of the entire HHV-6 genome into the subtelomere may indirectly stimulate TERRA transcription and lead to telomere shortening. This effect, however, may vary depending on the site of integration. Unfortunately, due to large amounts of chromosomal rearrangements in HEK293 cell lines, FISH analysis was not able to identify the specific site of integration for HEK293/HHV-6A single cell clones. The effect of HHV-6 integration on TERRA transcription and telomerase activity in vivo in infected patients has not been investigated, but is an interesting and important subject which should be explored.

Loss of the viral genome after subsequent growth in cell culture was also noted in seven of the ten originally established HEK293/HHV-6A single cell clones. We hypothesize that if the viral genome integrates close to the physical end of the chromosome, rapid loss of HHV-6 genome may occur due to end replication problems. Failure to maintain the viral genomes close to the end of the chromosome may also be related to the loss of telomerase function proposed previously, with the affected cell eventually losing the viral genome.
The third outcome, maintenance of the viral genome over time, shows that HEK293 cells can be used as an in vitro model for HHV-6 infection, latency, and reactivation. There have been no in vitro HHV-6 models established thus far. This model will prove useful for testing of recombinant/mutant of HHV-6 strains in vitro, and the effect of individual viral genes on the viral integration and reactivation.

Reactivation of HHV-6

An additional phenotypic difference seen with HEK293/HHV-6A clones was the inducibility with epigenetic modifiers TSA, sodium butyrate, carbamazepine, or valproic acid. Of the three clones tested, only one was inducible, and of the epigenetic modifiers inhibitors only TSA was capable of inducing viral reactivation from its integrated form. All of the epigenetic modifiers tested are histone deacetylases (HDACS). Histone deacetylation results in compaction of heterochromatin and the repression of transcription; thus, inhibition of HDACs typically leads to an increased gene expression. The most probable explanation for differences observed in the efficacy of the HDAC inhibitors is specificity. HDACs fall into five classes. Four of the classes include the classical or zinc-dependent HDACS (Table 14). The class III HDACs are made up by the sirtuin family of proteins. None of the HDAC inhibitors tested in this study were active against class III HDACs. However, TSA inhibits all zinc-dependent histone deacetylases (class I, IIa, IIb, and IV); while the other HDAC inhibitors tested are more specific (130,131). Valproic acid sodium butyrate acid specifically inhibits class I and IIa. Carbamazepine has some selectivity against class I, IIa, and IIb, but to date has only been shown to be active against three HDACS: HDAC3, 6, and 7 (132,133).
When HEK293/HHV-6A single cell clones were treated with TSA, reactivation resulted in concatemer formation by PCR amplification and Southern blot hybridization (Figure 19). Interestingly, during reactivation the length of the direct repeat was variable as indicated by the presence of different sized bands when the Southern blot was probed with a radiolabeled DR probe. The DR regions of the viral genome are variable and ranges in size from 8kbp to 13kbp, with up to 1kbp of this being comprised of telomere repeats. Presumably, homologous recombination may occur at any location within the telomere-like repeats; thus, this is one probable explanation for the variation

Table 14: Zinc-dependent Histone Deacetylases (130,131).

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</tr>
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</tr>
<tr>
<td></td>
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<td>Nucleus/cytosol</td>
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</tbody>
</table>
in DR length seen after circularization. It is also possible that there deletions occur in
the DR region during viral reactivation, as the smallest size DR detected by Southern
Blot is about half the size of the HHV-6 DR length previously recorded in the literature
(5,6,9). Large deletions of up to 5.4kb at internal regions in the internal regions of the
DRs have been observed during passage of HHV-6 viruses in T-cell in vitro (134),
resulting in DR lengths similar to those seen in this study. The exact regions of deletions
have not been elucidated in this study, but could be accomplished by cloning and
sequencing of the PCR amplified products.

**Proposed Mechanism of HHV-6 Latency & Reactivation**

It has been well established that HHV-6 establishes latency in vitro by integration
into the telomeres of host chromosomes. In order for homologous recombination to
occur, DNA strands must be cleaved. The most probable candidate for this activity is
the HHV-6 protein U94, which is homologous to the Rep78/68 protein of the adeno-
associated virus type 2(AAV-2). AAV-2 and HHV-6 are similar in that both specifically
integrates into the host chromosomes, however, AAV-2 integration has only been found
to occur in chromosome 17 and integration occurs in a region outside of the telomere.
The Rep78/68 is important for AAV-2 integration and possesses DNA binding,
endonuclease, and helicase activity. The HHV-6 protein U94 has confirmed DNA
binding activity and complements the activity of Rep deficient viruses and likely
performs a similar function((135,136). However, to date there are no reports of
endonuclease and helicase activity for this protein.
The model proposed here for integration and latency begins with cleavage of the viral telomere repeats by protein U94. Following cleavage, strand exchange and homologous recombination would occur, resulting in the integration of the entire viral genome into the subtelomere of the host chromosome. In this study, TSA was shown to induce the reactivation of latent HHV-6 from its integrated form; we propose that TSA treatment leads to the expression of a viral gene, which we propose is protein U94, which aids in excising the viral genome from the subtelomere and facilitates circularization of the viral genome. Replication of the circular intermediate results in the formation of concatemers of the viral genome (Figure 20).

**Nucleotide and Amino Acid Variation of HHV-6**

An HHV-6A virus isolated from Family 1/Sibling and completely sequenced was compared at the nucleotide and amino acid level to both HHV-6A GS and U1102, and our results indicate that the virus is distinct from either of the HHV-6A strains which were previously sequenced. Analysis of the whole genome sequence results clearly indicate that some regions of the reactivated virus are highly conserved when compared to the two fully sequenced HHV-6A variants. Interestingly, the reactivated virus from Family 1/Sibling 2 was >99% identical to the HHV-6A GS strain. We suspect that the two viruses may be similar because of the geographic region of isolation. Both the reactivated virus and the GS strain were isolated in North America, while the U1102 isolate was obtained from an individual in Uganda. The close sequence similarity between the GS virus and the Family 1/Sibling 2 virus, which were both isolated in
North America, and their divergence from the U1102 strain isolated in Uganda suggests that HHV-6 viruses may be further segregated into geographical subtypes.

The current study indicates that intrastrain variation may be as high as 3% at the nucleotide level and 8% at the amino acid level, and this is higher than previously recognized variation. These differences may pose difficulties when designing PCR primers and may lead to failure to identify individuals with HHV-6 in routine clinical assays. The divergence seen between the core gene blocks and towards the right end of the genome are not surprising, as these regions of the genome seem to be hotspot for variation (5,6). These regions are most divergent when comparing HHV-6A strains and HHV-6B viruses. Nevertheless, high levels of conservation at some open reading may indicate biological significance and these genes may prove useful in development of HHV-6 PCR assays for detection. Three of the most highly conserved genes were not located in the herpesvirus core gene blocks and were not unique to roseoloviruses (Table 11): U47A, U60, and U63. No work has been done to characterize the function of either of these genes, and future studies should address this issue. Open reading frame U47A is located immediately downstream of the hypervariable U47 gene region, and is presumed to encode an envelope glycoprotein. However, no work has been done to further characterize this gene. The U60 spliced late gene which encodes a DNA packaging terminase, is the most conserved gene when comparing HHV-6A and HHV-6B viruses, although it is sometimes designated U66 in the B variants (5,6). Finally, U63 is an uncharacterized protein, but relationship to HCMV UL92 has been proposed. CMV UL92 is a betaherpesvirus-specific gene that is essential for CMV replication in fibroblasts; however, it also has not been fully characterized (137).
Figure 20: Proposed Mechanism for HHV-6 Integration and Reactivation

Homologous recombination between the host telomeres and viral telomeres are facilitated by the viral protein U94, which probably cleaves the viral telomeres to allow strand exchange and recombination. Reactivation, induced by TSA, results in expression of viral genes, such as U94, that function to allow recombination of the viral telomere repeats and result in the circularization of the viral genome. The circularized viral genome is replicated, and results in the formation of concatemers.
The predicted amino acid sequences of two proteins, U18 and U20, located between the betaherpesvirus core gene block and the first conserved herpesvirus core gene block, were shorter than the amino acid sequences for HHV-6A GS and U1102. The U18 envelope glycoprotein was 66 amino acids shorter than the amino acid for HHV-6A GS and HHV-6A U1102, and the U20 protein was 188 amino acids shorter than the laboratory strains, GS and U1102. Both of these proteins are preferentially expressed in HHV-6B chronically infected T-cell lines. However, the function of neither of these proteins in HHV-6A viruses has been fully characterized (138). Recent reports indicate that for HHV-6B viruses, U20 is involved in inhibition of tumor necrosis factor receptor induced apoptosis in infected cells (139). However, the function of U20 encoded by HHV-6A, which bears only a 90% amino acid identity to HHV-6B U20, has not been investigated. The HHV-6A and HHV-6B U18, U19, U20 genes (previously named EJFL6, EJFL4, and EJFL3) differ in temporal regulation and splicing patterns. Both U18 or U20 may be expressed as full length proteins or a splice product of the three open reading frames may be produced (138,140). For HHV-6A, the U19 is excluded by splicing and produces a transcript shorter than the HHV-6B transcript (526bp vs 1.9kbp) expressed during the first 8 hours of infection, while the HHV-6B splice product is expressed up to 24 hours post infection.

Inherited Herpesvirus Syndrome (IHS)

Although inheritance of HHV-6 occurs infrequently, by using previously reported prevalence estimates approximately 2.5 million people in the United States have inherited the virus. This is a conservative estimate, as wide scale studies assessing prevalence have not been performed, and increased prevalence has been noted in
hospitalized individuals and individuals displaying neurological dysfunction (40,42–
49,86,92). The patients in this study represent a unique population of individuals with
chronic fatigue syndrome that have inherited HHV-6. Approximately 1.25 million
individuals in the United States suffer from chronic fatigue syndrome; if the CIHHV-6
prevalence reported in this study holds true (over 2%), this unique population would be
made up of more than 25,000 people. The increased CIHHV-6 prevalence in this
population suggests a role of HHV-6 in CFS pathology. In support of this hypothesis, in
this study we documented late viral mRNA production in all four unrelated patients with
CIHHV-6 and chronic fatigue syndrome-like neurological symptoms. In contrast, viral
mRNA was reported in only 8% of asymptomatic CIHHV-6 (34).

Surprisingly, the sequence of the late mRNAs markedly differed from the
inherited viral genome sequences in CIHHV-6 positive patients with CFS-like
neurological symptoms. The presence of an exogenous persistently replicating HHV-6
virus suggests that patients with CIHHV-6 exhibit immune tolerance or
immunosuppression. It has been shown that the humoral response to inherited HHV-6
differs from primary infection. A study by Tanaka-Taya et al. supports the notion of
immune tolerance in CIHHV-6 patients (38). There, only 14% had detectable antibodies
directed at glycoprotein B, the major neutralizing epitope, compared to 60% detection in
healthy adult controls (38). Reduced gB titers suggest that individuals with CIHHV-6 may
have a reduced ability to fight a secondary HHV-6 infection. Additionally, several studies
of cellular response to HHV-6 indicate that there is a low frequency of circulating HHV-6
responsive T-cells (105–107). Currently it is unclear if this phenomenon holds true for
individuals harboring CIHHV-6, but low frequency of responding T-cells may contribute to repeated HHV-6 infections.

To date, there have been no studies on the rate of superinfection in patients with inherited HHV-6. This study suggests that, in symptomatic CIHHV-6 patients, infection with an exogenous HHV-6 virus may be a frequent occurrence. Additionally, we propose that superinfection is the differentiating factor between symptomatic and asymptomatic individuals with CIHHV-6; however, this was not directly investigated in this study. Taken together, we propose that some CIHHV-6 individuals acquire and are persistently infected with exogenous HHV-6 strains that lead to a wide range of neurological symptoms. We propose to name this condition inherited herpesvirus 6 syndrome or IHS. The virus isolated and sequenced from Family 1/Sibling 2 differed from the inherited virus at five open reading frames analyzed and this individual also suffered from CFS-like neurological symptoms; according to the above criteria, this individual also suffers from IHS (Table 13).

Results indicate that patient response to antiviral therapy using oral valganciclovir or intravenous foscarnet was largely dependent on treatment length. A three week treatment with valganciclovir was ineffective in preventing virus reactivation, as indicated by the recurrent expression of U100 mRNA. We recently documented the long-term benefit of antiviral drug therapy of two patients with IHS, one of which was Family 1/Sibling 2 (83). Both of these patients have suffered debilitating neurological symptoms but antiviral therapy resulted in marked and long-lasting improvement also documented by quantitative EEG (83). Years after cessation of antiviral therapy, we
were unable to detect detectable U100 mRNA in their PBMCs and these individuals are also free of neurological symptoms.

**Reactivation of Inherited HHV-6**

There have been previous reports of reactivation of integrated HHV-6. Arbuckle *et al.* 2010 demonstrated *in vitro* reactivation of integrated HHV-6 by chemical inducers, such as TPA and trichostatin. More recently, Gravel *et al.* reported the transmission of a CIHHV-6 strain transplacentally from mother to child, in the absence of inheritance (15,36). In the first, sequencing of the direct repeat and the U94 gene revealed that the reactivated and inherited viruses were identical (15). In the latter study, evidence for this transmission comes from nucleotide sequence comparison of a portion of the U39 envelope glycoprotein B, and the presence of unique polymorphisms in the DNA sequences of both the mother and infant (36). Here, we employed a similar strategy by sequencing genomic DNA and cDNA isolated from the same patient. However, our results indicate that the detected mRNA arose from only an exogenous HHV-6 virus in all the patient samples evaluated, rather than the inherited viruses. We did not see evidence of reactivation of the CIHHV-6 virus. However, it is possible that this occurs as well and that the copy number is too low to be detected. Contradictions between this study and previously mentioned studies suggest that the ability of CIHHV-6 viruses to reactivate is a complex process, and may vary from one individual to another. Immunological status may play a role in the ability of individuals to be infected with exogenous HHV-6 or for the reactivation of the latent inherited virus. No studies have
conclusively shown the lack of neutralizing antibodies in CIHHV-6 individuals, and antibodies directed against the HHV-6 IE-A gene were detected at a higher rate in CIHHV-6 individuals (38). This indicates that some CIHHV-6 individuals are capable of mounting an immune response to HHV-6; these individuals may be less susceptible to continuous reactivation of the virus or reinfection by exogenous strains of the virus. The differences in observed immune responses may be due to the ability of the immune system of some individuals to override immune tolerance. Alternatively, the degree of differences between the sequence of dominant antigen epitopes of the inherited viral genes and contemporary virus isolates may also impact immune response.
FUTURE DIRECTIONS

The current study demonstrated reactivation of integrated HHV-6 in *in vitro* models and the propensity for infection with exogenous HHV-6 strains in individuals with an inherited form of the virus. Although HHV-6 and its inheritance have been recognized by a number of studies, there are limited studies focusing on CIHHV-6 and the characterization of HHV-6 strains. Below are suggestions of future studies which are imperative for the understanding of HHV-6 latency and inheritance.

**Prevalence of CIHHV-6 in Neurological Disorders**

Large multi-site studies should focus on determining the prevalence of CIHHV-6 in chronic fatigue syndrome and other neurological disorders. In this study, there is limited evidence that the rate of HHV-6 inheritance may be greater than the proposed 0.8% in the United States population. Other studies have also demonstrated increased prevalence of inheritance in individuals hospitalized for a variety of conditions (40,42–49). Many hospitals and research institutions have repositories of biological specimens and testing for CIHHV-6 is simple and cost efficient.

Detection of CIHHV-6 can accomplished by quantitative real time PCR analysis performed on whole blood, hair follicle, and other biological specimens. Amplification of the U67 open reading frame has previously been used as a means of determining CIHHV-6 prevalence (66) and will be used here. A result of greater than $10^6$ viral copies
per ml would indicate inheritance of the virus. Prevalence will be calculated by dividing
the number of inherited cases by the total number of samples tested. Statistical
significance will be determined using the Fisher’s Exact test, as described previously
(66).

**Cellular and Humoral Responses to HHV-6 in CIHHV-6 Individuals**

Future studies should evaluate cellular and humoral response to HHV-6 in
individuals with an inherited form of the virus. More recently, several research groups
have focused on the cellular and humoral response to HHV-6 (105,107,141). However,
none of these studies have evaluated these responses in individuals with an inherited
form of HHV-6. This is an important area of research which needs to be addressed in
the near future, considering the implications of the current study. One of the most
variable genes noted in sequencing study was U11, an immunodominant tegument
protein (Table 13). A recently published article looked at the antibody response to this
protein in individuals suffering from CFS and found no differences in antibody response
when compared to healthy controls (88). However, one limitation of this study is that it
failed to look at individuals with an inherited form of the virus.

PBMCs will be isolated from CIHHV-6 or non-CIHHV-6 individuals by density
gradient centrifugation (Lymphoprep) and maintained in RPMI-1640 medium with 10%
FBS and 50µg/ml gentamicin. For T-cell expansion cells will be maintained in culture
medium containing phytohaemagglutinin (20µg/million cells) and 50U/ml interleukin-2. A
Low HHV-6 specific T-cell frequency has previously been reported (105–107), therefore,
ex vivo expansion with HHV-6 antigen may be necessary prior to functional assays.
HHV-6-specific T-cell response be assessed by interferon gamma enzyme-linked immunosorbent spot assay, and the functional characteristics of HHV-6 specific CD4+ and CD8+ T-cells will be determined by intracellular cytokine assays.

For evaluating humoral response, we propose measuring serum titer of anti-HHV6 neutralizing antibodies (gB) and anti-U11 antibodies by ELISA. Statistical significance of differences in responses between CIHHV-6 and non-CIHHV-6 individuals will be assessed using the student’s p-test.

**Functional Characterization of HHV-6 Encoded Proteins**

Considering that HHV-6A and HHV-6B are important human pathogens, there have been limited studies characterizing the function of HHV-6 encoded proteins. The present study highlighted a number of open reading frames which are either well conserved or highly variable from one strain to another. Characterization of genes which fall into either of these categories will allow researchers to understand the molecular basis for interstrain and intrastrain differences observed for HHV-6 strains.

Our group previously used an HHV-6A BAC vector to created a chloramphenicol-selectable green fluorescent-protein (GFP) expressing HHV-6A virus (15); this vector allows for manipulation of the viral genome in an *E. coli* system. First, PCR will be used for generating deletion cassettes which contain the flanking region of individual open reading frames, the kanamycin gene, and loxP (locus of X-over P1) sites. The purified deletion cassette will then be transformed into *E. coli* cells containing the HHV-6A BAC vector and mutants will be selected for by LB agar plates containing chloramphenicol and kanamycin. Deletion of selected open reading frames will be confirmed by
restriction enzyme digestion and southern blot analysis. To generate revertant mutants the recombinant BAC vector can be transformed into *E. coli* cells expressing the Cre (Cyclization recombinase) site-specific recombinase. Recombination will occur, result in the deletion of the kanamycin cassette and result in an intact copy of the viral gene.

After generation of the knockout BAC vector, virus reconstitution will be accomplished by transfecting the BAC vector into HEK293 cells and growing the cells until cytopathic effect is visible. The recombinant or revertant wild-type virus will then be isolated by several freeze thaw cycles and harvesting of supernatant after high speed centrifugation to remove cellular debris.

Once generated, the knockout genes can be assessed for their ability to establish latency and be reactivated from latency. Establishment of latency can easily be tracked flow cytometry or fluorescent microscopy when working with a GFP expressing mutant virus. In our experience cells infected with an HHV-6 GFP expressing viruses fluoresce brightly during early times of infection and fluorescence intensity lowers as latency is established. Fluorescence should again increase after addition of TSA, and induction of viral replication.

**Characterization of Inherited Herpesvirus-6 Genomes**

There is little known about the genetic variability of inherited HHV-6 viruses, and future studies should focus on characterizing inherited HHV-6 viruses in detail. Restriction fragment analysis and sequencing number of viral genes, suggests that inherited HHV-6 viruses are divergent when compared to each other and previously characterized HHV-6 viruses. Full genome sequencing of the inherited genome may
reveal critical antigen epitope variability in addition to mutations with the potential to cause viral replication defects. Sequence variation may also affect the ability of inherited viruses to reactivate or determine whether inherited viral genome expression occurs. Furthermore, it is also possible that some HHV-6 viruses that have entered the germline viruses differ in tropism than other viruses and can more efficiently infect gonad or pre-gonad cells. These differences may be a contributing factor to why CIHHV-6 is present in only a small fraction of the population, although HHV-6 viruses are ubiquitous.

To characterize inherited HHV-6 sequences, whole genome sequencing can be performed on DNA isolated from whole blood of CIHHV-6 positive patients. We propose the use of 454 sequencing methodology to accomplish whole genome sequencing of the inherited viral genome. We expect that sequencing will be partially incomplete in the portions of the viral genome containing repetitive sequences and that PCR amplification and cloning will be necessary to complete the viral genome. Assembly of the sequenced viral genome will be performed using HHV-6A U1102 or HHV-6B Z29 as reference strains. Nucleotide sequences and translated amino acid sequences for individual open reading frames will be aligned with prototype sequences, and evaluated for potential truncations and significant differences in coding sequences.
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Persistent Human Herpesvirus-6 Infection in Patients With an Inherited Form of the Virus

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Human herpesvirus-6 (HHV-6) A and B are ubiquitous betaherpesviruses viruses with lymphotropic and neurotropic potential. As reported earlier, these viruses establish latency by integration into the telomeres of host chromosomes. Chromosomally integrated HHV-6 (CIHHV-6) can be transmitted vertically from parent to child. Some CIHHV-6 patients are suffering from neurological symptoms, while others remain asymptomatic. Four patients with CIHHV-6 and CNS dysfunction were treated with valganciclovir or foscarnet. HHV-6 replication was detected by reverse transcriptase polymerase chain reaction amplification of a late envelope glycoprotein. In this study we also compared the inherited and persistent HHV-6 viruses by DNA sequencing. The prevalence of CIHHV-6 in this cohort of adult patients from the USA suffered from fatigue syndrome; integration

INTRODUCTION

Human herpesvirus-6A and 6B (HHV-6A and HHV-6B) are ubiquitous betaherpesviruses viruses known for their lymphotropic and neurotropic potential [Hall et al., 1998; Santoro et al., 1999]. Primary HHV-6B infection results in exanthema subitum and typically occurs during the first 2 years of life [Yamanishi et al., 1988]. These viruses persist after primary infection; viral reactivation is associated with a variety of adult conditions and complications including encephalitis, drug-induced hypersensitivity or drug rash with eosinophilia and systemic symptoms, and transplant rejection [Tohyama et al., 2007; Watanabe et al., 2008].
cohort of adult patients from the USA suffering from a wide range of neurological symptoms including long-term fatigue were found significantly greater than the reported 0.8% in the general population. Long-term antiviral therapy inhibited HHV-6 replication as documented by loss of viral mRNA production. Sequence comparison of the mRNA and the inherited viral genome revealed that the transcript is produced by an exogenous virus. In conclusion, the data presented here document that some individuals with CIHHV-6 are infected persistently with exogenous HHV-6 strains that lead to a wide range of neurological symptoms; the proposed name for this condition is inherited herpesvirus 6 syndrome or IHS. J. Med. Virol.

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KEY WORDS: human herpesvirus 6; HHV-6; antiviral drug treatment; chronic Watanabe et al., 2008).

Unlike other human herpesviruses, HHV-6 viruses establish latency by integration into the telomeres of host chromosomes and may be inherited [Arbuckle et al., 2010]. In a screen of blood donors in the United States and the United Kingdom, the prevalence of inherited HHV-6 in the general population is approximately 0.8% [Hall et al., 2004, 2008; Leong et al., 2007; Hoddinott et al., 2008]. The prevalence of CIHHV-6 is increased in children referred for encephalitis, solid organ, and stem cell transplant recipients.

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as well individuals with lymphoproliferative disorders [Torelli et al., 1995; Griffiths et al., 1999; Kidd et al., 2000; Ward et al., 2007; Potenza et al., 2009; Lee et al., 2011, 2012; Zerr et al., 2011; Hubacek et al., 2012; Pellett et al., 2012]. In these populations, the prevalence of CIHHV-6 averages 2%.

In vitro studies and clinical reports indicate that HHV-6 may be reactivated from its integrated form [Arbuckle et al., 2010]. However, detection of reactivation is difficult because patients with inherited HHV-6 consistently present with viral DNA copy number above 0.5 million/ml in whole blood [Pellett et al., 2012]. There have been suggestions that elevated anti-HHV6 IgG levels are indicative of reactivation [Ablashi et al., 2000]; however, antibody response is variable among patients and may yield inconclusive results. Detection of mRNA, however, seems to be a promising marker for viral reactivation and several research groups have detected active HHV-6 replication using reverse transcriptase polymerase chain reaction (RT-PCR) [Norton et al., 1999; Van et al., 2001; Caserta et al., 2007; Ihira et al., 2012]. Institutional Review Board. All subjects presented with more than 0.5 million DNA copy numbers of HHV-6 in whole blood, with concomitant positive results of HHV-6 in hair follicles as detected by Viracor-IBT Laboratories, Inc. Patients presented with fatigue and neurological symptoms including, but not limited to, depression, hypersomnia, memory, and cognitive impairment. In this study, patients received therapeutic antiviral therapy consisting of either twice daily valganciclovir (Patients 1, 2, and 3) or 60 mg/kg foscarnet (Patient 4) per day. Patients receiving short-term treatment received 900 mg valganciclovir twice daily; patients receiving long-term treatment received 900 mg valganciclovir twice daily for 3 weeks and 450 mg twice daily for 3 weeks or longer. Blood samples were collected in Paxgene DNA, RNA, and heparin tubes before and during treatment.

A two-tailed Fisher exact test was used to determine the significance of CIHHV-6 prevalence among this cohort of US adult patients suffering from neurological symptoms.
A recent publication described the successful treatment of two CIIHV-6 patients with detectable HHV-6 glycoprotein mRNA in whole blood [Montoya et al., 2012]. Prior to treatment, both patients exhibited neurological symptoms including cognitive impairment and depression with concomitant abnormal quantitative EEG readings. Six weeks of foscarnet treatment resulted in the resolution of neurological symptoms and normalization of brain waves; however, symptoms returned after cessation of antiviral treatment.

Previous studies reported reduced antibody titers to HHV-6 glycoprotein B in the serum of individuals with CIIHV-6, when compared to those of healthy controls [Tanaka-Taya et al., 2004]. This suggests that there is an immune tolerance in these individuals, and the central hypothesis of this study is that repeated reactivation or exogenous infection may contribute to illness in symptomatic patients with CIIHV-6.

In this study, nested RT-PCR assay was utilized to amplify U100 envelope glycoprotein mRNA as a means of detecting HHV-6 replication in blood samples isolated from symptomatic individuals with CIIHV-6 [Norton et al., 1999]. The goal of this study is to use mRNA detection as a means of determining the efficacy of antiviral treatment and optimal treatment time. Additionally, sequence analyses on HHV-6 late mRNA and inherited viral DNA were performed to investigate the persistence of exogenous HHV-6 in individuals with CIIHV-6.

**METHODS**

**Study Subjects, Treatment, Statistical Analysis**

Four patients diagnosed with inherited HHV-6 provided written informed consent prior to the start of this study, and the study was approved by the

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**Detection of Viral mRNA by Nested RT-PCR Assay**

Nested RT-PCR was used to amplify HHV-6 glycoprotein U100 mRNA in intracellular RNA from whole blood. Blood samples were collected in PAXgene blood RNA tubes and RNA was isolated using Trizol reagent. Total RNA was converted to cDNA using the GoScript Reverse Transcription System (Promega, Madison, WI) and 5 ng of total RNA was used for PCR. Two rounds (30 cycles each) of PCR were performed using RedTaq polymerase (Sigma, St. Louis, MO), as described previously [Norton et al., 1999]. Primers used for amplification are as follows: U100Round1F: CAAAATTTCTACCTCCGAAAATGT; U100Round1R: GAGTCCAT GAGTTAGAAGATT; U100Round2F: ACTACTACCAGAAGATATAG; U100Round2R: AAGC GCGTGCAGGTITCCCAA [Norton et al., 1999]. RNA isolated from uninfected or infected MoI3 T lymphocytes was used as a negative or positive control, respectively. Reverse transcription and subsequent PCR amplification were conducted independently for each patient sample. Additionally, a reverse transcriptase null reaction was performed to ensure the absence of DNA in RNA samples. For a cellular control, beta actin was amplified with 30 cycles of PCR. Amplified products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized using UV light in the Gel Doc Molecular Imaging System (Bio-Rad, Hercules, CA).

**PCR Amplification, Cloning, Sequencing, and Virus Isolation**

A portion of the U100 gene was amplified by PCR amplification using RedTaq Polymerase (Sigma). DNA was isolated from agarose gel using Wizard SV Gel and PCR Clean-Up System (Promega) and TA
cloned using the pGEM-T-Easy cloning system.
Cloned genes were sequenced using the ABI3130XL capillary sequencing instrument. Sequencing was performed at the Moffitt Cancer Center Molecular Genomics Core Facility.

**Restriction Enzyme Digestion and Southern Blot Hybridization**

Five micrograms of cellular DNA from patient PBMCs or 1 ng of virion DNA was digested with SacI and separated using agarose gel electrophoresis. DNA fragments were transferred to nitrocellulose by vacuum blotting and hybridized with 32P-labeled HHV6A U1102 cloned into a BAC vector [Tang et al., 2010].

**Alignment of Nucleotide Sequences**

To obtain alignment scores, nucleotide sequences were aligned using ClustalW2 using default alignment parameters (DNA weight matrix: TUB, Gap open: 10, Gap extension: 0.2, Gap distance: 5, Number: 1). Alternatively, multiple sequences were aligned using the Mauve algorithm and visualized using Jalview in the Virus Pathogen Database and Analysis Resource (ViPR).

on blood samples isolated from symptomatic patients prior to the administration of antiviral medications; follow up samples were taken during or after treatment. For each RT-PCR reaction, 5 μg of total RNA isolated from peripheral blood was used. In all cases, U100 mRNA was detected in blood samples, in the absence of antiviral treatment (Fig. 1). On the other hand, ten control single donor samples obtained from the local blood bank were negative for U100 mRNA (data not shown). In the absence of reverse transcriptase, PCR products were not detected (Fig. 1). Long-term (>6 weeks) administration of foscarnet (Patient 4) or valganciclovir (Patient 3) resulted in the abrogation of U100 mRNA expression (Fig. 1), while short-term (3 weeks) administration of valganciclovir was not sufficient to eliminate viral gene expression in two unrelated patients (Patients 1 and 2). Five weeks after the cessation of intravenous foscarnet treatment (Patient 4), U100 mRNA was detected in the whole blood of Patient 4. In all cases, resolution of symptoms was concurrent with the reduction of mRNA expression. This suggests that U100 mRNA detection is a reliable method of detecting HHV-6 persistence. However, treatment efficacy is variable and appears to be dependent on the length of treatment, with treatment length of greater than or equal to 6 weeks being optimal for valganciclovir and
APPENDIX 2 (continued)

RESULTS

Prevalence of CIEHV-6 in Cohort of Patients With Neurological Symptoms

To reveal the prevalence of CIEHV-6 in 337 US patients suffering from a wide range of neurological symptoms, including long-term fatigue, peripheral blood samples were tested by quantitative PCR by Viracor-IBT Laboratories. Seven patients had higher than 0.5 million HHV-6 copies per milliliter of blood. A two-tailed Fisher exact test was used to determine the significance of CIEHV-6 prevalence among these patients. The CIEHV-6 rate among this cohort of US adult patients suffering from neurological symptoms is 2.1% (7/337). This is significantly greater (P = 0.06) than the expected value of 0.8% reported in the general US population (Hall et al., 2004, 2008).

Long-Term Antiviral Treatment Abrogates Viral mRNA Production

The four patients enrolled in this study harbored over 0.5 million copies of HHV-6 DNA copy numbers per milliliter of peripheral blood. Patients presented with a variety of neurological symptoms including, but not limited to, headache, blurred vision, and memory impairment, as well as other symptoms such as generalized pain and long-term fatigue.

To determine if the symptoms were due to replicating HHV-6, nested RT-PCR was employed to detect the late envelope glycoprotein U100 mRNA in whole blood samples from patients with CIEHV-6 (Norton et al., 1999). Initial RT-PCR assays were performed foscarin (Table 1).

The Late U100 Viral mRNA Originates From an Exogenous HHV-6 Strain

Amplified U100 cDNA was sequenced to confirm that the RT-PCR products were derived from mature mRNA that lacks introns rather than the inherited viral DNA. Sequencing was also performed to determine if the detected mRNA originated from the...
inherited virus, or from an exogenous virus. The U100 gene of the inherited viral genome was also amplified and both cDNA and inherited HHV-6 DNA sequences of U100 were cloned and sequenced. For sequences from the inherited viral DNA fragments, introns were removed using previously accepted splice sites prior to alignment using ClustalW2. Isolated cDNA sequences were also compared to the U100 sequences for HHV-6A U1102 and GS, or HHV-6B Z29. All patient cDNA sequences were more similar to HHV-6A U1102 than HHV-6A GS and differed from HHV-6A U1102 by only a CG inversion at bases 193/194 (Fig. 2). On the other hand, the inherited genomic sequences were more similar to HHV-6A GS. The inherited HHV-6A sequences were greater than 99% identical to each other for three patients, as were all the reactivated viral sequences.

The cDNA and inherited genomic sequences differed from each other in all cases. U100 cDNA shared only an average of 96.3% nucleotide sequence identity with the inherited HHV-6A viral genome. U100 cDNA from Patient 1 was more similar to HHV-6A U1102, while the inherited HHV-6 DNA sequence was 96% identical to HHV-6B Z29. In addition, the U100 mRNA sequence and inherited viral DNA sequences shared only a 90% nucleotide sequence identity. Differences in the genomic DNA and cDNA sequences demonstrate that these symptomatic patients with inherited HHV-6 harbor more than one HHV-6 virus, and the U100 mRNA detected by RT-PCR originates from an exogenous virus.

The inherited HHV-6 banding patterns, Southern blot hybridization shows distinct banding patterns for each inherited virus and a significantly diverged banding pattern from HHV-6A GS, indicating that all inherited viruses evaluated in this experiment markedly differ from HHV-6A GS and also uniquely distinct from each other.

**DISCUSSION**

Inheritance of HHV-6 infrequently occurs in the healthy, general US population and the reported prevalence is around 0.8% [Hall et al., 2004, 2008]. However, there is a noted increased prevalence in hospitalized individuals and individuals displaying neurological dysfunction [Pellet et al., 2012]. Importantly, the prevalence CIHHV-6 in this patient population suffering from a wide range of neurological symptoms is significantly higher (over 2%), suggesting a possible role of CIHHV-6 in pathology. In support of this hypothesis, late viral mRNA production was documented in all four unrelated patients with CIHHV-6 and neurological symptoms. In contrast, viral mRNA was reported in only 8% of asymptomatic CIHHV-6 individuals [Hall et al., 2008].

Unexpectedly, the sequence of late mRNAs and markedly differed from the inherited viral genome sequences. The presence of an exogenous persistently replicating HHV-6 virus suggests that patients with CIHHV-6 exhibit immune tolerance or a weakened immune response to the virus. It has been shown...
Inherited HHV-6 Viral Genome Sequences Are Heterogeneous

The results clearly indicate that the reactivated virus from these patient samples did not emanate from the inherited virus; in all cases the reactivated viruses were more similar to HHV-6A U1102 than HHV-6A GS. Additionally, there was a high level of similarity between the U100 DNA sequences of the inherited viruses. In order to compare the genome of the inherited HHV-6 viruses to the HHV-6A GS virus, restriction fragment length analysis was performed on DNA from the PBMCs of four patients or GS virion DNA. Briefly, DNA was subjected to restriction enzyme digestion using SacI, fragments were separated by electrophoresis, blotted onto nitrocellulose and probed with a radiolabeled HHV-6A U1102 BAC clone (Fig. 3). Despite similarities among immune response to the virus, it has been shown that the humoral response to inherited HHV-6 differs from primary infection. A previous study supporting the notion of immune tolerance in CIHV-6 patients [Tanaka-Taya et al., 2004] reported that only 14% had detectable antibodies directed at glycoprotein B, the major neutralizing epitope. In contrast, there was 60% detection in healthy adult controls [Tanaka-Taya et al., 2004]. Reduced gB titers suggest that individuals with CIHV-6 may have a reduced ability to fight a secondary HHV-6 infection. To date, there have been no studies on the rate of superinfection in patients with inherited HHV-6. This study suggests that, in symptomatic CIHV-6 patients, infection with an exogenous HHV-6 virus may be a frequent occurrence. Additionally, one may propose that superinfection is the differentiating factor between symptomatic and asymptomatic individuals with CIHV-6; however, this was not investigated in this study.

Fig. 2. Nucleotide alignment of cDNA and genomic sequences. PCR amplified HHV-6 glycoprotein cDNA or corresponding genomic DNA fragments were TA cloned and sequenced. Obtained sequences were aligned using the Mauve algorithm and visualized in JalView in the Vipr database.

exogenous HHV-6 virus in all the patient samples evaluated, rather than the inherited viruses. There was no evidence of reactivation of the CHHHV-6 virus. However, it is possible that this occurs as well and that the copy number is too low to be detected.

To date, there have been no reports on HHV-6-specific CD4 or CD8 responses in CHHHV-6 patients. Two independent groups recently reported that the large structural phosphoprotein, U111, is an immuno-dominant CD4 and CD8 epitope for HHV-6B [Martin et al., 2012; Nastke et al., 2012]. Future studies could reveal whether patients with IHS fail to develop adequate T cell-mediated protective immunity.

Another finding of this study was that in all cases, the partial U100 sequence inherited by three of the patients was more similar to HHV-6A GS than HHV-6A U1102. This finding may be attributed to the fact that the GS strain was isolated in the United States, the location of all patients. To date, the GS strain is the only HHV-6A virus isolated in the United States and little is known as to the variation of HHV-6 viral sequences by geographic location.

Differences between the inherited and reactivated viruses may pose difficulties when designing PCR primers, and may lead to failure to identify individuals with CHHHV-6 in routine clinical assays.

The SacI restriction enzyme cleavage profiles of the inherited strains were highly heterogeneous as compared to strain GS. This may reflect significant sequence divergence; however, it is also possible that the inherited viruses suffered deletions and/or rear-

Fig 3. Restriction fragment analysis of inherited HHV-6 viruses. Cellular DNA from patient PBMCS or virion DNA from HHV-6 GS was digested with SacI. Fragments were separated by electrophoresis, blotted onto nitrocellulose, and probed with a radiolabeled HHV-6A U1102 BAC clone. Lane 1: HHV-6A GS virion DNA, lane 2: Patient 2, lane 3: Patient 3, lane 4: Patient 4, lane 5 & Patient 6. Patient 5 is sibling 1 from Family 1, diagnosed with IHS [Arimokhe et al., 2010, 2013].
APPENDIX 2 (continued)

Taken together, it may be proposed that some CIHHV-6 individuals acquire and are infected persistently with exogenous HHV-6 strains that lead to a wide range of neurological symptoms. A fitting name for this condition is infected herpesvirus 6 syndrome or IHS.

The results indicate that patient response to antiviral therapy using oral valganciclovir or intravenous foscarnet was largely dependent on treatment length; a 3-week treatment with valganciclovir was ineffective in preventing virus reactivation, as indicated by the recurrent expression of U100 mRNA. Recent reports document the long-term benefit of antiviral drug therapy of two patients with IHS [Montoya et al., 2012]. Both of these patients have suffered debilitating neurological symptoms but antiviral therapy resulted in marked and long-lasting improvement also documented by quantitative EEG [Montoya et al., 2012]. Currently, the two patients have no detectable U100 mRNA in their PBMCs and are free of neurological symptoms.

There have been previous reports of in vitro reactivation of integrated HHV-6 by chemical inducers, such as TPA and trichostatin A [Arbuckle et al., 2010]. However, it remains unclear as to whether or not inherited HHV-6 strains retain their ability to reactivate and cause persistent infection. In this study, sequencing of genomic DNA and cDNA indicated that the detected mRNA arose only from arrangements resulting in a markedly different restriction enzyme cleavage profile.

Another question that remains unanswered is at what point during human evolution did HHV-6 enter the germ line? Inheritance of CIHHV-6 follows Mendelian genetics and the sequence of inherited and current HHV-6 isolates are quite divergent suggesting that inheritance of the virus in some families dates back hundreds or thousands of years.

Diagnosis of IHS requires detection of CIHHV-6 status and detection of persistent viral infection. Several commercial companies offer quantitative PCR assays that may reveal the presence of CIHHV-6 but there are no clinical laboratories offering specific tests to detect persistent HHV-6 infection. The study presented here shows that the RT-PCR assay for the HHV-6 U100 mRNA offers a means of distinguishing latent infections from lytic/persistent infections. Since HHV-6 has been also recognized as an agent involved in organ rejection of transplant recipients the RT-PCR assay could guide a physician’s decision on whether administration of antiviral drugs is warranted.

REFERENCES

APPENDIX 2 (continued)

Peristent HHV-6 in Patients With CIHVV-6


APPENDIX 2 (continued)


