Replacement Mutagenesis of the Human Cytomegalovirus Genome: US10 and US11 Gene Products Are Nonessential

THOMAS R. JONES,* VIERA P. MUZITHRAS, AND YAKOV GLUZMAN

Molecular Biology Section, Medical Research Division, American Cyanamid Co., Pearl River, New York 10965

The US6 gene family, located within the unique short region (US) of the human cytomegalovirus (HCMV) genome, contains six open reading frames (US6 through US11) which may encode glycoproteins, such as gcII (D. Gretch, B. Kari, R. Gehrz, and M. Stinski, J. Virol. 62:1956–1962, 1988). By homologous recombination, several different recombinant HCMV were created which contain a marker gene, β-glucuronidase, inserted within this gene family. It was demonstrated that β-glucuronidase has utility as a marker gene for the identification of recombinants in this herpesvirus system, without the occurrence of deletions in other regions of the viral genome. DNA and RNA blot analyses attested to the fidelity of the recombination. Immunoprecipitation experiments using monospecific polyclonal antisera indicated that the US10 and/or US11 gene products were not expressed in the recombinants, as predicted. These results, along with single-cycle growth analyses, indicated that the US10 and US11 gene products are nonessential for virus replication and growth in tissue culture. HCMV recombinants expressing β-glucuronidase seemed to be genetically stable.

Human cytomegalovirus (HCMV) is an important opportunistic pathogen of immunocompromised adults and can cause neurological abnormalities in some infants infected in utero (1). The 230-kb genome of HMCV is similar in structure to that of herpes simplex virus (HSV), as reviewed by Stinski (59). It has long and short unique regions, UL and US, respectively, each bounded by inverted repetitions. Sequencing of the entire HCMV genome was completed recently by Barrell and coworkers (6). Their results indicate that the cytomegalovirus genome contains more than 200 significant open reading frames. To date, relatively few of these open reading frames have been studied as to the function(s) of the protein which they may encode.

In the HSV system, the analysis of viral mutants has proven to be invaluable in the study of the function of many viral genes. These studies employed temperature-sensitive mutants (9, 39, 45, 48, 62, 63), site-directed insertional mutants by exploiting the selectable thymidine kinase (*tk*) gene (31, 32, 40, 41), and mutants created by random mutagenesis procedures utilizing mini-Mu phage and transposon Tn*5* (23, 61). More recently, the prokaryotic β-galactosidase gene has been used for site-directed insertion into the HSV genome, allowing for the easy identification of viral mutants as blue plaques after overlay with a chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (11, 12, 29). In contrast to conditionally lethal mutants in which mutations lie in essential genes, the other methodologies offer the advantage that mutants in nonessential genes can be created and identified. These successful strategies for the creation of HSV mutants have not been employed to any great extent in the HCMV system. Probably because of the long replication cycle of this virus, there have only been a few reports concerning the creation or analysis of HCMV temperature-sensitive mutants (19, 42, 56, 65, 66). Because of the limited host range of HCMV in tissue culture (human diploid fibroblasts), mutagenesis by exploitation of a *tk* gene would be exceedingly difficult. To date, only Spaete and Mocarski (55) have reported the

successful site-directed insertion of a marker gene (β-galactosidase) into the genome of cytomegalovirus, within one copy of the repeats flanking UL. However, this virus contained an unexpected deletion adjacent to the site of the insertion. Reasons for the infrequent use of such an powerful genetic approach to study gene function in HCMV may include difficulties in the preparation or transfection of the viral DNA and the genetic instability of β-galactosidase marker-expressing recombinants (55).

We report here the construction of β-glucuronidase marker-expressing HCMV recombinants by site-directed insertional mutagenesis and show that several members of the US6 gene family are nonessential for growth in tissue culture. The US6 gene family is located in the US and comprises six open reading frames (US6 through US11) which share limited amino acid homology (64) and may encode glycoproteins, such as gcII (15, 16). Although it has been shown to have wide application in plant and nematode systems (21, 22), the use of β-glucuronidase as a marker gene in mammalian systems has not been reported previously. US11 and/or US10 have been replaced by the marker gene in separate virus isolates without any other detected deletions in the viral genome. Recombinant virus lacking either or both of these genes were not impaired in their ability to replicate in tissue culture compared with that of wild-type virus.

MATERIALS AND METHODS

Cells and virus. Human foreskin fibroblast (HFF) cells were isolated in this laboratory and used for fewer than 20 passages. They were grown in Dulbecco's modified Eagle medium (Mediatech) containing 10% fetal calf serum (GIBCO) and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid). Vero cells were grown in the same media, but with 10% calf serum instead of fetal calf serum. Human cytomegalovirus strain AD169 was obtained from the American Type Culture Collection and propagated according to standard protocols. For HCMV-infected HFF cells, 5% calf serum (Hyclone)–5% fetal calf serum was used in the media instead of 10% fetal calf serum. HSV type 1

---

* Corresponding author.

(HSV-1) strain Patton was obtained from R. Hyman (The Pennsylvania State University College of Medicine, Hershey, Pa.).

DNA sequence. The HCMV US numerical base designation used throughout this manuscript is that which was published previously (64).

Plasmid constructions. The HCMV strain AD169 genomic HindIII X and G DNA fragments were cloned into pAT153 yielding pHind-X and pHind-G, respectively (37). The HCMV strain AD169 XbaI P DNA fragment was cloned from virion DNA into the XbaI site of pACYC184 to yield pXbaI-P. pBam-U contains the HCMV BamHI U fragment, subcloned from pHind-G, into the BamHI site of pACYC184. pBgl-I and pBgl-M contain the HSV-1 strain Patton genomic BglII I or M DNA fragments, respectively, cloned into the BglII site of pKC7 vector (24). pEco-N contains the EcoRI N DNA fragment, from the same strain of HSV-1, cloned into pACYC184 (24). The protein A fusion protein vector used (pT7protA) was constructed from pRIT2T (Pharmacia) by B. Rasmussen (American Cyanamid Co., Pearl River, N.Y.). In pT7protA, the protein A fusion gene is under the control of bacteriophage T7 gene 10 promoter and termination signals (44). All plasmid DNA manipulations were done according to standard protocols (46). Plasmids for recombination into the HCMV or HSV-1 genome were constructed in a pT7-1 (U.S. Biochemical) backbone. The starting plasmid was pBgluc which contains the 1.9-kb prokaryotic β-glucuronidase gene (Clontech, Palo Alto, Calif.) inserted into the SmaI site within the pT7-1 polylinker. The order of the restriction sites within the polylinker of pBgluc relative to the β-glucuronidase gene is: HindIII, PstI, SalI, XbaI, BamHI, 5'-β-glucuronidase-3', SstI, and EcoRI. To facilitate homologous recombination into the viral genome, the appropriate viral sequences were cloned 5' and 3' to the β-glucuronidase gene in pBgluc. To insert β-glucuronidase in place of tk in the HSV-1 genome, pBgdTK was constructed. Unlike the constructs for recombination in the HCMV genome (below), the starting plasmid was pBglucpolA, which is essentially the same as pBgluc, except that it has a 0.8-kb fragment containing the simian virus 40 t splice and polyadenylation signal at the 3' end of the β-glucuronidase gene. Sequentially, pBgdTK contains the 2.5-kb SalI-BglII fragment (from pBgl-I; contains the tk promoter and 5' untranslated sequences [nearest to the BglII site {40}] and 5' flanking sequences [to the SalI site at map unit 0.33 {17}]), β-glucuronidase and polyadenylation signal, and the 2.3-kb SstI-BamHI fragment (from pBgl-M, containing sequences encoding the C-terminal 228 amino acids of tk and 3' flanking region [36, 40]). The endogenous HSV-1 tk early promoter controls β-glucuronidase gene expression in this construct. To insert β-glucuronidase in the HCMV US10-US9 intergenic region, p2.7EBgpAUS10/9 was constructed (Fig. 1B). Sequentially, this plasmid contains the 1.3-kb ApaI-ApaI fragment (bases 14834 to 13527, containing US11 and US10 sequences; from pHind-X), the 768-b SspI-XmaIII (−713 to +55) 2.7E promoter fragment (from pBam-U [14]), β-glucuronidase; the 180-b SmaI-HaeIII HSV-1 tk polyadenylation signal fragment (36), and the 2.0-kb ApaI-BamHI fragment (bases 13527 to 11548, containing US9 and US8 sequences; from pHind-X). In order to replace HCMV US11, pBgdUS11 was constructed (Fig. 1C). Sequentially, this plasmid contains the 1.8-kb PstI-XbaI fragment (bases 16713 to 14897, containing US13, US12, and US11 promoter sequences; from pXbaI-P), β-glucuronidase, and the 1.5-kb SalI-SstII fragment (bases 14677 to 13215, containing C-terminal US11 sequences and US10 sequences;

from pHind-X). The endogenous US11 early promoter controls β-glucuronidase gene expression in this construct. In order to replace HCMV US10, pBgdUS10 was made (Fig. 1D). Sequentially, this plasmid contains the 1.2-kb HindIII-XhoI fragment (bases 15362 to 14189, containing US11 coding sequences and US10 promoter sequences; from pHind-X), β-glucuronidase, and the 1.8-kb SmaI-EcoRI fragment (bases 13782 to 11949, containing C-terminal US10 sequences, US9 sequences, and N-terminal US8 sequences; from pHind-X). The endogenous, weak, US10 promoter controls β-glucuronidase expression in this plasmid. To replace HCMV US10 and US11 simultaneously, pBgdUS11/10 was constructed (Fig. 1E). This plasmid is similar to pBgdUS10, except that it has the 1.8-kb PstI-XbaI fragment (bases 16713 to 14897, containing US13, US12, and US11 promoter sequences; from pXbaI-P) inserted upstream of β-glucuronidase, instead of the 1.2-kb HindIII-XhoI fragment. A schematic of the relevant regions of the plasmids for recombination into the HCMV genome are shown in Fig. 1. p2.7EBgpAUS10/9 was used in the creation of RV134, pBgdUS11 was used in the creation of RV699, pBgdUS10 was used in the creation of RV131, and pBgdUS11/10 was used in the creation of RV67. Prior to transfection for recombination into the viral genome, the plasmids were linearized at a unique restriction site at one end of the viral sequences flanking β-glucuronidase.

Viral DNA. Infectious HCMV DNA was isolated from partially purified nucleocapsids after sorbitol cushion density gradient centrifugation (57, 58). The nucleocapsids were resuspended in 50 mM Tris (pH 7.5)–1 mM MgCl$_2$. The nucleocapsid suspension was made 0.1 M Tris (pH 8.0)–0.1 M EDTA–0.1 M NaCl and lysed by the addition of Sarkosyl to a final concentration of 0.5%. After digestion with 100 μg of proteinase K per ml (3 h at 50°C), phenol-chloroform extraction was done and the resulting nucleic acids were precipitated with ethanol. The spooled DNA was dried briefly and resuspended overnight in 1× TE (10 mM Tris [pH 8.0]–1 mM EDTA). Miniprep recombinant virus DNA (for DNA blot analysis) was isolated from the cytoplasm of infected cells. The procedure used was similar to that used for the isolation of total cytoplasmic RNA (see below), except that modified 2× PK buffer was used (containing 1% Sarkosyl instead of 2% sodium dodecyl sulfate [SDS]) and there was a treatment with RNase A (25 μg/ml at 37°C for 20 min) prior to the proteinase K treatment.

Transfection for recombinant HCMV. HFF cells were split so that they were 70 to 80% confluent on the day of transfection. The cells were trypsinized and suspended to 5.6 × 10^5 cells per ml in Dulbecco's modified Eagle medium–10% fetal calf serum–25 mM HEPES. The DNA was transfected by using a modified calcium phosphate coprecipitation technique. Briefly, 1.5 μg of infectious HCMV DNA, 2.5 μg of linearized plasmid DNA, and 2 μg of sonicated salmon sperm DNA were mixed in the calcium chloride solution (300 μl containing 10 mM Tris [pH 7.0]–250 mM calcium chloride) and chilled on ice. To initiate the coprecipitation, the DNA was removed from the ice and 300 μl of 2× HeBS (pH 6.95; at room temperature; 1× HeBS is 19.2 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.8 mM sodium phosphate, 0.1% glucose) was added dropwise with gentle mixing. As soon as the slightest precipitate was visible, the precipitate was placed on ice (to prevent further precipitate from forming). The precipitate was mixed with 3 × 10^6 cells (in suspension) and placed in an 82-mm tissue culture plate. After 6 h at 37°C, the medium was removed and the cells were shocked with 20% dimethyl sulfoxide in 1× HeBS for

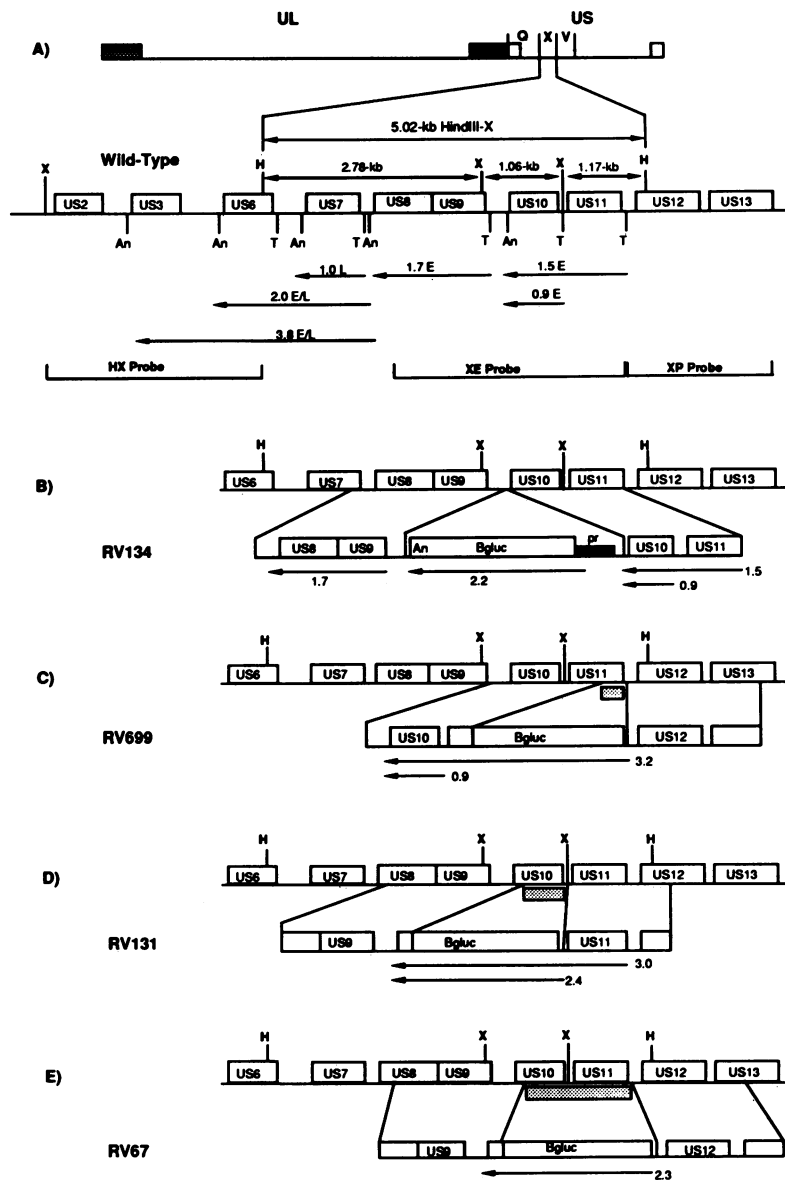FIG. 1. Organization of recombinant virus genomes. (A) The first line depicts the location of the US6 family containing HindIII X DNA fragment in the HCMV strain AD169 (wild-type) genome. The shaded boxes are the inverted repeats which bound the UL, and the clear boxes are those which bound the US. Below is a schematic of the organization of the US6 family of genes and neighboring genes. The locations of HindIII (H) and XhoI (X) restriction endonuclease sites and the size (in kilobases) of the fragments are given. Some of the major transcripts from this region, which were mapped previously (25), are indicated, along with their kinetic class (E, early; L, late) and their size (in kilobases). The direction of transcription is right to left. T, transcription initiation site; An, polyadenylation site. The location of probes used in Fig. 2 DNA blot analyses is shown. (B) Genomic organization of RV134. This recombinant contains a 2.83-kb insertion of a β-glucuronidase (Bgluc) expression cassette in the US10-US9 intergenic region as shown. No HCMV genome sequences were deleted by this insertion. The second line (in this panel, as well as in panels C through E) shows the organization of the relevant region of the linearized plasmid constructed to make the recombinant virus. The expected transcripts from this region are shown and their size given in kilobases. The black box is the 2.7E promoter (pr); the tk polyadenylation signal adjacent to the β-glucuronidase gene (An) is also shown. (C) Genomic organization of RV699. The β-glucuronidase gene was inserted at the US11 transcription initiation site and replaces 220 b downstream from there, including the sequences encoding the N-terminal 62 (of 215 total) amino acids of US11. This replacement results in a 1.68-kb increase in genome size. The region deleted is shown by a shaded box just below the first line. The endogenous US11 promoter controls transcription of β-glucuronidase in this construct. Clear boxes adjacent to β-glucuronidase (without notation) are the remaining portions of partially deleted open reading frames. For example, just downstream of the β-glucuronidase are the sequences encoding the C-terminal 153 amino acids of the US11 open reading frame. Both the 3.2- and 0.9-kb transcripts utilize the endogenous polyadenylation site downstream of US10. (D) Genomic organization of RV131. The β-glucuronidase gene was inserted at the US10 transcription initiation site and replaces 407 b downstream from there, including the sequences encoding the N-terminal 120 (of 185 total) amino acids of US11. The XhoI site located between US11 and US10 in the wild-type genome is lost as result of the insertion. This replacement results in a 1.49-kb increase in genome size. The region deleted is shown by the shaded box. Only the sequences encoding the C-terminal 65 amino acids remain (clear box adjacent to β-glucuronidase). β-Glucuronidase gene expression is controlled by the endogenous US10 promoter. The 3.0- and 2.4-kb transcripts are 3' coterminal at the polyadenylation site downstream of US10. (E) Genomic organization of RV67. The β-glucuronidase gene was inserted at the US11 transcription initiation site and replaces 1.12 kb downstream from there, including all of the US11 coding region and all but the sequences encoding the C-terminal 65 amino acids of US10 (clear box adjacent to β-glucuronidase). The region deleted is shown by the shaded box. This replacement results in a 0.78-kb increase in genome size. β-Glucuronidase gene expression is controlled by the US11 promoter. Only a 2.3-kb transcript is predicted.

2 min. The cells were washed twice with phosphate-buffered saline, and growth medium was added. The media was changed every 4 to 7 days. When cytopathic effect was extensive (14 to 21 days posttransfection), virus was harvested. The resulting virus stock was serially diluted and placed on HFF cells. After 10 to 12 days, the infected cells were overlaid with 0.5% agarose in modified Eagle medium (GIBCO) containing 100 μg of X-Glu (5-bromo-4-chloro-3-indolyl-β-D-glucuronide; Biosynth) per ml. Blue (recombinant virus-containing) plaques were picked 3 h to several days after adding the overlay (depending on the strength of the promoter controlling β-glucuronidase expression). In some cases, the original transfection plate was overlaid for the first blue plaque identification. Recombinant viruses were plaque purified four times (one round of plaque purification after all plaques on a plate appeared blue).

Transfection for recombinant HSV. Essentially, the procedure given above for HCMV was followed, except that HSV-1 strain Patton DNA and Vero cells were used.

RNA extraction. Total cytoplasmic RNA was isolated by the Nonidet P-40 lysis method as described previously (46). HCMV kinetic class RNA was isolated after infection of HFF cells at a multiplicity of infection of 5. Immediate-early RNA was isolated at 8 h postinfection from cells in the presence of either 25 μM anisomycin or 100 μg of cycloheximide per ml, as indicated, from 1 h before infection until harvest. Early RNA was isolated from cells in the presence of 100 μg of phosphonoformate per ml at 24 h postinfection. Late RNA was isolated from cells at 72 h postinfection.

RNA blot analysis. Fifteen micrograms of total cytoplasmic RNA was electrophoresed through 1.2% formaldehyde agarose gels according to standard protocol (46). The RNA was blotted to Nytran (Scheicher and Schuell), UV cross-linked, and prehybridized for 4 to 16 h in 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt, 50 mM sodium phosphate (pH 6.5), 1% glycine, 100 μg of denatured sonicated salmon sperm DNA per ml, and 0.1% SDS at 42°C. Using high-specific-activity riboprobes (46), the blots were hybridized in 50% formamide, 5× SSC, 1× Denhardt, 20 mM sodium phosphate (pH 6.5), 1 mM EDTA, 100 μg of denatured sonicated salmon sperm DNA per ml, 50 μg of yeast tRNA per ml, and 0.1% SDS for 18 h at 60°C. Riboprobe blots were washed twice in 2× SSC at room temperature for 5 min and then treated with 1 μg of RNase A per ml in 2× SSC for 15 min. This was followed by three high-stringency washes in 0.05× SSC–0.1% SDS at 55°C for 20 min. The washed blots were exposed to XAR5 film (Kodak) with Dupont Cronex intensifying screens at −70°C.

DNA blot analysis. Viral DNA was electrophoresed through 0.6% agarose gels. The DNA was blotted to Nytran, UV cross-linked, and prehybridized as described above for the RNA blots. By using DNA probes (made by using a random priming kit [Bethesda Research Laboratories]), the blots were hybridized at 42°C in the buffer given for the RNA blot analysis, except that the buffer lacked the yeast tRNA. The blots were washed twice in 2× SSC–0.1% SDS at room temperature for 5 min. Three high-stringency washes were done in 0.1× SSC–0.1% SDS at 50°C for 20 min each. The washed blots were exposed as described for the RNA blots.

Production of polyclonal antisera. US10 and US11 genes products were expressed as protein A fusion proteins in *Escherichia coli* BL21 (44). The pT7protA fusion vector (described above) contains the unaltered pRIT2T polylinker 3′ of the protein A coding region. For the US10 or US11 fusions, bases 14119 to 13127 or bases 14834 to 14189,

respectively, were inserted in frame into the polylinker. Accordingly, the US10 fusion contained all except the N-terminal eight amino acids of the US10 open reading frame and the US11 fusion contained all except the N-terminal 11 amino acids of the US11 open reading frame. The fusion proteins were solubilized and purified from inclusion bodies of bacteria induced with isopropyl-β-D-thiogalactopyranoside (IPTG). New Zealand White rabbits were immunized at 6-week intervals with these fusion protein antigens by subcutaneous injection using either Freund's complete adjuvant (primary immunization) or Freund's incomplete adjuvant (secondary immunizations). Serum was obtained after the fifth boost.

Immunoprecipitation. Uninfected or infected-cell proteins (8 to 12 h after infection at a multiplicity of 5) were radiolabelled with methionine and cysteine as follows. Cells were depleted of methionine and cysteine for 45 min prior to labelling by incubation in serum-free DMEM lacking these amino acids (Specialty Media, Lavalette, N.J.). Metabolic radiolabelling of proteins was done by adding [^{35}S]methionine and cysteine (1,100 Ci/mmol; ExpreSS; New England Nuclear) to a final concentration of 200 μCi/ml in the same media, but containing dialyzed fetal calf serum (GIBCO) at a final concentration of 1%. After labelling, cell lysates were prepared by solubilization in triple-detergent lysis buffer (46). The cleared lysates (supernatant after centrifugation for 5 min at 15,000 × g and 4°C) were retained for immunoprecipitation (46). Briefly, the radiolabelled extracts were precleared by using preimmune serum and killed *Staphylococcus aureus* (Bethesda Research Laboratories). Proteins binding to immune serum were pelleted by using protein A sepharose (Pharmacia). The washed immunoprecipitates were boiled in the presence of 2-mercaptoethanol and electrophoresed in denaturing polyacrylamide gels. The gels were fixed and soaked in salicylate fluor (46) prior to drying and autoradiography.

Single-cycle growth analysis. Confluent monolayers of HFF cells in 35-mm plates were infected at a multiplicity of infection of 2 with either wild-type or recombinant HCMV. After adsorption for 2 h at 37°C, the inoculum was removed and fresh medium was added to each plate. The plates were incubated at 37°C until the indicated day postinfection. The plates were frozen at −70°C, thawed at 37°C, and infected cells were scraped into the medium. The medium–infected-cell suspension was sonicated for 1 min. Total infectious virus was quantitated by plaque assay with a 0.5% agarose overlay.

RESULTS

β-Glucuronidase is a useful marker gene for recombinant herpesviruses. In order to study HCMV gene function by the genetic approach of site-directed insertion and replacement mutagenesis, a small marker gene, β-glucuronidase, was investigated for its utility in herpesvirus systems. Two likely reasons why β-glucuronidase has not been used as a marker gene in animal virus systems are the successful usage of β-galactosidase in many systems (including HSV) and the thought that endogenous cellular β-glucuronidase in mammalian cells would preclude the usefulness of this gene as a marker (11, 12, 29, 38). To test whether β-glucuronidase would serve as a suitable marker in a herpesvirus system, recombination into the known nonessential HSV-1 *tk* gene was performed (52). Following cotransfection of pBgdTK and HSV-1 DNAs into Vero cells, β-glucuronidase-expressing virus from seven blue plaques were purified and their
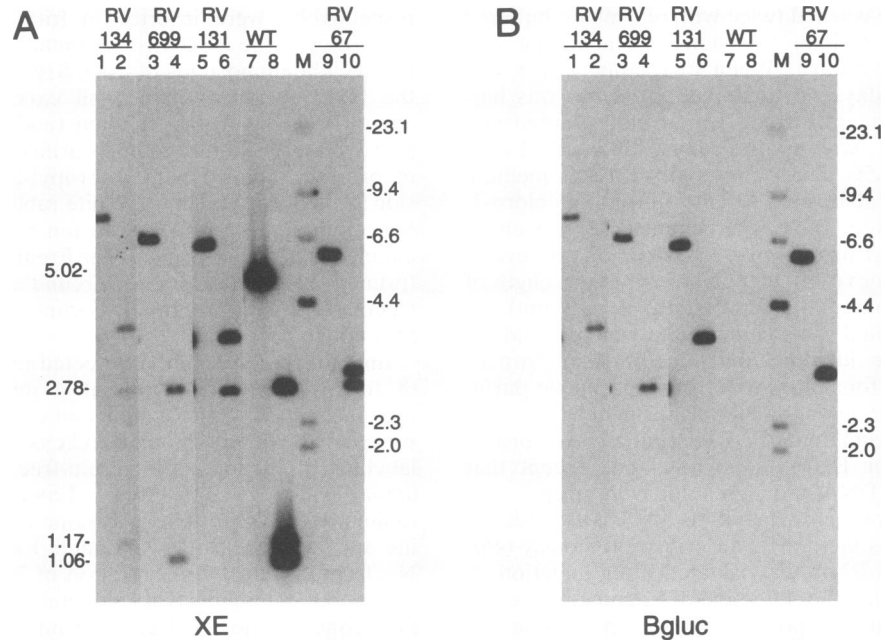
FIG. 2. DNA blot analysis of recombinant HCMV DNA. Viral DNAs were digested with HindIII (lanes 1, 3, 5, 7, and 9) or HindIII-XhoI (lanes 2, 4, 6, 8, and 10) and electrophoresed in a 0.6% agarose gel. After transfer to a nylon membrane, the DNAs were hybridized with either an HCMV HindIII X fragment-derived 2.95-kb XbaI-EcoRI (XE) probe (A) or a 0.6-kb β-glucuronidase (Bgluc) gene probe (B). The location of the XE probe is shown in Fig. 1A. The DNAs were from RV134 (lanes 1 and 2), RV699 (lanes 3 and 4), RV131 (lanes 5 and 6), wild-type strain AD169 (lanes 7 and 8), and RV67 (lanes 9 and 10). Lanes M, radiolabelled λ HindIII DNA markers whose size is given in kilobases to the right of each panel. The location of the hybridizing DNA fragments from wild-type virus is indicated to the left of each panel by size (in kilobases). All lanes were from the same gel, but different (optimal) autoradiogram exposures were used to make the figure.

DNAs were analyzed. Each was shown to contain the expected insertion of the β-glucuronidase gene in place of *tk* within the HSV-1 EcoRI N fragment (data not shown). This was the only recombinant virus DNA fragment which displayed altered electrophoretic mobility compared with that of wild-type virus (data not shown). These results substantiated that β-glucuronidase can be used as a marker gene for the identification of recombinant virus in a herpesvirus system.

The next step was to determine whether β-glucuronidase could be used in a similar fashion with HCMV. HCMV is permissive in very few cell types in tissue culture (including HFF cells), and infection by this virus stimulates the expression of some cellular genes (4, 8, 20, 47, 58). We determined that both uninfected and wild-type HCMV-infected HFF cells expressed very little endogenous cellular β-glucuronidase, no more than uninfected Vero cells (data not shown) which were used for the HSV-1 recombinants (above). Since there have not been any published reports of nonessential genes in the HCMV system, our strategy to test whether β-glucuronidase could serve as a marker in this system was to design a plasmid that would direct β-glucuronidase recombination into the US10-US9 intergenic region. We have recently mapped transcripts expressed from the HCMV US6 gene family (25). Low-abundance read-through RNAs are transcribed from an intergenic region between US10 and US9. p2.7EBgpAUS10/9 was constructed such that when it recombined into this region, the β-glucuronidase gene would be expressed under the control of another HCMV promoter, 2.7E (14), and that transcription would be terminated by the HSV-1 *tk* polyadenylation signal engineered into this plasmid. By homologous recombination due to the HCMV flanking sequences, this β-glucuronidase expression cassette

should be inserted just downstream of the US10 polyadenylation signal and about 275 b upstream of the US9 transcription start sites. It was hoped that this insertion would not interfere with expression directed by the US9 promoter. After transfection of linearized p2.7EBgpAUS10/9 with wild-type HCMV genomic DNA, the primary plaques were harvested and replated on HFF cells for screening as described in Materials and Methods. Blue plaques were obtained at a frequency of about 5%.

By DNA blot analysis (Fig. 2), the fidelity of the insertion was determined. The recombinant genome of RV134, a virus purified from one of the blue plaques, had the β-glucuronidase expression cassette inserted at the predicted location within the HindIII X fragment of the HCMV genome (Fig. 1B). As shown in Fig. 2A, the 2.95-kb XbaI-EcoRI fragment probe (XE [Fig. 1A]) from HindIII X probe hybridized to the 5.02- and 7.85-kb HindIII DNA fragments in wild-type and RV134 DNA digests, respectively. The HCMV HindIII X DNA fragment increased in size by 2.83 kb because of the insertion of the β-glucuronidase expression cassette (Fig. 1B). In HindIII-XhoI double digests, only the predicted 1.06-kb wild-type fragment showed altered mobility (to 3.9 kb) in RV134 (Fig. 2A). Only the 7.85-kb HindIII and 3.9-kb HindIII-XhoI DNA fragments from RV134, and not any wild-type DNA fragments, hybridized with a β-glucuronidase probe (Fig. 2B). Similar DNA blot hybridizations using the HX and XP probes (Fig. 1A) established that the regions surrounding the β-glucuronidase expression cassette insertion (e.g., HindIII Q and V fragments [Fig. 1A]) were not altered in RV134 compared with wild-type virus (data not shown). Also, using a total HCMV genomic DNA probe and a probe from UL, no unexpected alterations of the RV134 genome were revealed (data not shown). The data indicated
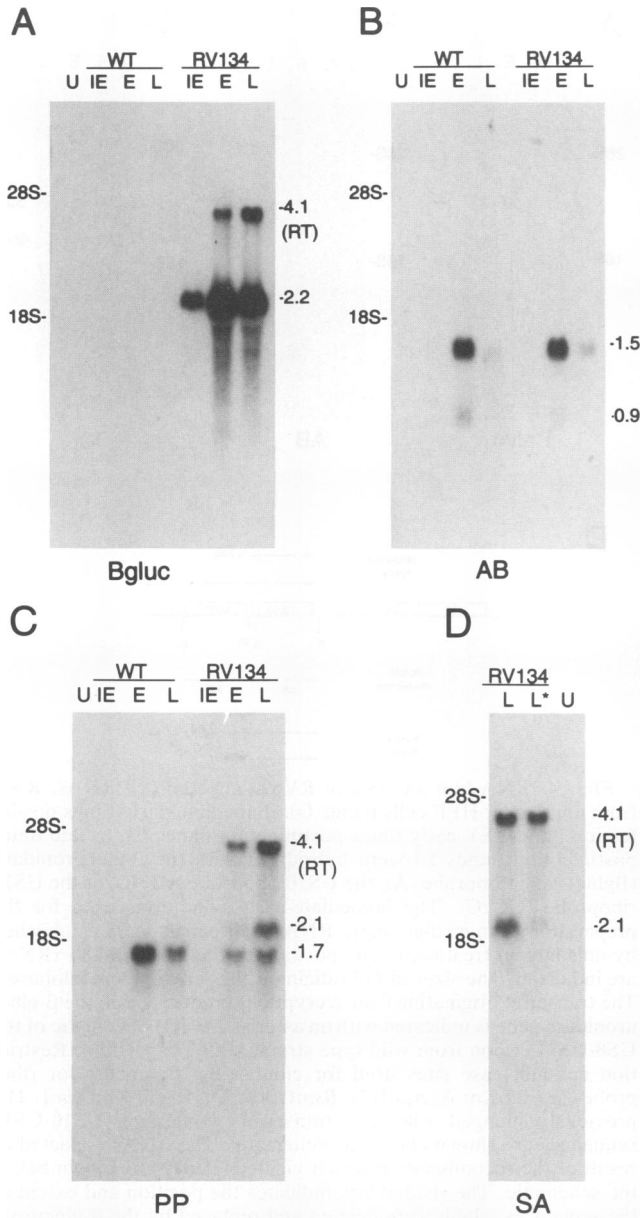
FIG. 3. RNA blot analysis of RV134-infected cell RNAs. RNA from uninfected HFF cells (lanes U), immediate-early times postinfection (lanes IE), early times postinfection (lanes E), or late times postinfection (lanes L) were hybridized with the β-glucuronidase (Bgluc) gene riboprobe (A), the US10 riboprobe AB (B), the US8-US9 riboprobe PP (C), or the US9-US10 intergenic riboprobe SA (D). Lane L* in panel D contains RNA from cells which were infected and maintained in the presence of 100 μg of phosphonoformate per ml until late times (72 h) postinfection. The cells were infected with either wild-type (WT) HCMV strain AD169 or RV134

that the recombination of the β-glucuronidase expression cassette into the RV134 genome had occurred correctly.

RNA blots of kinetic class transcripts demonstrated that the expected 2.2-kb β-glucuronidase message was present in very abundant amounts at early and late times postinfection in RV134-infected HFF cells (Fig. 3A), as would be predicted from transcripts under the control of the strong 2.7E promoter (14, 35, 55). A 4.1-kb readthrough transcript was also detected by using this β-glucuronidase riboprobe (Fig. 3A). The β-glucuronidase riboprobe did not hybridize with RNA from wild-type virus-infected cells. Except for a slight reduction in the steady-state level of the 1.7-kb US9-US8 transcript at early times postinfection, the insertion of the β-glucuronidase expression cassette into the US10-US9 intergenic region in RV134 did not have an effect on RNA expression from the adjacent upstream or downstream transcription units, either US11-US10 (1.5- and 0.9-kb RNAs [Fig. 3B]) or US9-US8 (1.7-kb RNA [Fig. 3C]), respectively, compared with that in the wild type. The cumulative results from the DNA and RNA blot analyses suggested that insertion of the β-glucuronidase expression cassette does not significantly alter expression from neighboring transcription units and allows for predictable construction, as well as the identification, of HCMV recombinant mutants.

It was previously reported that the HSV-1 late glycoprotein H promoter sequences are closely associated with the polyadenylation signal of the upstream *tk* gene (51). This late promoter region is within the *tk* polyadenylation signal fragment used to terminate transcription in the β-glucuronidase expression cassette used in the RV134 mutant of HCMV. This HSV-1 late promoter was also activated at late times postinfection in RV134-infected HFFs, as evidenced by the 2.1-kb late transcript which hybridized with the US8-US9 probe (Fig. 3C). The HSV-1 glycoprotein H promoter was regulated as a true late promoter when incorporated into the HCMV genome (of RV134) since the 2.1-kb transcript was detected (by using a US9-US10 intergenic region probe [Fig. 3D]) most abundantly only at late times postinfection in the absence, but not in the presence, of phosphonoformate, a viral DNA synthesis inhibitor. As expected for a message under the control of a promoter active both at early and late times postinfection, the 4.1-kb β-glucuronidase readthrough RNA was detected equally under both conditions (Fig. 3D).

Replacement of US10 and US11 open reading frames. As a

as indicated. The position of the 28S and 18S rRNAs are shown. The sizes of hybridizing RNAs are given in kilobases. The β-glucuronidase gene-containing readthrough transcript (RT) is indicated. The schematic is of the US8-US11 region from wild-type strain AD169 of HCMV. The previously mapped wild-type transcripts containing US10-US11 sequences are shown above the schematic. The position of the β-glucuronidase gene expression cassette insertion is indicated by #. The position of the HCMV gene-derived riboprobes are indicated. Restriction endonuclease sites, some of which were used for cloning the fragments for riboprobes, are: A, *Apa*I; B, *Bsm*I; Ps, *Pst*I; Pv, *Pvu*II; Xb, *Xba*I; and Xh, *Xho*I. The immediate-early conditions used for the preparation of immediate-early RNA in panels A and B were established by cycloheximide treatment (described in Materials and Methods). The 2.2-kb β-glucuronidase message (from the strong 2.7E early promoter) detected in panel A under the cycloheximide immediate-early conditions was not detected when anisomycin treatment was used to establish immediate-early conditions (data not shown). The immediate-early conditions used for the preparation of immediate-early RNA in panel C were established by anisomycin treatment.

first step to examine the role played by the US10 and US11 gene products in the viral infectious cycle, plasmids which would replace part of either gene by the β-glucuronidase marker gene under the control of the endogenous US10 or US11 promoters which were mapped previously (25) were constructed. In pBgdUS11 and pBgdUS10, the β-glucuronidase gene was inserted at the transcription initiation site and replaced the normal 5′ untranslated sequences and sequences encoding the first (amino-terminal) 62 or 120 amino acids of either the US11 or US10 open reading frames, respectively (Fig. 1C and D). These plasmids were designed such that only the open reading frame containing the β-glucuronidase insertion would be mutated and expression from the adjacent open reading frames should not be altered. Cotransfection of the plasmids with wild-type HCMV DNA and then plaque purification of β-glucuronidase-expressing virus yielded recombinant viruses RV699 and RV131 from the transfections involving pBgdUS11 and pBgdUS10, respectively. Therefore, in RV699, US11 gene expression should have been abolished, and in RV131, US10 gene expression should have been abolished. DNA blot analysis of the viral DNAs demonstrated that the β-glucuronidase gene was inserted in the predicted location within HindIII X region of the genome (Fig. 2). The 2.95-kb XbaI-EcoRI fragment (XE [Fig. 1A]) and β-glucuronidase probes both hybridized to 6.7- and 6.5-kb HindIII DNA fragments in digests of RV699 and RV131 DNAs, respectively. The increase in size of the HindIII X DNA fragment (5.02 kb in wild-type virus) reflects the net increase in genome size (1.68 kb in RV699 and 1.49 kb in RV131) predicted by the insertion (Fig. 1C and D). The correct location of the β-glucuronidase insertion within the HindIII X region was confirmed by hybridization of these probes to HindIII-XhoI digests of the viral DNA. The 2.95-kb XbaI-EcoRI (XE) fragment hybridized to 1.17-, 1.06-, and 2.78-kb fragments from wild-type virus DNA (Fig. 2A). In RV699, only the 1.17-kb fragment was altered and increased in size to 2.85 kb (and comigrated with the 2.78-kb fragment in the gel used for this blot; confirmed by SstI and HindIII-PstI digests [data not shown]). In RV131, the β-glucuronidase gene insertion resulted in the loss of the XhoI restriction site that was at the junction of the 1.17- and 1.06-kb fragments (Fig. 1A). The resulting fragment was 3.73 kb; the 2.78-kb fragment remained unaltered. Both the 2.85-kb fragment from RV699 and the 3.73-kb fragment from RV131 hybridized with the 2.95-kb XbaI-EcoRI fragment and β-glucuronidase gene probes (Fig. 2A and B). In DNA blot experiments identical to that described above for RV134, no other alterations of the RV699 or RV131 genomes, compared with that of wild-type virus, were detected (data not shown). This included the regions neighboring the HindIII X fragment (HindIII Q and HindIII V fragments).

Previous fine mapping of transcripts from the wild-type virus (25) in conjunction with RNA blot analysis of kinetic class RNAs confirmed the correct insertion of the β-glucuronidase gene within the US11 and US10 transcription units in RV699 and RV131, respectively. Briefly, in wild-type virus, a 1.5-kb early transcript initiates upstream of US11 and terminates at the polyadenylation site downstream of US10. Also, a 0.9-kb early transcript initiates upstream of US10 and is 3′ coterminal with the 1.5-kb RNA (thus, the US10 riboprobe hybridizes to both transcripts [Fig. 3B]). Because of the β-glucuronidase gene insertion in RV699, the 1.5-kb US11-US10 transcript of wild type increased in size to 3.2 kb (Fig. 1C and 4D) and hybridized with the β-glucuronidase (Fig. 4A), US10 (Fig. 4B), and US11 (Fig. 4C) ribo-
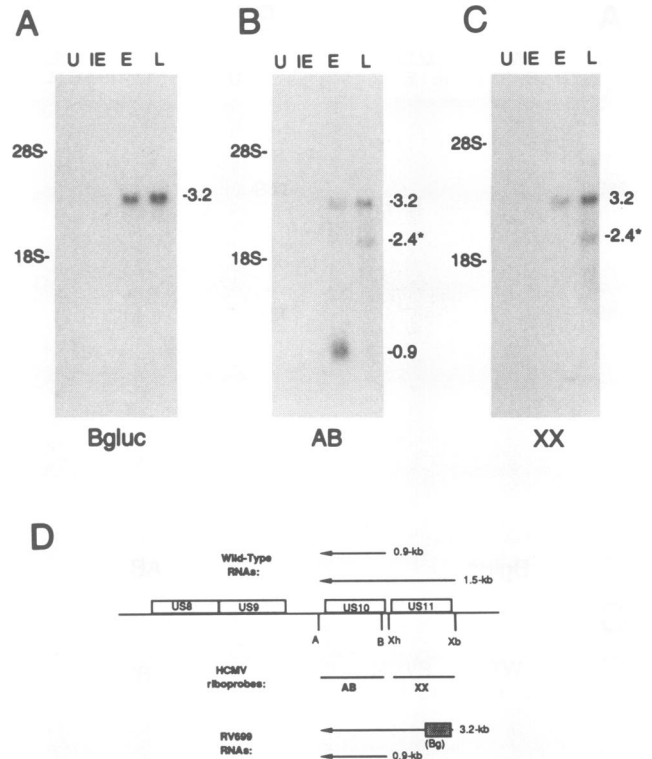

FIG. 4. RNA blot analysis of RV699-infected cell RNAs. RNA from uninfected HFF cells (lanes U), immediate-early times postinfection (lanes IE), early times postinfection (lanes E), or late times postinfection (lanes L) were hybridized with the β-glucuronidase (Bgluc) gene riboprobe (A), the US10 riboprobe AB (B), or the US11 riboprobe XX (C). The immediate-early conditions used for the preparation of immediate-early RNA in all panels were established by anisomycin treatment. The position of the 28S and 18S rRNAs are indicated. The sizes of hybridizing RNAs are given in kilobases. The transcript originating from a cryptic promoter within the β-glucuronidase gene is indicated with an asterisk (∗). (D) Schematic of the US8-US11 region from wild-type strain AD169 of HCMV. Restriction endonuclease sites used for cloning the fragments for riboprobes are shown: A, ApaI; B, BsmI; Xb, XbaI; and Xh, XhoI. The previously mapped wild-type transcripts containing US10-US11 sequences are shown above the schematic. The RNAs expected as result of the recombination which yielded RV699 are shown below the schematic. The shaded box indicates the position and extent of the sequences which were deleted and replaced by the β-glucuronidase gene (not drawn to scale) in RV699-infected cell RNAs. The position of the HCMV gene-derived riboprobes are indicated.

probes. The 0.9-kb US10 message was unaltered in RV699 and hybridized only with the US10 riboprobe (Fig. 4B). Because of the β-glucuronidase gene insertion in RV131, both the 1.5-kb US11-US10 and 0.9-kb US10 RNAs increased in size to 3.0 and 2.4 kb, respectively (Fig. 1D and 5D). Both of these transcripts hybridized with the β-glucuronidase (Fig. 5A) and US10 (Fig. 5B) riboprobes, but only the former hybridized with the US11 (Fig. 5C) riboprobe.

The RNA blot analyses of Fig. 4 and 5 revealed additional transcripts, most abundant in late RNAs, by using probes which hybridized downstream of the β-glucuronidase gene. For example, the US11 and US10 probes hybridized to a late transcript from RV699 which migrated at about 2.4 kb (Fig. 4B and C). Also the US10 probe hybridized to two smaller late RNAs (approximately 1.3 and 1.5 kb) in from RV131
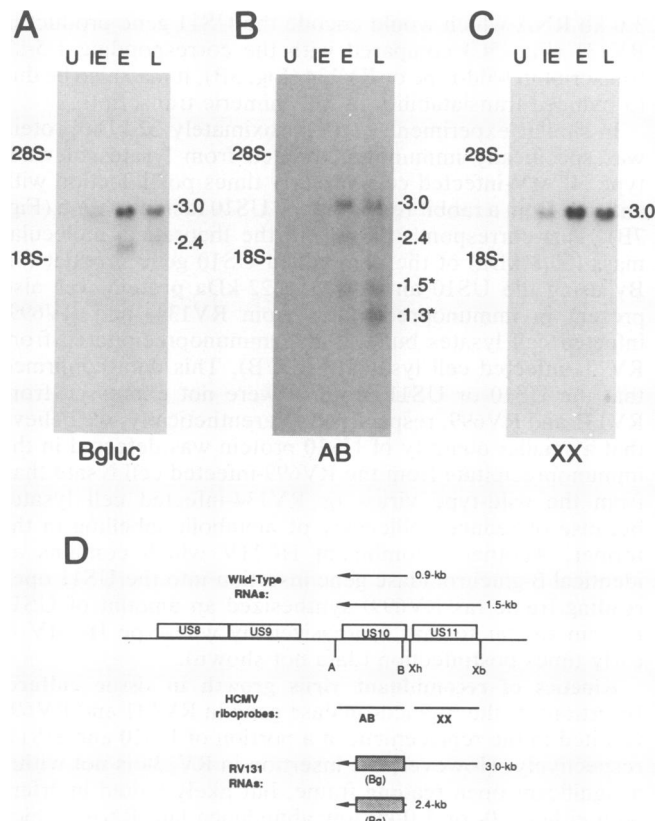
FIG. 5. RNA blot analysis of RV131-infected cell RNAs. RNA from uninfected HFF cells (lanes U), immediate-early times postinfection (lanes IE), early times postinfection (lanes E), or late times postinfection (lanes L) were hybridized with the β-glucuronidase (Bgluc) gene riboprobe (A), the US10 riboprobe AB (B), or the US11 riboprobe XX (C). The immediate-early conditions used for the preparation of immediate-early RNA in panels A and C were established by cycloheximide treatment and in panel B by anisomycin treatment. The position of the 28S and 18S rRNAs are indicated. The sizes of hybridizing RNAs are given in kilobases. Those transcripts originating from a cryptic promoter within the β-glucuronidase gene are indicated with an asterisk (*). (D) Schematic of the US8-US11 region from wild-type strain AD169 of HCMV. Restriction endonuclease sites used for cloning the fragments for riboprobes are shown: A, ApaI; B, BsmI; Xb, XbaI; and Xh, XhoI. The previously mapped wild-type transcripts containing US10-US11 sequences are shown above the schematic. The RNAs expected as result of the recombination which yielded RV131 are shown below the schematic. The shaded box indicates the position and extent of the sequences which were deleted and replaced by the β-glucuronidase gene (not drawn to scale) in RV131-infected cell RNAs. The position of the HCMV gene-derived riboprobes are indicated. The small amount of RNA detected under immediate-early conditions in panels A and C was due to leakage under the cycloheximide conditions.

(Fig. 5B). Since these transcripts did not hybridize with a US9 riboprobe (data not shown), we believe that they terminated at the first polyadenylation signal encountered, just downstream of US10. Therefore, in both cases, in the absence of splicing, the 5' end of the transcripts would lie within the β-glucuronidase gene, about 1.1 to 0.9 kb downstream from its amino terminus. These RNAs were not detected by the β-glucuronidase probe, since it contains only the 0.6 kb from the amino terminus of that gene. The data
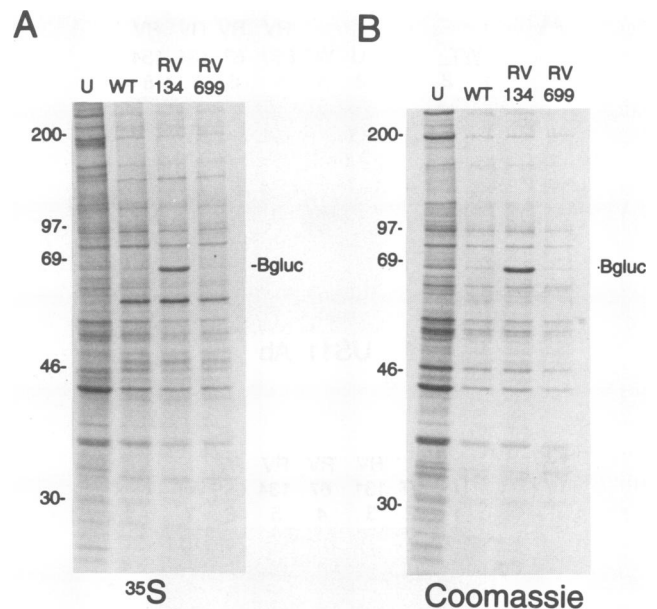

FIG. 6. Total protein analysis of virus-infected cells. HFF cells were uninfected (lanes U) or infected at a multiplicity of infection of 5 with wild-type strain AD169 (lanes WT), RV134, or RV699. From 68 to 72 h postinfection (or mock infection), proteins were metabolically labeled with [35S]methionine and [35S]cysteine as described in Materials and Methods. Equal volumes of the cell lysates were electrophoresed in SDS–10% PAGE, and the proteins were stained with Coomassie blue. The gel was exposed to X-ray film (A) and photographed (B). The position of the 68-kDa β-glucuronidase (Bgluc) protein is indicated.

inferred that there is a cryptic promoter within the β-glucuronidase gene that is activated at late times postinfection by HCMV.

Protein analysis of recombinant HCMV. Proteins synthesized by RV134 were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) of total infected-cell lysates. A 68-kDa protein, which is the size of the β-glucuronidase monomeric subunit (21), was easily detected in lysates of cells infected with RV134 (Fig. 6). Corresponding to very abundant amounts of β-glucuronidase RNA in RV134-infected cells (Fig. 3A), this protein was the most abundant protein which was synthesized (Fig. 6A) or accumulated (Fig. 6B) by late times postinfection. The 68-kDa protein was not detected in uninfected-cell lysates or those from wild-type virus-infected cells. This protein was abundant in RV134-infected cells since in that virus β-glucuronidase is under the control of the strong 2.7E promoter (14, 35). The 2.7E promoter was previously utilized to drive β-galactosidase production in HCMV recombinant RC256 (55). β-Galactosidase protein accumulated to similar high levels in cells infected by RC256. β-Glucuronidase accumulation was not detected in RV699-infected cells (Fig. 6B). This was because of the comparatively weak US11 promoter which controls β-glucuronidase expression in RV699 (25) (Fig. 1C) and therefore the relatively low steady-state level of the 3.2-kb β-glucuronidase-encoding RNA (Fig. 4A) compared with the 2.2-kb message in RV134-infected cells (Fig. 3A).

Immunoprecipitation analysis was used to determine whether the genes disrupted by the insertions in RV131 and RV699 were expressed (Fig. 7). US10 or US11 fusion proteins were expressed in bacteria from the protein A
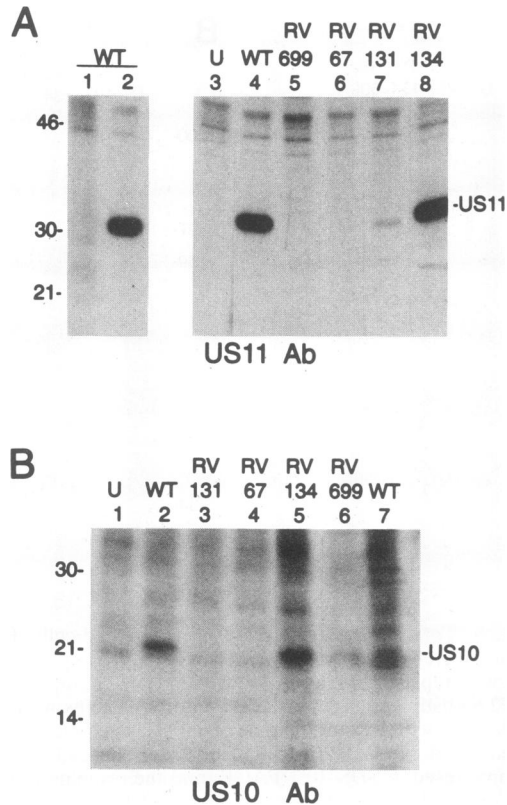
FIG. 7. Immunoprecipitation of US10 and US11 proteins from recombinant virus-infected cell lysates. HFF cells were uninfected (U) or infected at a multiplicity of infection of 5 with wild-type strain AD169 (WT), RV134, RV699, RV131, or RV67. From 8 to 12 h postinfection (or mock infection), proteins were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine as described in Materials and Methods. (A) Immunoprecipitation of cell lysates with US11 immune antisera (lanes 2 to 8). Preimmune serum from the same rabbit was used in lane 1. Lanes 1 and 2 and lanes 3 through 8 are from different SDS-10% PAGE gels. (B) Immunoprecipitation of cell lysates with US10 immune antisera. An 15% SDS-PAGE gel was used. The position of the Amersham ^{14}C-protein molecular weight markers are indicated to the left of each panel. The position of the US11 or US10 immunoprecipitated proteins is also indicated.

fusion vector pT7protA (described in Materials and Methods). Monospecific polyclonal antisera were obtained from rabbits after immunization with the fusion proteins. Immune antisera from a rabbit receiving the US11 fusion protein immunoprecipitated a 31-kDa protein from lysates of wild-type HCMV-infected cells at early times postinfection (Fig. 7A, lanes 2 and 4). This protein was not precipitated with preimmune sera (from the same animal) or from uninfected-cell lysates (Fig. 7A, lanes 1 and 3, respectively). The theoretical molecular mass of the unmodified US11 gene product is 25.3 kDa (6). The 31-kDa protein recognized by the US11 antisera is a glycosylated form of the US11 gene product (26). As expected, the 31-kDa US11 gene product was immunoprecipitated from lysates of RV134- and RV131-infected cells and was absent from lysates of RV699-infected cells (Fig. 7A) using that antisera. Although infected at the same multiplicity, smaller quantities of US11 protein were immunoprecipitated from lysates from RV131-infected cells than from lysates of wild-type virus- or RV134-infected cells. Although this may be due to slightly reduced levels of the

3.0-kb RNA which would encode the US11 gene product in RV131 (Fig. 5C) compared with the corresponding 1.5-kb transcript in wild-type or RV134 (Fig. 3B), it may also be due to reduced translatability of the chimeric transcript.

In similar experiments, an approximately 22-kDa protein was specifically immunoprecipitated from lysates of wild-type HCMV-infected cells at early times postinfection with antisera from a rabbit receiving the US10 fusion protein (Fig. 7B). This corresponds closely to the theoretical molecular mass (20.8 kDa) of the unmodified US10 gene product (6). By using the US10 antisera, the 22-kDa protein was also present in immunoprecipitates from RV134- and RV699-infected cell lysates but was not immunoprecipitated from RV131-infected cell lysates (Fig. 7B). This data confirmed that the US10 or US11 proteins were not expressed from RV131 and RV699, respectively. Parenthetically, we believe that a smaller quantity of US10 protein was detected in the immunoprecipitate from the RV699-infected cell lysate than from the wild-type virus- or RV134-infected cell lysates because of reduced efficiency of metabolic labelling in the former. Another recombinant HCMV which contains an identical β-glucuronidase gene insertion into the US11 open reading frame (as RV699) synthesized an amount of US10 protein similar to that synthesized by wild-type HCMV at early times postinfection (data not shown).

Kinetics of recombinant virus growth in tissue culture. Insertions of the β-glucuronidase gene in RV131 and RV699 resulted in the replacement of a portion of US10 and US11, respectively. However, the insertion in RV134 is not within a significant open reading frame, but likely would interfere with either 3.0- or 1.0-kb low-abundance late RNAs which are transcribed from the opposite DNA strand as the US6 family genes within this region (data not shown). In order to assess the effect of these insertions, single-cycle growth analyses were performed in HFF cells (Fig. 8). The eclipse period for each of the recombinant viruses was 2 to 3 days. This, as well as the rate of infectious virus production by each of the recombinants, was similar to that of wild-type virus (Fig. 8). These results are in agreement with those published previously for wild-type strains of HCMV (43, 53). The data suggested that interruption of the transcription units from the strand opposite to the US6 family (RV134), as well as the lack of expression of the US10 or US11 proteins, does not impair virus growth kinetics. In low-multiplicity infections, the size and appearance of recombinant virus plaques were similar to wild-type plaques (not shown).

US10 and US11 genes can be replaced simultaneously. The viral growth studies of RV131 and RV699 have suggested that the lack of either US10 or US11 gene expression, individually, does not result in a defect in the ability of these viruses to grow in tissue culture. US10 and US11 genes are adjacent to each other in HCMV US, both are expressed most abundantly at early times postinfection (25) (Fig. 3B), and they share limited homology at the amino acid level (64). On the basis of the latter, it was implied that the protein products of these genes may have a common function(s). Therefore, HCMV may be able to tolerate loss of either one of these genes, but not both. To address this question, RV67 in which a 1.11-kb region containing all of the US11 coding region and most of the US10 coding region was replaced by β-glucuronidase under the control of the US11 promoter was constructed (Fig. 1E), resulting in a net increase in size of the HindIII X fragment of 0.78 kb. DNA blot analysis, similar to that described for the previous recombinant viruses, revealed that only the anticipated alteration in the HCMV genome had occurred. The 2.95-kb XbaI-EcoRI
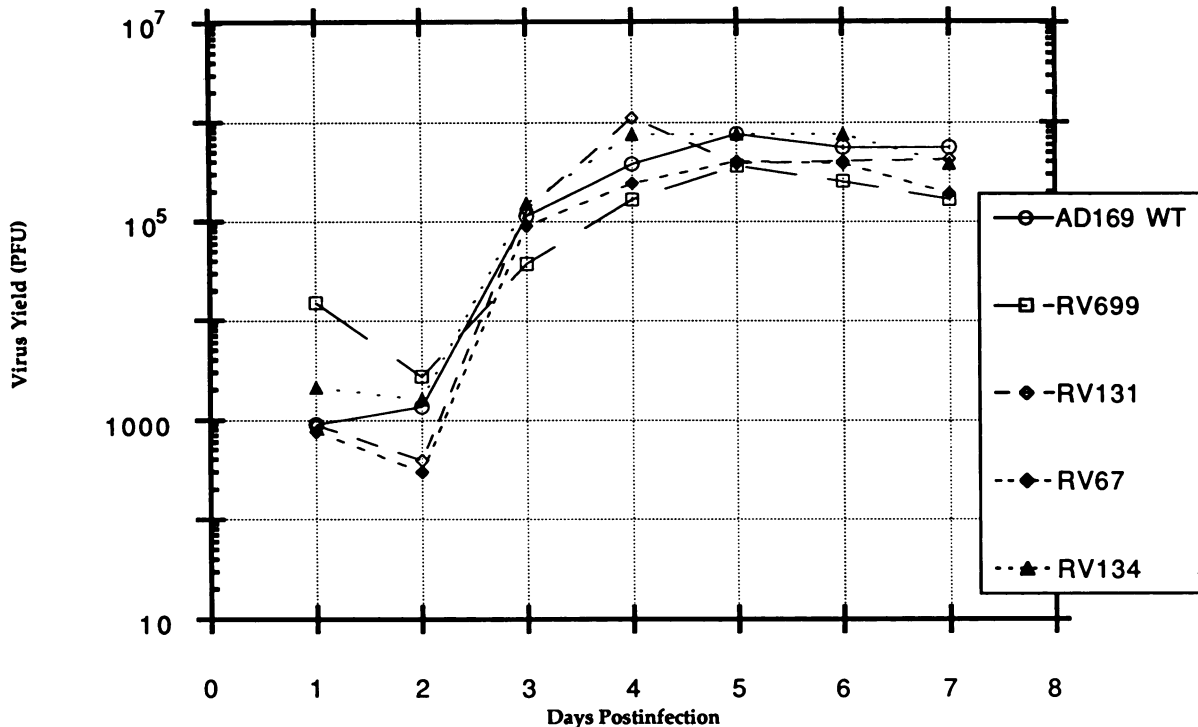
FIG. 8. Single-cycle growth analysis of recombinant viruses.

(XE) fragment and β-glucuronidase probes (Fig. 2A and B, respectively) hybridized to 5.8-kb HindIII and 3.0-kb HindIII-XhoI fragments. The latter represents the insertion within the fused (since the XhoI site was part of the removed sequences) wild-type 1.06-kb XhoI and 1.17-kb HindIII-XhoI fragments (Fig. 1A and E). As before, no alterations were detected in the neighboring HindIII Q or V regions or in any other region of the RV67 genome (data not shown). By RNA blot analysis, a 2.3-kb transcript, which hybridized with either β-glucuronidase (Fig. 9A) or US10 (Fig. 9B) riboprobes, but not to the US11 riboprobe (since these sequences were completely deleted [blot not shown]), was expressed from this region in RV67. A 4.1-kb readthrough transcript was also detected. The low-abundance late transcripts arising from a cryptic promoter within the β-glucuronidase gene detected in RV131-infected cell transcripts were also detected in RV67-infected cell RNA with the US10 probe (Fig. 9B). As expected, neither the US10 nor the US11 protein was detected by immunoprecipitation (Fig. 7). Like deletion of the US11 or US10 genes individually, single-cycle growth analysis did not reveal a defect in the ability of RV67 to replicate in tissue culture compared with that of the wild-type virus (Fig. 8).

DISCUSSION

In this article, the utility of the prokaryotic β-glucuronidase marker gene to identify recombinant herpesviruses, especially HCMV, has been demonstrated. This gene has been used as a marker in plant and some lower eukaryotic systems but has not reported to be useful in mammalian systems previously (21, 22, 33). β-Galactosidase is commonly utilized as a marker gene for the identification of HSV recombinants and also in the one reported HCMV recombinant. However, in the latter case, this HCMV recombinant

(RC256) contained a 5.5-kb deletion adjacent to the site of β-galactosidase insertion (55); one hypothesis that the authors proposed was that the deletion occurred because of the large size of the β-galactosidase insert coupled with the DNA size packaging limits of the HCMV capsid. In this regard, β-glucuronidase may be advantageous to β-galactosidase as a marker gene in the HCMV system because of its relative small size (1.9 versus 3.1 kb). Like β-galactosidase, functional β-glucuronidase is likely tetrameric, consisting of identical monomeric 68-kDa subunits, and is very stable (21). The types of chromogenic substrates (which yield soluble, insoluble, or fluorescent products) which are available for β-galactosidase are also available for β-glucuronidase. Another characteristic of the β-galactosidase-expressing HCMV recombinant RC256 was that it was very unstable, with 10% of plaques appearing white (55). We have isolated and characterized recombinant HCMV which contain and express the prokaryotic β-glucuronidase gene from either of three locations within US. All of the β-glucuronidase-expressing HCMV recombinants that have been isolated and reported in this article seemed to be genetically stable.

It was demonstrated that the US10 and US11 genes can be disrupted, either individually (RV131 and RV699, respectively) or simultaneously (RV67), resulting in loss of protein expression from these open reading frames, without significant impairment in the ability of these recombinant viruses to replicate in a tissue culture system. These genes products were not supplied in trans, as such, by the host cell. We concluded that the protein products of the US10 and US11 genes are nonessential for HCMV replication and growth in human fibroblasts. Data from several laboratories have demonstrated that many HSV-1 genes, including 11 of 12 genes from the HSV US region, can be deleted without effect on tissue culture growth of these HSV mutants (30–32, 41,

A

U IE E L

28S·

-4.1
(RT)

18S·

-2.3

B

U IE E L

28S·

-4.1
(RT)

18S·

-2.3

·1.5*

·1.3*

C

Wild-Type
RNAs:

0.9-kb

1.5-kb

US8   US9   US10   US11

A   B Xh   Xb

HCMV
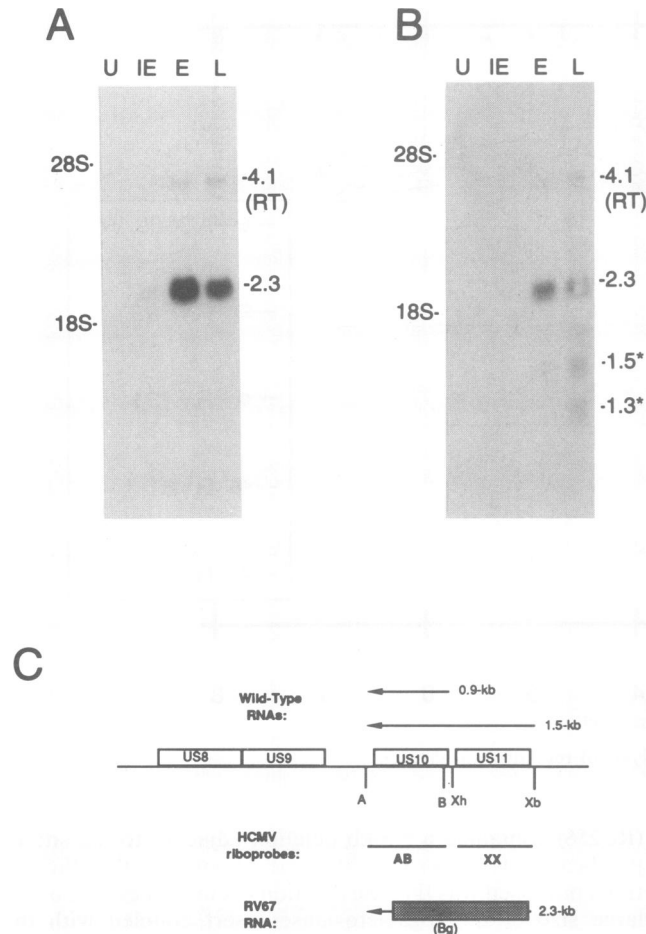riboprobes: AB   XX

RV67
RNA: 2.3-kb
(Bg)

FIG. 9. RNA blot analysis of RV67-infected cell RNAs. RNA from uninfected HFF cells (lanes U), immediate-early times postinfection (lanes IE), early times postinfection (lanes E), or late times postinfection (lanes L) were hybridized with the β-glucuronidase gene riboprobe (A) or the US10 riboprobe AB (B). The immediate-early conditions used for the preparation of immediate-early RNA in both panels were established by anisomycin treatment. The position of the 28S and 18S rRNAs are indicated. The size of hybridizing RNA is given in kilobases. The β-glucuronidase gene-containing readthrough transcript (RT) is indicated. Those transcripts originating from a cryptic promoter within the β-glucuronidase gene are indicated with an asterisk (∗). (C) Schematic of the US8-US11 region from wild-type strain AD169 of HCMV. Restriction endonuclease sites used for cloning the fragments for riboprobes are shown: A, *Apa*I; B, *Bsm*I; Xb, *Xba*I; and Xh, *Xho*I. The previously mapped wild-type transcripts containing US10-US11 sequences are shown above the schematic. The message expected as result of the recombination which yielded RV67 is shown below the schematic. The shaded box indicates the position and extent of the sequences which were deleted and replaced by the β-glucuronidase gene (not drawn to scale) in RV67-infected cell RNA. The position of the HCMV gene-derived riboprobes are indicated.

50, 61). More recently, Manning and Mocarski (34) reported that the murine cytomegalovirus *ie*2 gene product is dispensable for virus growth in NIH 3T3 cells. Also, many of the HCMV US open reading frames are members of families of genes with limited amino acid homology (64). If there is conservation of function within the protein products of a family, it may be possible to delete many family members without an apparent effect. Another alternative is that there

may be clusters of tissue culture dispensable genes in the HCMV US just as in the HSV-1 US (32, 61). These possibilities are being addressed currently.

Mainly on the basis of hydropathy analysis of proteins deduced from DNA sequence data, it was proposed that the US10 and US11 gene products are membrane glycoproteins (64). This hypothesis was supported by data which demonstrated that a monoclonal antibody which reacts with HCMV virion glycoprotein complex II (called gcII) also immunoprecipitates US10 and US11 gene products synthesized in vitro (15). Results from our experiments utilizing tunicamycin suggested that the US11 gene product was glycosylated (26). Through the use of viral recombinants in the HSV-1 system, it has been shown that several of the virion envelope glycoprotein genes are nonessential for virus growth in tissue culture (18, 30–32, 49). These include gC, gE, gG, and gI. Except for gC, these dispensable glycoproteins lie in the US region of HSV-1, just as US10 and US11 map in the US region of HCMV. The only essential gene within the HSV-1 US encodes the virion glycoprotein gD (29). To date, it remains unclear as to what extent the products of the US10 and US11 genes are present in the HCMV-infected cell membrane or the virion envelope (27).

Analysis of the β-glucuronidase gene-containing RNA expression in the HCMV recombinants has revealed several notable results. Transcripts which were expressed at late times postinfection often did not efficiently terminate at the first encountered polyadenylation signal and can be called readthrough RNAs. β-Glucuronidase gene expression in RV134 is under the control of the 2.7E promoter, which is very active at both early and late times postinfection (14, 35). When early and late transcripts were compared, the amount of the 4.1-kb readthrough transcript increased about threefold at late times (Fig. 3A). The readthrough of β-glucuronidase gene-containing RNAs observed in RV134 is not simply explained by the heterologous HSV *tk* polyadenylation signal not being recognized as efficiently as a HCMV polyadenylation signal, since an increase of readthrough was observed at late times in β-glucuronidase gene-containing RNAs which should terminate at the endogenous US10 proximal polyadenylation signal from RV67 (Fig. 9A). This increased propensity for readthrough RNAs at late times postinfection was also observed for entirely endogenous HCMV US6 and US7 late messages (25).

Another observation is that the chimeric β-glucuronidase gene-containing RNAs did not display the same kinetics as their wild-type counterparts. For example, steady-state cytoplasmic levels of the 3.2-kb message from RV699-infected cells (Fig. 4A), the 3.0-kb transcript from RV131-infected cells (Fig. 5A), and the 2.3-kb RNA from RV67-infected cells (Fig. 9A) were only slightly, if at all, reduced at late times compared with early times postinfection. Like the 1.5-kb US11-US10 transcript from wild-type-infected cells, these chimeric messages were transcribed under the control of the US11 promoter. In wild-type virus-infected cells, this 1.5-kb message was greatly reduced at late times compared with early times postinfection (25) (Fig. 3B). There are at least two possibilities for this discrepancy. First, the presence of β-glucuronidase gene sequences on a transcript may stabilize it so it has a longer cytoplasmic half-life than wild-type RNAs. Second, negative intragenic (*cis*) transcriptional regulatory sequences may be abrogated as result of the β-glucuronidase gene insertion into the transcription unit.

A third observation is that the HSV-1 true late gH promoter is also regulated as a HCMV late promoter when it was incorporated into the genome of RV134 (Fig. 3D). This

suggests that a similar mechanism(s) regulates late expression of these related viruses. For example, HCMV may encode a HSV-1 ICP27-like protein, which is essential for the expression of, at least, some HSV-1 late genes (34a, 45). Also, a single-stranded DNA-binding protein of HSV-1, ICP8, was reported to be involved in the stimulation of late gene expression in that viral system (10). An early DNA-binding protein with similarity to ICP8 has been identified as the product of the HCMV open reading frame UL57 (2, 6, 28). Recognition of heterologous virus signals has been reported previously between HSV and HCMV: HSV-1 recognizes the HCMV *a* DNA packaging and cleavage sequence (54), and HCMV functions can complement an HSV-1 ICP0 mutant (60).

Several genes determined to be nonessential for tissue culture growth of HSV increase the virulence of that virus in animal model systems (7, 50). It has been hypothesized that the tissue culture dispensable HSV proteins, including glycoproteins, may be important for the survival and maintenance of the virus in its ecological niche, the human host (11, 30). Unfortunately, as of yet, there is not a good animal model system for HCMV to see if this may also be the case for the tissue culture dispensable US11 and US10 gene products. Interestingly, several members of the tissue culture dispensable adenovirus E3 transcription unit were shown to be important in evasion of the host's immune system (3, 5, 13). One of these was a 19-kDa membrane glycoprotein that is localized in the endoplasmic reticulum and can bind with major histocompatibility complex class 1 antigens, blocking its transport to the cell surface.

Attempts to disrupt and replace other HCMV US6 gene family members by insertion of the β-glucuronidase marker gene are in progress. If these mutants are obtained, they can be used to assess the contribution of the products of these open reading frames on virus viability and also on glycoprotein content of the HCMV virion. Specifically, the mutants could be used to assess the contribution of the US6 gene family members protein products to gcII, as has been proposed (15, 27).

REFERENCES

1. **Alford, C. A., and W. J. Britt.** 1990. Cytomegalovirus, p. 1981–2010. *In* D. M. Knipe and B. N. Fields (ed.), Virology, 2nd ed. Raven Press, New York.
2. **Anders, D. G., and W. Gibson.** 1988. Location, transcript analysis, and partial nucleotide sequence of the cytomegalovirus gene encoding an early DNA-binding protein with similarities to ICP8 of herpes simplex virus type 1. J. Virol. **62**:1364–1372.
3. **Andersson, M., S. Paabo, T. Nilsson, and P. A. Peterson.** 1985. Impaired intracellular transport of class 1 MHC antigens as a possible means for adenovirus to evade immune surveillance. Cell **43**:215–222.
4. **Boldogh, I., S. AbuBakar, C. Deng, and T. Albrecht.** 1991. Transcriptional activation of cellular oncogenes *fos, jun,* and *myc* by human cytomegalovirus. J. Virol. **65**:1568–1571.
5. **Burgert, H. G., and S. Kvist.** 1985. An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class 1 antigens. Cell **41**:987–997.
6. **Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchinson, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. Weston, and B. G. Barrell.** 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. **154**:125–169.
7. **Chou, J., E. R. Kern, R. J. Whitley, and B. Roizman.** 1990. Mapping of herpes simplex virus-1 neurovirulence to γ₁34.5, a gene nonessential for growth in culture. Science **250**:1262–1266.
8. **Colberg-Poley, A. M., and L. D. Santomenna.** 1988. Selective induction of chromosomal gene expression by human cytomegalovirus. Virology **166**:217–228.
9. **DeLuca, N. A., M. A. Courtney, and P. A. Schaffer.** 1984. Temperature-sensitive mutants in herpes simplex virus type 1 ICP4 permissive for early gene expression. J. Virol. **52**:767–776.
10. **Gao, M., and D. M. Knipe.** 1991. Potential role for herpes simplex virus ICP8 DNA replication protein in stimulation of late gene expression. J. Virol. **65**:2666–2675.
11. **Goldstein, D. J., and S. K. Weller.** 1988. Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterization of an ICP6 *lacZ* insertion mutant. J. Virol. **62**:196–205.
12. **Goldstein, D. J., and S. K. Weller.** 1988. An ICP6::*lacZ* insertional mutagen is used to demonstrate that the UL52 gene of herpes simplex virus type 1 is required for virus growth and DNA synthesis. J. Virol. **62**:2970–2977.
13. **Gooding, L. R., L. W. Elmore, A. E. Tollefson, H. A. Brady, and W. S. M. Wold.** 1988. A 14,700 mw protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. Cell **53**:341–346.
14. **Greenaway, P. J., and G. W. G. Wilkinson.** 1987. Nucleotide sequence of the most abundantly transcribed early gene of human cytomegalovirus strain AD169. Virus Res. **7**:17–31.
15. **Gretch, D. R., B. Kari, R. C. Gehrz, and M. F. Stinski.** 1988. A multigene family encodes the human cytomegalovirus glycoprotein complex gcII (gp47-52 complex). J. Virol. **62**:1956–1962.
16. **Gretch, D. R., B. Kari, L. Rasmussen, R. C. Gehrz, and M. F. Stinski.** 1988. Identification and characterization of three distinct families of glycoprotein complexes in the envelopes of human cytomegalovirus. J. Virol. **62**:875–881.
17. **Holland, L. E., R. M. Sandri-Goldin, A. L. Goldin, J. C. Glorioso, and M. Levine.** 1984. Transcriptional and genetic analyses of the herpes simplex virus type 1 genome: coordinates 0.29 to 0.45. J. Virol. **49**:947–959.
18. **Holland, T. C., F. Homa, S. Marlin, M. Levine, and J. Glorioso.** 1984. Herpes simplex virus type 1 glycoprotein C-negative mutants exhibit multiple phenotypes, including secretion of truncated glycoproteins. J. Virol. **52**:566–574.
19. **Ihara, S., K. Hirai, and Y. Watanabe.** 1978. Temperature-sensitive mutants of human cytomegalovirus: isolation and partial characterization of DNA-minus mutants. Virology **84**:218–221.
20. **Isom, H. C.** 1979. Stimulation of ornithine decarboxylase by human cytomegalovirus. J. Gen. Virol. **42**:265–278.
21. **Jefferson, R. A., S. M. Burgess, and D. Hirsh.** 1986. β-Glucuronidase from *Escherichia coli* as a gene fusion marker. Proc. Natl. Acad. Sci. USA **83**:8447–8451.
22. **Jefferson, R. A., M. Klass, N. Wolf, and D. Hirsh.** 1987. Expression of chimeric genes in *Caenorhabditis elegans*. J. Mol. Biol. **193**:41–46.
23. **Jenkins, F. J., M. J. Casadaban, and B. Roizman.** 1985. Application of the mini Mu phage for target sequence specific insertional mutagenesis of the herpes simplex virus genome. Proc. Natl. Acad. Sci. USA **82**:4773–4777.
24. **Jones, T. R.** 1985. Ph.D. thesis. The Pennsylvania State University College of Medicine, Hershey.
25. **Jones, T. R., and V. P. Muzithras.** 1991. Fine mapping of the transcripts expressed from the US6 gene family of human cytomegalovirus strain AD169. J. Virol **65**:2024–2036.
26. **Jones, T. R., and V. P. Muzithras.** Unpublished data.
27. **Kari, B., R. Goertz, and R. Gehrz.** 1990. Characterization of cytomegalovirus glycoproteins in a family of complexes designated gC-II with murine monoclonal antibodies. Arch. Virol. **112**:55–65.
28. **Kemble, G. W., A. L. McCormick, L. Pereira, and E. S. Mocarski.** 1987. A cytomegalovirus protein with properties of herpes simplex virus ICP8: partial purification of the polypeptide and map position of the gene. J. Virol. **61**:3143–3151.
29. **Ligas, M. W., and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62**:1486–1494.
30. **Longnecker, R., S. Chatterjee, R. J. Whitley, and B. Roizman.**

1987. Identification of a herpes simplex virus 1 glycoprotein gene within a gene cluster dispensable for growth in cell culture. Proc. Natl. Acad. Sci. USA **84:**4303–4307.

31. **Longnecker, R., and B. Roizman.** 1986. Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction *a* sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the α47 gene. J. Virol. **58:**583–591.

32. **Longnecker, R., and B. Roizman.** 1987. Clustering of genes dispensable for growth in culture in the S component of the HSV-1 genome. Science **236:**573–576.

33. **Luckow, V. A., and M. D. Summers.** 1989. High level expression of nonfused foreign genes with *Autographa californica* nuclear polyhedrosis virus expression vectors. Virology **170:**31–39.

34. **Manning, W. C., and E. S. Mocarski.** 1988. Insertional mutagenesis of the murine cytomegalovirus genome: one prominent α gene (ie2) is dispensable for growth. Virology **167:**477–484.

34a. **McCarthy, A. M., L. McMahan, and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. J. Virol. **63:**18–27.

35. **McDonough, S. H., S. I. Staprans, and D. H. Spector.** 1985. Analysis of the major transcripts encoded by the long repeat of human cytomegalovirus strain AD169. J. Virol. **53:**711–718.

36. **McKnight, S. L.** 1980. The nucleotide sequence and transcript map of the herpes simplex virus thymidine kinase gene. Nucleic Acids Res. **8:**5949–5964.

37. **Oram, J. D., R. G. Downing, A. Akrigg, A. A. Dollery, C. J. Duggleby, G. W. G. Wilkinson, and P. J. Greenaway.** 1982. Use of recombinant plasmids to investigate the structure of the human cytomegalovirus genome. J. Gen. Virol. **59:**111–129.

38. **Paigen, K.** 1979. Genetic control of acid hydrolases. Annu. Rev. Genet. **13:**417–466.

39. **Parris, D. S., R. A. F. Dixon, and P. A. Schaffer.** 1980. Physical mapping of herpes simplex virus type 1 *ts* mutants by marker rescue: correlation of the physical and genetic maps. Virology **100:**275–287.

40. **Post, L. E., S. Mackem, and B. Roizman.** 1981. Regulation of α genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with α gene promoters. Cell **24:**555–565.

41. **Post, L. E., and B. Roizman.** 1981. A generalized technique for deletion of specific genes in large genomes: α gene 22 of herpes simplex virus 1 is not essential for growth. Cell **25:**227–232.

42. **Radsak, K., C. J. Kaiser, D. Haustein, and F. Rapp.** 1988. Polypeptide synthesis in human fibroblasts infected with DNA-negative mutants of human cytomegalovirus. Intervirology **29:**101–107.

43. **Rapp, F.** 1983. The biology of cytomegaloviruses, p. 9. *In* B. Roizman (ed.), The herpesviruses, vol. 2. Plenum Press, New York.

44. **Rosenberg, A. H., B. N. Lade, D. Chui, S. Lin, J. J. Dunn, and F. W. Studier.** 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene **56:**125–135.

45. **Sacks, W. R., C. C. Greene, D. P. Aschman, and P. A. Schaffer.** 1985. Herpes simplex virus type 1 ICP27 is an essential regulatory gene. J. Virol. **55:**796–805.

46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

47. **Santomenna, L. D., and A. M. Colberg-Poley.** 1990. Induction of cellular *hsp70* expression by human cytomegalovirus. J. Virol. **64:**2033–2040.

48. **Schaffer, P. A., G. M. Aron, N. Biswal, and M. Benyesh-Melnick.** 1973. Temperature-sensitive mutants of herpes simplex virus type 1: isolation, complementation, and partial characterization. Virology **52:**57–71.

49. **Schranz, P., H. Neidhardt, C. H. Schroder, and H. C. Kaerner.** 1989. A viable HSV-1 mutant deleted in two nonessential major glycoproteins. Virology **170:**273–276.

50. **Sears, A. E., I. W. Halliburton, B. Meignier, S. Silver, and B. Roizman.** 1985. Herpes simplex virus 1 mutant deleted in the α22 gene: growth and expression in permissive and restrictive cells and establishment of latency in mice. J. Virol. **55:**338–346.

51. **Sharp, J. A., M. J. Wagner, and W. C. Summers.** 1983. Transcription of herpes simplex virus in vivo: overlap of a late promoter with the 3' end of the early thymidine kinase gene. J. Virol. **45:**10–17.

52. **Smiley, J. R.** 1980. Construction in vitro and rescue of a thymidine kinase deficient deletion mutation of herpes simplex virus. Nature (London) **285:**333–335.

53. **Smith, J. D., and E. De Harven.** 1973. Herpes simplex virus and human cytomegalovirus replication in WI-38 cells. I. Sequence of viral replication. J. Virol. **12:**919–930.

54. **Spaete, R. R., and E. S. Mocarski.** 1985. The *a* sequence of the cytomegalovirus genome functions as a cleavage/packaging signal for herpes simplex virus defective genomes. J. Virol. **54:**817–824.

55. **Spaete, R. R., and E. S. Mocarski.** 1987. Insertion and deletion mutagenesis of the human cytomegalovirus genome. Proc. Natl. Acad. Sci. USA **84:**7213–7217.

56. **Stenberg, R. M., A. S. Depto, J. Fortney, and J. A. Nelson.** 1989. Regulated expression of early and late RNAs and proteins from the human cytomegalovirus immediate-early region. J. Virol. **63:**2699–2708.

57. **Stinski, M. F.** 1976. Human cytomegalovirus: glycoproteins associated with virions and dense bodies. J. Virol. **19:**594–604.

58. **Stinski, M. F.** 1978. Sequence of protein synthesis in cells infected by human cytomegalovirus: early and late virus-induced polypeptides. J. Virol. **26:**686–701.

59. **Stinski, M. F.** 1990. Cytomegalovirus and its replication, p. 1959–1980. *In* D. M. Knipe and B. N. Fields (ed.), Virology, 2nd ed. Raven Press, New York.

60. **Stow, E. C., and N. D. Stow.** 1989. Complementation of a herpes simplex virus type 1 Vmw110 deletion mutant by human cytomegalovirus. J. Gen. Virol. **70:**695–704.

61. **Weber, P. C., M. Levine, and J. C. Glorioso.** 1987. Rapid identification of nonessential genes of herpes simplex virus type 1 by Tn5 mutagenesis. Science **236:**576–579.

62. **Weller, S. K., D. P. Aschman, W. R. Sacks, D. M. Coen, and P. A. Schaffer.** 1983. Genetic analysis of temperature-sensitive mutants of HSV-1: the combined use of complementation and physical mapping for cistron assignment. Virology **130:**290–305.

63. **Weller, S. K., K. J. Lee, D. J. Sabourin, and P. A. Schaffer.** 1983. Genetic analysis of temperature-sensitive mutants which define the gene for the major herpes simplex virus type 1 DNA-binding protein. J. Virol. **45:**354–366.

64. **Weston, K., and B. G. Barrell.** 1986. Sequence of the short unique region, short repeats, and part of the long repeats of human cytomegalovirus. J. Mol. Biol. **192:**177–208.

65. **Yamanishi, K., and F. Rapp.** 1977. Temperature-sensitive mutants of human cytomegalovirus. J. Virol. **24:**416–418.

66. **Yamanishi, K., and F. Rapp.** 1979. Induction of host DNA synthesis and DNA polymerase by DNA-negative temperature-sensitive mutants of human cytomegalovirus. Virology **94:**237–241.