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Identification, Characterization, and Sequence Analysis of a cDNA Encoding a Phosphoprotein of Human Herpesvirus 6

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Human herpesvirus 6 (HHV-6)-specific monoclonal antibody (MAb) 9A5D12 reacted with the nucleus of HHV-6 strain GS-infected cells and immunoprecipitated a phosphorylated polypeptide with an approximate size of 41 kDa, designated HHV-6 P41. A 110-kDa polypeptide was also immunoprecipitated by the MAb. These polypeptides were synthesized early in infection, and the synthesis was greatly reduced by phosphonoacetic acid. Polypeptides with identical sizes were recognized by the MAb from cells infected with an additional eight HHV-6 strains. A 2.1-kb cDNA insert was identified from an HHV-6(GS) cDNA library constructed in the λgt11 expression system by using MAb 9A5D12. This cDNA insert hybridized specifically with viral DNA from HHV-6 strains GS and Z-29 and with two predominant transcripts with approximate sizes of 2.5 and 1.2 kb from infected cells. The reactivity of the MAb with a fusion protein expressed in the prokaryotic vector suggested that the cDNA encodes a 62- to 66-kDa protein. Analysis of the nucleotide sequence of the cDNA insert revealed a 623-amino-acid-residue single open reading frame of 1,871 nucleotides, with an open 5′ end. The predicted polypeptide is highly basic and contains a long stretch of highly hydrophobic residues localized to the carboxy terminus. The amino-terminal half of the predicted HHV-6 protein from the cDNA shows significant homology with the UL44 gene product of human cytomegalovirus, coding for the ICP36 family of early-late-class phosphoproteins. Two TATA boxes are located at nucleotide positions 668 and 722 of the cDNA. In vitro translation of RNA transcribed in vitro from the cDNA resulted in the synthesis of a 41-kDa polypeptide only. This polypeptide was readily immunoprecipitated by MAb 9A5D12, and its partial peptide map was identical to that of the 41-kDa polypeptide detected in infected cells. Together, these results indicate that the HHV-6 P41 is encoded within a gene coding for a larger protein.

Human herpesvirus 6 (HHV-6) is a newly identified herpesvirus, initially isolated from peripheral blood lymphocytes (PBL) of patients with lymphoproliferative disorders and AIDS (43). Several related herpesviruses were subsequently isolated from PBL of children with exanthem subitum (25, 39, 47, 49) and AIDS (2, 4, 15, 34, 48), from kidney and liver transplant patients (5, 37, 52, 53), and from an infant with fatal fulminant hepatitis (6). Isolation and seroconversion have strongly implicated its etiological role in exanthem subitum, and the seroprevalence in the normal population is >80% (20, 23, 25, 26, 38, 47, 49). HHV-6 has also been isolated from the cell-free saliva of healthy adults and human immunodeficiency virus type 1-seropositive individuals (19, 31). Newer virus isolates were initially identified as HHV-6 by their hybridization to a 9-kb DNA probe (pZVH14) from the HHV-6 prototype strain GS [HHV-6(GS)] (1, 2, 4, 15, 25, 34, 48, 49), and restriction site heterogeneity among isolates has been reported (1, 4, 24, 25, 34). HHV-6 isolates infect $CD4^+$ human T cells and T-lymphocyte-derived cell lines and do not infect cultured monolayer fibroblast or epithelial cells from humans or animals. The various isolates have been shown to differ in growth properties and in the phenotype of T-cell lines that were infected (1, 3, 4, 15, 34, 54).

The DNA of HHV-6(GS) is estimated to be approximately 170 kb (24), sufficient to code for more than 70 proteins. Knowledge about the structure of the HHV-6 genome, gene organization, gene expression, regulation, coding proteins, and their functions is limited. Recently, a sequence of 21,858 bp from the genome of HHV-6 strain U1102 [HHV-6(U1102)] has been determined (29). The sequence has a mean composition of 41% G:C, and 17 open reading frames have been predicted. The predicted gene products of nine of these open reading frames have been shown to be homologous to a set of gene products which are conserved in all other herpesviruses sequenced, and sequence analysis shows that HHV-6 is closely related to cytomegalovirus (CMV) (29, 33).

We have previously identified several proteins and glycoproteins specific to HHV-6(GS)-infected cells, and monoclonal antibodies (MAbs) to HHV-6 proteins have been generated (7). Identification of the genes encoding these proteins is an initial step towards a better understanding of the structure and function of these proteins. In this report, we describe the characterization of a 41-kDa phosphoprotein of HHV-6 strain GS (HHV-6 P41) that is conserved among all eight HHV-6 strains examined to date. We also report the characterization of a cDNA identified from an HHV-6(GS) cDNA library constructed in the λgt11 expression system. The cDNA insert was used to map the gene on the genomes of HHV-6 strains GS and Z29 and to determine the number and size of transcripts in infected cells. We have determined the nucleotide sequence of the cDNA insert and have characterized the cDNA further by in vitro transcription and translation and by the expression of fusion protein in *Escherichia coli*. Our data suggest that the HHV-6 P41 is encoded within a gene coding for a larger protein. Furthermore, the amino-terminal half of the predicted HHV-6 protein from the cDNA shows strong homology with the human CMV (HCMV) UL44 gene product, coding for the ICP36 family of proteins.

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MATERIALS AND METHODS

Cells and viruses. Suspension cultures of human T-cell lines HSB-2 (ATCC CCL 120.1. CCRF-HSB-2), Molt-3 (ATCC CRL 1552), and phytohemagglutinin-stimulated human cord blood lymphocytes (CBL) (3) were used for virus propagation. Cells were grown in RPMI 1640 medium (Sigma, St. Louis, Mo.) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Nine HHV-6 strains were used in this study. HHV-6 prototype strain GS [HHV-6(GS)] was a gift from R. C. Gallo, National Cancer Institute, Washington, D.C., and HHV-6(Z29), originally isolated from a Zairian AIDS patient (34), was a gift from P. Pellett, Centers for Disease Control, Atlanta, Ga. HHV-6 strains DA, OK, DC, Co2, Co3, Co5, and Co6 were gifts from D. V. Ablashi, National Cancer Institute. HHV-6(DA) was isolated from a patient with chronic fatigue syndrome (2). HHV-6(OK) was from a child with exanthem subitum (25), and HHV-6(DC) was isolated from a leukopenia patient. HHV-6 Co2, Co3, Co5, and Co6 were originally from the laboratory of G. R. Krueger, University of Cologne, and were isolated from PBL of patients with unclassified collagen vascular disease (Co2), systemic lupus erythematosus with facial rash (Co3), chronic fatigue syndrome (Co6), and a healthy adult (Co5).

HSB-2 cells were used for routine propagation of HHV-6 strains GS, DA, Co2, Co3, Co5, and Co6; these strains did not replicate in Molt-3 cells. CBL were used for routine propagation of HHV-6 strains Z29, OK, and DC. HHV-6(Z29) was also grown in Molt-3 cells. Infection was carried out by mixing 10^6 uninfected cells per ml with HHV-6-infected cells at a ratio of 5:1 (7). Infected cells were collected at the peak of cytopathic effect (day 6 to 12 postinfection [p.i.]), and 10^7 cells were suspended in 1 ml of medium and frozen and thawed twice. Cell debris was removed by centrifugation, and the supernatant fluid was used as the virus inoculum for radiolabeling procedures. Infectivity titers were measured as described before (7) and are expressed as 50% tissue culture-infective dose ($TCID_{50}$). HHV-6(GS) purified by continuous-flow centrifugation on 10 to 60% sucrose gradients (24) was a gift from S. F. Josephs, National Cancer Institute, Washington, D.C.

Antibodies and indirect immunofluorescence assay. The production and characterization of MAbs and rabbit polyclonal antibodies against HHV-6(GS)-infected cells have been described earlier (7). High-titer ascitic fluids of MAb 9A5D12 (immunoglobulin G2a [IgG2a] isotype) were used in these studies. Acetone-fixed, uninfected, and HHV-6-infected cells were used for the indirect immunofluorescence assay (IFA). Cells were collected, washed in phosphate-buffered saline (PBS, pH 7.4), air-dried on slides (5 mm inner diameter, 10 circles per slide; Roboz Surgical Instrument Co., Washington, D.C.), and fixed in cold acetone for 10 min. Fixed cells were incubated with twofold dilutions of MAbs beginning at 1:10 for 30 min at 37°C. After incubation, slides were washed with PBS and further incubated with a prestandardized dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (HyClone Laboratories, Logan, Utah) for 30 min at 37°C. After washing, slides were mounted with 50% (vol/vol) glycerol in PBS and examined under an Olympus fluorescence microscope.

Radiolabeling procedures, RIP, and SDS-PAGE. Infection was carried out at 10 $TCID_{50}$/cell; 10^6 uninfected cells were mixed with virus and incubated at 37°C for 2 h. Unabsorbed virus was removed by centrifugation, and the cells were further incubated at 37°C. On day 3 p.i., uninfected and infected cells were washed once with PBS, and 10^7 cells were labeled for 20 h with 250 μCi of [^{35}S]methionine (specific activity, 1,072 Ci/mmol; NEN Du Pont, Wilmington, Del.) or with 1 mCi of $^{32}P_i$ (H_3PO_4; specific activity, 8,500 Ci/mmol; NEN Du Pont). In studies to determine the kinetics of HHV-6(GS) protein synthesis, after virus absorption for 2 h (time zero), infected cells were labeled for different lengths of time. In studies with viral DNA synthesis inhibitor, after virus absorption, HHV-6(GS)-infected cells were incubated for various times with phosphonoacetic acid (PAA; 300 μg/ml; Sigma).

Radioimmunoprecipitation (RIP) was carried out essentially as described previously (7, 9, 10). Cells were solubilized with lysing buffer (0.05 M Tris hydrochloride, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 100 U of aprotinin per ml, 0.1 mM phenylmethylsulfonyl fluoride), sonicated, and centrifuged at 100,000 × *g* for 1 h. Equal amounts of trichloroacetic acid-precipitable radioactivity (5×10^5 cpm) from control and virus-infected cell lysates were mixed with 10 μl of antibodies and 100 μl of protein A-agarose beads (Genzyme, Boston, Mass.), and samples were mixed continuously at 4°C for 2 h. The immunoprecipitates were collected, washed, dissociated by boiling in sample buffer, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 9% acrylamide cross-linked with 0.28% *N*,*N*′-diallyltartardiamide (DATD). Molecular weight markers (Sigma) were electrophoresed in parallel lanes. Gels were stained, destained, infused with 2,5-diphenyloxazole, dried on filter paper, and placed in contact with XAR-5 film at −70°C for fluorography.

Cleveland partial proteolysis of proteins. Immunoprecipitated proteins were electrophoresed as described above, the unfixed gels were dried on a filter paper, and polypeptide bands were located by using X-ray film. Polypeptide bands were excised and rehydrated, and samples were treated with 2 and 10 μg of V8 protease (Sigma), followed by SDS-PAGE in 15% acrylamide (17). Gels were infused with 2,5-diphenyloxazole, dried on filter paper, and placed in contact with XAR-5 film at −70°C for fluorography.

Western immunoblot. Protein samples were separated by SDS-PAGE in acrylamide cross-linked with DATD and electrophoretically transferred to nitrocellulose sheets (7, 9). Standard prestained molecular weight markers (Sigma) were included in parallel lanes. The nitrocellulose sheets were treated overnight with blocking buffer (10 mM Tris-HCl [pH 7.2], 0.15 M NaCl, 5% skimmed milk) and reacted for 2 h at room temperature with blocking buffer containing MAbs. The sheets were washed with washing buffer (10 mM Tris-HCl [pH 7.2], 0.15 M NaCl, 0.3% Tween 20) and finally incubated for 3 h with alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (HyClone Laboratories). Bound anti-mouse IgG antibodies were detected by using 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Sigma) (7, 9).

Construction of cDNA library and screening of λgt11 expression system. cDNA was constructed from polyadenylated RNA obtained from HHV-6(GS)-infected HSB-2 cells with a commercial cDNA synthesis system according to the manufacturer's recommendations (Invitrogen, San Diego, Calif.). Internal *Eco*RI sites were methylated with *Eco*RI methylase and *Eco*RI linkers were attached to the cDNAs. The cDNA was then ligated into the *Eco*RI sites of predigested λgt11 arms. The ligated cDNAs were packaged into phage heads and amplified by infection of *E. coli* Y1090 (r^-) (22). The cDNA library was screened with MAbs by the

procedures described by Huynh et al. (22). Immunoreactive phages were picked and purified by four subsequent steps of cloning and screening. Phage DNA containing the cDNA insert was prepared from plate lysates as described by Maniatis et al. (35). This recombinant phage DNA was digested with *Eco*RI, and the cDNA was subcloned directly into pUC13 (Pharmacia, Piscataway, N.J.).

Induction of fusion protein. *Eco*RI-digested cDNA insert from pUC13 was ligated into prokaryotic expression vector pGEMEX-1 (Promega, Madison, Wis.) and transformed into *E. coli* JM109(DE3). The vector plasmid contained the bacteriophage T7 gene *10* promoter, which controls expression of recombinant genes (46). The amino-terminal portion of the fusion protein expressed from this construct contained the leader peptide of T7 gene *10*, with an approximate size of 26 kDa. The cDNA insert was also digested with restriction enzymes *Asu*II, *Nsi*I, and *Pst*I, followed by *Bam*HI, blunt-ended with the Klenow fragment of *E. coli* DNA polymerase, and religated with T4 DNA ligase. The resultant constructs had inserts of approximately 0.7, 1.1, and 1.4 kb and were termed pCD41A, pCD41B, and pCD41C, respectively. Bacterial cultures were grown to an OD_{550} of 0.5, and expression of fusion protein was induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. Bacterial pellets from 20 ml of culture were resuspended in 2 ml of 50 mM Tris buffer (pH 7.5) containing 5 mM EDTA and 4 mg lysozyme and incubated at 37°C for 15 min, then lysed with 150 μl of 10% Triton X-100–5 mM NaCl at 4°C for 30 min. The lysates were sonicated for 20 s and centrifuged at 10,000 rpm. The final pellets were resuspended in 10 mM Tris buffer (pH 7.5), and protein concentrations were estimated. Equal amounts of protein (10 μg) were boiled in SDS-PAGE sample buffer, and proteins were analyzed by SDS-PAGE and Western blot.

DNA and RNA extraction and hybridization. For the isolation of viral DNA from infected cells, 10^7 HHV-6-infected cells were washed once with PBS, suspended in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.5]), incubated at 65°C for 15 min, and then lysed in 1% SDS–20 mg of proteinase K per ml at 65°C for 20 min (21). After this incubation, NaCl was added to a final concentration of 1.0 M, and the samples were kept at 4°C overnight. Samples were then centrifuged at 12,000 rpm in a Beckman Ti 80 rotor for 1 h, and the supernatants were extracted with chloroform and phenol and precipitated with ethanol (35). For the isolation of host cell DNA, 10^7 uninfected cells were suspended in 1 ml of TE buffer, incubated at 65°C for 15 min, and then lysed in 1% SDS–20 mg of proteinase K per ml at 65°C for 20 min. Samples were then extracted with chloroform and phenol and precipitated with ethanol (21, 35). Viral DNA from purified HHV-6(GS) virions was isolated by the cesium chloride method described by Josephs et al. (24). Total unfractionated RNA was isolated from uninfected and HHV-6-infected (96 p.i.) cells by lysing cells in a solution containing 5 M guanidine isothiocyanate, 0.1 M 2-mercaptoethanol, and 0.1% SDS (13, 16). The lysate was centrifuged through CsCl at 35,000 rpm in a Beckman SW55 Ti rotor for 18 h at 20°C. The RNA pellet was recovered by ethanol precipitation (13, 16, 35).

Viral DNA and host cell DNA from the following sources were used: (i) viral DNA from purified HHV-6(GS) virions; (ii) HHV-6(GS) viral DNA from infected HSB-2 cells; (iii) viral DNA from HHV-6(Z29)-infected Molt-3 cells; and (iv) host cell DNA from uninfected HSB-2 and Molt-3 cells. DNAs were digested to completion with restriction enzymes *Eco*RI and *Bam*HI. Detailed restriction maps of the HHV-6 genome have not yet been determined. We have elected to use *Eco*RI and *Bam*HI enzymes since restriction enzyme bands generated by these two enzymes have been used previously to estimate the size of the HHV-6(GS) genome (24). *Eco*RI-digested viral DNA from cells infected with herpes simplex virus type 1 (HSV-1), HSV-2, CMV, and Epstein-Barr virus (EBV) were also included in the Southern analysis. Restriction endonuclease fragments of viral and host cell DNA were separated on a 1% agarose gel and transferred to GeneScreen Plus with 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Radiolabeled cDNA probes were synthesized by nick translation with [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol; NEN DuPont) and the Klenow fragment of DNA polymerase I. Filters containing DNA were hybridized with the labeled probe and washed in aqueous conditions at 65°C as described by Maniatis et al. (35) and Southern (45). Denaturing agarose gels containing 0.66 M formaldehyde were used for Northern (RNA) blots, and the blots were hybridized at 65°C in a solution containing 1% SDS, 1 M sodium chloride, 10% dextran sulfate, and denatured salmon sperm DNA (35).

In vitro transcription of cDNA. *Eco*RI-digested cDNA inserts were cloned into the *Eco*RI cloning site of the pcDNAII vector (Invitrogen) containing SP6 and T7 promoters or into the pGEMEX-1 vector (Promega) containing SP6 and T3 promoters. Synthesis of sense and antisense RNA transcripts with SP6, T7, and T3 RNA polymerases and capping of RNA at the 5′ end were carried out as described in the Riboprobe system instruction manual (Promega).

In vitro translation and SDS-PAGE. RNA samples from in vitro transcription experiments were translated in vitro with [^{35}S]methionine and rabbit reticulocyte lysate preparations (Promega) according to the manufacturer's recommendations. Samples of in vitro-translated products were boiled with sample buffer and analyzed by SDS-PAGE. In vitro-translated products mixed with equal volumes of lysing buffer were used for immunoprecipitation with monoclonal and rabbit polyclonal antibodies. Immunoprecipitated samples were analyzed by SDS-PAGE.

DNA sequence analysis. The cDNA insert from pUC13 was digested with *Eco*RI and subcloned directly into the M13mp18 vector (Pharmacia). A series of overlapping clones of both orientations were generated by Erase-A-Base systems (Promega), and sequencing was performed by the dideoxynucleotide chain termination method (44). Sequence data were analyzed by using the IBI-Pustell GENEric sequence analysis programs, and hydropathic analysis was conducted with an algorithm of Kyte and Doolittle (28) contained in the software, with a nine-amino-acid window. Amino acid homology analysis was conducted with the FASTA program of Pearson and Lipman (32).

Nucleotide sequence accession number. The sequence data presented here have been assigned GenBank accession number M62700.

RESULTS

MAb 9A5D12 recognizes a phosphoprotein specific for HHV-6(GS)-infected cells. MAb 9A5D12 was selected initially based on its specific reactivity with HHV-6-infected cells in enzyme-linked immunosorbent assays (ELISAs) and RIP assays (7). Granular nuclear and occasional cytoplasmic fluorescence were observed by immunofluorescence with acetone-fixed HHV-6(GS)-infected cells (Fig. 1B). The MAb did not react with the surfaces of intact infected cells (data not shown). Uninfected and HHV-6-infected cells collected

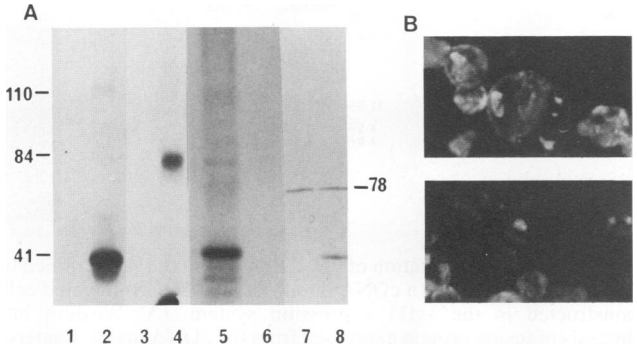

FIG. 1. (A) SDS-PAGE analysis of HHV-6(GS) polypeptides recognized by MAb 9A5D12. Reactivity of MAb in immunoprecipitation reactions (lanes 1 to 6). Samples were reduced with 2-mercaptoethanol (lanes 1, 2, and 5 to 8) or not reduced (lanes 3 and 4). Lanes 1 and 3, [^{35}S]methionine-labeled uninfected HSB-2 cells. Lanes 2 and 4, [^{35}S]methionine-labeled HHV-6 strain GS-infected HSB-2 cells. Lane 5, $^{32}P_i$-labeled HHV-6(GS)-infected HSB-2 cells. Lane 6, $^{32}P_i$-labeled uninfected HSB-2 cells. Reactivity of MAb with Western-blotted total-cell extracts of uninfected (lane 7) and HHV-6-infected (lane 8) cells. Samples were analyzed on 9% acrylamide cross-linked with DATD, and standard prestained and unstained molecular size markers were included in parallel lanes. (B) Reactivity of MAb 9A5D12 with HHV-6(GS)-infected cells in IFA. HHV-6-infected HSB-2 cells were collected at 3 days p.i., fixed in acetone, and reacted with MAb 9A5D12 followed by FITC-labeled anti-mouse IgG antibodies.

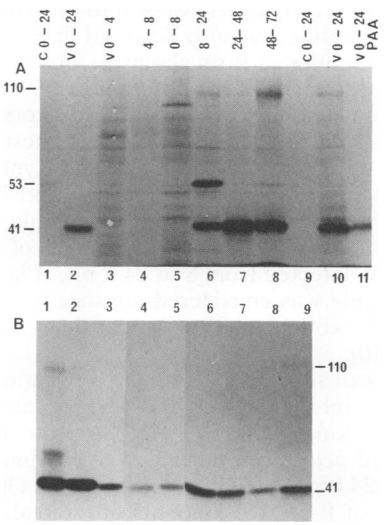

FIG. 2. (A) Kinetics of synthesis of HHV-6 protein P41. Uninfected HSB-2 cells (C, lanes 1 and 9) and HHV-6-infected cells (V, lanes 2 to 8, 10, and 11) labeled with [^{35}S]methionine were used for immunoprecipitation reactions with MAb 9A5D12. HSB-2 cells were infected with HHV-6 at 10 $TCID_{50}$/cell, and after 2 h of absorption, cells were washed (time zero) and labeled with [^{35}S]methionine between 0 and 4, 4 and 8, 0 and 8, 0 and 24, 24 and 48, and 48 and 72 h p.i. Infected cells were also incubated with 300 μg of PAA between 0 and 24 h p.i. (lane 11) (B) Reactivity of MAb 9A5D12 with cells infected with different HHV-6 strains. Infected cells labeled with [^{35}S]methionine were used in immunoprecipitation reactions. Lanes 1 to 9, HHV-6 strains GS, DA, Z-29, OK, DC, Co2, Co3, Co5, and Co6, respectively. HHV-6 strains GS, DA, Co2, Co3, Co5, and Co6 were grown in HSB-2 cells. HHV-6 strain Z-29 was grown in Molt-3 cells. HHV-6 strains OK and DC were grown in CBL.

3 days p.i. were labeled for 20 h with [^{35}S]methionine and used for immunoprecipitation assays. MAb 9A5D12 immunoprecipitated a major HHV-6(GS)-specific polypeptide with an approximate size of 41 kDa and additional but less intense polypeptides of 45, 84, and 110 kDa (Fig. 1A, lane 2) (7). The less intense polypeptides were better resolved after longer exposure of the autoradiographs (data not shown). No reactivity was seen with uninfected cells (Fig. 1A, lanes 1 and 3). When immunoprecipitated samples were electrophoresed without reduction by 2-mercaptoethanol, the 41-kDa polypeptide band was replaced by a polypeptide of 84 kDa, suggesting that the high-molecular-weight form may be a disulfide-linked dimer of the 41-kDa polypeptide (Fig. 1A, lane 4). From cells labeled with $^{32}P_i$, a prominent HHV-6-specific phosphorylated polypeptide of 41 kDa was immunoprecipitated (Fig. 1A, lane 5), and no reactivity was seen with uninfected cells (Fig. 1A, lane 6). Several less intense phosphorylated polypeptides of 38, 84, and 110 kDa were also immunoprecipitated. In phosphoamino acid analysis of immunoprecipitated proteins, the 41-kDa polypeptide was predominantly phosphorylated at serine residues (data not shown). The 41-kDa polypeptide was also the major phosphorylated polypeptide recognized by human sera (8).

In Western blot experiments with HHV-6(GS) proteins, only a polypeptide of 41 kDa was recognized by the MAb (Fig. 1A, lane 8). This result demonstrates that the MAb recognized an epitope presents on the 41-kDa polypeptide and further suggests that the other polypeptides detected by the MAb in immunoprecipitation reactions might result from their molecular association with the 41-kDa polypeptide by a protein-protein interaction. Alternatively, the common epitope recognized by the MAb in the other polypeptides may be sensitive to denaturing conditions. A polypeptide of 78 kDa detected in the Western blot reaction from both infected and uninfected cells (Fig. 1, lanes 7 and 8), and absorption of MAb with uninfected cells did not remove the reactivity with this polypeptide (data not shown). The reactivity with the 78-kDa polypeptide was considered nonspecific, since a similar-size protein was also detected by all other MAbs as well as by alkaline phosphatase-conjugated goat anti-mouse IgG second antibodies alone (data not shown). The MAb did not immunoprecipitate any [^{3}H]glucosamine-labeled polypeptides (7), and the mobility of the [^{35}S]methionine-labeled polypeptides immunoprecipitated by the MAb did not change after treatment with glycosidic enzymes or when extracts from tunicamycin (5 μg/ml)-treated cells were used for immunoprecipitation (8). The phosphoprotein recognized by MAb 9A5D12 was designated P41.

Kinetics of synthesis of HHV-6 P41. The kinetics of synthesis of P41 was examined by using HHV-6(GS)-infected cells labeled with [^{35}S]methionine for different time periods. Electron microscopic examination of infected cells at 72 h p.i. revealed large quantities of enveloped virus particles in cytoplasmic vacuoles and in extracellular compartments (data not shown). P41 was readily immunoprecipitated by the MAb from HHV-6(GS)-infected cells labeled between 0 and 24 h p.i. Of potential importance, an additional specific polypeptide of 110 kDa was also detected with this MAb during this time (Fig. 2A, lanes 2 and 10), which was resolved better after longer exposure of the autoradiograph. Synthesis of P41 was undetectable from 0 to 8 h p.i. (Fig. 2A, lanes 3 to 5). P41 was first detected between 8 and 24 h p.i., and the synthesis continued throughout the observation

period, to 72 h p.i. (Fig. 2A, lanes 6 to 8). Synthesis of the 110-kDa polypeptide was also detected first between 8 and 24 h p.i. and continued throughout the observation period. Densitometric tracings of the autoradiographs and counting of radiolabel in excised portions of the gels corresponding to the different bands demonstrated that synthesis of P41 was maximal between 24 and 48 h p.i. and synthesis of the 110-kDa protein was maximal between 48 and 72 h p.i. (data not shown). Six times less 110-kDa polypeptide was detected than P41. A host cell-specific polypeptide of 53 kDa was inconsistently detected from 8 to 24 h p.i. (Fig. 2A, lane 6). This polypeptide was considered nonspecific, as it was not detected in all extracts taken at similar time points (Fig. 2A, lanes 2 and 10).

PAA has been shown to inhibit the replication of HHV-6, presumably by inhibiting HHV-6 DNA replication (2, 14). To determine the kinetic class of HHV-6 protein P41, HHV-6(GS)-infected cells were labeled with [^{35}S]methionine between 0 and 24 h p.i. with and without PAA (300 μg/ml). In the presence of PAA, the amounts of radiolabeled P41 and 110-kDa polypeptides immunoprecipitated were significantly reduced but not abolished (Fig. 2A, lane 11). Under these conditions, HHV-6 glycoproteins were completely inhibited by PAA (data not shown). Similar findings have been reported previously for HHV-6(Z-29) with MAb 9A5D12 (14). These data suggested that the continued synthesis of P41 and the 110-kDa polypeptide depends on viral DNA replication and these HHV-6 polypeptides probably belong to the early-late class of HHV-6 proteins.

HHV-6(GS) protein P41 is conserved among HHV-6 strains. When tested with cells infected with eight additional HHV-6 strains, MAb 9A5D12 showed granular nuclear fluorescence and immunoprecipitated a polypeptide of 41 kDa (Fig. 2B, lanes 2 to 9). Polypeptide bands of 45 and 110 kDa were also detected after longer exposure of the autoradiographs. Under nonreducing conditions, the 41-kDa polypeptide band immunoprecipitated from the different HHV-6 strains was also replaced by an 84-kDa polypeptide band (data not shown). These results demonstrate that HHV-6 P41 is conserved among the HHV-6 strains tested. MAb 9A5D12 did not cross-react with cells infected with HSV-1 (strain KOS), HSV-2 (strain 333), CMV (AD169), or EBV (B95-8) in IFA and ELISA, demonstrating that the MAb is specific for HHV-6.

Identification of a cDNA encoding the HHV-6 phosphoprotein P41. To identify the gene encoding HHV-6 protein P41, a cDNA library of HHV-6(GS)-infected cells was constructed in the λgt11 expression system and screened with MAb 9A5D12. One immunoreactive recombinant phage producing a β-galactosidase fusion protein was identified. Agarose gel electrophoresis of the insert excised from λgt11 with *Eco*RI revealed a cDNA insert of 2.1 kb. The cDNA insert subcloned into pUC13 was designated pCD41. In a Western blot assay, MAb 9A5D12 recognized several polypeptides from the lysates of insert-bearing phage ranging from 130 to 36 kDa (data not shown). Rapid proteolysis of the β-galactosidase fusion protein may account for the generation of smaller polypeptides. Since this hampered the estimation of the size of the protein coded by the cDNA insert, the pCD41 insert was cloned in the prokaryotic expression vector pGEMEX-1 and transformed into *E. coli* JM109(DE3). Recombinant protein was induced by the addition of IPTG for 4 h, and the Western-blotted bacterial lysates were reacted with MAb 9A5D12. Several proteins were recognized by the MAb from two of the lysates with inserts, and among these, a fusion protein of 88 to 92 kDa was prominent

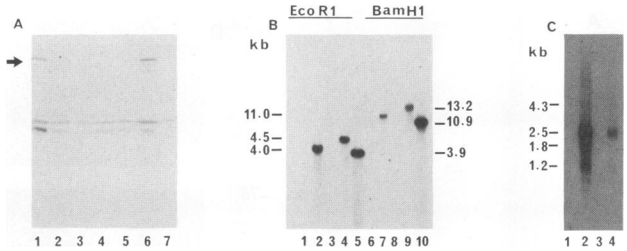

FIG. 3. Characterization of the cDNA insert pCD41 identified by the MAb 9A5D12 from a cDNA library of HHV-6(GS)-infected cells constructed in the λgt11 expression system. (A) Western blot analysis of fusion protein expressed from the cDNA insert. Bacterial colonies containing the pCD41 insert (lanes 1 to 6) or the vector alone (lane 7) in the pGEMEX-1 expression system were induced with IPTG for 4 h. Bacterial lysates obtained were electrophoresed on 12% SDS-PAGE, transferred to nitrocellulose sheets, and reacted with MAb 9A5D12. The arrow shows a major 88- to 92-kDa fusion protein band detected in lanes 1 and 6. (B) Southern blot hybridization of pCD41 insert with HHV-6 DNA and uninfected-cell DNA digested with *Eco*RI (lanes 1 to 5) and *Bam*HI (lanes 6 to 10). Lanes 1 and 6, DNA from uninfected HSB-2 cells. Lanes 3 and 8, DNA from uninfected Molt-3 cells. Lanes 2 and 7, HHV-6(GS) DNA from infected HSB-2 cells. Lanes 4 and 9, HHV-6(Z29) DNA from infected Molt-3 cells. Lanes 5 and 10, HHV-6(GS) DNA from purified virions. The sizes (in kilobases) of HHV-6-specific DNA fragments hybridized with the ^{32}P-labeled 2.1-kb pCD41 insert are indicated. Standard lambda marker DNAs of known sizes were included in parallel lanes. (C) RNA transcripts identified by the cDNA insert. Uninfected and HHV-6 (GS and Z29 strains)-infected-cell total RNAs were subjected to electrophoresis in a formaldehyde-agarose gel, transferred to GeneScreen Plus, and probed with the ^{32}P-labeled 2.1-kb pCD41 insert. Each lane was loaded with 10 μg of RNA. Lane 1, uninfected HSB-2 cell RNA. Lane 2, HHV-6(GS)-infected HSB-2 cell RNA. Lane 3, uninfected Molt-3 cell RNA. Lane 4, HHV-6(Z29)-infected Molt-3 cell RNA. The sizes (in kilobases) of HHV-6-specific RNAs that hybridized with the 2.1-kb pCD41 insert are indicated.

(Fig. 3A, lanes 1 and 6). However, Coomassie staining of the gel did not reveal any specific protein. No reactivity was seen with other insert-containing (Fig. 3A, lanes 2 to 5) and non-insert-containing (Fig. 3A, lane 7) bacterial lysates. Nonreactive clones were later found to contain the insert in the wrong orientation. Since the amino terminus of the fusion protein contained the gene *10* protein of T7 approximately 26 kDa, this suggested that the pCD41 insert codes for a protein of 62 to 66 kDa.

Southern blot analysis with cDNA insert pCD41. To determine the viral specificity of the cDNA insert and to map the coding gene on the viral genome, [α-^{32}P]dCTP-labeled 2.1-kb pCD41 was examined by Southern blot analysis. The pCD41 insert hybridized specifically with the *Eco*RI- and *Bam*HI-digested DNA fragments from HHV-6 virions (Fig. 3B, lanes 5 and 10) and from HHV-6-infected cells (Fig. 3B, lanes 2, 4, 7, and 9). No hybridization was seen with DNA from uninfected cells (Fig. 3B, lanes 1, 3, 6, and 8) or with any of the *Eco*RI-digested viral DNA fragments from HSV-1, HSV-2, CMV, and EBV (data not shown). Specific hybridization was seen with 3.9-kb *Eco*RI (Fig. 3B, lane 5) and 10.9-kb *Bam*HI (Fig. 3B, lane 10) DNA fragments from purified virions of HHV-6(GS) and with 4.0-kb *Eco*RI (Fig. 3B, lane 2) and 11.0-kb *Bam*HI (Fig. 3B, lane 7) DNA fragments from HHV-6(GS)-infected cells. Hybridization to slightly larger sizes of viral DNA fragments from infected cells could be due to the retardation of viral DNA mobility by host cell DNA. Nevertheless, these results clearly dem-

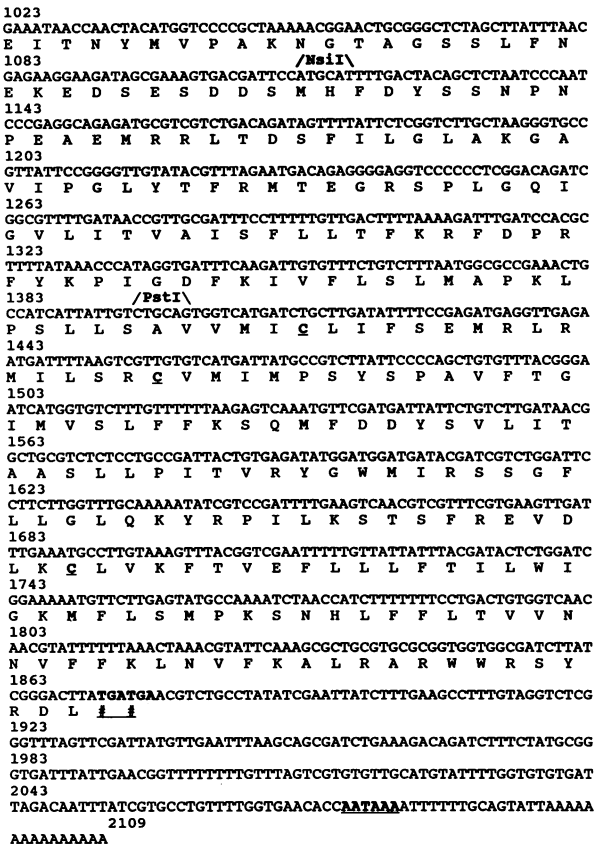

FIG. 4. Nucleotide sequence of pCD41 and the deduced amino acid sequence of the single ORF. The nucleotide position is numbered on the left, and the amino acid position is numbered on the right. The termination codons and the polyadenylation signal sequences are underlined. Asterisked sequences indicate possible TATA boxes, and the underlined methionines are the presumed start sites for in vitro translation. Restriction sites for *Asu*II, *Nsi*I, and *Pst*I are marked on the nucleotide sequence. The three underlined sequences indicate potential sites for N-linked glycosylation (NXT/S), and cystine residues are indicated by underlining.

onstrate the HHV-6 specificity of the cDNA insert identified by MAb 9A5D12. The pCD41 insert also hybridized with 4.5-kb *Eco*RI (Fig. 3B, lane 4) and 13.2-kb *Bam*HI (Fig. 3B, lane 9) DNA fragments from HHV-6(Z29)-infected Molt-3 cells. Hybridization with different sizes of DNA fragments from the two strains of HHV-6 further supports the previous reports of restriction site heterogeneity among HHV-6 strains (1, 4, 24, 25, 34).

Northern blot analysis with cDNA insert pCD41. To define further the viral specificity of pCD41, equal quantities of total unfractionated RNA isolated from HHV-6(GS)-infected and HHV-6(Z29)-infected and uninfected cells were tested by Northern blot analysis with a ^{32}P-labeled 2.1-kb pCD41 insert. Hybridization was detected with RNA from infected cells only (Fig. 3C, lanes 2 and 4), and the sizes of HHV-6 transcripts were calculated by comparing their mobility with RNA marker molecules (Bethesda Research Laboratories, Gaithersburg, Md.). Two prominent transcripts with estimated sizes of 2.5 and 1.2 kb were identified by pCD41 from HHV-6 strain GS (Fig. 3C, lane 2) and from strain Z29 (Fig. 3C, lane 4). Two less intense bands of 1.8 and 4.3 kb were also detected (Fig. 3C, lanes 2 and 4). The intensity of the 2.5-kb transcript was about 3.3 times that of the 1.2-kb band. Even though equal amounts of total cellular RNA (10 μg) were loaded per sample well, the hybridization intensity was stronger with HHV-6(GS) RNA than with HHV-6(Z29) RNA. Nevertheless, these data further confirm the specificity of the pCD41 insert and demonstrate that P41 is conserved between these two HHV-6 strains.

DNA sequence analysis of cDNA insert pCD41. DNA sequence analysis revealed that the cDNA insert consisted of 2,109 bp and the sequence has a mean composition of 41% G:C. The sequence was further verified by restriction digestion analysis with the predicted restriction enzyme sites. The complete sequence and the predicted amino acid residues are shown in Fig. 4. Computer analysis revealed a single open reading frame (ORF) of 1,871 nucleotides, beginning at nucleotide 3 at the 5′ end, and the stop condons (TGA TGA) are located at nucleotide positions 1872 to 1877 at the 3′ end (Fig. 4). The 238 nucleotides at the 3′ end are untranslated. There is one potential polyadenylation signal (AATAAA), located at nucleotide positions between 2077 and 2082 at the 3′ end. The predicted protein is 623 amino acid residues in length, with a calculated molecular weight of 70,863, and is highly basic, with an estimated pI of 10.99. Hydropathic analysis of the pCD41 ORF indicates a major stretch of hydrophobic domains clustered at the carboxy region between amino acids (aa) 402 and 602 (Fig. 5). At least six regions in the carboxy terminus of the ORF show a hydrophobic score of greater than +1.6. Since sequences with a high probability to be membrane-spanning usually have a hydrophobic score greater than +1.6 (28), this suggests membrane association of the predicted pCD41 protein. There are nine cysteine and 25 methionine residues in the

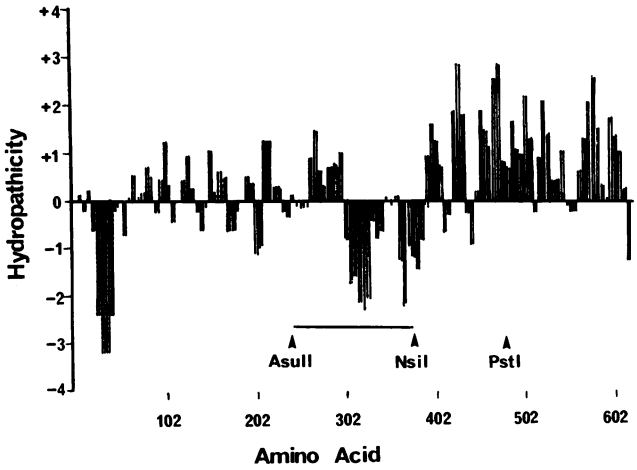

FIG. 5. Hydropathic analysis of the deduced amino acid sequence of pCD41. The predicted amino acid sequence was analyzed for hydrophobic and hydrophilic characteristics by using the Kyte and Doolittle algorithm (28). Scores were averaged within a sliding window of nine. The vertical axis represents a relative hydropathic score in which positive value are hydrophobic and negative values are hydrophilic. Sequences with a high probability to be membrane-spanning have a score greater than +1.6. The amino acid number of the pCD41 sequence and the location of restriction sites for *Asu*II, *Nsi*I, and *Pst*I are given. The solid line between *Asu*II and *Nsi*I represents the putative antigenic site recognized by the MAb 9A5D12, spanning a highly hydrophilic region of the protein.

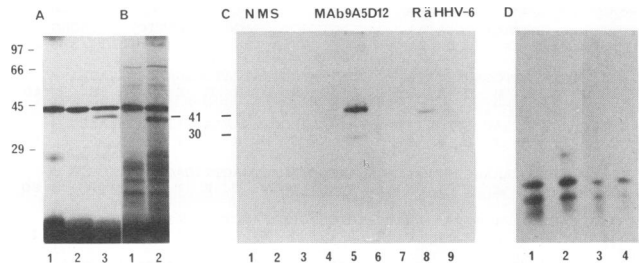

FIG. 6. In vitro transcription and translation of the cDNA insert. Sense and antisense transcripts prepared in vitro from the cDNA insert pCD41 were translated in vitro with rabbit reticulocyte lysates and [^{35}S]methionine. Samples were analyzed on 12% SDS–PAGE and by RIP followed by SDS-PAGE. (A) In vitro translation of transcripts from the pCD41 insert cloned in the pCDNAII vector. Lane 1, translation without the addition of RNA. Lane 2, antisense RNA transcribed from the Sp6 promoter. Lane 3, sense RNA transcribed from the T7 promoter. (B) In vitro translation of transcripts from the pCD41 insert cloned in the pGEMEX-1 vector. Lane 1, translation without the addition of RNA. Lane 2, sense RNA transcribed from the T3 promoter. (C) RIP of in vitro-translated products. Transcripts from the pCD41 insert cloned in the pCDNAII vector were translated in vitro and used for immunoprecipitation with normal mouse serum (NMS, lanes 1 to 3), MAb 9A5D12 (lanes 4 to 6), and rabbit polyclonal antibody (RäHHV-6) against HHV-6(GS)-infected cells (lanes 7 to 9). Lanes 1, 4, and 7, translation without RNA. Lanes 2, 5, and 8, translation and antisense RNA transcribed from the Sp6 promoter. Lanes 3, 6, and 9, translation with sense RNA transcribed from the T7 promoter. (D) Product made by the pCD41 sense transcript is the HHV-6 protein P41 immunoprecipitated from HHV-6(GS)-infected HSB-2 cells. The sense RNA transcribed in vitro from the cDNA in pGEMEX-1 vector was translated in vitro, and the products were used for RIP with MAb 9A5D12. The [^{35}S]methionine-labeled 41-kDa in vitro-translated polypeptide (lanes 3 and 4) and the P41 immunoprecipitated from HHV-6(GS)-infected cells (lanes 1 and 2) were separated by SDS-PAGE, subjected to partial proteolysis with 10 μg (lanes 1 and 3) and 2 μg (lanes 2 and 4) of V8 protease, and analyzed on 15% SDS–PAGE. Sizes are shown in kilodaltons.

predicted protein, and 20 of the methionine residues are clustered between aa 229 and 623 (Fig. 4). There are three potential sites for the addition of N-linked oligosaccharides, all of which are associated with the amino-terminal part of the predicted protein within aa position 253. (Fig. 4).

In vitro transcription and translation of cDNA insert pCD41 result in the synthesis of a 41-kDa polypeptide. The predicted size of the protein encoded by the pCD41 ORF is much larger than that (41 kDa) of the protein recognized by the MAb from the infected cell. Detection of a large fusion protein (Fig. 3) indicated that the entire length of the cDNA was transcribed in the prokaryotic expression vector pGEMEX-1. Reactivity of the MAb with the fusion protein expressed in both λgt11 and pGEMEX-1 could be due to a cross-reactive determinant in the protein coded by pCD41. To determine the relationship between HHV-6 P41 and the protein coded by pCD41, the cDNA insert was cloned in pcDNAII and pGEMEX-1 vectors. Sense and antisense transcripts were synthesized by using SP6 and T7 promoters (pcDNAII) and sense RNA by using the T3 promoter (pGEMEX-1). The resulting RNA transcripts were capped, translated in vitro, and then analyzed by SDS-PAGE. No specific polypeptides were synthesized from RNA transcribed from the SP6 promoter (antisense RNA; Fig. 6A, lane 2) or from a control in vitro translation without RNA (Fig. 6A, lane 1, and 6B, lane 1), and no large polypeptide predicted to be coded by the pCD41 insert was translated. Instead, a prominent [^{35}S]methionine-labeled polypeptide of 41 kDa was synthesized from the sense RNA transcribed from the T7 promoter (Fig. 6A, lane 3) as well as from the T3 promoter (Fig. 6B, lane 2).

To determine the specificity of the polypeptide synthesized, the in vitro-translated products were immunoprecipitated with MAb 9A5D12 and rabbit polyclonal antibodies against HHV-6(GS)-infected cells. Both these antibodies immunoprecipitated a polypeptide of 41 kDa translated from the sense RNA (T7 promoter). Longer exposure of the autoradiograph revealed several less-intense lower-molecular-weight polypeptides, including a 30-kDa polypeptide (Fig. 6C, lanes 5 and 8). No reactivities were seen with translated products from antisense RNA or control RNA (Fig. 6C, lanes 3, 6, 7, and 9). Normal mouse serum (Fig. 6C, lanes 1 to 3) and normal rabbit serum (data not shown) did not show any reactivity. V8 protease was used to define the partial proteolytic peptide mapping of the in vitro-synthesized 41-kDa polypeptide (pGEMEX-1 vector transcript) and the HHV-6 P41 from infected cells. Both polypeptides showed identical peptide profiles (Fig. 6D, lanes 1 to 4), and similar profiles were also seen with the 41-kDa polypeptide translated from the sense transcript from the pcDNAII vector (data not shown). These results clearly demonstrated that the pCD41 insert predicted to code for a larger protein also encoded the complete HHV-6 phosphoprotein P41.

Reactivity of MAb 9A5D12 with fusion proteins expressed from deletion constructs of the cDNA insert. The SDS-PAGE mobility of the 41-kDa polypeptide remained unaltered when inhibitors of proteolytic enzymes were included in the in vitro translation reaction mixes (data not shown). This suggested that the 41-kDa polypeptide was not derived by proteolytic cleavage (data not shown). We next considered

the possibility of internal initiation of translation of the transcript leading to the in vitro synthesis of the 41-kDa polypeptide. Careful analysis of pCD41 nucleotide sequences revealed two TATA boxes, TATAAAA and TATATTT, located at nucleotide positions 668 to 674 (aa 223 to 225) and 722 to 728 (aa 241 to 243), respectively (Fig. 4). In context to these regions, there are three ATG codons, beginning at nucleotide positions 688, 784, and 789, respectively (aa positions 227, 248, and 263, respectively; Fig. 4). Translation initiation at any of these three codons could result in the synthesis of polypeptides of 45, 42, and 41 kDa, respectively. According to this model, the antigenic epitope recognized by the MAb is predicted to be located after aa 227.

To test this possibility, the cDNA insert in the pGEMEX-1 expression vector was digested with restriction enzymes *Asu*II, *Nsi*I, and *Pst*I. The restriction sites for these enzymes are located at nucleotide positions 704, 1102, and 1395, respectively, from the 5′ end of the insert (Fig. 4). The resulting 234-aa, 371-aa, and 466-aa ORFs are predicted to code for 30-, 42-, and 52-kDa polypeptides, and the deletion constructs were named pCD41A, pCD41B, and pCD41C, respectively. The three enzymes were selected for the following reasons. (i) These are the only enzymes with only one restriction site in the insert, with no other restriction sites either in the polylinker region or in the vector. This kept the 5′ end of the fusion protein intact. (ii) The *Asu*II site is located between the two putative TATA boxes, and a fusion protein induced from this construct would contain only the first 234 amino acids from the amino terminus of the pCD41 ORF. (iii) The amino acids encoded by nucleotides between the *Asu*II and *Nsi*I restriction sites are highly hydrophilic (aa 234 to aa 371, Fig. 5) and contain the two ATG codons at aa positions 248 and 263. Translation initiation at any of these two codons should result in polypeptides of 42 and 41 kDa, which is closer to the observed size of 41 kDa immunoprecipitated by the MAb.

Fusion proteins from the deletion constructs were induced with 0.5 mM IPTG for 4 h, and equal quantities of bacterial lysates (10 μg) were analyzed by SDS-PAGE and by Western blot. No fusion protein was detected by Coomassie staining or by Western blot of bacterial lysates with the non-insert-bearing control vector (Fig. 7A, lane 1; Fig. 7B, lane 1). Bacterial lysates with the pCD41 insert ligated in the wrong orientation also did not show any reactivity (data not shown). From bacterial lysates with the full-length cDNA insert (2.1 kb, 623 aa), a major fusion protein of >84 kDa together with several minor more rapidly migrating bands were recognized by MAb 9A5D12 (Fig. 7B, lane 2). In contrast, no specific protein was detected in the stained gel (Fig. 7A, lane 2). A prominent specific band of 63 kDa was detected from pCD41A insert-containing bacterial lysate by Coomassie staining (Fig. 7A, lane 3). However, MAb 9A5D12 did not react with this fusion protein in the Western blot (Fig. 7B, lane 3). In contrast, a fusion protein of 74 kDa and several minor bands were recognized by the MAb from bacterial lysates with the pCD41B insert (371 aa of pCD41; Fig. 7B, lane 4), and only faintly stained similar molecular weight bands were observed from the same lysates (Fig. 7A, lane 4). MAb 9A5D12 reacted very strongly with a fusion protein of 78 kDa and several other faster migrating bands from bacterial lysates with the pCD41C insert (466 aa of pCD41; Fig. 7B, lane 5). Similar proteins were also observed from the Coomassie-stained gel (Fig. 7A, lane 5). These results demonstrate that the first 377 aa in the pCD41 ORF most probably contain the antigenic site recognized by the MAb and suggest that the site is probably located between aa positions 235 and 371, which include the two potential start sites (aa 248 and 263). This also supported the possibility that internal initiation of translation resulted in the in vitro synthesis of the 41-kDa polypeptide.

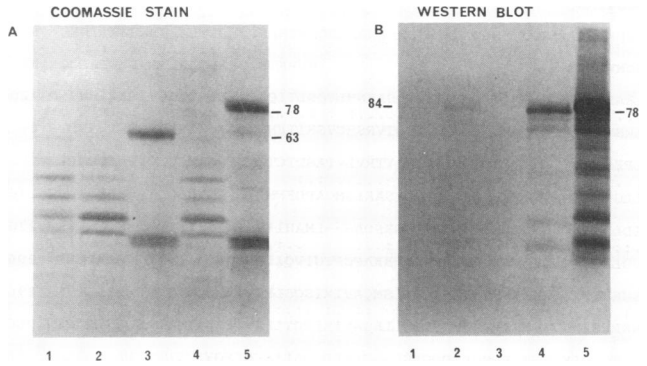

FIG. 7. Reactivity of MAb 9A5D12 with the fusion proteins expressed from cDNA deletion constructs. Deletions in the cDNA insert were generated by restriction enzymes *Asu*II, *Nsi*I, and *Pst*I, and the resulting constructs in the pGEMEX-1 vector were named pCD41A, pCD41B, and pCD41C. Bacterial colonies containing each of the deletion constructs were induced with IPTG for 4 h, and proteins were extracted and analyzed on 12% SDS–PAGE. Gels were either stained with Coomassie stain (A) or transferred onto a nitrocellulose sheet and reacted with MAb 9A5D12 (B). Lanes A1 and B1, pGEMEX-1 vector alone. Lanes A2 and B2, pCD41 containing the full-length insert. Lanes A3 and B3, pCD41A deletion construct. Lanes A4 and B4, pCD41B deletion construct. Lanes A5 and B5, pCD41C deletion construct. The approximate sizes of fusion proteins are indicated in kilodaltons.

Predicted ORF of HHV-6 pCD41 insert shows amino acid homology with the HCMV UL44 gene product. Using the FASTA program of Lipman and Pearson (32), we compared the amino acid sequences of the predicted pCD41 ORF with the protein data base libraries and a protein library of all available herpesvirus sequences. The only significant match detected was with the predicted protein product of HCMV gene UL44. There is 41.8% identity in a stretch of 282 amino acids (Fig. 8), with a FASTA score (ktup = 1) of 623. The predicted HCMV UL44 gene product consists of 433 aa, with a predicted molecular weight of 46,234 and an estimated pI of 9.9. The HCMV UL44 gene has been shown to encode a group of early-late DNA-binding nonstructural proteins that include phosphorylated and glycosylated species and are referred to as the ICP36 protein family of HCMV (11, 12, 30). The homology detected occurred only in the amino-terminal region of the pCD41 ORF, between aa positions 48 and 320. Four of the nine cystine residues in the pCD41 ORF are conserved in the HCMV UL44 gene ORF (Fig. 8).

DISCUSSION

In this report, we have characterized in detail a phosphoprotein encoded in the HHV-6 genome of 41 kDa which is specifically recognized by MAb 9A5D12. HHV-6 P41 is one of the most abundant proteins produced early in the replicative cycle. Since PAA reduced the quantity of P41 synthesized, it is tentatively assigned to the early-late class of HHV-6 proteins.

A variety of HHV-6 strains have been isolated from different geographical locations as well as from patients with various pathological conditions. These HHV-6 strains show

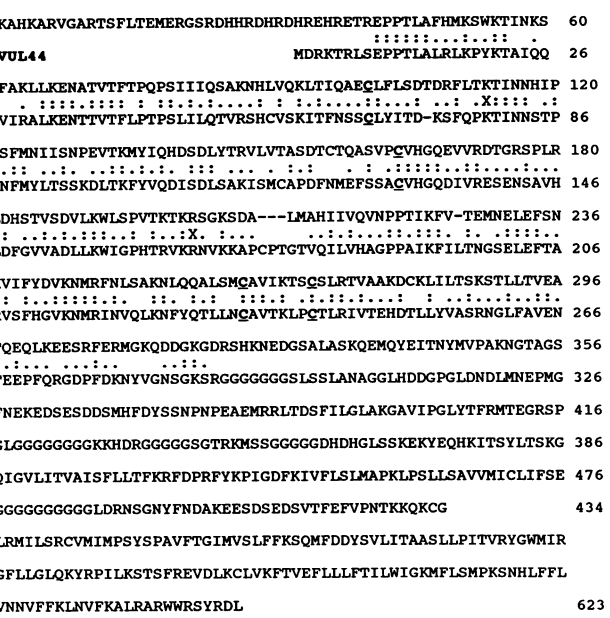

FIG. 8. Amino acid homology between the HHV-6 pCD41 ORF (pCD41AA) and the HCMV UL44 gene product. Amino acid comparison (ktup = 1) was performed by using the FASTA program of Lipman and Pearson (32). Identical amino acids are marked by two dots, and conservative changes are marked by single dot. The positions of four conserved cystine residues are indicated by boldfacing and underlining.

DNA restriction site heterogeneity (1, 24, 25, 34) and differ considerably in their in vitro tropism (1, 3, 4, 15, 34, 54) and antigenic properties (1, 54). Our preliminary studies suggest that these viruses are antigenically closely related and yet different. Among the many MAbs tested, MAb 9A5D12 is one of the few that reacts with all HHV-6 strains tested and has been of considerable value in monitoring in vitro HHV-6 infection (1, 14, 54). MAb 9A5D12 also showed similar reactivity in our recent testing with 12 additional HHV-6 strains (data not shown). This MAb is therefore a potentially ideal diagnostic reagent for the identification of newer HHV-6 isolates and in the detection of HHV-6 antigen in clinical specimens. We have recently used this antibody for the detection of HHV-6 viral antigens in salivary glands and in bronchial glands from healthy individuals and patients with various disorders. In these studies, antigen detection by MAb 9A5D12 was found to be more sensitive than in situ hybridization assays (27). Since human sera also reacted with a 41-kDa polypeptide of HHV-6(GS) (7, 10), HHV-6 protein P41 may also be useful in the serological measurement of HHV-6 infection.

Sequence analysis of the cDNA insert pCD41 predicted a product of about 70 kDa, and the MAb recognized a fusion protein of about 88 to 92 kDa which included the 26-kDa portion of the T7 gene *10* leader protein. The difference from the predicted 96-kDa fusion protein may be due to the migration anomalies of the protein in SDS-PAGE. Alternatively, this could be due to rapid proteolysis of the fusion protein, which is suggested by the detection of several smaller polypeptides which react with the MAb.

The stability of a fusion protein is dependent upon the host bacterial strain, the expression vector system, and the incubation conditions. In the λgt11 expression system, proteolysis of the β-galactosidase fusion protein containing the pCD41 insert appeared to be very rapid. Expression of the fusion protein in the pGEMEX-1 system also appeared to depend on the amino acid sequences in the pCD41 ORF. The highly hydrophobic carboxy terminus of the ORF appears to affect the stability of the fusion protein, as the full-length fusion protein was detected only in the Western blot. Removal of part of the carboxy terminus appears to stabilize the fusion protein from the pCD41C insert. Even in this deletion construct, detection of several smaller bands in the Western blot suggests rapid proteolysis, which increased considerably after 24 h of induction with IPTG (data not shown).

Several features of the cDNA identified and characterized in this study merit additional comment. Even though the cDNA is predicted to encode a large protein, evidence presented here indicates that the cDNA insert also encodes the HHV-6 phosphoprotein P41. Localization of MAb reactivity to a region carboxy terminal to aa 235 of the pCD41 ORF, together with the absence of other reading frames in the cDNA sequence, absence of high-molecular-weight polypeptides in the in vitro translation, in vitro translation of a 41-kDa polypeptide from the sense transcript of the cDNA insert, immunoprecipitation of this polypeptide by the MAb, peptide map identical to that of HHV-6 P41, stop and polyadenylation signals at the 3′ end of the insert, and the presence of two TATA boxes in the cDNA sequence, strongly suggest that HHV-6 P41 is encoded in a gene with the coding potential for a larger protein.

One of the predominant transcripts detected in the Northern blot analysis has a calculated size of 1.2 kb, which is sufficient to encode the 41-kDa peptide. Another major 2.5-kb RNA was also detected in Northern blot analysis with the cDNA insert. Since the predicted ORF of the 2.1-kb cDNA is open at the 5′ end, the 2.5-kb RNA probably represents the full-length transcript. In addition to the 623 aa predicted from the cDNA insert, the 2.5-kb RNA could code for an additional 100 or more amino acids at the amino terminus. Besides HHV-6 P41, the MAb also immunoprecipitated a 110-kDa polypeptide in lower quantities from HHV-6-infected cells, with similar kinetics of synthesis (Fig. 2). In preliminary studies, translation of an HHV-6(GS) RNA hybrid selected by pCD41 resulted in the synthesis of a 41-kDa polypeptide and a high-molecular-mass (>80 kDa) polypeptide. Preliminary studies also show partial identity between the 110-kDa and 41-kDa polypeptides. These observations suggest that the 110-kDa polypeptide may be encoded by the 2.5-kb transcript. With a single potential polyadenylation site at the 3′ end, it is probable that the 2.5-kb and 1.2-kb RNAs have a common 3′ end. Even though the 2.5-kb message is 3.3 times more abundant than the 1.2-kb RNA, the 41-kDa polypeptide was more abundantly detected than the 110-kDa polypeptide. The reason for this discrepancy is not known at present; it could be due to the stability of the 110-kDa polypeptide synthesized and/or to the nonaccessibility of the epitope in the 110-kDa polypeptide in immunoprecipitation reactions. Nonreactivity of the MAb with the 110-kDa polypeptide in Western blot reactions suggests such nonaccessibility of the epitope in the 110-kDa polypeptide under Western blot conditions. Alternatively, if the 2.5-kb RNA serves as a bicistronic message, then the abundance of 41-kDa polypeptide could be due to its synthesis from the 2.5-kb RNA as well as from the 1.2-kb RNA. Whether the full-length 2.5-kb RNA can serve as a potential bicistronic message for the synthesis of these

polypeptides and the regulation of transcription and translation of these messages merits further study.

Overlapping in-frame ORFs with coterminal 3′ ends may also give rise to these polypeptides, and the detection of TATA boxes in the cDNA sequence provides support for this concept. Overlapping mRNAs with distinct 5′ ends from multiple promoters and common 3′ ends have been well documented for a number of genes in herpesviruses (11, 12, 18, 30, 36, 40–42, 50, 51). Using a cDNA library, Robson and Gibson have identified a 1,050-bp cDNA insert coding for a 40-kDa assembly protein of primate CMV (41). This insert hybridized to 1-kb and 1.8-kb transcripts from the infected cells, and the sequence analysis showed a single ORF with an open 5′ end (41). These investigators have recently demonstrated that the 40-kDa assembly protein is encoded by a gene at the 3′ end of a 2-kb ORF that has three transcriptional start sites. Three proteins of 64, 45, and 37 kDa are shown to be encoded by 2-kb, 1.6-kb, and 1-kb RNA, respectively, and these RNAs have coterminal 3′ ends (15a). Our studies suggest that HHV-6 phosphoprotein P41 is encoded by a gene at the 3′ end of an ORF present in the 2.5-kb RNA. Further support for this suggestion requires primer extension, hybrid arrest, and hybrid selection analyses together with the characterization of the full-length genomic DNA coding for these proteins. Such studies are in progress.

MAb 9A5D12 did not react with HSV-1, HSV-2, CMV, and EBV in ELISA and IFA assays, and the HHV-6 pCD41 insert did not cross-hybridize with other herpesviruses under the conditions used. However, a homology search showed that the amino-terminal half of the predicted ORF of the HHV-6 cDNA insert has a significant match with the HCMV UL44 gene product, belonging to the ICP36 gene family. This finding further supports the concept that HHV-6 is closely related to CMV (29, 33). Comparison of the HCMV UL44 ORF and HHV-6 pCD41 ORF with HSV-1, varicella-zoster virus, and EBV sequences has not revealed any significant homologies. The HHV-6 pCD41 ORF shows divergence from the HCMV UL44 ORF in the amino and carboxy termini. Even though the glycine-rich stretches in the carboxy terminus of the HCMV UL44 ORF have been shown to match numerous reading frames as a result of compositional bias (12), no such homology can be detected with the carboxy terminus of HHV-6 pCD41 ORF. The long stretch of multiple hydrophobic domains in the carboxy region of pCD41 ORF is suggestive of membrane association, and such multiple hydrophobic domains are absent from the HCMV UL44 ORF. The tentative MAb-reactive site located between aa 235 and 371 of the pCD41 ORF shows only limited homology with the HCMV UL44 ORF.

In spite of these differences, there are several similarities between the HHV-6 pCD41 ORF and the HCMV UL44 gene product. The HCMV ICP36 (UL44) coding sequences have been mapped to the 5′ 1.5 kb of the 4.5-kb ICP36 RNA, and the 3′ end contains multiple potential ORFs (12, 30). The HCMV UL44 gene has been shown to encode a group of early-late DNA-binding nonstructural proteins that include both phosphorylated and glycosylated species. One of these is a 52-kDa phosphoprotein, translated from a 4.5-kb RNA (12, 30). HCMV UL44 gene expression has been shown to occur from different TATA boxes, in both early and late replicative cycles of HCMV (12, 30). Similar to HHV-6 P41, HCMV UL44 gene products are also localized to the nucleus of HCMV-infected cells (12, 30). Whether the 41- and 110-kDa polypeptides of HHV-6 also have DNA-binding properties remains to be determined, and the highly basic nature of the HHV-6 pCD41 ORF is certainly suggestive of such a property. Sequence homology, similar pattern of synthesis, phosphorylation, and nuclear localization suggest a close evolutionary relationship between HHV-6 protein(s) coded by the pCD41 ORF and the HCMV UL44 gene product. Studies are under way to define the antigenic cross-reactivity between these proteins and its relevance in the serological measurement of infections by these viruses.

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