Identification and Mapping of the Gene Encoding the Glycoprotein Complex gp82-gp105 of Human Herpesvirus 6 and Mapping of the Neutralizing Epitope Recognized by Monoclonal Antibodies

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Monoclonal antibodies (MAbs) 2D4, 2D6, and 13D6 against human herpesvirus 6 (HHV-6) variant A strain GS recognized virion envelope glycoprotein complex gp82-gp105 and neutralized the infectivity of HHV-6 variant A group isolates. A 624-bp genomic fragment (82G) was identified from an HHV-6 strain GS genomic library constructed in the λ gt11 expression system by immunoscreening with MAb 2D6. Rabbit antibodies against the fusion protein expressed from the genomic insert recognized glycoprotein complex gp82-gp105 from HHV-6-infected cells, thus confirming that the genomic fragment is a portion of the gene(s) that encodes gp82-gp105. This genomic insert hybridized specifically with viral DNAs from HHV-6 variant A strains GS and U1102 under high-stringency conditions but hybridized with HHV-6 variant B strain Z-29 DNA only under low-stringency conditions. DNA sequence analysis of the insert revealed a 167-amino-acid single open reading frame with an open 5' end and a stop codon at the 3' end. Hybridization studies with HHV-6A strain U1102 DNA localized the gp82-gp105-encoding gene to the unique long region near the direct repeat at the right end of the genome. To locate the neutralizing epitope(s) recognized by the MAbs, a series of deletions from the 3' end of the gene were constructed with exonuclease III, and fusion proteins from deletion constructs were tested for reactivity with MAbs in a Western immunoblot assay. Sequencing of deletion constructs at the reactive-nonreactive transition point localized the epitope recognized by the three neutralizing MAbs within or near a repeat amino acid sequence (NIYFNIIY) of the putative protein. This repeat sequence region is surrounded on either side by two potential N-glycosylation sites and three cysteine residues.

Human herpesvirus 6 (HHV-6) is a newly identified lymphotropic herpesvirus initially isolated from the peripheral blood lymphocytes of patients with lymphoproliferative disorders and AIDS (27). Several studies have shown that HHV-6 is the etiological agent of exanthem subitum (roseola) (26, 29, 32). Primary infection occurs early in childhood, and 90% of the population is seropositive for HHV-6 by the age of 3 years (2; reviewed in reference 26). HHV-6 isolates differ in their *in vitro* growth properties, exhibit DNA restriction site polymorphism, and differ in their antigenic properties (1, 3, 8, 11, 14, 17, 19, 21, 28, 31). These properties segregate HHV-6 isolates into two groups. A consensus was reached among the investigators who met at the 17th International Herpesvirus Workshop, Edinburgh, Scotland (August 1992), to designate these two groups tentatively HHV-6 variants A (HHV-6A) and B (HHV-6B). The HHV-6A group contains most of the isolates from adults, which includes isolate GS from a patient with lymphoproliferative disorder, U1102 from a Ugandan AIDS patient, isolates from patients with various immune disorders, and two isolates from children with roseola (1, 3, 8, 11,

17, 28). The HHV-6B group contains most of the isolates from children with roseola (1, 3, 8, 11, 17, 28) as well as isolates from adults, such as HHV-6 strain Z-29 from a Zairian AIDS patient (21), an isolate from a leukopenia patient, and an isolate from a bone marrow transplant patient (1, 8). Reactivities of adult sera suggest that individuals may be infected with viruses from one group or both (8).

Reactivities of monoclonal antibodies (MAbs) against HHV-6A strain GS show that the two groups of HHV-6 isolates are antigenically closely related, exhibiting group-common and group-specific epitopes. MAbs 2D4, 2D6, and 13D6 reacted with all HHV-6A isolates in radioimmunoprecipitation, Western immunoblot, enzyme-linked immunosorbent, and indirect immunofluorescence assays and recognized envelope-associated major glycoprotein complex gp82-gp105 (4, 7). In Western blot reactions, these MAbs recognized several polypeptides of about 105, 92, 82, 62, and 58 kDa. Polypeptides of about 105 and 82 kDa were recognized from [³H]glucosamine-labeled and ¹²⁵I-labeled virions (4, 7). Pulse-chase experiments and assays with *N*-glyconase and tunicamycin suggest two precursor polypeptides of about 58 and 84 kDa for the various polypeptides in the gp82-gp105 complex (7). The three MAbs reacted with HHV-6A strain GS-infected cells in surface immunofluorescence and immune electron microscopy assays and neutralized the infectivity of all HHV-6A isolates (4, 7). Although these MAbs did not react with HHV-6B isolates in neutral-

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ization, Western blot, enzyme-linked immunosorbent, immunofluorescence, or immune electron microscopy assays, they immunoprecipitated polypeptides of about 78 and 72 kDa from HHV-6B isolates (7). Identification of the gene(s) that encodes glycoprotein complex gp82-gp105 is an initial step towards a detailed analysis of the structure and functions of these glycoproteins and will lead to a better understanding of the diversity of these glycoproteins among the two groups of HHV-6 isolates.

In this report, we describe the identification and characterization of a 624-bp genomic fragment of HHV-6A strain GS representing a part of the gene that encodes the gp82-gp105 polypeptides. The genomic insert was used to map the gene on the HHV-6A strain U1102 genome. We determined the nucleotide sequence of the insert, which revealed a single open reading frame (ORF) of 501 nucleotides, with an open 5' end and a stop codon at the 3' end. The predicted protein is 167 amino acid residues long and contains four potential N-glycosylation sites and six cysteine residues. We also report the mapping of the neutralizing epitope recognized by the MAbs with various exonuclease III deletion constructs of the gp82-gp105-encoding gene fragment. The epitope is localized within or near a repeat amino acid sequence (NIYFNIY) of the putative protein.

MATERIALS AND METHODS

Viruses and cells. HHV-6A strains GS and U1102 were gifts from R. C. Gallo, National Cancer Institute, Bethesda, Md., and from the late R. W. Honess, National Institute for Medical Research, Mill Hill, London, England, respectively. HHV-6B strain Z29 was a gift from P. Pellett, Centers for Disease Control, Atlanta, Ga. Suspension cultures of human T-cell lines HSB-2 (ATCC CCL 120.1, CCRF-HSB-2) and Molt-3 (ATCC CRL 1552) 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. HSB-2 cells were used for routine propagation of HHV-6A strains GS and U1102, and HHV-6B strain Z29 was grown in Molt-3 cells. Infection of cells and assay procedures have been described previously (5, 8).

MAbs. The production and characterization of MAbs against HHV-6A strain GS-infected cells has been described previously (5). High-titer ascitic fluids of MAbs were raised by injecting hybridoma clones intraperitoneally into pristane-primed BALB/c mice.

Radiolabeling procedures, radioimmunoprecipitation, and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Infection was carried out at a multiplicity of infection of 10 tissue culture infective doses per cell. Uninfected cells (10^6) were mixed with HHV-6A strain GS 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 postinfection, 10^7 uninfected and infected cells were labeled for 20 h with 250 μ Ci of [35 S]methionine (specific activity, 1,072 Ci/mmol; Du Pont NEN, Wilmington, Del.) and the lysates were used for immunoprecipitation reactions as described previously (5, 8). The precipitates were washed, dissociated by boiling in sample buffer, and analyzed by SDS-PAGE in 9% acrylamide cross-linked with 0.28% *N,N'*-diallyltartardiamide. Molecular weight markers (Sigma) were electrophoresed in parallel channels. 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.

Construction and screening of cDNA and genomic libraries and subcloning of insert 82G. Construction of a cDNA library from HHV-6A strain GS-infected cells in the λ gt11 expression system has been described previously (9). An HHV-6A strain GS genomic DNA library was also constructed in the λ gt11 expression system. Briefly, purified viral DNA was sonicated for 3 min and the staggered ends of the sonicated DNA were filled by the Klenow fragment of DNA polymerase I. The DNA fragments were methylated and ligated with *Eco*RI linkers. The DNA fragments were separated by electrophoresis in a 0.8% agarose gel, and fragments of 0.5 to 2.0 kb were ligated to the *Eco*RI sites of predigested λ gt11 arms. The ligated DNAs were packaged into bacteriophage heads and amplified by infection of *Escherichia coli* Y1090 (r^-) (9, 22). The cDNA and genomic libraries were screened with MAbs as described previously (9). Immunoreactive phage plaques were picked and purified by four subsequent steps of cloning and screening. Phage DNA containing the HHV-6A strain GS DNA insert was prepared from plate lysates as described by Maniatis et al. (22). This recombinant phage DNA was digested with *Eco*RI, separated in a 0.8% agarose gel, and purified from the agarose by phenol-chloroform extraction. This insert, designated 82G, was then subcloned directly into pUC13 (Pharmacia, Piscataway, N.J.), pBluescript [pBSK(+); Stratagene, La Jolla, Calif.], and pGEMEX-1 (Promega, Madison, Wis.) vectors. Proper orientation in prokaryotic expression vector pGEMEX-1 was verified by restriction analysis and by the reactivities of MAbs with the Western blotted fusion proteins from transfected JM109(DE3) or BL21(DE3)pLysS bacteria induced by 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG).

Raising rabbit antibodies against the fusion protein. An *Eco*RI-digested genomic insert from plasmid pUC13 was ligated into prokaryotic expression vector pATH (a plasmid amenable to the making of tryptophan hybrids) and transformed into *E. coli* RR1 for recombinant protein synthesis (30). The amino-terminal portion of the fusion protein expressed from this construct contained the leader peptide of protein TrpE, with an approximate molecular mass of 38 kDa (30). Plasmid construction and orientation were verified by restriction digest analysis. Plasmid-containing bacteria grown in pATH medium were induced with β -indoleacrylic acid, and insoluble recombinant protein was isolated as described elsewhere (30). Briefly, plasmid-transformed RR-1 bacteria were cultured overnight in pATH medium containing tryptophan. Samples were then diluted 40 times and subcultured at 30°C to a final optical density at 600 nm of 0.1 in pATH medium without tryptophan. A 200-ml volume of this culture was induced with 1 mg of indoleacrylic acid for 3 to 4 h. Harvested bacterial pellets were washed with 10 mM Tris (pH 7.5), treated with 5 ml of lysozyme (2 mg/ml in 50 mM Tris, 5 mM EDTA) for 1 h on ice, and then kept in 750 μ l of 10% Triton X-100-700 μ l of 5 M NaCl for 30 min on ice. The lysed bacteria were sonicated briefly and centrifuged at 10,000 rpm for 10 min in a Beckman JA-20 rotor, and the pellets were washed twice with 10 mM Tris (pH 7.5) containing 1 M NaCl. The final insoluble protein pellet was suspended in 1 ml of 10 mM Tris (pH 7.5). To check expression of the fusion protein, 10 μ l of the insoluble protein was dissolved in SDS-PAGE loading buffer and separated by SDS-PAGE. These were either stained with Coomassie brilliant blue or Western blotted and reacted with MAbs. New Zealand White male rabbits were first immunized subcutaneously and intramuscularly with 100 μ g of fusion protein in Freund's complete adjuvant. At 20 and 40 days later, rabbits were immunized with the same amount of

antigen in Freund's incomplete adjuvant. The rabbits were boosted 2 months after the last injection and bled 10 days later. Immunoglobulin G antibodies (R82G) were purified from the sera by affinity chromatography on a protein A-agarose column.

Western blotting. Bacterial strains JM109(DE3) and BL21(DE3)pLysS were transfected with an insert containing plasmid pGEMEX-1 by the CaCl_2 method (22). Induction of fusion proteins was accomplished by adding IPTG (1 mM) to bacterial cultures grown to an optical density at 600 nm of 0.2 to 0.5, and insoluble fractions were collected 4 h later as described above. Protein samples were separated by SDS-PAGE in acrylamide cross-linked with N,N' -diallyltartardiamide and electrophoretically transferred to nitrocellulose sheets (5, 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% skim milk) and then reacted for 3 h at room temperature with blocking buffer containing MABs or rabbit antisera. The sheets were washed with washing buffer (10 mM Tris-HCl [pH 7.2], 0.15 M NaCl, 0.3% Tween 20) and incubated for 1 h with alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit antibodies (HyClone Laboratories). Bound, enzyme-labeled antibodies were detected with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium substrate (Sigma) (5, 9).

DNA and RNA extraction and hybridization. Viral DNA from infected cells, host cell DNA, and total unfractionated RNA from uninfected and HHV-6A strain GS-infected (96 h postinfection) cells were isolated as described previously (9). DNAs were digested to completion with restriction enzymes. *EcoRI*-digested viral DNAs from cells infected with herpes simplex virus type 1 (HSV-1), HSV-2, cytomegalovirus, and Epstein-Barr virus were also included in the Southern analysis. Restriction endonuclease fragments of viral and host cell DNAs were separated on 1% agarose gel and transferred to GeneScreen Plus with $5\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate). A radiolabeled 82G DNA probe was synthesized by nick translation with [α - ^{32}P]dCTP (specific activity, 3,000 Ci/mmol; DuPont NEN) and the Klenow fragment of DNA polymerase I. Membranes containing DNA were hybridized with the labeled probe and washed under high-stringency aqueous conditions at 65°C and under low stringency at 37°C as described by Maniatis et al. (22). Denaturing agarose gel containing 0.66 M formaldehyde was 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 (22).

To map the location of the gene that encodes gp82-gp105 on the HHV-6A strain U1102 genome, restriction enzyme-digested viral DNA from U1102-infected HSB-2 cells and cosmid and plasmid clones containing the entire U1102 genome were used. Construction and characterization of these cosmids and plasmids have been described previously (25). DNA was transferred to a nylon hybridization membrane by the Southern blot procedure, and the membranes were prehybridized for 6 h at 55°C in prebuffer (50% formamide, $5\times$ Denhardt's reagent, 0.5% SDS, $6\times$ SSPE, 5 mg of boiled salmon sperm DNA). The membranes were then hybridized overnight in hybridization buffer containing 1.5×10^7 cpm of ^{32}P -labeled probe 82G. After hybridization, the membranes were washed twice for 30 min each time in $2\times$ SSC at room temperature, for 1 h at 55°C in $2\times$ SSC-1% SDS, and then twice for 15 min each time in $0.1\times$ SSC at room temperature. Membranes were air dried, covered with

cellophane, and exposed to Kodak XAR-5 film. Sizes of hybridized DNA fragments were determined by comparing developed film with standard DNA markers run in parallel lanes.

DNA sequence analysis. The genomic HHV-6 strain GS DNA insert from pUC13 was digested with *EcoRI* and subcloned directly into vector M13mp18 (Pharmacia). A series of overlapping clones of both orientations was generated by the Erase-A-Base system (Promega), and sequencing was performed by the dideoxynucleotide chain termination method (22). Sequencing of HHV-6A strain GS insert deletion mutants was performed by the dideoxynucleotide chain termination method directly from double-stranded, linearized pGEMEX-1 mutant clones. Sequence data were analyzed by the IBI-Pustell GENEric sequence analysis programs, and hydropathic analysis was conducted by using an algorithm of Kyte and Doolittle (18) contained in the software, with a nine-amino-acid window. Nucleic acid and amino acid homology analyses were conducted with the FASTA program of Lipman and Pearson (20).

Deletion constructs and mapping of the epitope recognized by MABs. A nested set of deletion mutants was constructed from the genomic insert (82G) with the Erase-a-Base system (Promega) in accordance with manufacturer specifications. Briefly, the *EcoRI*-digested 82G insert from λ gt11 was subcloned into prokaryotic expression vector pGEMEX-1. This plasmid, designated p82G, contained the bacteriophage T7 gene 10 promoter, which controls expression of the recombinant gene (9) (see Fig. 7). The amino-terminal portion of the fusion protein expressed from this construct contained the leader peptide of T7 gene 10, with an approximate molecular mass of 26 kDa. Vector p82G was digested with enzymes *ApaI* and *XhoI*, which restrict only within the polylinker region, to give 3' and 5' overhangs, respectively. The linearized p82G insert was then digested with exonuclease III, and aliquots of the reaction were stopped at 10- to 20-s intervals by using S1 nuclease and the Klenow fragment of DNA polymerase I. Blunt ends were then religated with T4 ligase, and plasmids were transformed into competent bacterial strain JM109(DE3) or BL21(DE3)pLysS. Both bacterial strains contain an IPTG-inducible T7 RNA polymerase gene which directs transcription of the fusion protein from the T7 promoter of pGEMEX-1. Epitope mapping was performed by testing the reactivities of MABs with the Western blotted fusion proteins from the various deletion clones.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the GenBank data base and assigned accession no. L18985.

RESULTS

Identification of the gene fragment that encodes gp82-gp105 of HHV-6. MABs 2D6, 2D4, and 13D6 neutralized the infectivity of HHV-6A isolates in the absence of complement and recognized glycoprotein complex gp82-gp105 of HHV-6A isolates in Western blot and radioimmunoprecipitation reactions (4, 7). To identify the gene(s) that encodes gp82-gp105, cDNA and genomic DNA libraries of HHV-6A strain GS were constructed in the λ gt11 expression system and screened with MABs. From the genomic library, one immunoreactive phage clone was identified by MAB 2D6. This clone was purified by four successive steps of screening and cloning. In a Western blot assay with the insert-bearing phage lysate, MABs 2D4, 2D6, and 13D6 recognized a fusion protein of approximately 135 kDa. The data obtained with

FIG. 1. Identification of the 82G insert from the HHV-6 genomic λ gt11 expression library. Lanes: 1 to 3, reactivities of MAb 2D6 in Western blot reactions with IPTG-induced phage lysates; 1, control λ gt11 phage lysate without insert; 2, lysate from phage containing an insert identified by MAb 2D6 from the genomic λ gt11 library of HHV-6A strain GS DNA; 3, lysate from phage containing a cDNA insert that encodes HHV-6 protein P41 identified from an HHV-6 cDNA library in λ gt11. The designation 135 indicates the approximate size (in kilodaltons) of the fusion protein recognized by MAb 2D6. Lanes 4 to 9, rabbit antiserum against the genomic insert fusion protein recognizes gp82-gp105. [35 S]methionine-labeled uninfected (lanes 4, 6, and 8) and HHV-6A strain GS-infected (lanes 5, 7, and 9) cells were used in immunoprecipitation reactions. Lanes 4 and 5, rabbit preimmune serum (PB). Lanes 6 and 7, rabbit antiserum against the fusion protein expressed from the genomic insert. Lanes 8 and 9, MAb 2D6. Samples were analyzed on 9% acrylamide cross-linked with N,N' -diallyltartardiamide. Standard prestained and unstained molecular size markers were included in parallel lanes. The numbers indicate the approximate molecular masses (kilodaltons) of the polypeptides recognized from HHV-6-infected cells.

2D6 are shown in Fig. 1, lane 2. No reactivity with the control phage lysate (Fig. 1, lane 1) or with the lysate from phage containing a 2.1-kb cDNA insert that encodes HHV-6 nuclear phosphoprotein P41 (9) (Fig. 1, lane 3) was seen. Since the 135-kDa fusion protein included a β -galactosidase protein of approximately 116 kDa, this suggested that the genomic insert identified should code for a protein of about 19 kDa. Agarose gel electrophoresis of the insert excised from λ gt11 with *Eco*RI revealed a genomic insert of approximately 650 bp. This insert, designated 82G, was subcloned directly into pUC13, pBluescript, and prokaryotic expression vectors pGEMEX-1 and pATH.

To determine whether the 82G genomic insert identified by the MAb indeed codes for gp82-gp105 of HHV-6A strain GS, rabbit antibodies were raised against the fusion protein expressed in the pATH vector. Immunoglobulins G from immune sera (R82G) were purified and tested with HHV-6A strain GS-infected and uninfected cells in radioimmunoprecipitation and Western blot reactions. Preimmune serum showed no reactivity with [35 S]methionine-labeled uninfected or HHV-6A strain GS-infected HSB-2 cells (Fig. 1, lanes 4 and 5). In contrast, rabbit anti-82G (R82G) antibodies reacted specifically with polypeptides of about 82 and 105 kDa from HHV-6A strain GS-infected cells but showed no

FIG. 2. HHV-6 specificity of the genomic insert identified by MAb 2D6. (A) Southern blot hybridization with radiolabeled genomic insert 82G under low-stringency conditions. Uninfected and human herpesvirus-infected cell DNAs were digested with *Eco*RI (E) (lanes 1 to 7, 8, 10, and 11) or *Bam*HI (B) (lanes 9 and 12). The DNAs used were BHK-21 cell DNA (lane 1), HSV-1 DNA (lane 2), HSV-2 DNA (lane 3), cytomegalovirus DNA (lane 4), Epstein-Barr virus DNA from Raji cells (lane 5), Epstein-Barr virus DNA from B95-8 cells (lane 6), HSB-2 cell DNA (lane 7), HHV-6A strain GS DNA from infected HSB-2 cells (lanes 8 and 9), Molt-3 cell DNA (lane 10), and HHV-6B strain Z29 DNA from infected Molt-3 cells (lanes 11 and 12). The sizes (in kilobases) of the HHV-6-specific DNA fragments hybridized are indicated. Standard lambda marker DNAs of known sizes were included in parallel lanes. (B) Northern blot hybridization using radiolabeled genomic insert 82G with total RNA from uninfected HSB-2 cells (lane 1) and HHV-6A strain GS-infected HSB-2 cells (lane 2). The sizes (in kilobases) of HHV-6-specific RNAs that hybridized with the insert are indicated.

specific reactivity with uninfected cells (Fig. 1, lanes 6 and 7). The mobilities of these polypeptides were similar to those of HHV-6A strain GS polypeptides recognized by MAb 2D6 (Fig. 1, lane 9). Cross-immunoprecipitation reactions showed that MAb and rabbit antibodies recognized the same gp82-gp105 complex from HHV-6A strain GS (data not shown). In Western blot analysis, R82G antibodies reacted with polypeptides of about 82 and 105 kDa, as well as with the precursor forms of these proteins from HHV-6A strain GS-infected cells, which were identical to the polypeptides recognized by MAb 2D6 (data not shown). These data demonstrate that the genomic 82G insert represents a portion of the gene(s) that encodes gp82-gp105 of HHV-6A strain GS.

HHV-6 specificity of the genomic insert that encodes gp82-gp105. To ascertain the viral specificity of the 82G insert further, the [α - 32 P]dCTP-labeled 82G insert was used in a Southern blot analysis. Detailed restriction mapping of the HHV-6A strain GS genome has not been reported. We elected to use enzymes *Eco*RI and *Bam*HI, since restriction enzyme bands generated by these two enzymes have been previously used to estimate the size of the HHV-6A strain GS genome (16). The 82G insert hybridized specifically with the *Eco*RI- and *Bam*HI-digested DNA fragments from HHV-6A strain GS-infected cells under both high- and low-stringency conditions, and hybridization was detected only under low-stringency conditions with DNA from HHV-6B strain Z-29-infected cells. Figure 2A shows hybridization profiles obtained under low-stringency conditions. No hybridization with *Eco*RI-digested DNA from BHK-21

FIG. 3. (A) Termination codon and ORF analysis of 624-bp genomic insert 82G in all six reading frames. 82G ORF indicates the single potential ORF with an open 5' end. The positions of the six cysteine (C) residues and the potential N-glycosylation (N) sites in the ORF are indicated. The open arrow shows the location of the epitope region identified by deletion analysis (see Fig. 7 to 9). The numbers indicate nucleotide positions. (B) Hydropathy analysis of the deduced amino acid sequence of the above sequence by the Kyte and Doolittle algorithm. Scores were averaged within a sliding window of nine. The vertical axis represents a relative hydropathy score in which negative values show hydrophilicity and positive value show hydrophobicity. The numbers indicate amino acid positions.

cells (Fig. 2A, lane 1); viral DNA fragments from HSV-1, HSV-2, cytomegalovirus, and Epstein-Barr virus (Fig. 2A, lanes 2 to 6); or DNA from uninfected HSB-2 (Fig. 2A, lane 7) or Molt-3 (Fig. 2A, lane 10) cells was seen. Specific hybridization with 12-kb *Eco*RI (Fig. 2A, lane 8) and 6.3-kb *Bam*HI (Fig. 2A, lane 9) DNA fragments from HHV-6A strain GS-infected HSB-2 cells was seen. Furthermore, specific hybridization with two equal-intensity *Eco*RI DNA fragments of 12.5 and 10.5 kb (Fig. 2A, lane 11) and a 10.5-kb *Bam*HI DNA fragment (Fig. 2A, lane 12) from HHV-6B strain Z-29-infected Molt-3 cells was seen. These results confirmed the HHV-6 specificity of the genomic insert identified by MAbs. The fact that hybridization was seen only under low-stringency conditions indicates the diversity in the region of the HHV-6B strain Z-29 genome corresponding to the HHV-6A strain GS genome region that encodes gp82-gp105. Furthermore, hybridization with different-size DNA fragments from the variant A and B strains of HHV-6 confirms the reported restriction site heterogeneity among the two groups of HHV-6 (1, 3, 11, 14, 17, 28). In a Northern blot analysis with equal quantities of total unfractionated RNAs isolated from HHV-6A strain GS-infected and uninfected cells, the 32 P-labeled 82G insert hybridized specifically with RNA from HHV-6A strain GS-infected cells (Fig. 2B, lanes 1 and 2). The sizes of HHV-6 transcripts were

calculated by comparing their mobilities with those of RNA marker molecules (Bethesda Research Laboratories, Gaithersburg, Md.). Two prominent transcripts with estimated sizes of 2.5 and 3.5 kb and an additional, less intense band of 1.7 kb were identified by the 82G insert from HHV-6A strain GS.

DNA sequence analysis of genomic insert 82G. DNA sequence analysis revealed that the genomic insert consisted of 624 bp with a mean composition of 43.9% G + C. ORF analysis revealed a single ORF of 501 nucleotides (Fig. 3A). The complete sequence and the predicted amino acid residues are shown in Fig. 4. The 82G ORF has an open 5' end and a translational stop codon (TAA) at positions 502 to 504 (Fig. 3A and 4). A potential polyadenylation signal sequence (AAATAA) is located between nucleotide positions 557 and 563, near the 3' end of the insert (Fig. 4). The sequence was further verified by restriction digestion analysis with the predicted restriction sites.

The predicted protein is 167 amino acid residues long and has a calculated molecular weight of 19,214. Analysis of the putative primary amino acid sequence revealed six cysteine residues and four potential N-glycosylation sites (Fig. 3A and 4). Hydropathy analysis of the 82G ORF indicated two short stretches of hydrophobic domains (amino acids 5 to 14 and 42 to 52) and a hydrophilic region at amino acids 52 to

1 CTTGTTTATAGTCTATTAAACATCATCTTCTTTACTCCATGTCTGTTTCTCCGCGCA 60
 L V Y S L F N I I F L Y S M S C F S R A
 21 TACGAAATTTTACAGGACTGAGACGGAGAATGGCAACCGCAAGACTGGGCGTTATGAGA 120
 Y E I F T G L R R R M A T A R L G V M R
 41 CCGCGGAGATCTTGTGCGCTGATATTTTGTGCGCGTTTTCGATGGCGACGGCCCGCAGC 180
 P P R S C A L I F L C A F S M A T A P T
 61 AACGCTACGGCCACCGCGTGGCGGAAACCGTGAATCCACACCGCGCCAGGAGCAAA 240
 N A T A H R R A G T V K S T P P P E D K
 81 GGCAACTATACGCAAAATACTATGACAAAAACATTTTCAACATTTACGAAGGTAGA 300
 G N Y T A K Y Y D K M I Y F M I Y E G R
 101 AATTCTACGCGAGGAGACGCGCTATGGAAATCATCTCTAAATTTTCTACCTCCGAA 360
 M S T P R R R T L W E I I S K F S T S E
 121 ATGTTAAGTTTAAAGAGTTAAAGCTTTCGTCGCCGTAGATGATACCCGACTACTACC 420
 M L S L K R V K A F V P V D D M P T T T
 141 TTAGAAGATATAGCAGACATCCTGAACACGCGTTTTCGATGACAATAGCTGCGGTGT 480
 L E D I A D I L N Y A V C D D N S C G C
 161 ACCATGAGACACAAGCAGCGTAAGGATGCAGCGTTTCATCTTGTGTTATCCGATGTCGGA 540
 T I E T Q A R -

 ATCTACTGAGATCATAAATAAATGAATAGATAAATAATCATATCTAATTTATCGCAGCATC 600
 624
 ATGTTTCGGAGATATCATAATTGG

FIG. 4. Nucleic acid sequence of genomic fragment 82G and the deduced ORF. The nucleotide position is numbered on the right, and the amino acid position is numbered on the left. The termination codon and the polyadenylation signal sequence are marked by asterisks. Putative N-glycosylation sites (N-X-T/S) and cysteine residues are indicated by boldface type. The positions of the last six nucleotides at the 3' end in deletion clones 20D (CAAAAA) and 20C (CAACAT) (see Fig. 8A and B) are indicated by boldface type and by the symbol # above the last nucleotide. The amino acids (NIYFNIY) in the putative antigenic epitope within the boundaries of the 15-nucleotide differences of deletion clones 20C and 20D are shown in boldface type and by underlining.

112 (Fig. 3B). Comparison of the predicted amino acid sequence of the 82G insert with the protein data bank libraries and a protein library of all available herpesvirus sequences revealed no significant homology.

Mapping of the 82G insert on the HHV-6 strain U1102 genome. Since a restriction map for the HHV-6A strain GS genome was not available, the 82G insert was mapped on the HHV-6A strain U1102 genomic restriction map. The 82G insert was hybridized with restriction enzyme-digested HHV-6A strain U1102 viral DNA from infected HSB-2 cells and with the overlapping cosmid and plasmid clones containing the entire genome of HHV-6A strain U1102 (25). DNAs from the cosmids and plasmids were digested with *HindIII* and *PstI*, transferred to nylon membranes, and probed with the labeled 82G insert. Under high-stringency hybridization conditions, the 82G insert hybridized only with fragments of 2.1 and 1.1 kb from *HindIII*-*PstI*-digested cosmid clone pMF402-27 (Fig. 5A, lane 1). Since this clone contains a 43-kb *SmaI* fragment overlapping the boundary of the right terminal direct repeat (DR_R) of the HHV-6A strain U1102 genome (24), these data localized the gene that encodes gp82-gp105 to the unique long segment near the right end of the HHV-6 genome (Fig. 6).

To map the location of the insert further, HHV-6A strain U1102 DNA from infected HSB-2 cells was digested with enzymes that were used to generate the restriction map of the U1102 genome (24). The sequence of the 82G insert (624 bp) does not contain *BamHI*, *EcoRI*, *KpnI*, *PstI*, *SalI*, *SmaI*, or *XhoI* restriction sites but does contain a *HindIII* restriction site at position 383 (Fig. 6). Specific hybridization with a 9.5-kb *BamHI* fragment, 9.5- and 2.1-kb *HindIII* fragments, a 6.2-kb *PstI* fragment, 2.2- and 1.1-kb *HindIII*-*PstI*

FIG. 5. Hybridization of the HHV-6A strain GS 82G insert with HHV-6A strain U1102 and GS viral DNAs. (A) Southern blot hybridization with *HindIII*- and *PstI*-digested cosmid clone pMF402-27 of HHV-6A strain U1102. (B) Southern blot hybridization with HHV-6A strain U1102 DNA from infected HSB-2 cells. Lanes: 1 to 7, DNA digested with *BamHI*, *HindIII*, *PstI*, *HindIII* and *PstI*, *SalI*, *EcoRI*, and *DraI*, respectively. (C) Southern blot hybridization with HHV-6A strain GS DNA. Lanes 1 to 3, DNA digested with *HindIII*, *EcoRI*, and *BamHI*, respectively. The sizes (in kilobases) of the HHV-6-specific DNA fragments hybridized are indicated. Standard lambda marker DNAs of known sizes were included in parallel lanes.

fragments, a 6.7-kb *SalI* fragment, a >13-kb *EcoRI* fragment, and 1.25- and 0.43-kb *DraI* fragments was seen (Fig. 5B, lanes 1 to 7). The 82G insert hybridized with two identical fragments of 2.1 and 1.1 kb from cosmid clone pMF402-27, as well as the U1102 viral DNA digested with *HindIII* and *PstI* (Fig. 5A, lane 1, and B, lane 4). With HHV-6A strain GS DNA, specific hybridization with 9.5- and 1.5-kb *HindIII* DNA fragments (Fig. 5C, lane 1), a 12-kb *EcoRI* fragment (Fig. 5C, lane 2), and a 6.3-kb *BamHI* fragment (Fig. 5C, lane 3) was seen. The differences in the sizes of the HHV-6A strain U1102 and GS and HHV-6B strain Z-29 DNA fragments that hybridized (Fig. 2A) may be due to the reported heterogeneity in this region of the genome (24, 26).

Comparison of the sizes of fragments that hybridized with the 82G fragment from cosmid clones of U1102 and the other viral DNA fragments with the U1102 restriction map (24) localized the 82G insert in a region spanning *HindIII* fragments W and F, *PstI* fragment N, *SalI* fragment G, *BamHI* fragment G, *EcoRI* fragment A, and *SmaI* fragment A (Fig. 6). The reported sizes of these U1102 fragments are 2.05 and 9.4 kb for *HindIII* fragments W and F, respectively; 5.6 kb for the *PstI* N fragment; 9.5 kb for the *BamHI* G fragment; 6.7 kb for the *SalI* G fragment; and 14.5 kb for the *EcoRI* A fragment. These results further localized the gene that encodes gp82-gp105 near the right direct repeat (DR_R) region in the unique long segment of the HHV-6A strain U1102 genome (Fig. 6).

Mapping of the neutralizing epitope recognized by MAbs 2D4, 2D6, and 13D6. Since the 167-amino-acid ORF encoded by the 82G insert was recognized by the three neutralizing MAbs and since these MAbs showed variable reactivity with the two HHV-6 groups (7, 8), it was of interest to identify the epitope(s) recognized by these MAbs. The small 167-amino-acid ORF and the absence of convenient restriction sites within the 82G insert for construction of deletion plasmids prompted the use of an alternative approach to identify the epitope(s) recognized. A nested set of deletion mutants with

FIG. 6. Mapping of HHV-6A strain GS genomic insert 82G on the HHV-6A strain U1102 genomic restriction map. The top line shows the U1102 genome. The hybridization pattern shown in Fig. 5 localized the insert in a region spanning *Hind*III fragments W and F, *Pst*I fragment N, *Sal*I fragment G, *Bam*HI fragment G, *Eco*RI fragment A, and *Sma*I fragment A. The letter designations of fragments that hybridized with the 82G fragment and the sizes indicated in kilobases were taken from the genomic map described by Martin et al. (24). The location of the 82G insert in relation to the *Hind*III W and F fragments is shown at the bottom. DR_L and DR_R, left and right terminal direct repeats, respectively.

various sequence lengths deleted from the 3' end of the ORF was constructed from the genomic insert (82G) by using the Erase-a-Base system (Promega; Fig. 7). Progressively digested p82G plasmids from 25 different time points of exonuclease III digestion were checked by agarose gel electrophoresis and used to transform competent JM109(DE3) bacteria. Transformants were selected for ampicillin resistance, and plasmid preparations of the colonies were digested with *Eco*RI and *Nsi*I to release the insert (Fig. 7). Colonies with appropriate-size inserts were selected and numbered by decreasing insert lengths.

Bacterial clones containing these 82G deletion plasmids were induced with IPTG, and the lysates were collected after 4 h. These were separated by SDS-PAGE, Western blotted onto nitrocellulose sheets, and reacted with MAb 2D6 (Fig. 8A). MAb 2D6 reacted with a fusion protein of about 46 kDa from the p82G insert-bearing clone (Fig. 8A, lane 1). Since the termination codon of the p82G insert was eliminated by exonuclease III digestion in the higher-numbered clones, those with decreasing insert lengths (Fig. 8A, lanes 9 to 26), the stop signal for these fusion proteins was provided by the vector plasmid, giving rise to fusion proteins of various

FIG. 7. Schematic diagram for construction of exonuclease III deletion mutants for mapping of the neutralizing epitope recognized by MAb 2D6. The top lines represent the orientation of the 82G insert in the pGEMEX-1 vector (p82G). The amino-terminal portion of the fusion protein expressed from this construct contained the leader peptide of T7 gene 10 under control of the T7 promoter. The positions of the promoters and restriction sites are indicated. The p82G vector was digested with enzymes *Apa*I and *Xho*I, which restrict only within the polylinker region, to give 3' and 5' overhangs, respectively. The linearized p82G insert was then digested with exonuclease III, and aliquots of the reaction were stopped at short intervals by using S1 nuclease and the Klenow fragment of DNA polymerase I. Blunt ends were then religated with T4 ligase, and plasmids were transformed into competent bacterial strain JM109(DE3) or BL21(DE3)pLysS. Fusion proteins induced from these constructs were used to map the epitope recognized by the MAbs (see Fig. 8).

sizes. Positive MAb reactivity was seen with fusion proteins from all clones containing an insert longer than that of clone 21 (Fig. 8A). MAb 2D6 also reacted with faster-migrating common proteins in the Western blots from reactive clones and could represent degraded fusion proteins (Fig. 8A, lanes 1 to 20). To narrow the epitope boundaries, many deletion clones with insert sizes intermediate between those of the positive (clone 20) and negative (clone 21) reactive transition points (20A to 20D) were selected. Fusion proteins were induced from these clones and tested with a MAb in the Western blot assay. MAb 2D6 reacted with the fusion protein extract from clone 20C (Fig. 8B, lane 6) but not with that from clone 20D or any clone with a smaller insert (Fig. 8B, lanes 7 to 12).

The reactive (20C) and nonreactive (20D) clones were propagated further, and the inserts were sequenced. The larger, reactive 20C insert consists of 287 bp ending at the sequence CAACAT, and the smaller, nonreactive 20D insert consists of 272 bp ending at the sequence CAAAAA (Fig. 4). This suggested that the epitope recognized by MAb 2D6 is

FIG. 8. Mapping of the epitope recognized by MAb 2D6. (A) Lanes 1 to 26, bacteria containing p82G deletion mutants with different insert lengths were induced with IPTG for 4 h. The fusion proteins were extracted, run on SDS-15% PAGE in order of decreasing insert size, transferred onto a nitrocellulose sheet, and reacted with a 1:100 dilution of MAb 2D6. FP indicates the various sizes of fusion proteins recognized by the MAb. Numbers indicate the molecular masses (kilodaltons) of the standard prestained markers run in parallel lanes. Positive reactivity with the MAb was seen with fusion proteins from all of the clones containing an insert longer than that of clone 21. (B) Lanes 1 to 13, deletion clones with insert sizes around the positive (clone 20) and negative (clone 21) reactive transition points (A) were selected. Fusion proteins were induced from these clones and tested with the MAb in a Western blot assay. MAb 2D6 reacted with the fusion protein extract from clone 20C (lane 6) but not with that from clone 20D or any other clone with a smaller insert (lanes 7 to 13).

probably within or close to the boundaries of the 15-nucleotide difference. Within the boundaries of the potential epitope identified by this method, there is an amino acid repeat consisting of asparagine, isoleucine, and tyrosine (NIY) with a separating phenylalanine (NIYFNIY). The larger, reactive 20C insert encodes the NIYFN amino acids, which are absent in the nonreactive 20D clone (Fig. 4). This region is within the hydrophilic domain between amino acids 52 and 112 (Fig. 3B). It is interesting that this repeat sequence region recognized by neutralizing MAb 2D6 is surrounded on either side by two potential N-glycosylation sites and three cysteine residues (Fig. 3 and 4).

Although sufficient fusion protein was produced from the deletion clones of p82G for testing in the Western blot assay, in some clones the quantity was insufficient for visualization with Coomassie blue staining (data not shown). To verify that a fusion protein was indeed induced from the clones which were unreactive with MAb 2D6 in the Western blot reactions (Fig. 8A and B), the yield of fusion protein needed to be increased. A plasmid from each of the p82G deletion clones was purified and transformed into competent BL21(DE3)pLysS cells. This bacterial strain contains a plasmid that codes for T7 lysozyme, which has been shown to inhibit the activity of the uninduced levels of T7 DNA polymerase present in BL21(DE3) cells, thus allowing the bacteria to grow to a higher number before IPTG induction. The transformed cells were induced with IPTG, and the insoluble protein fractions were collected. Aliquots of these insoluble proteins were separated by SDS-PAGE and either stained with Coomassie blue or transferred to nitrocellulose sheets to test their reactivity with MAb 2D6. Fusion protein from all of the clones was indeed detected, including clones 20D, 21, and 22, which were nonreactive with MAb 2D6 (Fig. 9A). The reactivity of MAb 2D6 with these fusion proteins in Western blots (Fig. 9B) was identical to the pattern obtained previously with fusion protein expressed from vector pGEMEX-1 in JM109(DE3), as shown in Fig. 8A and B, thus demonstrating that the absence of MAb reactivity was not due to absence and/or degradation of the fusion protein.

MAbs 2D4 and 13D6 also neutralized the infectivity of the HHV-6 variant A isolates (7), and these MAbs also recognized the 135-kDa fusion protein expressed from the λ gt11 clone identified by MAb 2D6 (data not shown). This indi-

FIG. 9. Reactivities of MAbs 2D6, 2D4, and 13D6 with the fusion proteins expressed from the p82G deletion constructs in BL21(DE3)pLysS bacteria. The fusion proteins were induced with IPTG for 4 h, extracted, and analyzed on SDS-15% PAGE. Gels were either stained with Coomassie blue (A) or transferred onto a nitrocellulose sheet and reacted with a 1:100 dilution of MAb 2D6 (B), 2D4 (C), or 13D6 (D). The arrowheads indicate the various sizes of fusion proteins (A) and the fusion proteins recognized by the MAbs (B, C, and D). Lane 1, pGEMEX-1 vector alone. Lane 2, fusion protein from the full-length p82G clone. Lanes 3 to 8, fusion proteins from deletion clones. Lane 9, BL21(DE3)pLysS bacterial lysate not containing the 82G insert or vector pGEMEX-1. Lanes 5 and 6 correspond to clones 20C and 20D, respectively, as shown in Fig. 8B, lanes 6 and 7.

cated that the epitopes recognized by these antibodies were also present in the 167-amino-acid ORF encoded by the 624-bp 82G insert. To map the epitope(s) recognized by these MAbs, experiments with the deletion constructs of p82G described above were used. MAbs 2D4 and 13D6 recognized fusion proteins (Fig. 9C and D), while no reactivity was seen with MAbs against other HHV-6 glycoproteins and proteins (5) (data not shown). The reactivities of MAbs 2D4 and 13D6 with the fusion proteins (Fig. 9C and D) were identical to the reactivities seen with MAb 2D6 (Fig. 9B), indicating that these three neutralizing MAbs recognized a similar epitope within or close to the boundaries of the repeat amino acid sequence NIYFNIY.

DISCUSSION

Immunoscreening of λ gt11 libraries is a powerful tool for identification and cloning of specific genes. By using neutralizing MAbs against envelope glycoprotein complex gp82-gp105 of HHV-6A strain GS, we identified a clone from a λ gt11 HHV-6A strain GS genomic expression library. Southern and Northern blots with viral DNA and RNA verified the virus specificity of the 624-bp insert identified, and the reactivity of rabbit antibodies against the fusion protein further confirmed the authenticity of the gene identified. In the deduced amino acid sequence of the 624-bp genomic insert, the open 5' end, the stop codon, and the potential polyadenylation signal sequence near the 3' end indicate that the 82G insert represents the 3' end of the gene and therefore could code for the carboxy terminus of its protein product. This would place the epitope recognized by the MAbs at the carboxy end of the protein. The reactivities of MAbs with the surfaces of infected cells in an immunofluorescence assay and in immune electron microscopy and the neutralization of infectivity (4, 7) clearly localized the epitope recognized by MAbs on the outer surfaces of virion envelopes. These data, together with the lack of a distinct hydrophobic membrane-spanning region in the predicted 167-amino-acid protein, suggest that gp82-gp105 of HHV-6A strain GS is a class II glycoprotein, like the influenza virus neuraminidase protein, with the carboxy terminus exposed on the exterior of the envelope. Hydrophobic analysis of the full-length gene sequence would provide information regarding the potential membrane-spanning regions and, together with testing of additional MAbs against the different regions of gp82-gp105, would support and/or clarify the class II glycoprotein designation. However, the possibility that this ORF is part of an exon that encodes glycoprotein complex gp82-gp105 cannot be ruled out.

Various strategies have been used to map the epitopes recognized by MAbs (6, 10, 12, 13, 15, 23). The method we used for epitope mapping took advantage of the fact that the genomic insert identified was small and that the MAbs recognized the Western blotted fusion proteins. This procedure involved the generation of a nested set of deletion mutants by exonuclease III digestion. With this method, we were able to map the neutralizing epitope recognized by MAb 2D6 to within five amino acids of the fusion protein. This epitope included the repeat amino acid sequence NIYFNIY (Fig. 3 and 4). Further confirmation of the neutralizing epitope requires the use of synthetic peptides corresponding to this region and rabbit antibodies against these peptides. Such studies and testing of the reactivities of human sera are in progress. The epitope identified here lies within a region of relative hydrophilicity. The domain surrounding this amino acid repeat is symmetrical, containing

three cysteine residues and two potential N-glycosylation sites on either side of the epitope. The significance of this symmetry is not known.

The gp82-gp105 complex of HHV-6 variant A strains is composed of at least three major polypeptides of about 105, 92, and 82 kDa, as well as a few minor bands of about 38 and 36 kDa. The reactivity of MAbs with these polypeptides in Western blot reactions suggests the sharing of antigenic epitopes. Peptide mapping of the individual bands with *Staphylococcus aureus* V8 protease has demonstrated that the gp82 and gp105 polypeptides are closely related yet nonidentical (7). The 624-bp insert hybridized to at least two separate RNA species from HHV-6 strain GS-infected cells. The relatedness of these two RNA species remains to be elucidated. However, they likely share the same 3' ends, since the 624-bp insert represents the coding sequence for the carboxy terminus of a polypeptide. These data suggest that these polypeptides are related to each other at the genetic level, perhaps as members of a 3'-coterminal family, with the various mRNAs formed either through differential splicing or by internal start sites. Our results localized the gene that encodes gp82-gp105 near the right end of the unique long region of the HHV-6A strain U1102 genome (Fig. 6), and preliminary studies showed that the 5' end of the ORF in the 82G insert extends into the direct repeat region. Work is in progress to sequence the entire gene that codes for gp82-gp105, which would provide important insight into the relatedness of the glycoproteins within the gp82-gp105 complex which share the same immunoreactive region. Furthermore, this will also help in the detailed analysis of the structure and functions of these glycoproteins and contribute to a better understanding of the diversity of these glycoproteins among the two groups of HHV-6 isolates and the genomic heterogeneity exhibited.

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