AN ABSTRACT OF THE THESIS OF

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Title: Analysis of cDNA and gDNA Libraries of Human Herpesvirus 6 By Monoclonal Antibodies To Virus Infected Cells

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A novel herpesvirus, human herpesvirus-6 (HHV-6) has recently been isolated from patients with AIDS, chronic fatigue syndrome, and certain leukemias. Reactive serum to HHV-6 has also been detected in high titers from patients with other diseases and in low titers from healthy populations. Sero-epidemiological data indicate that this virus is common in human populations and is encountered early in life. Of significance is the fact that it may play a direct and/or indirect role in specific disease processes. If so, diagnostic measures must be found in order to develop a clinical regimen for HHV-6 associated disease. To assay viral components, viral proteins must be generated in large quantities. Using copy DNA (cDNA) and genomic DNA (gDNA) libraries, two monoclonal antibodies have been found that react with viral antigen. These antibodies (13D6 and 2D6) appear to bind the same antigen from the genomic library and may be identical in their idiotypic structure. A third antibody (9A5) failed to react with a fusion product on Western blot. Because the libraries represent the entire genome of HHV-6, other viral proteins can also be studied and location of their genes mapped.

Analysis of cDNA and gDNA Libraries of Human Herpesvirus 6 By Monoclonal Antibodies To Virus Infected Cells

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INTRODUCTION

In the course of immunosuppressive disease, novel infectious agents are often discovered. Such is the case for human herpesvirus 6 (HHV-6). Salahuddin et al. (1986) discovered the virus while studying fresh peripheral blood mononuclear cells from patients with lymphoproliferative disorders. These included a patient with the acquired immunodeficiency syndrome (AIDS), Kaposi's sarcoma, and B-cell lymphoma, a patient with a T-cell lymphoma, and a patient with immunoblastic lymphoma. In each case, a small number of short lived refractile mononucleated or binucleated cells containing intranuclear and/or intracytoplasmic inclusion bodies expressing viral antigens were identified. When fresh peripheral blood cells from healthy individuals were mixed with culture supernatants from these atypical cells, similar large refractile cells appeared. The viral particles that were isolated contained a double stranded DNA of 170 kilobases (kb) sufficient to code for at least 70 proteins (Josephs et al., 1988). Electron microscopy revealed the presence of membrane bound bodies with icosahedral capsids. These data indicated that HHV-6 belongs to the family Herpesviridae.

Initially, the virus was shown to only infect cells of B-cell lineage and was named human B cell lymphotrophic virus (HBLV). Later, other cell lines were shown to be infected by the virus including HSB-2 (a T-cell line) and HEL (a megakaryocyte line). The virus has since been designated human herpesvirus 6 (HHV-6) (Ablashi et al., 1987).

The virus has since been associated with various disease states including sarcoidosis, exanthem subitum, Sjogren's syndrome, Hodgkin's lymphoma, Burkitt's lymphoma, AIDS, and chronic fatigue syndrome (CFS)

(Biberfeld et al., 1988; Ablashi et al.,1988A). In most of these conditions, viral association has been based upon sero-epidemiological studies. Biberfeld et al. (1988) demonstrated a high occurrence of antibody to HHV-6 in patients with sarcoidosis and malignant lymphomas, but less antibody with Sjogren's syndrome. Ablashi et al. (1988A) detected HHV-6 DNA in some B-cell lymphoma patients with Sjogren's syndrome. In this same study, one patient with Burkitt's lymphoma showed 70% cellular coinfection of HHV-6 with Epstein Barr virus (EBV). Antibody titer in this patient was >1:1280 for EBV and >1:640 for HHV-6. These data suggest that HHV-6 may play a role in Burkitt's lymphoma.

Perhaps of most significance is the association of the virus with AIDS and CFS. Herpesviruses account for a significant proportion of the collective morbidity and mortality in AIDS (Fauci, 1986). In one study of homosexual men, all symptomatic AIDS patients possessed elevated antibody titers to HHV-6 (1:80 - >1:640). Seventy percent of asymptomatic HIV-1 seropositive individuals were also seropositive for HHV-6 (1:8 - >1:640), while 46% HIV-1 seronegative individuals were seropositive for HHV-6 (<1:40). Prevalence of the virus is increased through all stages of AIDS (Ablashi et al., 1988A). Given the inherent immunocompromised state of AIDS patients, the normally mild pathogenic recurrence of a herpesvirus proves life threatening due to viral blooming within the host.

Lusso et al. (1989) demonstrated by two color immunofluorescence that both viruses can infect the same cell simultaneously. Complete viral pathways were observed through maturation. Expression of viral messenger RNA (mRNA) showed no increase, but HIV-1 antigen expression occurred more rapidly with co-infection. The cells simultaneously expressed HHV-6 antigen and induction of cytopathology was also accelerated. Mathematical

analysis suggested a synergistic cytocidal action of HHV-6 and HIV-1.

It is of interest that HIV-1 and HHV-6 gain entry into a host cell by binding to CD4, a receptor that normally binds MHC-II products (Lusso et al., 1989). After binding to the receptor, the viruses invade the cell by receptor mediated endocytosis (Lusso et al., 1989). Ablashi et al. (1988B) demonstrated mutualistic symbiosis between the two viruses. They infected a CD4+ cell culture with both viruses separately and observed limited viral replication and cytopathology. However, infecting the same culture type with both viruses simultaneously resulted in increased levels of reverse transcriptase, and HIV-1 and HHV-6 antigens.

As yet, the mechanisms which are responsible for triggering HIV expression are not known. With the information provided by Ablashi et al. (1988A) and Lusso et al. (1989), Horvat, Wood, and Balachandran (1989) constructed a plasmid containing the long terminal repeat (LTR) of HIV and the gene for chloramphenicol acetyl transferase (CAT). The LTR is responsible for initiating transcription in HIV and the gene for CAT provides a visual technique in determining expression of the LTR. This plasmid was inserted into HHV-6 targeted cells and followed by HHV-6 infection. This led to an increase of the LTR and CAT activity of 42-66 times that of uninfected control cells. This suggests that some as yet undefined component of HHV-6 replication stimulates HIV expression. This phenomenon, where products of one infectious agent activates the promoters of other genes or agents, is called transactivation. Based on this information, it has been hypothesized that HHV-6 may play a role in the progression from HIV seropositive to full blown AIDS. Other herpesviruses have been shown to increase HIV activity also (Mosca et al, 1987) and as herpesviruses are ubiquitous among human populations, this theory is not

without foundation.

HHV-6 has also been strongly implicated as the etiological agent of CFS. Symptoms of the disease include fever, recurrent headaches, and prolonged fatigue. Caligiuri et al. (1987) showed an increase in natural-killer (NK) cell populations with CFS. High levels of NK cells provide a barometer for viral infections, thereby lending support for viral etiology. Initially, it was thought that EBV was the primary etiological agent because of the prevalence of EBV antibodies. However, a causative link has yet to be developed for this virus.

In 332 cases of CFS from various areas of the United Sates, seroprevalence of HHV-6 antibody was greater than 80% (range: 1:80 - >1:2560). Control sera from the same areas were 35-45 percent (range: 1:20 - <1:160). HHV-6 was isolated from two patients afflicted with the disease. Persistent infection is one characteristic of herpesviruses and could account for the longevity of the disease (Ablashi et al., 1987). One group, realizing interleukin-1 (IL-1) also causes symptoms of CFS, suggested that HHV-6 may stimulate IL-1 production. However their subsequent study showed no increase in IL-1 activity (Morte et al., 1989).

HHV-6 distribution appears to be very wide. Seropositivity has been found in North America, western Europe, and many countries in central Africa, including the Ivory Coast, Uganda, Gambia, and Zaire (Krueger and Ablashi, 1987; Agut et al., 1988; Downing et al., 1987; Tedder et al., 1987; Lopez et al., 1988). In the United States, Saxinger et al. (1988) examined sera for HHV-6 collected in 1978 from 500 healthy blood donors. Sera showing viral neutralization (>50%) were found in Minneapolis (81%), Kansas City (88%), and from three random population samples (97%).

Antibodies to early and late proteins of the other human

herpesviruses, EBV, CMV, HSV, and VZV did not react with HHV-6. Also, antibodies to these viruses could be removed from sera by reacting with appropriately infected cells without reducing antibody levels to HHV-6. Conversely, adsorption with HHV-6 infected cells did not alter antibody to the other viruses (Salahuddin et al., 1986). Sera from other animals (primates and non-primates) were tested in similar fashion with the same results (Ablashi et al., 1988A).

Efstathou et al. (1988) generated DNA probes to HHV-6 and tested for sequence homology to the other five human herpesviruses. Only one showed strong cross hybridization to one of the viruses, CMV, and weak hybridization to the herpes simplex viruses. Josephs et al. (1986) used probes from the other herpesviruses to ascertain hybridization to HHV-6, but these probes failed to react. Kishi et al. (1988) showed that an avian herpesvirus, Marek's Disease Virus, shared some weak homology to HHV-6. These data indicated HHV-6 to be a distinct herpesvirus.

Balachandran et al. (1989) generated a panel of monoclonal antibodies against HHV-6 infected cells. From this panel, three monoclonal antibodies reacted with polypeptides of 105 kilodaltons (kd), and 82 kd. These same monoclonal antibodies also precipitated glycoproteins of 116 kd, 38 kd, 36 kd, and 31 kd. The polypeptides may be precursors and/or cleavage products. Whether the peptides were viral, or viral-induced cellular peptides had not been determined.

The research presented here concentrated on determining if the peptides precipitated by the three reactive monoclonal antibodies are virus encoded peptides. This was accomplished by the utilization of copy DNA (cDNA) and genomic (gDNA) libraries. DNA libraries can be useful in analyzing protein sequences generated by particular fragments of DNA. The

system presented here utilized a genetic variant of the bacteriophage lambda known as gtll. This particular variant produces lytic infection and large quantities of fusion proteins are generated. With gtll fusion proteins contain peptides representing a gene segment of interest, plus the EcoRI flanking ends of the ß-galactosidase gene of the phage. This fusion product can then be characterized by reactive antibody to the peptide sequence of interest (Davis et al., 1986). By further understanding the molecular biology of HHV-6, diagnostic and clinical strategies can be developed for disease associated with the virus.

MATERIALS AND METHODS

Cell Line and Virus Stock

A human T-cell line (HSB-2: peripheral blood, acute lymphoblastic leukemia [ATCC CCL 120.1 CCRF-HSB-2]) was used as the cellular source of HHV-6 and viral induced antigens. Cells were grown in RPMI 1640 medium (Sigma Chemical Company) supplemented with antibiotics and 10% heat-inactivated serum supplement (CPSR-1; Sigma). Virus used in this study was human herpesvirus type 6 (prototype strain GS) and was a gift of R. Gallo, D. Ablashi, and S. Salahuddin of the National Cancer Institute. For infection, uninfected HSB-2 cells were centrifuged and the pellets suspended in medium with 5% CPSR-1 at a concentration of 10^6 cells per ml. Cells were mixed with intact HHV-6 infected cells at an uninfected cell-infected cell ratio of 10:1, or with frozen and thawed HHV-6 infected cells. HHV-6 genomic library (bacteriophage lambda gt11) was a gift of S. Josephs of the National Institutes of Health.

Preparation of RNA Materials

Due to the ubiquitous nature of RNases, precautions were taken to assure elimination of RNases with all materials to be used in messenger RNA (mRNA) purification. Mask and gloves were used in all handling of RNA. All glassware and metalware used were washed thoroughly, soaked 24 hr in 0.1% diethylpyrocarbonate (DEPC), autoclaved, and baked overnight at 250° C. These were stored separately from non-RNA use materials. New, individually packaged plasticware was used (pipettes, centrifuge tubes, etc.). Double distilled water (ddH_2O) was treated with DEPC for 24 hr,

autoclaved, and used to hydrate all reagents (Maniatis, Fritsch, and Sambrook, pg. 190, 1982). Chemical reagents purchased were of molecular biology grade and were tested for RNase activity by the manufacturer.

Purification of mRNA

Approximately 5 x 10^6 HHV-6 infected (4 dpi) HSB-2 cells were washed twice with phosphate buffered saline (PBS). Fifteen milliliters of proteinase K buffer (10 mM Tris-HCl, pH 7.5, 1% SDS, 5 mM EDTA, and 1.5 mg proteinase K [added immediately prior to use]) were used to resuspend the pellet, and placed in an ice bath. This was transferred to a Dounce homogenizer and homogenized with 10 strokes. The homogenate was transferred to a polypropylene tube and incubated for 1 hr after which 15 ml of 1 M NaCl were added. The sample was loaded onto a column containing 500 mg of oligo-dT cellulose which was saturated in equilibration buffer (0.5% SDS, 500 mM NaCl, 4 mM EDTA, and 10 mM Tris-HCl, pH 7.5), and heated to 45° C with a heat lamp. Non poly-A cellular material was removed by washing the column with elution buffer until optical density (OD) Å260 was zero. SDS was removed by washing with 20 ml of a solution of 500 mM NaCl, 4 mM EDTA, and 10 mM Tris-HCl, pH 7.5. EDTA was removed by washing with 20 ml of 500 mM NaCl. Poly-A mRNA was eluted from the column with dH_2O and collected into 15 one ml samples (Burnett and Rosenbloom, 1979). Aliquots of each sample were estimated by OD measurement at Å260, and peak samples were pooled (Davis, Dibner, and Battey, pg. 327, 1986). One tenth volume of 3 M sodium acetate (filter sterilized) and 2 volumes 95% ethanol were added and the solution was stored at -20° C overnight to precipitate mRNA. This was centrifuged at 9,000 rpm in a Sorvall HB4 rotor for 1 hr to pellet the precipitate, which was then washed twice with 70% ethanol,

allowed to dry, and stored at -70° C.

In Vitro Translation of mRNA

Nuclease treated rabbit reticulocyte lysate was used for in vitro translation of mRNA samples. Thirty five microliters of lysate, 1 mM amino acid mixture, 5 µl of [^{35}S] methionine (final concentration 1 mci/ml), 2 µl RNA substrate (1 µg/µl), 7 µl DEPC treated dH_2O, containing 7 units of RNAsin were added to the reaction vial. All reagents were handled at 4° C and mixed by inversion. Samples were incubated at 30° C for 30, 60, and 90 min, collected and stored at -70° C (Maniatis, Fritsch, and Sambrook, pg. 347, 1982).

Liquid Scintillation

For liquid scintillation, 2 µl of translate were incubated in 1 ml of 1 N NaOH with 1.5% H_2O_2 at 37° C for 10 min. Four milliliters of ice cold 25% trichloroacetic acid (TCA) were added and the mixture incubated on ice for 30 min to precipitate protein. The solution was passed over a Whatman GF/C filter with aspiration. The filter was next washed with 10 ml of cold TCA (8%), followed by 10 to 20 ml of acetone under vaccum (Burnett and Rosenbloom, 1979).

SDS-PAGE/Autoradiography

Ten microliters of translate were diluted into 40 µl of 1x sodium dodecyl sulfate (SDS) sample buffer (0.625 M tris base, 0.1% SDS, 0.0005% bromo phenol blue, 1% β-mercaptoethanol, and 2% glycerol), boiled for 2 min, and loaded onto SDS-polyacrylamide gel (SDS-PAGE) for electrophoresis and autoradiography (Balachandran, pers. comm.).

Reverse Transcription

On ice, 60 µl, containing 7 µg of purified mRNA, and 12.5 µl of 100 mM methylmercury hydroxide (MeHgOH) were pipetted into an RNase-free vial. The contents were mixed, centrifuged briefly, and allowed to stand 7 min at room temperature. Twenty five microliters of sterile water and 11 µl of β-mercaptoethanol were added. This was mixed, centrifuged briefly, and incubated at room temperature for 5 min. The vial was placed on ice, with the following added in order; 1 µl oligo-dT random primer, 2 µl placental RNase inhibitor, 50 µl 5x RT buffer, 5 µl 25 mM dNTP, 6 µl reverse transcriptase mixed by tapping and followed by a brief centrifugation. The vial was next placed in a 42° C water bath for 90 min.

Ten microliters of 500 mM EDTA and 250 µl of phenol/chloroform were added at 4° C. This was vortexed and centrifuged for 2 min. The bottom phenol/chloroform layer was removed and discarded so as to preserve the interface. To this was added 250 µl of 4 M ammonium acetate (NH_4OAc) and 1 ml of -20° C 100% ethanol. The solution was vortexed vigorously and then frozen on dry ice. After thawing, the solution was centrifuged at 4° C for 15 min. The supernatant was removed with a flamed Pasteur pipette and discarded. The pellet was resuspended by adding 100 µl of sterile water, vortexing, and centrifuging briefly. One hundred microliters of 4 M NH_4OAc and 400 µl of -20° C 100% ethanol were added, mixed, and frozen on dry ice. The solution was thawed, centrifuged for 15 min at 4° C, and the supernatant removed as described above. Five hundred microliters of 80% ethanol were added to the pellet, the tube rocked twice, and centrifuged 5 min. The ethanol supernatant was removed as above. Precaution was taken to prevent the pellet from drying.

Second Strand Synthesis

On ice, 5 µl of 10x second strand buffer, 2.5 µl of 1.0 mg/ml bovine serum albumin (BSA), 1 µl of 10 mM β-NAD^+, 1µl of 5 mM dNTP, 4 µl of RNase H/*Escherichia coli* DNA ligase, and 2 µl of DNA polymerase I were added. The contents were mixed and briefly centrifuged, followed by incubations of 1 hr at 15° C, and 1 hr at room temperature. Immediately after, the solution was heated to 70° C for 10 min, centrifuged briefly, then chilled on ice. Three and one half microliters of T4 DNA polymerase were added with mixing, followed by incubation for 10 min at 37° C. This reaction was stopped by the addition of 2 µl of 500 mM EDTA.

Fifty microliters of phenol/chloroform were added with mixing and centrifuged for 2 min. The lower phenol/chloroform layer was removed and discarded, leaving the supernatant and interface intact. Precipitation was done by the addition of 56 µl of 4 M NH_4OAc and 224 ul of 100% -20° C ethanol followed by freezing on dry ice. The solution was thawed, centrifuged for 15 min at 4° C, and the ethanol removed. Five hundred microliters of 80% ethanol were added to the pellet with rocking and this was centrifuged for 5 min. The ethanol was then removed by aspiration leaving the double stranded cDNA pelleted.

At this juncture many sized species of double stranded cDNA existed in the pellet. Only fragments between 1 kb and 6 kb were to be used for ligation into phage chromosomes. In order to separate cDNA fragments on the basis of size, the cDNA sample was electrophoresed on an agarose gel parallel to ethidium bromide stained markers to visualize size break points.

To the reaction tube was added 10 µl of Tris-RNase A. This was

vortexed, centrifuged briefly, and then incubated for 5 min at 37° C. Five microliters of loading dye were added to the sample and incubated for 5 min at room temperature. The sample was then loaded into the middle lane of the gel, with markers loaded three lanes away. After running the gel until the dye front was one half distance down, electrophoresis was stopped. An asymetrical cut between the sample and the marker lanes was made the length of the gel. The gel section containing the markers was stained in ethidium bromide for 30 min and then observed under UV. The points of 1 kb and 6 kb were marked and the marker gel placed back in its original position with the sample gel. Using the marks from the marker gel as a guide, the sample gel was cut so that only fragments between 1 kb and 6 kb remained in the gel section.

The remaining sample gel section was cut into 5 smaller pieces and loaded into an electroeluter. Electroelution was done at 150 volts for 3 hr. Caution was taken to insure that the gel buffer temperature did not exceed 37° C. At the completion of electroelution the ds cDNA fragments were immobilized on a nitrocellulose membrane. The membrane was washed several times with 350 µl of elution buffer and collected into siliconized tubes. The sample was lyophilized to less than 200 µl. Twenty two microliters of 2 M sodium acetate and 450 µl of -20° C 100% ethanol were added with vigorous vortexing. The sample was frozen on dry ice, thawed, and centrifuged 15 min at 4° C. Ethanol was removed and 100 µl of sterile water, 12 µl of 2 M sodium acetate, and 225 µl of -20° C 100% ethanol were added to the pellet. After thorough vortexing, the sample was frozen on dry ice, thawed and centrifuged for 15 min. After removal of the supernatant, 500 µl of 80% ethanol were added, rocked, and centrifuged for 15 min at 4° C. Ethanol was removed by aspiration.

T4 Polynucleotide Kinase Treatment

The sample pellet was resuspended in 34 µl of sterile water. Added to this was 4 µl of 10x kinase buffer and 2 µl of T4 polynucleotide kinase (5 µg/µl). The contents were mixed, centrifuged briefly, and incubated for 30 min at 37° C. The reaction was stopped by the addition of 2 µl of 400 mM EDTA. Forty microliters of phenol/chloroform were added and followed by a 3 min centrifugation. The phenol/chloroform layer was removed from the upper aqueous phase. Four and one half microliters of 2 M sodium acetate were added along with 90 µl of -20° C 100% ethanol. The sample was frozen, thawed, and centrifuged 15 min at 4° C. Five hundred microliters of 80% ethanol were added with rocking and centrifuged an additional 3 min, followed by aspiration of the ethanol. The cDNA pellet was resuspended in 20 µl of sterile water.

Vector Ligation and Packaging

Recombinant virions (bacteriophage lambda gt11) were assembled using 20 µl of cDNA sample, 8 µl of lambda DNA arms, 4 µl of 10x ligation buffer, 2 µl of T4 ligase, and 6 µl of water. The mixture was incubated overnight at 15° C, then added to 50 µl of packaging extract, and incubated for 2 hr at room temperature. The constructed recombinant phage particles were brought to 500 µl with phage dilution buffer (100 mM NaCl, 10 mM $MgSO_4$, 10 mM Tris-HCl, pH 7.5).

Amplification of Library

Amplification of the library was accomplished in the following manner.

E. coli Y1090 was grown to an O.D. of 0.8 (Å600). Phage were incubated at 5 x 10^5 pfu with 200 µl of the E. coli for 15 min, vortexed, and plated on NZB (20 plates). These were incubated for 9 hr at 37° C. Five milliliters of phage dilution buffer was put onto each plate and the plates sealed with parafilm. The plates were rocked overnight at 4° C to elute phage. Phage were then collected into one container. Each plate was washed with an additional 3 ml of phage dilution buffer and collected into the container. Eight milliliters of chloroform were added to the container with mixing and incubated 15 min at room temperature. Phage were then centrifuged 15 min at 3000 x g to separate chloroform and cellular debris from the phage suspension (Maniatis, Fritsch, and Sambrook, pg. 293, 1982).

Titrations were performed with logarithmic dilutions from 10^{-5} through 10^{-9} on NZY plates in the presence of 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-Gal). Incubation was done overnight at 37° C. Samples were aliquoted into 1.5 ml volumes and, for long term storage, into 15 ml volumes with 7% DMSO and stored at -70° C (Maniatis, Fritsch, and Sambrook, pg. 66, 1982).

Screening of Libraries

E. coli Y1090 was grown overnight at 37° C with aeriation in LB medium (Bacto-tryptone 10 g, Bacto-yeast extract 5 g, NaCl 10 g/l) supplemented with 0.2% filter sterilized maltose (Davis, Dibner, and Battey, pg. 40, 1986). Cells were centrifuged for 10 min at 400 x g. Two hundred microliters of pellet were placed into 4 ml of fresh LB/maltose and incubated 1 hr at 37° C. LB agar plates and top agar overlays were equilibrated to 42° C. Dilution of phage was 2 x 10^5 pfu/ml. Ten microliters of phage dilution and 200 µl of Y1090 were vortexed, then

added to 3 ml of liquid top agar, vortexed again, then poured onto plates. After solidifying the plates were incubated 3.5 hr at 42° C. During the interim, nitrocellulose disks were soaked 1 hr in 10 mM isopropyl-B-D thiogalactopyranoside (IPTG) and allowed to dry.

After incubation, a disk was overlayed onto each plate and its position marked. This was incubated for 3 hr at 37° C. At room temperature the disks were removed and washed 3x in washing buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.3% Tween-20). Blocking was allowed overnight (10 mM Tris pH 7.5, 150 mM NaCl, 5% powdered milk). The disks were washed again in washing buffer 3x, then primary antibody (2D6 or 9A5) was added (diluted 1:100 in washing buffer) and incubated for 2 hr with agitation. After recovering primary antibody, and another 3x washing was done, secondary antibody (alkaline phosphotase labeled goat anti-mouse diluted in washing buffer according to manufacturer's directions [HyClone, Corp.]) was added and incubated for 2 hr with agitation. Disks were washed and rinsed in rinse buffer (100 mM Tris pH 8.8, 1 mM MgCl) for 10 min. Substrate (50 µl 5-bromo-4-chloro-3-indoyl-phosphate [100 mg/ml DMSO], 10 mg nitro blue tetrazolium, 100 ml rinse buffer) was added and allowed to react. Distilled H_2O was added to stop the reaction (Huynh, Young, and Davis, pg. 73, 1984). Isolated clones were picked, vortexed in chloroform, then centrifuged briefly. Virus was removed and amplified in LB/Maltose as described above. This process was repeated three times to assure clonal isolation.

Generation of Fusion Protein Lysates

Bacteria were grown overnight in LB/maltose with agitation. One milliliter was added to 20 ml of fresh LB/maltose and incubated at 37° C

with agitation until O.D. was between 0.8 and 1.0 at Å600. Eighteen milliliters were centrifuged 10 min at 400 x g, the supernatant was decanted, and the pellet was resuspended in 8 ml of fresh LB/maltose equilibrated at 42° C. To this was added 2 x 10^{10} phage particles and vortexed thoroughly. Five milliliters of fresh broth equilibrated at 42° C were added and mixed. Next, 260 µl of 500 mM IPTG were added. At 30 and 60 min intervals, 1.4 ml were removed into each of four Eppendorph tubes. These were centrifuged at 12,000 x g in a microfuge for 5 min. Pellets were resuspended in sample buffer, boiled for 3 min, and stored at -70° C (Huynh, Young, and Davis, pg. 77, 1984) prior to Western blot.

Analysis of Fusion Proteins by Western Blot

Immediately after completing SDS-PAGE, the gel was removed from the glass plate sandwich and placed into cold (4° C) transfer buffer (12 g Tris base, 57.65 g glycine, 4 l H_2O, 1 l methanol, pH 8.3) for 10 min. On ice, gel was sandwiched with nitrocellulose in a cassette. Proteins were transferred for 2 hr at 4° C in transfer buffer. Nitrocellulose was soaked overnight in blocking buffer, followed by the addition of primary antibody (diluted 1:100 in blocking buffer) for 3 hr at room temperature. The filter was next washed for 1 hr in washing buffer with three changes of buffer. Secondary antibody was incubated for an additional 3 hr and then washed as before. Rinse buffer was incubated for 15 min, then substrate was allowed to react. Distilled H_2O was used to stop the reaction (Davis, Dibner, and Battey, pg 311, 1986).

RESULTS

Infection of the suspension culture progressed for four days. Light microscopy revealed cellular aggregation in the HHV-6 infected cultures in contrast to the uninfected cells which remained evenly dispersed. Cells from infected cultures showed enlargement as compared to uninfected controls.

Of the twelve samples of mRNA collected from the oligo-dT elution column, samples 3, 4, 5, 10, 11, and 12 had Å260 peaks greater than 0.006 (Table 1). These were pooled and, after pelleting, were resuspended into 1 ml of dH_2O. A second OD reading was taken and the Å260 was 0.121. This resulted in a net yield of 1.21 µg/µl mRNA.

Analysis of translates revealed that peak counts occurred at the 60 min interval. The positive control (brome mosaic virus mRNA) yielded the highest activity at nearly 140,000 cpm, with the experimental sample showing more reactivity (>80,000 cpm) than the negative control (<50,000 cpm). This pattern was consistent throughout all but the 90 min samples at which time the negative control was higher than the sample mRNA (Figure 1).

SDS-PAGE did indicate many translational products from the sample mRNA, ranging from below 45 kd to above 116 kd (Figure 2, Lane 2). The positive control (brome mosaic virus mRNA) also showed banding, but at different points and intensities than the sample (Figure 2, Lane 3). The negative control revealed 3 distinct bands (Figure 2, Lane 1).

Assembly of the cDNA library resulted in a titer of approximately 2.0 x 10^6 pfu/ml, with recombinants occurring at approximately 15 percent

Table 1

Optical density reading of mRNA samples collected from HHV-6/HSB-2 lysates.

Sample No.	Absorbance
1	0.001
2	0.002
3	0.006*
4	0.012*
5	0.008*
6	0.002
7	0.001
8	0.002
9	0.002
10	0.012*
11	0.021*
12	0.012*

Samples were eluted from oligo-dT column with dH_2O.
A260 readings greater than or equal to 0.006 were pooled into a single sample and concentrated by lyophilization.
* Samples collected into a single pool.

Figure 1

Translational activity of mRNA sample as measured by liquid scintillation. Results are expressed as counts per minute. +C - Brome mosaic virus mRNA; -C - No mRNA; Ex - mRNA sample purified according to Materials and Methods.

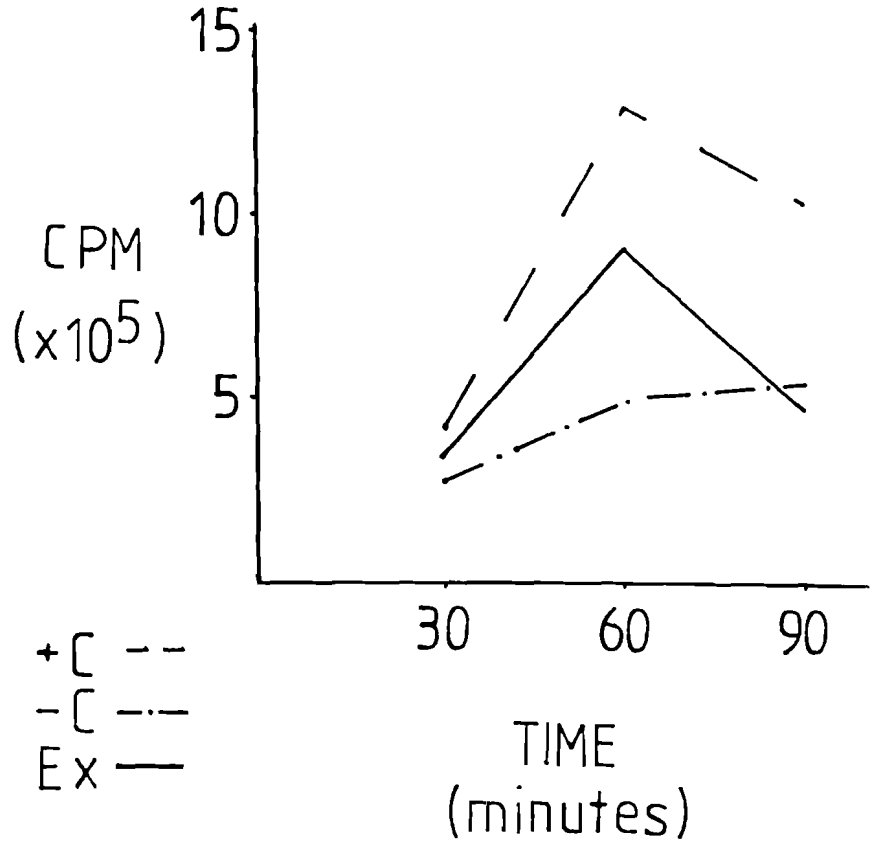

15
10
5
CPM
(x10^5)
30
60
90
TIME
(minutes)
+C
-C
Ex

Figure 2

Translational activity of mRNA sample as measured by SDS-PAGE/autoradiography. Exposure was 10 days at -70° C. White areas represent peptides that had incorporated [^{35}S] methionine. -C - No mRNA; Ex - mRNA sample purified according to Materials and Methods; +C - Brome mosaic virus mRNA. Weights are expressed in kilodaltons.

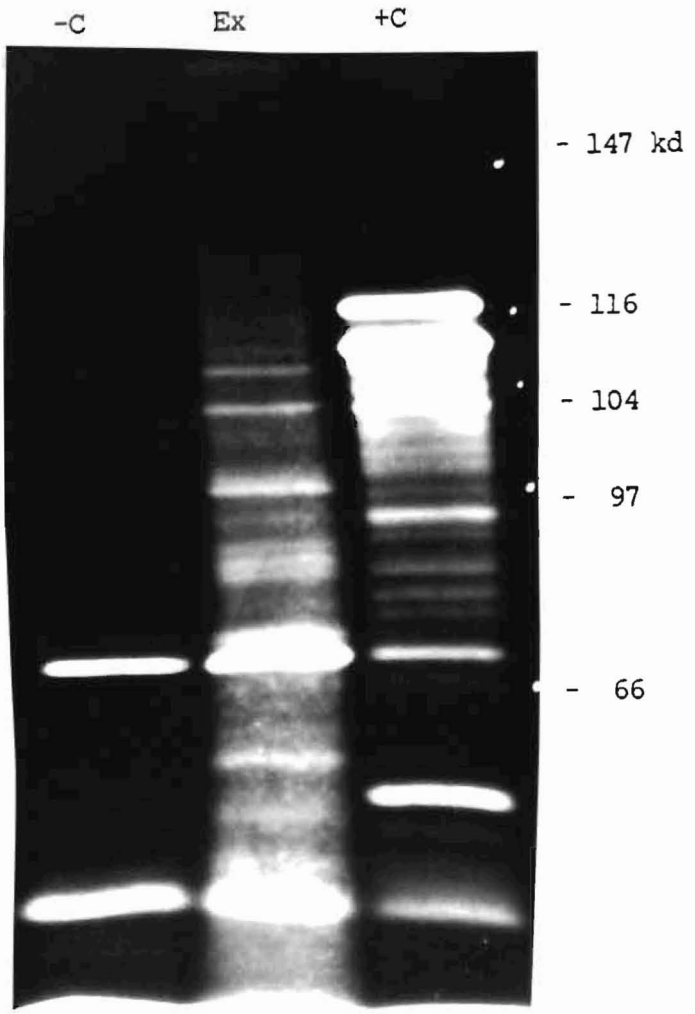

-C
Ex
+C
- 147 kd
- 116
- 104
- 97
- 66

Table 2

Occurrence of recombinant phage particles from cDNA library.

Group	Ratio	Percent
Control #1	0/197	0.0
Control #2	0/189	0.0
Sample #1	29/192	15.1
Sample #2	33/211	15.6

Plaque counts determined with phage particles grown overnight in the presence of X-gal. Ratio calculations: recombinant plaques (clear) / non-recombinant plaques (blue).

Table 3

Occurrence of recombinant phage particles from gDNA library.

Group	Count	Percent
Control #1	0	0.0
Sample #1	326	16.3
Sample #2	262	13.1

Plaque counts determined with phage particles grown overnight in the presence of X-gal. Ratio calculations: recombinant plaques (clear) / 2000 pfu per plate.

Table 4

Estimated occurrence of 2D6-reactive recombinant phage particles from gDNA library.

Group	Reactive	Percent
Control #1	1160	58
Sample #1	1240	62
Sample #2	1120	56

Counts determined with phage particles grown with nitrocellulose/IPTG saturated disk overlay. Primary antibody was diluted 1:100 and allowed to react for 2 hr prior to adsorbtion to reduce background. Secondary antibody was diluted according to manufacturer's instructions. Ratio calculations: recombinant plaques (clear) / 2000 pfu per plate.

Figure 3

Nitrocellulose disk from fourth screening with monoclonal antibody 2D6. Primary antibody was diluted 1:100 and allowed to react for 2 hr. Secondary antibody was diluted according to manufacturer's directions. Dark spots indicate reactive plaques.

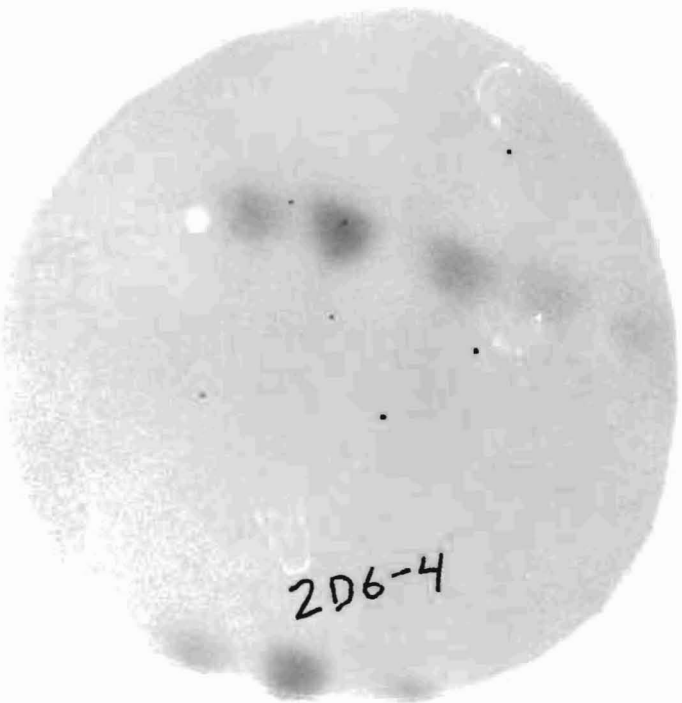

2D6-4

Figure 4

Nitrocellulose disk from fourth screening with monoclonal antibody 9A5. Primary antibody was diluted 1:100 and allowed to react for 2 hr. Secondary antibody was diluted according to manufacturer's directions. Dark spots indicate reactive plaques.

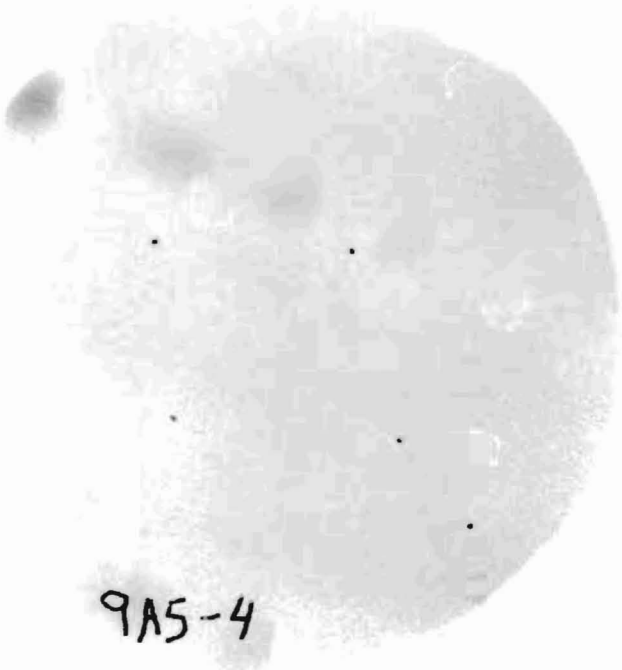

9A5-4

Figure 5

Western blot of fusion proteins with monoclonal antibody 2D6. Primary antibody was diluted 1:100 and allowed to react for 2 hr. Secondary antibody was diluted according to manufacturer's directions. NR1 - Non recombinant phage; gC2D6 - genomic clone 2D6; gC9A5 - genomic clone 9A5.

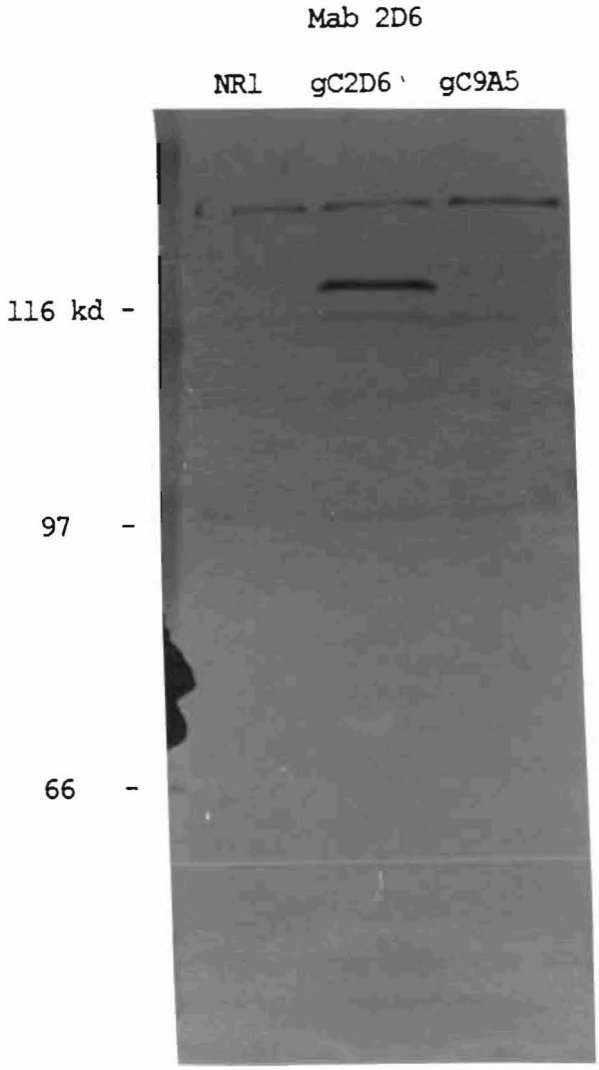

Mab 2D6
NR1
gC2D6
gC9A5
116 kd
97
66

Figure 6

Western blot of fusion proteins with monoclonal antibody 9A5. Primary antibody was diluted 1:100 and allowed to react for 2 hr. Secondary antibody was diluted according to manufacturer's directions. NR1 - Non recombinant phage; gC2D6 - genomic clone 2D6; gC9A5 - genomic clone 9A5.

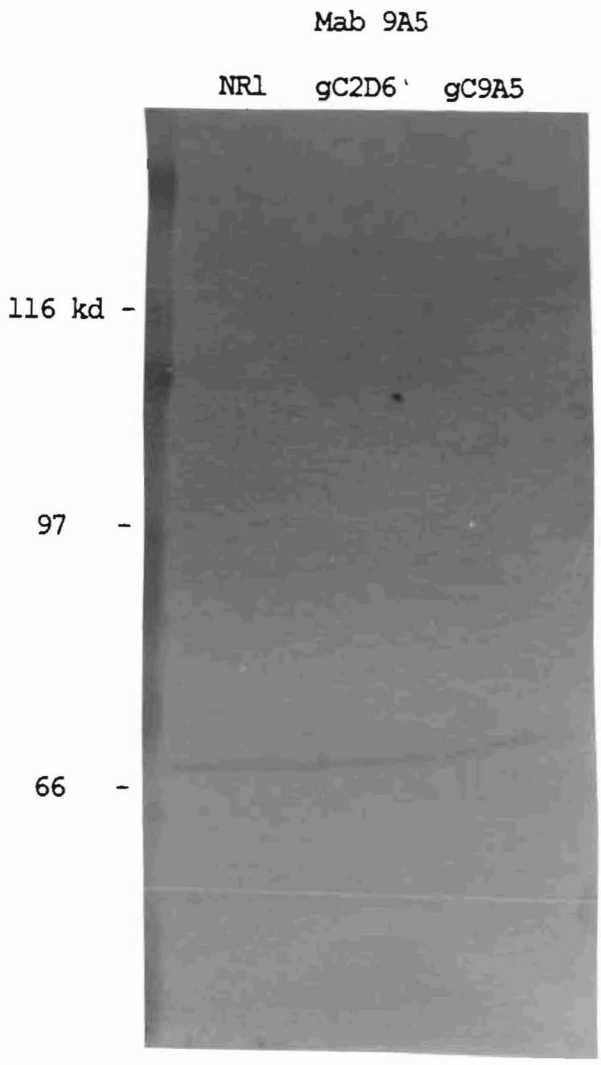
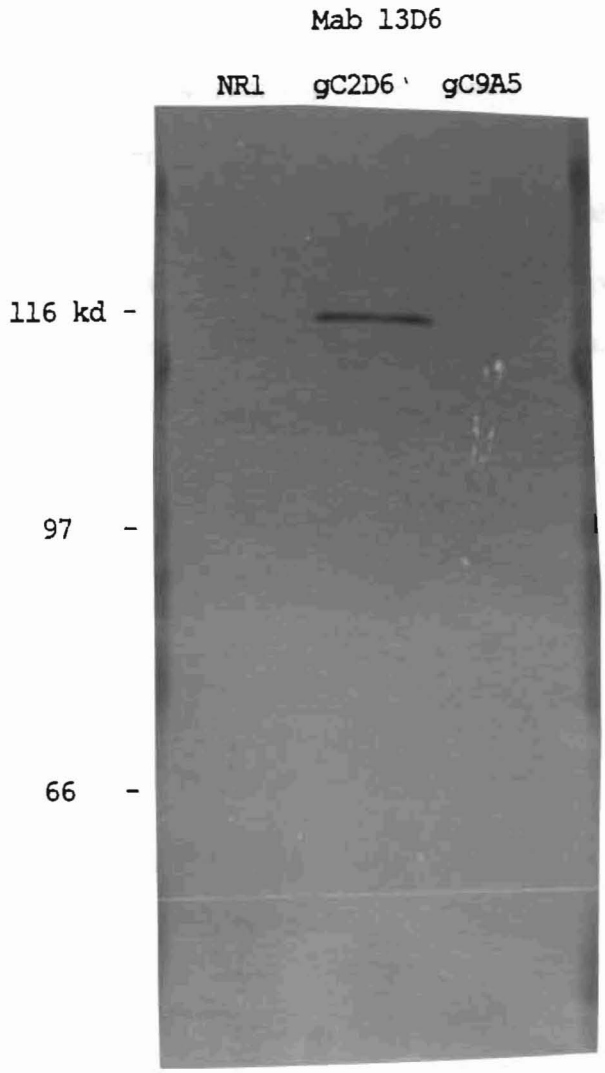

Mab 9A5
NR1
gC2D6
gC9A5
116 kd -
97 -
66 -

Figure 7

Western blot of fusion proteins with monoclonal antibody 13D6. Primary antibody was diluted 1:100 and allowed to react for 2 hr. Secondary antibody was diluted according to manufacturer's directions. NR1 - Non recombinant phage; gC2D6 - genomic clone 2D6; gC9A5 - genomic clone 9A5.


Mab 13D6
NR1
gC2D6
gC9A5
116 kd
97
66

(Table 2). Screenings of the cDNA library failed to reveal any clones that were reactive with 2D6 or 9A5.

Titration of the gDNA library showed recombinants in the approximate range of 13 to 16 percent (Table 3). First screenings of the gDNA library with antibody indicated many possible recombinant phages (Table 4). First screenings were repeated with the same antibody dilution and revealed several candidate clones. After four serial screenings, clones were isolated with both antibody species. Clone gC-2D6 was isolated by monoclonal antibody 2D6 (Figure 3), and clone gC-9A5 was isolated by monoclonal antibody 9A5 (Figure 4).

Western blots of fusion protein lysates showed antibody 2D6 reacted strongly with a gC-2D6 fusion product of 117 kd (Figure 5, Lane 2), but did not react with products of gC-9A5 (Figure 5, Lane 3) or a non-recombinant phage (NR1) (Figure 5, Lane 1). Antibody 9A5 failed to react with fusion products of the 3 lysates (Figure 6). Another monoclonal antibody (13D6) reacted identically to that of 2D6 (Figure 7).

DISCUSSION

The five previously identified human herpesviruses are classified in accordance to a variety of characteristics including differences in DNA structure, host range, and in vitro rapidity of growth (Roizman and Batterson, pp. 497-526, 1985). Although proteins from these viruses have differing structures and functions, they do possess common biological features such as morphology, mode of entry into host cells, nuclear location of DNA replication, and virus assembly from the nucleus (Balachandran et al. 1989). Human herpesvirus 6 is distinct yet shares these common biological features and has been classified as a herpesvirus (Josephs et al., 1986; Salahuddin et al., 1986; Ablashi et al., 1987).

Because HHV-6 shares these common biological features, it is likely that HHV-6 parallels the other herpesviruses in the molecular events that occur during viral replication. The replicative cycle of HHV-6 has yet to be clearly defined at the molecular level. However, some inferences may be drawn from the replicative cycle of the other herpesviruses.

Herpes simplex virus (HSV) replication has been well documented. After adsorption into the cytoplasm, the capsid is separated from HSV's core, and the core then enters the nucleus where viral replication commences. The eclipse period begins and the virus replicates exponentially. Each infected cell may produce 10^4 to 10^5 particles of which some 100 are infectious. Virus is shed slowly at first by budding, then rapidly as the cell succumbs to the pathological membrane stress caused by the exitting virions (Dulbecco and Ginsberg, pg. 1064, 1980.).

Replication of HSV is sequentially regulated into three stages, immediate early (alpha mRNA), delayed early (beta mRNA), and late (gamma mRNA). Alpha and beta transcripts code for enzymes that are responsible for carrying out replication. Gamma mRNAs code for the structural proteins that will be assembled into functional virions, some of which will be displayed at the surface of cells that the new virions infect. The targets of the monoclonal antibodies used in this research were the proteins encoded by gamma mRNAs. The occurrence of these transcripts is coordinately regulated, i.e., alpha mRNA must be transcribed before beta mRNA, and beta mRNA must be transcribed before gamma mRNA. With HSV, these events occur within 24 hr in cell culture (Dulbecco and Ginsberg, pg. 1064, 1980). It has yet to be demonstrated that HHV-6 replicates in precisely this manner, but it would be consistent with that of the other human herpesviruses.

Microscopic examination of HHV-6 infected cultures showed characteristic herpesvirus cytopathic effect at 4 days post infection (dpi). Because gamma mRNA is present at this point in HSV infected cells, and because little is known about HHV-6 replication, it was postulated that gamma mRNA would be present at this point in the HHV-6 cycle. Messenger RNAs were harvested and determined to contain virus encoded transcripts via autoradiography.

The mRNAs harvested from HHV-6 infected cells were converted into double stranded DNA, then inserted between two lambda gt11 arms cut at the sole EcoRI locus. This restriction site is within the β-gal gene, and the recombination event renders the β-gal product dysfunctional. The product is a fusion protein and contains a peptide representative of the cDNA flanked on either side by the β-gal protein.

The lambda phage host, E. coli Y1090, is lac-, and cannot synthesize the enzyme β-galactosidase (β-gal). Non-recombinant lamda phage gt11 possesses a functional β-gal gene that supplies Y1090 with the enzyme. When plated in the presence of X-gal, non-recombinant phage particles produce a blue plaque while recombinant phage particles, containing the dysfunctional β-gal product, will produce a clear plaque. This phenomenon can be utilized in determining recombinant phage particle percentages (Maniatis, Fritsch, and Sambrook, pg. 294, 1982).

Y1090's lac operon is thermoinducible. By incubating phage and bacteria at 42° C, the lac suppressor was active, preventing transcription but not affecting phage DNA replication. Many copies of the phage DNA were made during the 3.5 hr incubation. IPTG inhibits the suppressor. Its addition to the plate allowed transcription and translation to occur resulting in a burst of protein synthesis. The proteins that were synthesized after the introduction of the IPTG were liberated onto the nitrocellulose which has an affinity for protein. Monoclonal antibodies were used to detect the presence of antigen from the fusion product of the β-gal gene and the foreign cDNA segment (Huynh, Young, and Davis, 1984).

After several screenings of the cDNA library, no clones were detected with the monoclonal antibodies 9A5 or 2D6. Although autoradiograms of in vitro translates revealed the presence of many mRNAs, no reactive clones were detected with the monoclonal antibodies 9A5 or 2D6. Autoradiography is not capable of distinguishing between alpha, beta, or gamma mRNA. These data indicate that HHV-6 gamma mRNA synthesis does not commence until after 4 dpi. Subsequent data suggest this to be accurate (Balachandran, unpublished data). Because of the presence of recombinant clones in the cDNA library, it is likely that these particles contain alpha

and/or beta DNA and may be of use in future studies.

First screening of the gDNA library revealed many possible recombinant clones, some of which were false positives. The source of the monoclonal antibodies was mouse peritoneal ascitic fluid which naturally contains antibodies that react to E. coli, the host for the phage vectors. After adsorbing antibody on the first screening, subsequent screenings were free of false positives.

Three additional screenings were performed to isolate single clones of phage reactive to each monoclonal antibody. Clones were isolated by both antibodies tested, 2D6 and 9A5. These clones were amplified for further analysis by Western blot.

The Western blot of the fusion protein lysates gave strong indication of specific binding. Antibodies 13D6 and 2D6 reacted strongly with a fusion product of 117 kd, indicating that these two antibodies are probably identical in their antigen binding. Whether they bind the same determinant can not be ascertained with these data. Monoclonal antibody 9A5 failed to react on Western blot even though it detected antigen during screenings. Why this is so is not clear. Preparation of the fusion proteins for Western blot may have altered the peptide's tertiary structure after fusion protein generation thus preventing the antibody to recognize its determinant.

Balachandran et al. (1989) determined that these monoclonal antibodies bind many different sized peptides from infected cells. All of these peptides were smaller than 117 kd, indicating that there may be post translational processing of the peptides. Whether these peptides were viral encoded or viral influenced cellular products had not been determined in previous research. However, data presented here strongly indicate that

monoclonal antibodies 2D6 and 13D6 bind virus encoded protein and monoclonal antibody 9A5 probably binds virus encoded protein. It must be noted that the fusion protein used in the Western blot not only contains an HHV-6 peptide, but also the complete β-gal protein flanking both ends of the HHV-6 peptide which influences migration on SDS-PAGE. These results strongly indicate that the three monoclonal antibodies described by Balachandran et al. (1989) react with virus encoded proteins found at the surface of infected cells. Moreover, two of these antibodies (2D6 and 13D6) may be candidate immunological reagents for the diagnosis and/or assessment of HHV-6 infection by Western blot. The third antibody (9A5) may also be of use in detection of HHV-6.

It is intriguing that HHV-6 has eluded identification for so long. This may be related to its lymphotrophism, latency, frequency of reactivation, and detection systems used. In naturally occurring infections with herpesviruses, severe harm to the host is not generally associated with infection, and this appears to be congruent with HHV-6. In relation to AIDS, if these naturally occurring infections trigger HIV expression in vivo, the host's immune system could break down thus giving the herpesviruses greater opportunity to flourish at the host's expense. This, in turn, would further stimulate HIV production through transactivation and the resultant cascade from the viruses' synergism could result in mortality. It has yet to be determined whether the presence of HHV-6 in AIDS is a reactivation of a latent stage due to the immunocompromised state of a HIV infected individual or if the virus plays an active role in the disease progression.

In order to further understand the relationship HHV-6 has to human populations, more research must be performed. The site(s) at where HHV-6

establishes *in vivo* latency needs to be determined. Certain cells of the immune system do harbor HHV-6. However, there are probably other tissues susceptible to the virus. The DNA sequences contained within the DNA libraries can be used to generate nucleic acid probes which can precisely locate virus infected tissues.

Clearly, HHV-6 is at least associated with human disease and, at most, may be a root cause of some maladies. Evidence of HHV-6 infection has been shown in chronic fatigue syndrome, AIDS, and some forms of cancer. Whether HHV-6 is a cause or an effect in these diseases remains to be determined. If so, the reagents tested here may prove to be of diagnostic value in the management of HHV-6 infection.

SUMMARY

1. The cDNA library yielded some recombinant phage particles. However, none were reactive with the monoclonal antibodies 13D6, 2D6, or 9A5.

2. The gDNA library yielded many recombinant phage particles that expressed antigen strongly reactive on Western blot with antibodies 13D6 and 2D6, but no reactivity to antibody 9A5.

3. Antibodies 2D6 and 13D6 bind virus encoded protein and not virus influenced cellular protein.

4. Antibody 9A5 probably binds virus encoded protein, but definitive proof is lacking.

5. These antibodies may be of use for diagnostic testing for HHV-6 infections.

6. The libraries can be used to pinpoint loci of genes on the HHV-6 chromosome.

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Analysis of cDNA and gDNA Libraries of Human Herpesvirus 6 By Monoclonal Antibodies To Virus Infected Cells

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