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The UL16 Gene of Human Cytomegalovirus Encodes a Glycoprotein That Is Dispensable for Growth In Vitro

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The UL16 gene of human cytomegalovirus (HCMV) encodes a predicted translation product with features characteristic of glycoproteins (signal and anchor sequences and eight potential N-linked glycosylation sites). Antisera were raised against the UL16 gene product expressed in *Escherichia coli* as a β-galactosidase fusion protein. The antisera detected a 50-kDa glycoprotein in HCMV-infected cells that was absent from purified virions. The UL16 glycoprotein was synthesized at early times after infection and accumulated to the highest levels at late times after infection. A recombinant HCMV in which UL16 coding sequences were interrupted by a *lacZ* expression cassette was constructed by insertional mutagenesis. Analysis of the phenotype of the recombinant virus indicated that the UL16 gene product is nonessential for virus infectivity and growth in tissue culture.

Analysis of the coding content of the human cytomegalovirus (HCMV) genome has revealed the existence of a large number of genes that lack homologs in members of the alpha herpesvirus and gammaherpesvirus subgroups (7). Notable among these are a very large number of genes whose predicted translation products have the characteristics of glycoproteins. Thus, of 54 probable HCMV glycoprotein genes, only two encode predicted products with discernible homology with glycoproteins of other herpesviruses; UL55 encodes the homolog of herpes simplex virus type (HSV-1) gB (9), and UL75 encodes the homolog of HSV-1 gH (10, 32). These proteins are conserved in alpha-, beta-, and gammaherpesviruses (gB [5, 9, 11, 25, 33, 34] and gH [10, 14, 30]) and in HSV have been shown to be essential for virion entry (6, 13). The view that the HCMV homologs also function in virus entry is supported by the fact that antibodies specific for these proteins neutralize virus infectivity (9, 10, 23, 35, 37, 38).

Many studies have used HCMV-specific monoclonal antibodies or polyclonal sera to enumerate and characterize HCMV-specific glycoproteins (9, 10, 12, 16, 18, 23, 36, 37, 38), though a comparison of different studies is often difficult because of the use of different nomenclature. Gretch et al. (18) identified three distinct disulfide-linked glycoprotein complexes in the HCMV envelope which they designated gcI, gcII, and gcIII. gcI corresponds to the HSV-1 glycoprotein B homolog, which in HCMV is composed of disulfide-linked heterodimers generated by proteolytic cleavage of the UL55 gene product (2, 3, 16). Gretch et al. (17) proposed that gcII includes the products of the HXLF-1 and HXLF-2 genes (designated US11 and US10 by Chee et al. [7]), and gcII has recently been identified as a heparin-binding component of the virion envelope (22). The HXLF gene family (or US6 family) was analyzed by Weston and Barrell (44) and contains six consecutive open reading frames (ORFs) (US6 to US11) whose predicted translation products exhibit primary sequence homology and structural similarity. Replacement mutagenesis has shown that members of the US6 family are dispensable for HCMV growth in culture (20, 21). The major component of gcIII is gH (or p86), the UL75 gene product (10, 32, 38). HCMV gH is disulfide linked to the UL115 gene product (24), and gcIII corresponds to the gH:gL complex of HSV-1 (19). We know very little about the products of the remaining potential glycoprotein genes, and our only clue to their function comes from analysis of sequence homology with known mammalian genes. For example, the UL18 ORF encodes a class I major histocompatibility complex homolog (1, 4), and three genes (UL33, US27, and US28) encode products homologous with G-protein receptors (7).

In this report, we describe the characterization of the UL16 gene product. The gene is predicted to encode a class I transmembrane glycoprotein which has no homologs in current data bases. We have used two approaches to study the gene product. First, antisera raised against the gene product expressed in a prokaryotic system were used to characterize the protein synthesized in a eukaryotic system and in HCMV-infected cells. Second, the UL16 gene was inactivated by insertional mutagenesis, and the in vitro phenotype of the mutant virus was examined. We conclude that UL16 encodes a 50-kDa glycoprotein that is synthesized with early kinetics and is present in HCMV-infected cells but not in the virion envelope. The protein is dispensable and has no effect on the growth characteristics of HCMV in human fibroblasts in culture.

MATERIALS AND METHODS

Cells and viruses. MRC5 cells and 143 thymidine kinase-negative (TK^-) cells were grown in Glasgow's modified Eagle's medium containing 10% fetal calf serum (FCS). BHK-21 cells were maintained in the same medium supplemented with 10% newborn calf serum and 10% tryptose phosphate broth. HCMV strain AD169 was propagated and titrated in MRC5 cells according to standard protocols. The vaccinia virus WR strain and TK^- recombinant viruses derived from it were grown in BHK cells, and infectivity was determined by plaque assay on 143 TK^- cell monolayers as previously described (28). HCMV particles were purified

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from tissue culture medium by tartrate gradient centrifugation (41). Purified virus particles were examined by electron microscopy, and particle numbers were estimated by comparison with latex beads (approximately 250 nm in diameter) of known concentration, using the loop drop method (43).

Construction of a recombinant vaccinia virus expressing the HCMV UL16 gene. A recombinant vaccinia virus expressing HCMV UL16 was constructed by the general strategy described by Mackett et al. (27, 28) as follows. The entire UL16 ORF frame was isolated from plasmid pAT153 containing the 8.68-kb *Hin*dIII O fragment of the HCMV genome (31) as an *Nar*I fragment (nucleotides 22407 to 23331). The 924-bp *Nar*I fragment was end repaired and cloned into the *Sma*I site in the polylinker of pUC19, to allow the use of other restriction sites in the polylinker for subsequent cloning steps. The UL16 ORF was excised from the pUC19-based plasmid by digestion with *Sal*I (a restriction site in the polylinker 5′ to the UL16 gene); this procedure was followed by end repair using Klenow polymerase and then digestion with *Eco*RI (a site in the polylinker 3′ to the UL16 gene). The UL16 fragment was then cloned into *Sma*I-*Eco*RI-digested pRK19 (26) to generate pRK19-UL16, in which the initiating ATG (nucleotide 22414) was positioned downstream of the vaccinia virus 4b late promoter and the entire ORF was flanked by vaccinia virus TK coding sequences. A TK^- recombinant vaccinia virus was derived by recombination of vaccinia virus strain WR with pRK19-UL16 and selection in 5-bromodeoxyuridine. Recombinant plaques were identified by DNA hybridization and subjected to two rounds of plaque picking, and the genotype was confirmed by restriction enzyme analysis and Southern hybridization. The recombinant virus was named UL16Vac.

Production of polyclonal antisera. The UL16 gene product was expressed as a β-galactosidase fusion protein in *Escherichia coli*, using the pEX expression system (40). A 654-bp C-terminal fragment of the UL16 gene was isolated from pRK19-UL16 by digestion with *Hin*cII (nucleotide 22678) and *Eco*RI, end repaired, and cloned into the *Sma*I site of pEX3 such that the UL16 coding sequence was in frame with the *lacZ* coding sequence, under the control of the strong p_R promoter of bacteriophage lambda. The resulting plasmid, pEXUL16, was used to transform *E. coli* POP2136, and production of the *lacZ*-UL16 fusion protein was induced by heat shock. The fusion protein was purified by elution from sodium dodecyl sulfate (SDS)-polyacrylamide gels, and rabbits were immunized with the fusion protein by subcutaneous injection either with Freund's complete adjuvant (primary immunization) or Freund's incomplete adjuvant (secondary immunizations). Serum V88 was obtained after the fourth boost.

Affinity purification of polyclonal antisera V88. Affinity-purified antibodies were produced by a two-step purification method in which antibodies against β-galactosidase were first removed by using β-galactosidase linked to activated Sepharose CL-4B, and then UL16-specific antibodies were obtained by binding to the *lacZ*-UL16 fusion protein linked to the same support. UL16-specific antibodies were then eluted from this column with 0.1 M glycine (pH 2.1), and fractions were made 0.2 M Tris-Cl (pH 7.6) and 50% with respect to FCS and stored in aliquots at −20°C. The fractions were then analyzed by enzyme-linked immunosorbent assay and Western immunoblotting to determine which fractions contained the UL16-specific antibodies.

Western blot analysis. To prepare lysates for Western blot analysis, MRC5 or BHK-21 cells were infected with HCMV or vaccinia virus at a multiplicity of infection (MOI) of 5 or 30, respectively. HCMV-infected cells were washed with phosphate-buffered saline (PBS) and solubilized in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer 24, 48, and 96 h after infection. Vaccinia virus-infected cells were harvested, and lysates were prepared 15 h after infection. The proteins were separated on SDS-polyacrylamide gels (10% acrylamide) and electrophoretically transferred to nitrocellulose by the method of Towbin et al. (42). Following transfer, the membranes were incubated in blocking solution (consisting of 5% nonfat dried milk, 1% bovine serum albumin [BSA], and 1% FCS in PBS) for 2 h at 37°C. Polyclonal or affinity-purified antibodies were diluted 1/100 or 1/50 in blocking solution and incubated with preblocked membranes for 1 h at room temperature or at 4°C overnight. After being washed with 1% Nonidet P-40–1% FCS in PBS, the membranes were incubated with 0.1 μCi of [^{125}I]protein A (>30 mCi/mg; Amersham) per ml in blocking solution for 1 h at room temperature. Unbound [^{125}I]protein A was removed by washing with Nonidet P-40 solution, and the membranes were exposed to X-ray film at −70°C with an intensifying screen.

Preparation of cytoplasmic RNA. Confluent monolayers of MRC5 cells were infected with HCMV strain AD169 at an MOI of 10. For immediate-early RNA, the cells were maintained in growth medium containing 100 μg of cycloheximide per ml, and RNA was prepared 12 h after infection. For early RNA, the cells were maintained in growth medium containing 100 μg of phosphonoacetic acid per ml, and RNA was prepared 24 h after infection. For late RNA, cells were harvested at 72 h after infection with no drug treatment. To prepare RNA, cells were lysed on ice in 0.1 M NaCl–0.01 M Tris-HCl (pH 7.5)–1 mM EDTA containing 0.5% Nonidet P-40. Nuclei were removed by centrifugation; the supernatant was made 1% with respect to SDS and then extracted three times with phenol-chloroform and once with chloroform.

Northern (RNA) blot analysis. RNA samples (5 μg) dissolved in 20 mM morpholinepropanesulfonic acid (MOPS; pH 7)–1 mM EDTA–5 mM sodium acetate (MOPS buffer)–50% formamide–2 M formaldehyde were incubated at 60°C for 5 min prior to loading onto 1.5% agarose gels containing 2 M formaldehyde prepared in MOPS buffer. After electrophoresis, the separated RNA was transferred to nitrocellulose membranes, baked at 80°C for 2 h, and then prehybridized in 36% formamide–75 mM sodium citrate–0.75 M sodium chloride–0.1% BSA–0.1% Ficoll–0.1% polyvinylpyrrolidone–0.1% SDS–50 mM sodium phosphate (pH 6.5)–200 μg of calf thymus DNA per ml at 55°C for 6 h. Prehybridized membranes were then incubated with [^{32}P]dCTP-labeled probes prepared by using a Random Prime Kit (Boehringer Mannheim) in hybridization solution (36% formamide, 75 mM sodium citrate, 0.75 M sodium chloride, 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.1% SDS, 100 μg of calf thymus DNA per ml, 10% dextran sulfate) at 55°C overnight. Membranes were washed three times with 0.03 M sodium citrate–0.3 M sodium chloride–0.1% SDS at 65°C (5 min each time) and three times in 1.5 mM sodium citrate–15 mM sodium chloride–0.1% SDS at 65°C for (15 min each time) and then exposed to X-ray film at −70°C with an intensifying screen.

Transfection. Transfection of MRC5 cells was performed on subconfluent monolayers by the modified calcium phosphate procedure of Chen and Okayama (8). To construct a recombinant HCMV carrying the β-galactosidase gene, monolayers were transfected with 20 μg of HCMV (AD169)-infected cell DNA plus 2 μg of linearized plasmid DNA

containing the *lacZ* gene inserted into the desired site in the HCMV genome. After 10 to 12 days, the progeny were harvested and sonicated. The progeny yield was determined by an immunoperoxidase assay as follows. Dilutions of transfection progeny were plated on cell monolayers, and the cells were fixed after 6 days with 85% acetone at −20°C for 10 min. The fixed monolayers were incubated with a monoclonal antibody to the HCMV 72,000-molecular-weight immediate-early antigen for 1 h at room temperature, washed thoroughly with PBS, and then incubated with biotinylated protein A followed by streptavidin-conjugated horseradish peroxidase. HCMV plaques were then detected by incubation with PBS containing 600 μg of diaminobenzidine per ml and 0.003% hydrogen peroxide for 10 min at room temperature. β-Galactosidase-positive plaques were visualized by counterstaining with PBS containing 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 5 mM potassium ferricyanide-ferrocyanide, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (1 mg/ml). The proportion of *lacZ*-positive virus to wild-type HCMV was then calculated. Appropriate dilutions of transfection progeny were then replated, and after 10 days, recombinant plaques were detected by using an overlay of 1% low-melting-temperature agarose containing X-Gal (300 μg/ml). Blue plaques were picked and subjected to two rounds of plaque purification.

One-step growth cycle analysis. Confluent monolayers of MRC5 cells in 50-mm-diameter plates were infected at an MOI of 5 with either wild-type or recombinant HCMV. After adsorption for 1 h at 37°C, the inoculum was removed, the monolayers were washed twice with growth medium, and fresh medium was added to each dish. The plates were incubated at 37°C, and the growth medium was harvested at various times after infection and stored at −70°C. The titer at each time point was determined by plaque assay on MRC5 cells followed by immunoperoxidase assay or X-Gal assay for wild-type or recombinant virus as previously described.

Assay of infectious centers. Monolayers of MRC5 cells in 50-mm-diameter dishes were infected with AD169 or recombinant HCMV (100 PFU per dish). At various times after infection, the monolayers were washed with PBS and the cells were trypsinized and resuspended in 5 ml of growth medium. The number of infectious centers was determined by plating out dilutions of the infected cell suspensions and assaying for wild-type or recombinant HCMV (by immunoperoxidase or X-Gal assay as described above) 6 days later.

RESULTS

Identification and characterization of the UL16 gene product. To identify and characterize the product of the UL16 gene, we expressed the gene in mammalian cells by using a recombinant vaccinia virus and in *E. coli* as a *lacZ* fusion protein. Sera from rabbits immunized with the purified fusion protein (V88) were used in Western blot analysis of cells infected with the UL16-expressing vaccinia virus recombinant, UL16Vac (Fig. 1). V88 detected a heterogeneous protein species of approximately 50 kDa in cells infected with UL16Vac, whereas no species was detected in cells infected with a recombinant expressing the HCMV UL18 gene (4). Treatment of infected cells with tunicamycin, which inhibits N-linked glycosylation, reduced the molecular mass of the detected species to approximately 20 kDa, close to the size of the predicted primary translation product. The UL16 gene product is therefore heavily N glycosylated, consistent with the presence of eight potential N-linked glycosylation sites.

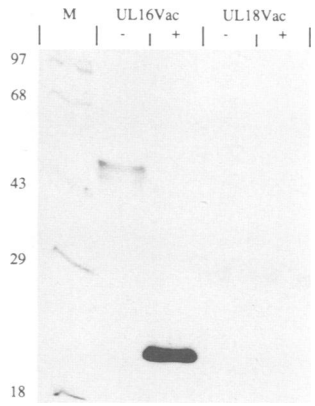

FIG. 1. Identification of the UL16 gene product. BHK cells were infected with UL16Vac or UL18Vac at an MOI of 30 in the absence (−) or presence (+) of 10 μg of tunicamycin per ml. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with polyclonal antiserum V88 diluted 1/100. Sizes of molecular weight standards (lane M) are shown in kilodaltons.

Initial attempts to detect the UL16 gene product in HCMV-infected cells by using serum V88 resulted in the appearance of multiple species in Western blots. Many of these species were detected nonspecifically because similar results were obtained with preimmune rabbit sera or with sera raised against other β-galactosidase fusion proteins. V88 serum was therefore affinity purified as described in Materials and Methods and used in Western blots to detect the UL16 gene product in HCMV-infected cells or in purified virions (Fig. 2). A 50-kDa species was detected in UL16Vac-infected cells and in HCMV-infected cells. The UL16 product was present at early times after infection and accumulated to maximum levels at late times after infection. However, we were unable to detect the UL16 product in a

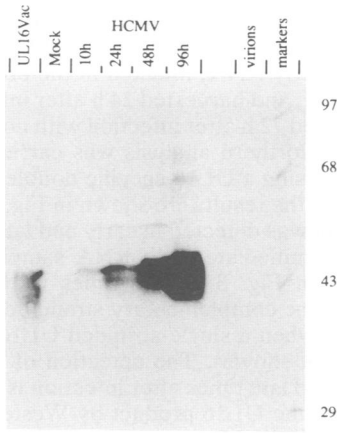

FIG. 2. Western analysis of HCMV-infected cell lysates and purified virions. MRC5 cells were infected with HCMV strain AD169 at an MOI of 5 or were mock infected. Cell lysates were prepared 10, 24, 48, and 96 h after infection. Lysates of infected cells or purified virions (approximately 10^{10} virus particles) were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with affinity-purified V88 diluted 1/50. Sizes of molecular weight standards are shown in kilodaltons.

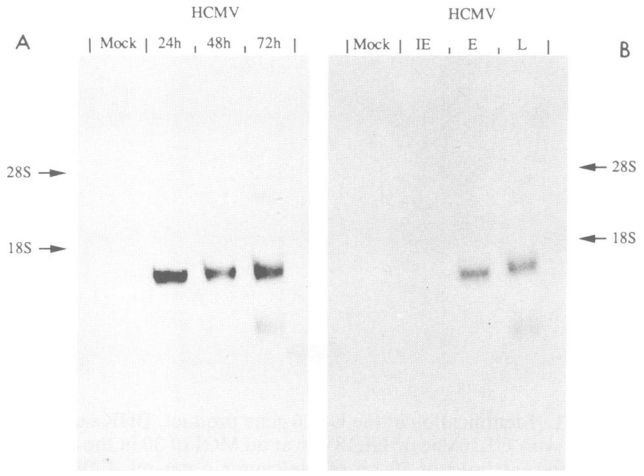

FIG. 3. Northern analysis of HCMV-infected cell RNA. MRC5 cells were infected with HCMV strain AD169 at an MOI of 10 or were mock infected. Cytoplasmic RNA was extracted 24, 48, and 72 h after infection (A). Immediate-early (IE) RNA was prepared 12 h after infection in the presence of cycloheximide (100 μg/ml), early (E) RNA was prepared 24 h after infection in the presence of phosphonoacetic acid (100 μg/ml), and late (L) RNA was prepared 72 h after infection with no drug treatment (B). RNA samples (5 μg per track) were separated by formaldehyde-agarose gel electrophoresis and transferred to nitrocellulose, and the filters were hybridized with a ^{32}P-labeled UL16-specific double-stranded DNA probe. Positions of 28S and 18S rRNAs are indicated.

track containing 10^{10} purified enveloped virus particles (equivalent to ~10 μg of protein). HCMV gH (the UL75 gene product) was easily detectable in these virions in a parallel experiment (not shown).

Northern blot analysis was performed on cytoplasmic RNA extracted from HCMV-infected cells, using a UL16-specific double-stranded DNA probe (Fig. 3A) as described in Materials and Methods. A 1.4-kb transcript was detected 24, 48, and 96 h after infection with HCMV and was absent from uninfected cells. To determine the kinetic class of the UL16 transcript, cells were infected with HCMV in the presence of cycloheximide and harvested 12 h after infection for immediate-early RNA, infected in the presence of phosphonoacetic acid, and harvested 24 h after infection for early RNA or harvested 72 h after infection with no drug treatment for late RNA. Northern analysis was carried out on these RNA samples, using a UL16-specific double-stranded DNA probe as before; the results are shown in Fig. 3B. The 1.4-kb UL16 transcript was detected in early and late RNA samples but not in the immediate-early RNA sample. The smaller band detected in Fig. 3 at late times is thought to be a transcript from the complementary strand because this band is not detected when a single-stranded UL16-specific probe is used (data not shown). The detection of the UL16 transcript at early and late times after infection is consistent with the detection of the UL16 product by Western blotting.

Generation of a UL16 deletion mutant. In an attempt to study the role of the UL16 glycoprotein in HCMV, we decided to generate a UL16 deletion mutant and compare the phenotype of the deletion mutant with that of wild-type HCMV in tissue culture. We used the β-galactosidase gene under the control of the HCMV major early promoter as an insertional inactivation cassette. The HCMV major early promoter is a strong promoter which is active at early and late times after infection (29). To insert the β-galactosidase expression cassette into the UL16 coding sequence, plasmid pEgalUL16 was constructed as follows. A 3.2-kb *Sac*I fragment (nucleotides 20874 to 24077) containing the UL16 gene was isolated from the *Hin*dIII O fragment of HCMV and cloned into the *Sac*I site of pSP64. A synthetic polylinker (*Sty*I-*Bgl*II-*Eco*RV-*Hin*dIII-*Sty*I) was cloned into a unique *Sty*I site (nucleotide 22968) within the UL16 coding sequence. A β-galactosidase expression cassette, isolated from pMVI (kindly donated by Gavin Wilkinson) as a *Bam*HI fragment containing the *E. coli* β-galactosidase gene under the control of the major early promoter of HCMV (15), was cloned into the *Bgl*II site in the polylinker in the UL16 gene. The resulting plasmid, pEgalUL16, contained the UL16 gene disrupted by the insertion of the β-galactosidase gene driven by the HCMV major early promoter, flanked by HCMV sequences (approximately 1.5 kb upstream and 1 kb downstream of the *lacZ* insertion site). pEgalUL16 does not contain a poly(A) site for the *lacZ* gene; however, we assumed that the UL16 poly(A) site would be utilized. Since the HCMV major early promoter contains translation termination stop codons in all three frames, any UL16 N-terminal product that was expressed would lack the predicted transmembrane anchor domain.

MRC5 cells were cotransfected with HCMV AD169 DNA and linearized pEgalUL16 DNA. After 10 days, when several plaques were visible, the monolayers were harvested, sonicated, and replated on monolayers of MRC5 cells. Recombinant HCMV expressing β-galactosidase was selected and cloned from the transfection progeny and described in Materials and Methods. Southern hybridization of the cloned virus (HCMVΔUL16) (Fig. 4) confirmed that the *lacZ* cassette had inserted at the correct site in the genome and that the virus was clonally pure and stable. To confirm that the recombinant virus HCMVΔUL16 did not express the UL16 gene product, Western blot analysis was carried out on cells infected with wild-type HCMV or with HCMVΔUL16, using affinity-purified V88 (Fig. 5). The 50-kDa UL16 gene product was detected in the control UL16Vac-infected cells and in wild type-infected cells; however, the 50-kDa species was not detected in HCMVΔUL16-infected cells, confirming that the UL16 gene was not expressed in the recombinant virus. The apparent difference in level of expression of UL16 product in wild-type-infected cells between Fig. 2 and 5 is merely a result of different exposure lengths of the autoradiographs. The higher-molecular-mass species (>100 kDa) in the HCMVΔUL16-infected cells is β-galactosidase detected by V88, which was raised against the *lacZ*-UL16 fusion. We have performed several experiments to confirm that the higher-molecular-mass band is in fact β-galactosidase. The same band is also detected with other β-galactosidase-specific antisera and is detected in another HCMV deletion mutant which contains the *lacZ* gene inserted at a different locus (data not shown). Although the polyclonal antisera had been affinity purified, it is apparent that some β-galactosidase-specific antibodies were still present.

Phenotypic characterization of HCMVΔUL16 on tissue culture. To assess the effects of the insertion of the β-galactosidase gene into the UL16 coding sequence, the ability of the recombinant virus to grow in single-cycle growth analysis (Fig. 6) and the rate of cell-to-cell spread (Fig. 7) were compared with results for wild-type HCMV. The recombinant HCMV was indistinguishable from the wild type both in one-step growth cycle analysis and in the rate of cell-to-cell spread. These results suggest that the glycoprotein encoded

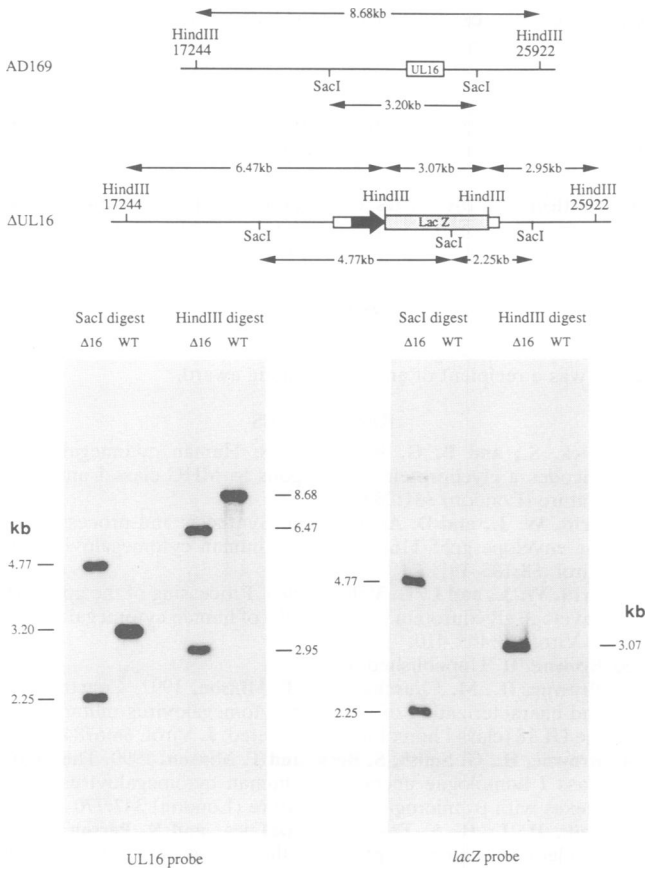

FIG. 4. Hybridization analysis of HCMVΔUL16 DNA. DNA samples from MRC5 cells infected with HCMVΔUL16 or HCMV AD169 were digested with *Sac*I or *Hin*dIII and subjected to agarose gel electrophoresis and Southern transfer to nitrocellulose. The filters were hybridized with ^{32}P-labeled probes comprising either the 3.2-kb *Sac*I subfragment of the HCMV *Hin*dIII O clone or a 3.07-kb *lacZ* probe. Positions of the *Sac*I and *Hin*dIII restriction sites in wild-type (WT; AD169) and recombinant (HCMVΔUL16 [Δ16]) DNA are shown.

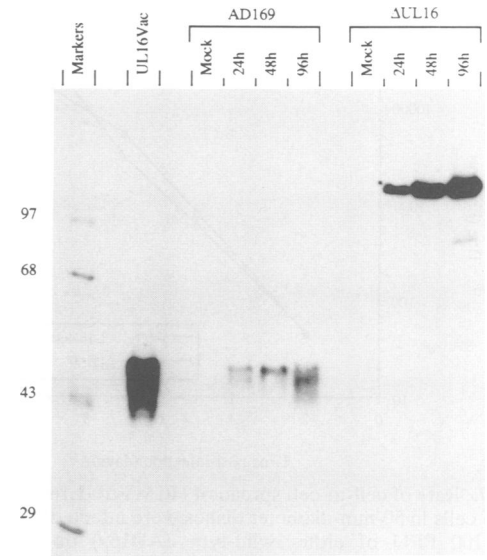

FIG. 5. Western analysis of HCMVΔUL16-infected cells. MRC5 cells were infected with HCMV AD169 or HCMVΔUL16 at an MOI of 5 or were mock infected. Cell lysates were prepared 24, 48, and 96 h after infection. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with affinity-purified V88 diluted 1/50. Sizes of molecular weight markers are shown in kilodaltons.

by UL16 is not involved in determining virus infectivity, cell-to-cell spread, or release of infectious virus in tissue culture. In low-multiplicity infections, recombinant virus plaques were similar in size and morphology to wild-type plaques (not shown). Particle counts were performed on stocks of AD169 and ΔUL16. Particle/infectivity ratios of 180:1 and 220:1 were calculated for AD169 and ΔUL16, respectively, and these values were not considered to be significantly different, given the errors in particle counting and plaque assays.

DISCUSSION

Chee et al. (7) suggested that the HCMV UL16 gene encoded a glycoprotein on the basis of a predicted N-terminal signal sequence, a potential transmembrane anchor close to the C terminus, and the presence of eight potential N-linked glycosylation sites. We report in this paper that the gene product is a heavily glycosylated species of 50 kDa that is synthesized in the early phase of the growth cycle and accumulates at late times. The protein is abundant in infected cells but not detectable in purified enveloped virions. Its absence from virions could be due to specific exclusion during virion budding or could indicate that different HCMV glycoproteins are targeted to different membrane components. To date, we have been unable to investigate the intracellular distribution of the UL16 protein because our antisera react only with denatured protein.

The inactivation of specific genes by insertion or deletion mutagenesis has been very widely used to investigate gene

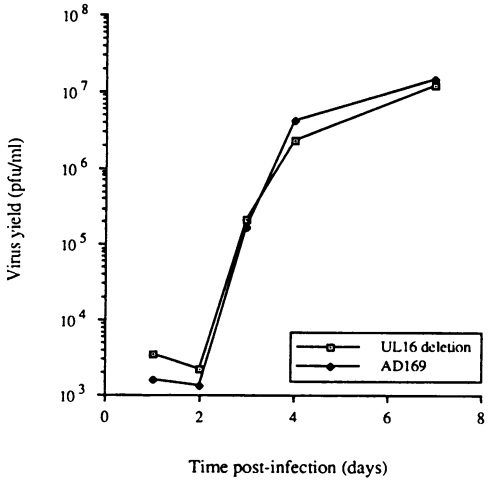

FIG. 6. Single-cycle growth analysis of HCMVΔUL16. MRC5 cells were infected with either wild-type (AD169) or recombinant (HCMVΔUL16) virus at an MOI of 5. After adsorption for 1 h at 37°C, monolayers were washed twice with medium and incubated with fresh growth medium. Extracellular virus was harvested 1, 2, 3, 4, and 7 days after infection, and titers were determined by plaque assay on MRC5 monolayers. Values shown are results of duplicate assays from duplicate infections.

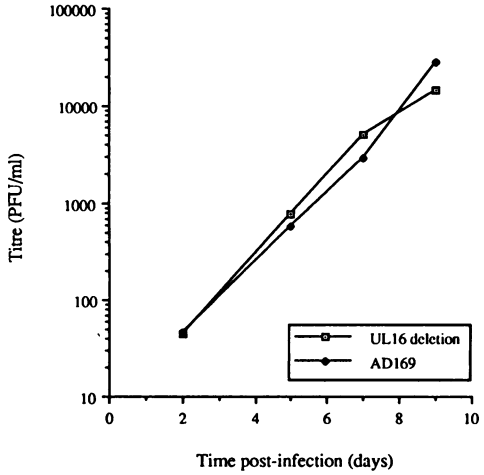

FIG. 7. Rate of cell-to-cell spread of HCMVΔUL16. Monolayers of MRC5 cells in 50-mm-diameter dishes were infected with approximately 100 PFU of either wild-type (AD169) or recombinant (HCMVΔUL16) virus per dish. Various times after infection (2, 5, 7, and 9 days), the monolayers were washed with PBS and cells were trypsinized and resuspended in 5 ml of growth medium. The numbers of infectious centers were determined by plaque assay on MRC5 monolayers. The results of duplicate assays are shown.

function in HSV, but few HCMV mutants of this type have been constructed, partly because of the poor transfection efficiency of HCMV DNA in permissive cells and the much longer time scale required for identification and cloning of potential mutants. Spaete and Mocarski (39) described the construction of a recombinant HCMV carrying the *lacZ* marker gene under the control of the HCMV major early promoter which disrupted one of the two copies of the major early gene (HCMVTRL4) within the L component of the HCMV genome, but this virus was found to contain an unselected deletion and rearrangements. The authors suggested that the size of the HCMV genome is close to the packaging size limit and that selection of viruses carrying *lacZ* inserts would favor the isolation of viruses with deletions. More recently, insertion of the β-glucuronidase gene has been used to inactivate genes of the US6 glycoprotein family (20, 21), and it is proposed that the success of this approach is due to the smaller size of the marker gene by comparison with *lacZ*. We have constructed a recombinant virus, HCMVΔUL16, in which the *lacZ* gene interrupts the UL16 ORF. This virus has a stable $lacZ^+$ phenotype, and although we cannot formally prove that it contains no deletions, examination of restriction digests has revealed no differences between AD169 DNA and HCMVΔUL16 DNA other than those resulting from the *lacZ* insertion. In our view, there is little evidence to suggest that packaging size constraints preclude the insertion of *lacZ* into the intact HCMV genome.

The UL16 gene is dispensable for growth in human fibroblasts in vitro, and HCMVΔUL16 is indistinguishable from the parental virus AD169 in its ability to grow in human fibroblasts or to spread from cell to cell. To date, therefore, the HCMV genes UL16 (this report), UL18 (3b) and UL33 (3a), UL20 (23a), IRS1, US1 to US5, and the US6 family (20, 21), many of which encode glycoproteins (7), have been shown to be dispensable for growth in culture. By analogy with HSV, the gB and gH:gL homologs of HCMV (the products of the UL55, UL75, and UL115 genes) can be expected to be essential for virion infectivity, but the majority of the remaining glycoproteins will probably be found to be dispensable. It is possible that some of these molecules are required for successful infection of different cell types, and it will be interesting to assess the growth of HCMVΔUL16 in a variety of cells. However, it is equally likely that these HCMV glycoproteins are involved in subtle interactions of the virus with its host in vivo, and in the absence of a convenient animal model, elucidation of the function of these molecules will be a formidable problem.

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