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A Cluster of Dispensable Genes within the Human Cytomegalovirus Genome Short Component: IRS1, US1 through US5, and the US6 Family

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By insertional mutagenesis, human cytomegalovirus recombinants deleted of each of the US6 glycoprotein family genes were isolated. A recombinant lacking IRS1, US1 through US5, and most of the US6 family was also isolated. The growth kinetics of these mutants were similar to that of the wild type. A dispensable cluster of genes was identified.

The genome of human cytomegalovirus is organized into long (L) and short (S) components (24). Each component contains unique-sequence DNA (UL and US) bounded by terminal repeats and internal inverted repeats (TR_S and IR_S, respectively, for those flanking US). The 35-kb US region of human cytomegalovirus (HCMV) is composed of several gene families (2, 27). Although the function(s) of these genes are unknown, several families may encode membrane glycoproteins or multiply hydrophobic transmembrane proteins, including potential G protein-coupled receptor homologs (3). The US6 family contains six open reading frames (US6 through US11) whose products may contribute to the heterogeneous abundant virion glycoprotein complex called gcII (6–8, 12, 13). At the RNA level, expression of members of the US6 family is differentially regulated at either early or late times postinfection (9). By insertional mutagenesis using the β-glucuronidase marker gene, we recently reported the isolation and characterization of recombinant HCMV mutants which are deleted of the US10 and/or US11 genes (11). These mutants grew in tissue-cultured human foreskin fibroblast cells with the same kinetics as did parental wild-type HCMV (strain AD169).

To complete our functional assessment of this gene family in the HCMV infectious cycle, similar β-glucuronidase-expressing viral recombinants which lack one or more of the remaining US6 gene family members have been constructed (US6 through US9; Fig. 1). The recombinant viruses were constructed by cotransfection of wild-type HCMV strain AD169 DNA with the appropriate linearized plasmid (Fig. 1), using a procedure which was detailed previously (11). Mutants with replacements of US9 (RV61), US9-US8 (RV80), US7 (RV725), and US6 (RV69) were purified. DNA blot analysis of plaque-purified recombinant virus DNA (11) verified the anticipated homologous recombination of the plasmids within the HCMV genome (Fig. 2). Although data from blots of *Hin*dIII- and *Hin*dIII-*Xho*I-digested viral DNA are shown, blots of *Xho*I- and *Eco*RI-digested viral DNA were also done (data not shown), and the results were consistent with the results stated below. The expected restriction fragments are indicated in Fig. 1 for each viral recombinant. The β-glucuronidase probe hybridized to the proper genomic DNA restriction fragments from each of the recombinants and did not hybridize to wild-type DNA (Fig. 2A). In *Hin*dIII digests, the XP probe (which contains sequences from portions of the *Hin*dIII X and V DNA fragments; Fig. 1) hybridized to the 5.4-kb *Hin*dIII V fragment in all recombinants as well as to the fragments which hybridized to the β-glucuronidase probe (Fig. 2B). This probe hybridized to the same fragments in *Hin*dIII-*Xho*I-digested mutant and wild-type DNAs. The PP probe (which contains sequences from the *Hin*dIII Q and X DNA fragments; Fig. 1) hybridized to the same fragment as did the β-glucuronidase probe as well as to the 2.8-kb *Hin*dIII-*Xho*I fragment from *Hin*dIII-Q in *Hin*dIII-*Xho*I-digested DNAs (Fig. 2C). An exception to the latter was RV69, in which the *Hin*dIII site at the Q/X junction was deleted as a result of the recombination. In this case, the PP probe hybridized to the predicted 7.1-kb *Xho*I-*Xho*I fragment, as did the β-glucuronidase probe. In *Hin*dIII-digested recombinant and wild-type virus DNAs, the PP probe hybridized to joint and terminal fragments and to the fragments which hybridized with the β-glucuronidase probe (Fig. 2C). The mutants' joint and terminal region fragments displayed heterogeneity that is typical of the corresponding fragments from the wild-type virus (25). The results of these DNA hybridizations, as well as those using other probes from the US, UL, and joint regions of the genome (data not shown), established that the predicted homologous recombination events had occurred and that no other alterations of the recombinant viral genomes were detected.

Plasmid pBgdUS11 (Fig. 3B) was used to create RV699 (11). While examining viral recombinants which resulted from a transfection involving pBgdUS11, we identified a mutant, called RV670, which had an unanticipated extensive deletion. In the creation of a β-glucuronidase-expressing recombinant mutant, two recombination events must occur: one on each side of the plasmid-borne marker gene with sequences in the HCMV genome. DNA blot hybridization studies indicated that one such event in the creation of RV670 was the expected homologous recombination between the plasmid and viral DNA within sequences upstream of US11. This conclusion was indicated since both the β-glucuronidase and XP probes hybridized to the proper-sized 8.2-kb fragment (Fig. 3B) in *Xho*I-digested RV670 DNA (Fig. 4A and B). In addition, the XP probe hybridized to the unaltered 5.4-kb *Hin*dIII V fragment in RV670, as well as in wild-type, viral DNA (Fig. 4B). However, the β-glucuronidase and XP probes unexpectedly hybridized with two joint region fragments (C′ and G′) and an approximately

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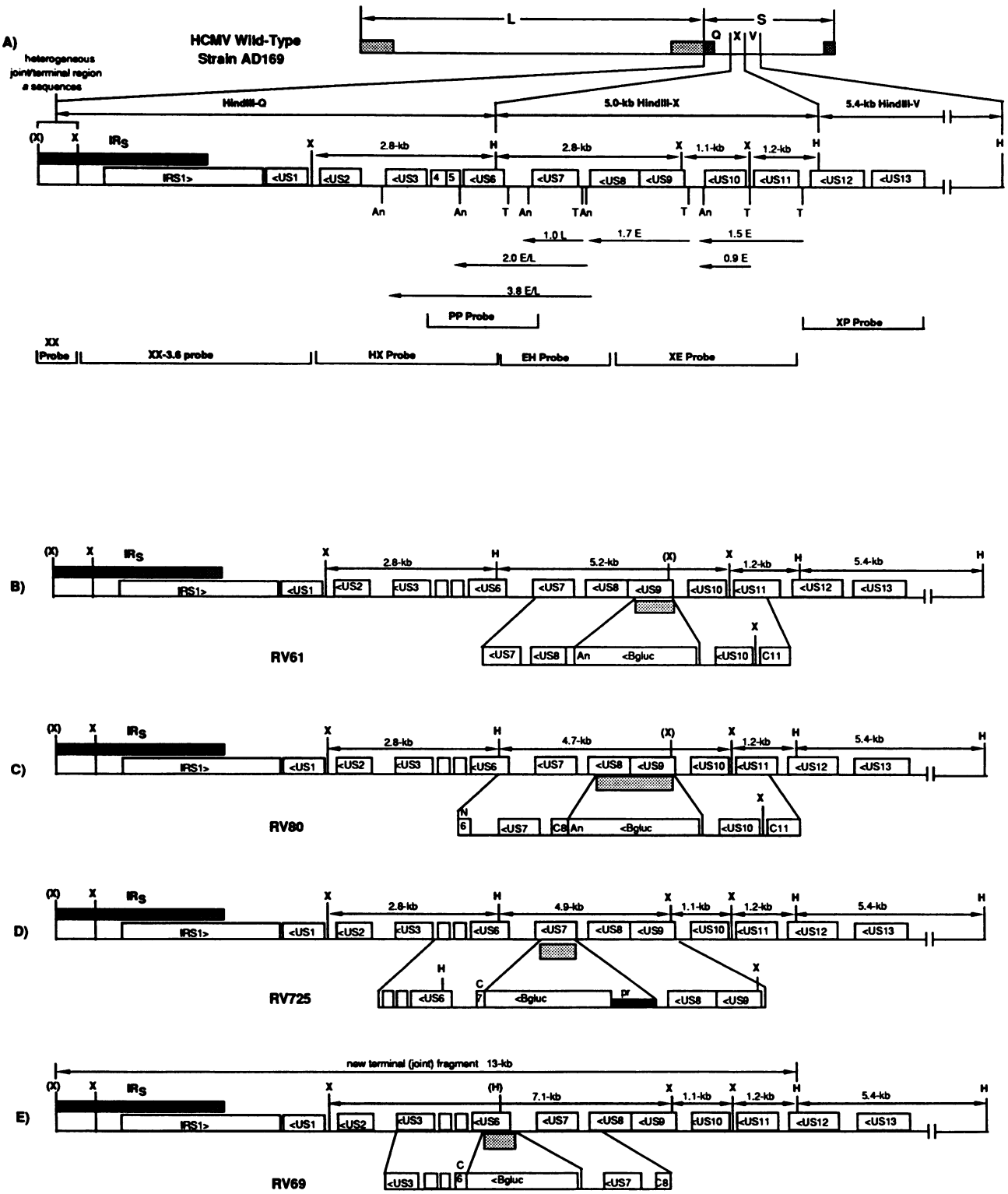

FIG. 1. Organization of recombinant virus genomes. (A) The first line is a schematic of the overall organization of the HCMV wild-type genome. Unique region sequences are shown by a line, while repeated region sequences are indicated by shaded boxes. Relevant *Hin*dIII fragments, within the L and S components, are indicated by letter designation (20). The second line is an expansion of the wild-type *Hin*dIII-Q, -X, and -V regions of the S component. The significant open reading frames, and their orientations, are shown as open boxes (2). The position of the IR_S repeated sequence is indicated by the shaded rectangle. The locations of *Hin*dIII (H) and *Xho*I (X) restriction endonuclease sites are shown. Some of the major transcripts from this region, which were mapped previously (9), are indicated, along with their kinetic class (E, early; L, late). The locations of the hybridization probes used for the DNA blot analyses of Fig. 2 (PP and XP) are indicated. The locations of probes from this region of the viral genome used for other DNA blot hybridization analyses (not shown) of these recombinants are given. The sequences (according to the numbering system of Weston and Barrell [27]) contained within the HCMV-derived

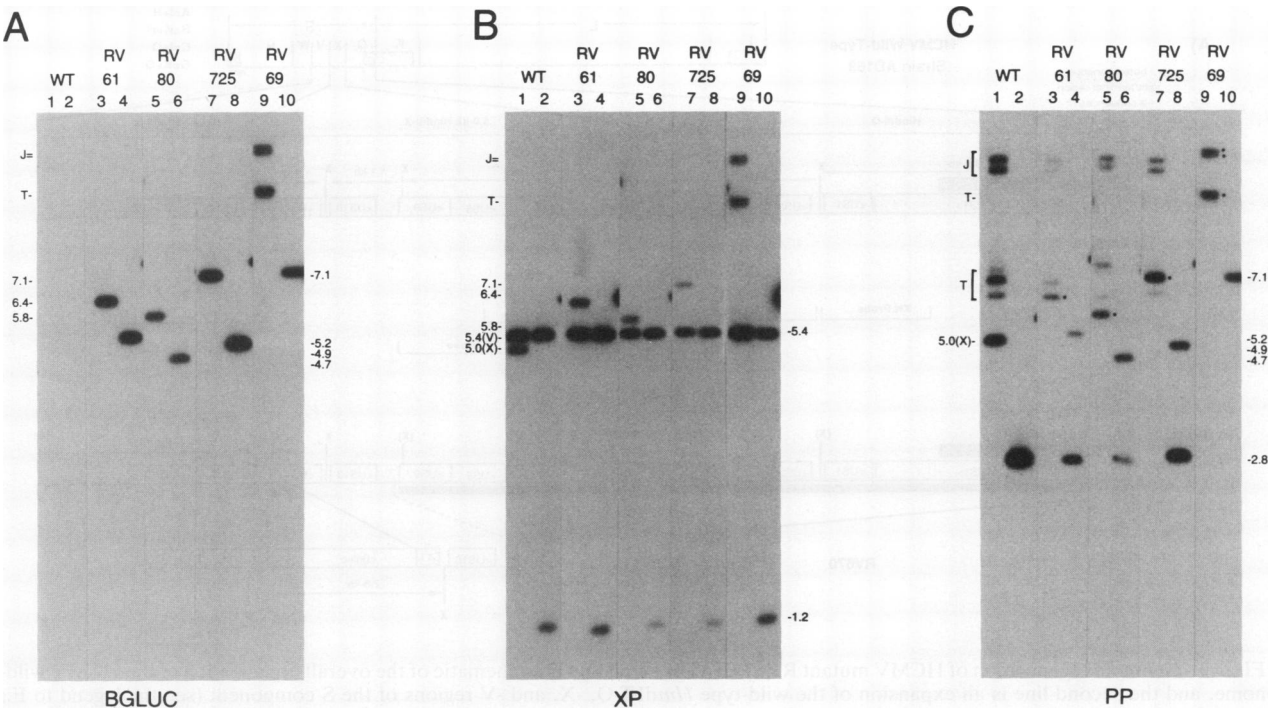

FIG. 2. Blot analysis of recombinant mutant genomic DNA. Either wild-type (WT) HCMV or the indicated recombinant virus (RV) DNA was digested with *Hin*dIII (odd-numbered lanes) or *Hin*dIII and *Xho*I (even-numbered lanes), electrophoresed and blotted (11), and hybridized with a β-glucuronidase probe (A), the XP probe (B), or the PP probe (C). The regions of the HCMV genome from which the XP and PP probes were derived are indicated in Fig. 1. The sizes (in kilobases) or designations of fragments hybridizing in *Hin*dIII digests are indicated to the left of each panel; the sizes of fragments hybridizing in *Hin*dIII-*Xho*I digests are indicated to the right. J, joint region fragment; T, terminal region fragment. In panel C, a dot adjacent to a hybridizing fragment in *Hin*dIII digests designates those fragments which also hybridized to the β-glucuronidase probe in panel A.

7.5-kb terminal region fragment (Q′) in *Hin*dIII digests of RV670 DNA (Fig. 4A and B). This finding meant that at least the *Hin*dIII site at the junction of the *Hin*dIII Q and X fragments (Fig. 3A) was deleted from this recombinant. To determine the extent of the deletion, blots of *Hin*dIII-digested RV670 DNA were sequentially hybridized with probes representing regions of DNA which normally are downstream of US11 in the wild-type HCMV genome. The results which follow were corroborated by hybridization of these probes to blots of *Hin*dIII-*Xho*I, *Xho*I, and *Eco*RI digests of RV670 DNA (data not shown). Probes XB (specific for US9 and US8), EH (specific for US8 and US7; data not shown), HX (specific for US6 through US2), and XN (specific for US1 and unique region sequences of IRS1) did not hybridize with any RV670 DNA fragments. Also, the AA probe (specific for the IR_S and TR_S repeat sequences found in both the IRS1 and TRS1 genes of the HCMV short component) did not hybridize to the RV670 Q′ (terminal) or C′/G′ (joint) region DNA fragments (Fig. 4F). The AA probe hybridized only with DNA fragments which contained TRS1 sequences (i.e., *Hin*dIII H [terminal] and *Hin*dIII A and B [joint] fragments) in this mutant. In confirmation, the AA

DNA hybridization probes are 3397 to 3950 (XX), 3950 to 7509 (XX-3.6), 7509 to 10343 (HX), 9247 to 10953 (PP), 10343 to 11949 (EH), 11949 to 14897 (XE), and 14897 to 16713 (XP). (B to E) The region of the wild-type genome (first line) deleted by recombination to create the mutant is shown by a shaded rectangle. The second line is the organization of the relevant region of the linearized plasmid used to make the recombinant virus. In each case, the plasmid contains 1.5 to 1.7 kb of viral sequences (derived from either pHind-X or pHind-G [11, 20]) flanking the β-glucuronidase (Bgluc) marker gene to direct homologous recombination (indicated by the slanted lines). The sizes of restriction fragments from each recombinant virus are given. (B) Genomic organization of RV61, deleted of US9. The β-glucuronidase gene was inserted within the 5′ untranslated region of US9 and replaces 0.68 kb downstream from there, including sequences encoding the N-terminal 221 (out of 247 total) amino acids of US9. The endogenous US9 early promoter controls β-glucuronidase gene expression in this construct. (C) Genomic organization of RV80, deleted of US9 and US8. The β-glucuronidase gene was inserted within the 5′ untranslated region of US9 and replaces 1.27 kb downstream from there, including sequences encoding US9 and the N-terminal 171 (out of 235 total) amino acids of US8. The endogenous US9 early promoter controls β-glucuronidase gene expression in this construct. (D) Genomic organization of RV725, deleted of US7. The β-glucuronidase gene, under the control of the HCMV 2.7E promoter (11) (pr; solid rectangle), was inserted after the N-terminal methionine codon of US7 and replaces 0.60 kb downstream from there, including sequences encoding US7 amino acids 2 through 201, out of 225 total amino acids of US7. (E) Genomic organization of RV69, deleted of US6. The β-glucuronidase gene was inserted at the early/late transcription initiation site of US6 (9) and replaces 0.38 kb downstream from there, including sequences encoding the N-terminal 120 (out of 183 total) amino acids of US6. The endogenous US6 early promoter controls β-glucuronidase gene expression in this construct. The approximately 13-kb new terminal fragment (after digestion with *Hin*dIII) created as a result of the recombination which deleted the *Hin*dIII site at the *Hin*dIII-Q/X junction is indicated. An, polyadenylation signal; T, transcription initiation site.

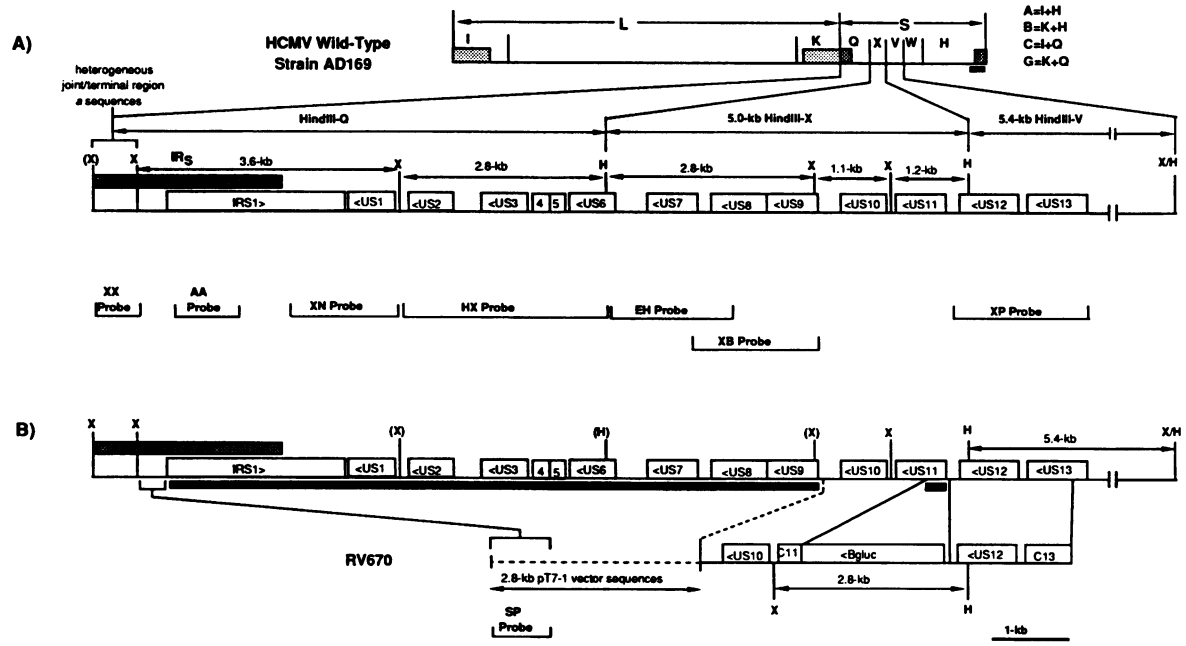

FIG. 3. Genomic organization of HCMV mutant RV670. (A) The first line is a schematic of the overall organization of the HCMV wild-type genome, and the second line is an expansion of the wild-type *Hin*dIII-Q, -X, and -V regions of the S component (see the legend to Fig. 1). The solid rectangle beneath the *Hin*dIII H fragment is the position of the 6.1-kb *Xho*I DNA fragment which contains TRS1, an open reading frame which spans the TR_S/US junction (2). Locations of the hybridization probes for the DNA blot analyses shown in Fig. 4 are indicated. The XX, HX, EH, and XP probes are described in the legend to Fig. 1. The sequences contained within the other HCMV-derived DNA hybridization probes are 4252 to 5390 (AA), 6336 to 7509 (XN), and 11548 to 13127 (XB). (B) The first line is the organization of the wild-type genome; the second line is a representation of the linearized plasmid, pBgdUS11 (11), which was used for recombination to create the RV670 mutant. The prokaryotic vector sequences (a pT7-1 backbone [U.S. Biochemical]) are shown as a dashed line. The position of the vector SP probe is indicated. In the creation of RV670, homologous recombination between the plasmid and genomic DNA occurred in the sequence of US12 or US13. The other recombination event was nonhomologous: between the distal end of the vector sequences and the region between the genomic *a* sequences and IRS1, as indicated by the bracketed regions connected by the line. The solid rectangles below the first line indicate the genomic sequences which were deleted as a result of the events which led to the creation of RV670. Bgluc, β-glucuronidase.

probe hybridized with the 6.1-kb *Xho*I fragment derived from the unaltered TRS1 end of the short component of the RV670 genome and not to the 3.6-kb *Xho*I fragment expected if the IRS1 end of the short component was also unaltered (Fig. 4G). However, a plasmid vector probe (SP; representing the distal end of the linearized plasmid) hybridized with the altered terminal and joint region DNA fragments of RV670, Q′ and C′/G′, respectively (Fig. 4H). These data suggested that the second recombination event in the creation of RV670 was nonhomologous, between the plasmid vector sequences and sequences within the HCMV IR_S, as indicated by the brackets in Fig. 3B. In addition to the replacement of US11, this resulted in the deletion of a 9-kb contiguous portion of the HCMV genome (IRS1 and US1 through US9) being replaced by prokaryotic vector sequences in RV670. On the basis that the vector distal (SP) probe hybridized, it was inferred that most of the 2.7 kb of vector sequences from pBgdUS11 were retained in the recombinant after the nonhomologous recombination event. The result is a net reduction in size of the short component by >6 kb. Since the 0.55-kb XX probe (specific for the *a*-like repeated sequences [25]) hybridized to all joint and terminal fragments in RV670 DNA (as well as in wild-type DNA; Fig. 4I), the HCMV *a*-like sequences were present on the altered S-component terminus containing the prokaryotic sequences. Thus, the HCMV mutant RV670 genome is deleted of most (at least 1.7 kb of a total of 2.0 kb) non-*a*-repeat sequences from one end of the short component but not the other. This altered short component still inverts, suggesting that the bulk of the non-*a*-repeat sequences of HCMV IR_S/TR_S are not required for short-component inversion. Accordingly, in herpes simplex virus (HSV), the *a*-repeat sequences were implicated to play an important role in component inversion (4, 16–19). The HCMV *a*-repeat sequences bear some sequence and functional resemblance to the HSV *a*-repeat sequences (22, 25).

To determine whether deletion of any of these open reading frames had an effect on the viral infectious cycle, single-cycle growth analyses were done. As shown in Fig. 5, the growth kinetics of each of the mutants was similar to that of wild-type virus (21), indicating that the deleted gene(s) was not required or beneficial for growth of the virus in the human fibroblast tissue culture system. In all cases, significant amounts of progeny virus were not detected until 3 days postinfection, and all accumulated to high titers by 5 days postinfection.

Throughout the course of infection of permissive human fibroblasts with HCMV, characteristic morphological alterations in cell size and shape occur (reviewed in reference 1). By 6 to 24 h postinfection, the cells contract to a rounded form (early rounding); this is followed by cell relaxation and enlargement (cytomegaly) at late times postinfection. Microscopic examination of cells infected with mutants RV61, RV80, RV725, and RV69 all displayed this progression of morphological alterations. In contrast, cells infected with mutant RV670 did not show typical early rounding, although

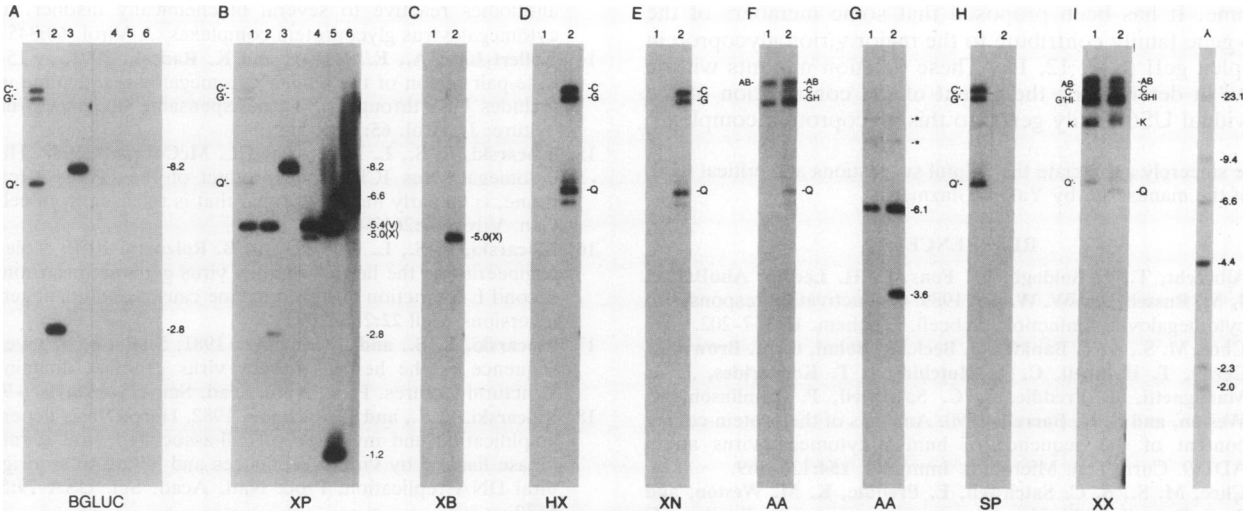

FIG. 4. Blot analysis of RV670 mutant genomic DNA. (A and B) RV670 DNA (lanes 1 to 3) or AD169 wild-type DNA (lanes 4 to 6) was digested with *Hin*dIII (lanes 1 and 4), *Hin*dIII-*Xho*I (lanes 2 and 5), or *Xho*I (lanes 3 and 6), electrophoresed and blotted, and hybridized with the β-glucuronidase probe (A) or XP probe (B). (C to F, H, and I) RV670 DNA (lanes 1) or AD169 wild-type DNA (lanes 2) was digested with *Hin*dIII, electrophoresed and blotted, and hybridized to the XB probe (C), HX probe (D), XN probe (E), AA probe (F), SP probe (H), or XX probe (I). (G) RV670 DNA (lane 1) or AD169 wild-type DNA (lane 2) was digested with *Xho*I, electrophoresed and blotted, and hybridized to the AA probe. Hybridizing fragments are indicated by size (in kilobases) or by conventional letter designation, as shown in Fig. 3. The asterisks indicate the positions of the 13.5- and 10.6-kb *Xho*I fragments from TR_L and IR_L, respectively, present in digests of both AD169 wild-type and RV670 mutant DNAs which weakly hybridized with the IR_S-derived AA probe (2). Lane λ shows the location of *Hin*dIII-digested, radiolabelled lambda bacteriophage molecular weight marker DNA.

at late times postinfection cytomegaly was observed (not shown). Investigation of which gene(s) is responsible for the phenotype is in progress. We recently isolated a mutant HCMV, RV35, in which all of the US6 family genes were simultaneously deleted (10). Unlike RV670, RV35 induces typical early rounding of infected fibroblasts.

Recently, Kollert-Jons et al. (14) reported that the viral genome of the HCMV temperature-sensitive mutant *ts*9 (28) had a rearranged US region, including the duplication of some genes and the apparent deletion of open reading frames US1 through US13. With use of a directed approach involving the construction of many β-glucuronidase marker-expressing recombinant insertion-deletion mutants, our data are in agreement with theirs. In addition to recombinants reported in this and our previous article (11), mutants with deletions of US12 and US13 have also been isolated in this laboratory (10). However, as result of our analysis of RV670, we add IRS1 to this dispensable cluster of open reading frames in the S component of the HCMV genome. In contrast to *ts*9, the RV670 genome did not contain duplications of S-component unique genes. Also, there was a decrease in S-component repetitive sequences and an overall reduction in the size of S, unlike the case for *ts*9 (14).

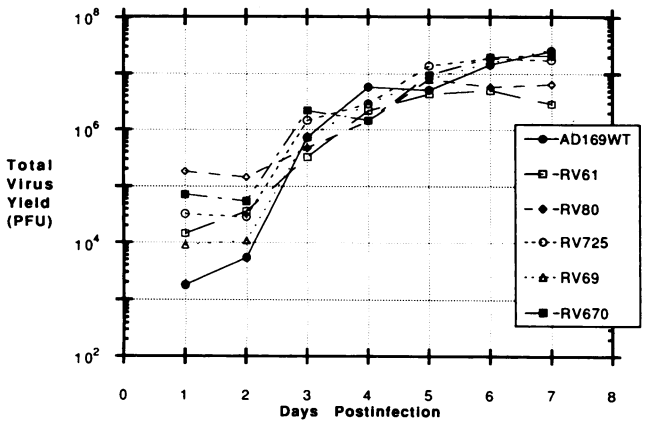

FIG. 5. Single-cycle growth analysis of HCMV recombinant mutants. Human foreskin fibroblast cells were infected with the indicated recombinant mutant at a multiplicity of 2. After a 2-h adsorption period at 37°C, the monolayers were washed with phosphate-buffered saline and fresh medium was added. Total (intracellular plus extracellular) virus was harvested daily, and titers were determined as described previously (11).

IRS1 is a member of the US22 gene family, composed of 13 open reading frames (2). One member of this family encodes ICP22, a protein which has been found in the nucleus, cytoplasm, and medium of infected cells (15). The IRS1 and TRS1 gene products are highly homologous, since the N-terminal 529 amino acids of each are encoded from the IR_S/TR_S repeat sequences (27). Deletion of one of these genes may not be detrimental, since their respective products may have common functions or targets. Accordingly, both IRS1 and TRS1 proteins have transcriptional transactivating activities and may cooperate with IE1 and IE2 proteins to activate the ICP36 promoter (23). The products of the US2 and US3 open reading frames have been proposed to be related glycoproteins. US3 RNA is expressed at immediate-early times and is differentially spliced at late times postinfection (26). A US3 gene product was reported to function in conjunction with the products of other HCMV immediate-early genes as a transcriptional transactivator of the cellular heat shock protein 70 promoter (5). We conclude that the US6 family of genes, as well as its joint proximal neighbors (US1 through US5) and IRS1, are nonessential for HCMV replication in tissue-cultured human foreskin fibroblasts and can be simultaneously deleted from the viral

genome. It has been proposed that some members of the US6 gene family contribute to the major virion glycoprotein complex gcII (6, 7, 12, 13). These deletion mutants will be useful in determining the extent of the contribution of the individual US6 family genes to this glycoprotein complex.

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