JOURNAL OF VIROLOGY, Dec. 1992, p. 7146–7152
0022-538X/92/127146-07$02.00/0

Vol. 66, No. 12

Point Mutations in the DNA Polymerase Gene of Human Cytomegalovirus That Result in Resistance to Antiviral Agents

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Received 22 June 1992/Accepted 31 August 1992

Three independently isolated mutants of human cytomegalovirus strain AD169 were found to be resistant to ganciclovir at a 50% effective dose of 200 μM. Phosphorylation of ganciclovir was reduced 10-fold in mutant-infected cells compared with AD169-infected cells. All three mutants were also determined to be resistant to the nucleotide analogs (*S*)-1-[(3-hydroxy-2-phosphonylmethoxy)propyl]adenine (HPMPA) and (*S*)-1-[(3-hydroxy-2-phosphonylmethoxy)propyl]cytosine (HPMPC) and hypersensitive to thymine-1-D-arabinofuranoside (AraT). Single base changes resulting in amino acid substitutions were demonstrated in the nucleotide sequence of the DNA polymerase gene of each mutant. The polymerase mutation contained in one of the mutants was transferred to the wild-type AD169 background. Ganciclovir phosphorylation in cells infected with the recombinant virus produced by this transfer was found to be equivalent to that of AD169-infected cells. The ganciclovir resistance of the recombinant was reduced fourfold compared with that of the parental mutant; however, the recombinant remained resistant to HPMPA and HPMPC and hypersensitive to AraT. The ganciclovir resistance of the mutants therefore appears to result from mutations in two genes: (i) a kinase which phosphorylates ganciclovir and (ii) the viral DNA polymerase.

Human cytomegalovirus (HCMV) is a member of the herpesvirus family. Primary HCMV infections are generally mild or asymptomatic except for congenital infections, which often have serious sequelae. A hallmark of herpesvirus infections is the establishment of latency following a primary infection. Reactivation of latent infection has increasingly become a factor contributing to the morbidity and mortality associated with AIDS and organ transplantation (9, 11). Ganciclovir (GCV) and phosphonoformic acid (PFA; foscarnet) are presently the only approved drugs for the treatment of HCMV disease, but isolates resistant to these drugs have been reported (2, 10, 18, 30, 31).

The viral DNA polymerase is the target of many of the antiviral agents which have been found to be effective inhibitors of HCMV replication. These antiviral agents fall into two groups. The first group, which includes GCV, acyclovir (ACV), and thymine-1-D-arabinofuranoside (AraT), requires monophosphorylation by a virus-encoded enzyme. Di- and triphosphorylation of GCV monophosphate are believed to be carried out by cellular enzymes (2, 23, 29). The triphosphate derivative is the active form which is the substrate for the viral DNA polymerase. Resistance to these drugs, therefore, can result from mutations in either of two viral genes: (i) the kinase that monophosphorylates the drugs (22, 29, 32), or (ii) the DNA polymerase (5, 12, 13).

The second group of antiviral agents includes the nucleoside monophosphate analogs (*S*)-1-[(3-hydroxy-2-phosphonylmethoxy)propyl]adenine (HPMPA) and (*S*)-1-[(3-hydroxy-2-phosphonylmethoxy)propyl]cytosine (HPMPC) and the pyrophosphate analogs PFA and phosphonoacetic acid (PAA). These agents do not require activation by a virus-encoded product to become substrates for the viral DNA polymerase, and therefore, resistance to these compounds is believed to result directly from mutations in the DNA polymerase gene (26, 28).

Aside from their clinical relevance, mutations conferring drug resistance can be important tools for mapping viral genes and determining the functions of the gene products. An extensive amount of work has been done on the mechanisms of antiviral resistance of herpes simplex virus (HSV). Many drug-resistant mutants have been used to study the viral DNA polymerase and thymidine kinase genes of HSV, and most of the mutations have been mapped to specific nucleotides (5, 8, 12, 13, 16, 21). In contrast to HSV, much less is known about mechanisms of resistance in HCMV. The drug-resistant mutants of HCMV that have been reported thus far appear to have mutations in the DNA polymerase gene (7, 31) or in the viral kinase (2, 30). Evidence for altered viral functions has been based primarily on the pattern of resistance to antiviral agents and reduced intracellular drug anabolism.

We have isolated three GCV-resistant mutants of HCMV strain AD169 which are also resistant to HPMPC and HPMPA. We have characterized these mutants by their susceptibilities to other antiviral agents, their intracellular GCV anabolic activities, and the nucleotide sequences of their DNA polymerase genes.

(Parts of this work were presented in preliminary form at the XVth International Herpesvirus Workshop, Washington, D.C., 1990, and the 31st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Ill., 1991.)

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MATERIALS AND METHODS

Cells and virus. Human foreskin fibroblasts (HFF) at passage 5 to 7 were purchased from Bartels, Bellevue, Wash., and propagated in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 50 μg of gentamicin per ml, and 2.5 μg of amphotericin B per ml (growth medium). Confluent monolayers were maintained in Eagle's MEM containing the same supplements except that the concentration of fetal calf serum was reduced to 1% (maintenance medium). HCMV strain AD169 is the GCV-sensitive wild-type virus. D1/3/4, D6/3/1, and D10/3/2 are GCV-resistant mutants derived from AD169. HP/A1B/4 is a recombinant resulting from transfer of the D1/3/4 DNA polymerase mutation to AD169.

Antiviral agents. GCV was obtained as a gift from Syntex, Palo Alto, Calif. HPMPA and HPMPC were gifts from Bristol-Myers Squibb, Wallingford, Conn. AraT and PFA were purchased from Sigma, St. Louis, Mo. ACV was purchased from Burroughs-Wellcome, Research Triangle Park, N.C.

Antiviral susceptibility measured by plaque reduction assay. All monolayers in a 24-well plate were inoculated with a single dilution of virus calculated to produce 30 to 40 plaques in the absence of drug. Four control wells were overlaid with maintenance medium containing 0.3% agarose and no drug. The remaining wells were overlaid with medium containing 0.3% agarose and a range of drug concentrations. Quadruplicate wells were exposed to each drug concentration. The 50% effective dose (ED_{50}) was determined as the micromolar concentration of each antiviral agent which reduced the number of plaques by 50% or greater relative to the number of plaques produced in the control wells. The assay results were read at 10 to 14 days postinfection.

Antiviral susceptibility measured by ELISA. Monolayers in 96-well microtiter plates were inoculated with wild-type or mutant strains of HCMV at a single dilution calculated to produce 50% (2+) cytopathic effect in 5 to 7 days. A set of six cell control wells were mock infected with maintenance medium alone. The inoculum was replaced with medium containing dilutions of the drug (six wells per dilution). Medium without drug was added to the cell control wells as well as one set of inoculated wells which served as the virus control. HCMV late antigen was detected by enzyme-linked immunosorbent assay (ELISA) as described previously (33). The ED_{50} is the micromolar concentration of each antiviral agent which reduced the absorbance of colored substrate product by 50% or greater.

HPLC analysis of GCV anabolism in virus-infected cells. HFF monolayers in 150-cm^2 flasks were inoculated at a multiplicity of infection of 1.0. One flask was mock infected to serve as an uninfected-cell control. At either 72 or 96 h postinfection, 25 μCi of [^{3}H]GCV (a gift from Syntex) was added to each flask.

After 24 h of incubation, the monolayers were trypsinized. Cells were washed three times with Dulbecco's phosphate-buffered saline (PBS). After the final wash, the cells were resuspended in PBS and extracted with 0.5 M perchloric acid. The extracts were centrifuged, and the supernatant was collected for high-pressure liquid chromatography (HPLC) analysis.

GCV and GCV monophosphate (a gift from Syntex) at concentrations of 100 mM were used as standards for HPLC analysis of the products of infected-cell GCV anabolism. HPLC was performed on a Partisil 10 ODS3 reverse-phase column (250 by 4.6 mm; Phenomenex, Rancho Palos Verdes, Calif.) with 0.02 M KH_2PO_4 (pH 3.65) as the mobile phase. One-hundred-microliter samples of the perchloric acid extracts were injected onto the column. Fractions were collected at 0.3-min intervals for 15 min. The radioactivity of each fraction was measured by scintillation counting.

DNA sequencing. Plasmid clones of the HCMV DNA polymerase genes were identified from genomic libraries of each of the three GCV-resistant mutants D1/3/4, D6/3/1, and D10/3/2, the recombinant HP/A1B/4, and the wild-type AD169 strain from which the mutants were derived. These plasmids were purified on CsCl gradients. For sequencing, 5 to 7 μg of double-stranded plasmid DNA was denatured in 200 mM NaOH–0.2 mM EDTA at room temperature for 5 min, neutralized with 200 mM ammonium acetate (pH 4.6), and ethanol precipitated. The denatured DNA was used as the template for sequence analysis by the Sequenase version 2.0 protocol (United States Biochemical, Cleveland, Ohio).

Transfections. Monolayers of HFF in 60-mm dishes were infected with wild-type AD169 virus at a multiplicity of infection of 0.5. The virus was allowed to adsorb for 3 h. Plasmid DNA (40 μg) carrying the DNA polymerase gene derived from a GCV-resistant mutant was mixed with 1 ml of growth medium and 10 μl of hexadimethrine bromide (Sigma) (27). The DNA solution was placed on the infected cells for 6 h. The cells were washed with Earle's balanced salt solution and treated with 30% dimethyl sulfoxide for 4 min. The dimethyl sulfoxide was removed; the cells were washed twice with Earle's balanced salt solution, and growth medium was added. After 72 h, the remaining viable cells were harvested and used to infect new HFF monolayers in six-well plates. HPMPA at a concentration of 1 μM was added to the maintenance medium for selection of recombinant virus. The supernatant fluid was harvested after 14 days and used to inoculate HFF monolayers in 24-well plates. The monolayers were overlaid with 0.3% agarose containing maintenance medium and 1.5 μM HPMPA. Titers were determined in the presence and absence of drug. Isolated plaques appearing in wells with drug were selected and passed to tubes of HFF cells, with continued selection in medium containing 1.5 μM HPMPA. Supernatant virus from the tube cultures was plaque purified again in the presence of HPMPA. Virus samples from isolated plaques were then propagated without selection, plaque purified, and maintained frozen at −70°C for further analysis.

RESULTS

Isolation of GCV-resistant mutants. The GCV-resistant mutants were isolated by exposure of the wild-type stock to increasing concentrations of GCV. Ten tube monolayers were inoculated with AD169 and maintained in medium containing 5 μM GCV. Each tube monolayer was passed separately and exposed to increasing concentrations of GCV in 10 μM increments up to 25 μM. Tube isolates which showed 100% cytopathic effect at 25 μM were tested at 50 and 100 μM GCV. Three of these tube isolates showed 100% cytopathic effect in the presence of 100 μM GCV. Each of these isolates was plaque purified three times. The plaque-purified strains have been designated D1/3/4, D6/3/1, and D10/3/2.

Initially, attempts were made to isolate GCV-resistant mutants from the wild-type AD169 stock by direct selection in the presence of 5, 10, or 25 μM GCV. Samples from the few plaques which appeared were retested for resistance to GCV. In all cases, no increased resistance relative to the parental wild-type AD169 could be detected even after

TABLE 1. Susceptibility testing results for HCMV strains[a]

Assay and virus	ED_{50} (μM)					
	GCV	ACV	PFA	AraT	HPMPA	HPMPC
Plaque reduction assay						
AD169	10	150	250	4,000	1.0	1.0
D6/3/1	200	150	300	200	4	8
D1/3/4	200	150	300	100	4	8
D10/3/2	200	150	300	<100	4	8
HP/A1B/4	50	100	300	400	4	8
In situ ELISA						
AD169	6.25	200	200	4,000	1.0	1.0
D6/3/1	200	100	200	200	5	10
D1/3/4	200	150	200	200	5	10
D10/3/2	200	150	150	100	10	15
HP/A1B/4	50	100	300	200	5	15

[a] Both the plaque reduction assay and the in situ ELISA were repeated two or more times, and the data presented are derived from a representative assay. Sensitive and resistant strains were always assayed simultaneously for comparison.

several rounds of plaque purification. Similar results have been reported by others (2, 31).

Antiviral susceptibility testing. The antiviral susceptibility profiles of mutants D1/3/4, D6/3/1, and D10/3/2 and the wild-type AD169 were determined by the plaque reduction assay (Table 1). These mutants were 10-fold more resistant to GCV than wild-type AD169. The mutants did not show increased resistance to the pyrophosphate analog PFA or the nucleoside analog ACV. Unlike AD169, the mutants were, however, resistant to the nucleoside monophosphate analogs HPMPA and HPMPC and hypersensitive to AraT.

An in situ ELISA (33) was used as an alternative method to determine the susceptibility profiles of HCMV strains. This assay detects a late viral antigen produced in infected fibroblast monolayers. Production of the antigen is dependent on DNA replication, which is inhibited by all of these antiviral agents. The results are shown in Table 1. The antiviral susceptibility profiles of mutant and wild-type strains demonstrated by the plaque reduction assay were confirmed by the ELISA.

Analysis of GCV anabolism by HPLC. GCV anabolism in mutant- and wild-type-virus-infected cells was analyzed by reverse-phase HPLC. Standard retention times for unphosphorylated GCV and GCV monophosphate averaged 10 and 4 min, respectively. No standards are available for the di- and triphosphate derivatives of GCV; however, other nucleotide di- and triphosphates had retention times of between 2 and 3 min in this assay system. It was assumed that the di- and triphosphates of GCV would likewise have shorter retention times than the GCV monophosphate on this reverse-phase column.

In uninfected fibroblasts (Fig. 1A), very low levels of phosphorylated GCV derivatives were present in the fractions with retention times that corresponded to those of the nucleoside phosphates (fractions 8 to 18). Unphosphorylated drug eluted in fractions 33 to 38. The levels of phosphorylated GCV derivatives in cells infected with wild-type virus are shown in Fig. 1C (fractions 8 to 18). The corresponding fractions from mutant-infected cells had 10-fold lower counts than those from wild-type-infected cells (Fig. 1B, D, and F). GCV anabolism in mutant-infected cells is therefore greatly reduced compared with that in wild-type-infected cells. These results suggest that each of the mutants has an alteration in the kinase that phosphorylates GCV (22, 32).

DNA sequence of mutant DNA polymerase genes. Although the HPLC data indicated that the GCV resistance of the mutants could result from reduced GCV anabolism, the resistance to HPMPA and HPMPC suggested that there was an additional mutation in the DNA polymerase gene of each of these strains. The nucleotide sequence of the entire DNA polymerase gene from each of the three GCV-resistant mutants was therefore determined and compared with the published sequence for the wild-type AD169 (20). Each mutant polymerase gene was found to contain a single base change that results in an amino acid substitution (Fig. 2). The position numbers in Fig. 2 correspond to those of the published HCMV AD169 DNA polymerase sequence (20). The mutations in both D1/3/4 and D6/3/1 are at position 2160, where A is substituted for C. This mutation results in a conservative amino acid change from leucine to isoleucine at amino acid residue 501. The mutation in D10/3/2 is at position 1893, where G is substituted for T, which changes amino acid residue 412 from phenylalanine to valine. A second, silent mutation in D10/3/2 at position 2111 (C to A) was also found. The DNA polymerase gene of the wild-type AD169 strain from which the mutants were derived was sequenced wherever mutations were found in D1/3/4, D6/3/1, and D10/3/2 in order to eliminate the possibility of AD169 strain-specific changes. The sequence of the strain of AD169 used in this study is identical to the published AD169 sequence (20) at every site at which a base substitution has been found in D1/3/4, D6/3/1, and D10/3/2.

Marker transfer of the D1/3/4 mutation to the wild-type AD169 background. The HPLC and sequence data demonstrated that there are two mutations in the GCV-resistant strains D1/3/4, D6/3/1, and D10/3/2. We therefore wanted to determine the contribution of the mutant DNA polymerase to GCV resistance. A plasmid carrying the D1/3/4 DNA polymerase gene was transfected into HFF infected with the wild-type strain AD169. Resistant virus was selected in the presence of 1.5 μM HPMPA, and strain HP/A1B/4 was obtained from this selection. The ED_{50} of GCV for HP/A1B/4 is 50 μM, which represents a fourfold reduction compared with that for the parental strain D1/3/4 (Table 1); however, HP/A1B/4 remains fivefold more resistant to GCV than the wild-type AD169. The ED_{50} of HPMPA (4 μM) for HP/A1B/4 is similar to that for D1/3/4. HP/A1B/4, like D1/3/4, is also hypersensitive to AraT and sensitive to PFA.

Although HP/A1B/4 is resistant to GCV, the anabolism of GCV in HP/A1B/4-infected cells is similar to that in wild-type AD169-infected cells (Fig. 1E). By comparison, GCV anabolism in cells infected with the parental D1/3/4 virus is at least 10-fold lower than that in cells infected with the wild type. These data indicate that the kinase that phosphorylates GCV is unaltered in HP/A1B/4.

Nucleotide sequence analysis of the HP/A1B/4 DNA polymerase gene demonstrated that the same base substitution at position 2160 (C to A) found in the parental mutant D1/3/4 is present in HP/A1B/4 (Fig. 3). HP/A1B/4, therefore, is a recombinant strain resulting from transfer of the D1/3/4 polymerase mutation to the wild-type AD169.

Relationship of mutations to conserved regions of other DNA polymerases. Comparison of the nucleotide sequences of the mutant polymerase genes with the sequences of other viral DNA polymerases shows a high degree of conservation in the regions where we have mapped mutations (Fig. 4). The leucine residue at position 501 which is changed to isoleucine in both D6/3/1 and D1/3/4 is conserved in all human herpesvirus DNA polymerases (34). The phenylalanine residue at position 412 which is changed to valine in D10/3/2 is con-

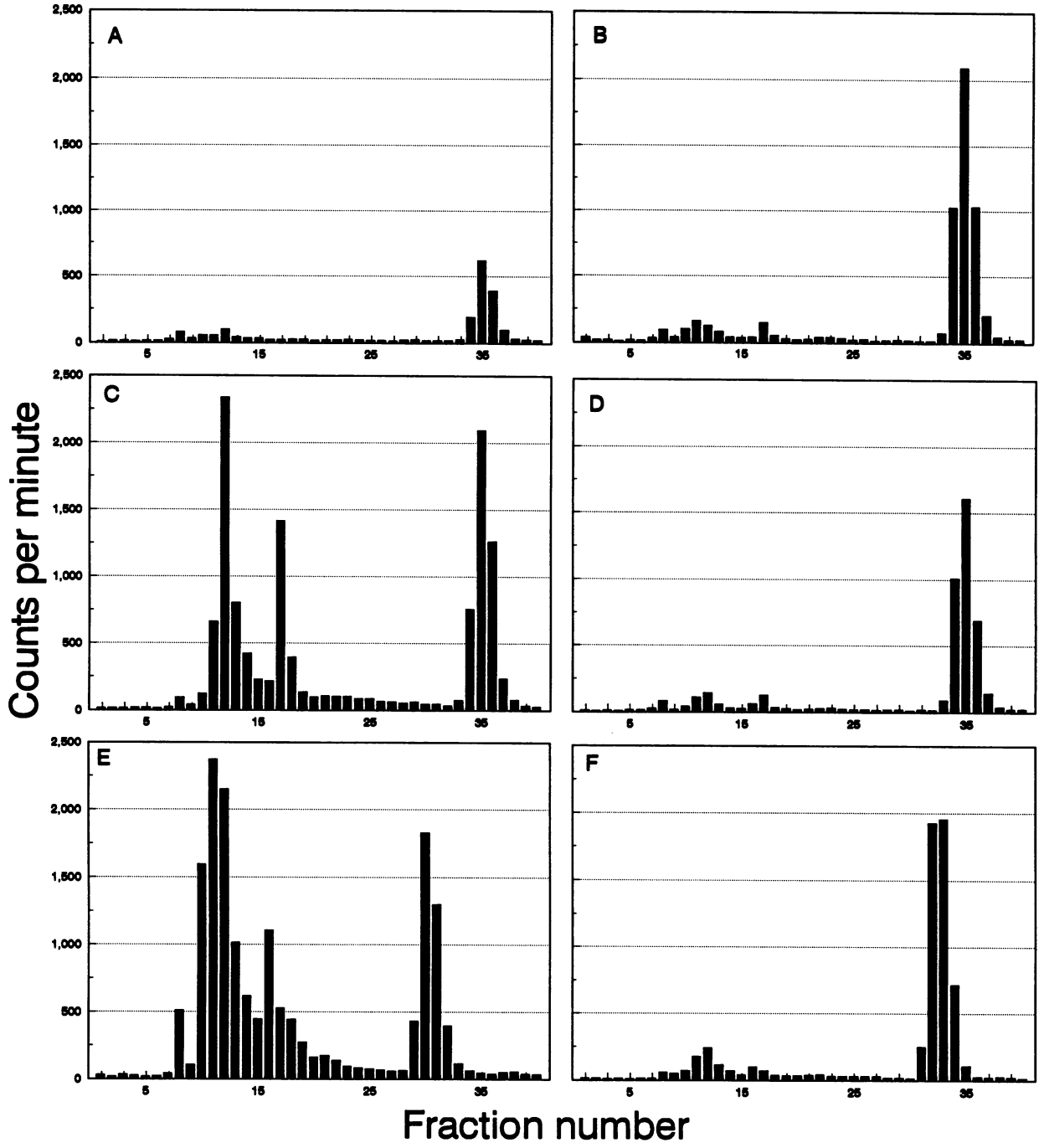

FIG. 1. HPLC analysis of virus-induced GCV phosphorylation. Fractions 33 to 38 contain unphosphorylated GCV, and fractions 8 to 18 contain the phosphorylated GCV derivatives. (A) Mock-infected human fibroblasts. (B) D1/3/4-infected fibroblasts. (C) AD169-infected fibroblasts. (D) D6/3/1-infected fibroblasts. (E) HP/A1B/4-infected fibroblasts. (F) D10/3/2-infected fibroblasts. Standards consisting of GCV and GCV monophosphate were run on the same column to determine relative retention times. The average retention time for GCV was 10 min (fractions 34 to 36), and that of GCV monophosphate was 4.5 min (fractions 15 and 16). (E) HP/A1B/4 was assayed at a later time. With this sample, the unphosphorylated GCV is contained in fractions 29 to 33 and the phosphorylated derivatives are in fractions 8 to 19. Standards and AD169-infected cells were run as controls. All results were reproduced in a minimum of three separate experiments.

served not only in all human herpesviruses (Fig. 4) but also in several other prokaryotic and eukaryotic DNA polymerases (1, 37). These mutations are within 300 bp of each other in the coding sequence of the HCMV polymerase gene.

Wong et al. (37) identified six regions of homology among a group of eukaryotic and prokaryotic DNA polymerases. These regions were labeled I through VI in order of decreasing degree of homology. An additional region displaying homology among several viral DNA polymerases but not found in human polymerase α was described by Gibbs and

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                                                                    1940
AD169     AACATCAACTCTTTTGACTTGAAGTACATCCTCACGCGTCTCGAGTACCTGTATAAGGTG
D1/3/4    ------------------------------------------------------------
D6/3/1    ------------------------------------------------------------
D10/3/2   ------------G-----------------------------------------------

                                                                    2000
AD169     GACTCGGCAGCGCTTCTGCAAGTTGCCTACGGCGCAGGGCGGCCGTTTCTTTTTACACAGC
D1/3/4    ------------------------------------------------------------
D6/3/1    ------------------------------------------------------------
D10/3/2   ------------------------------------------------------------

                                                                    2060
AD169     CCGGCCGTGGGTTTTAAGCGGCAGTACGCCGCCGCTTTTCCCTCGGCTTCTCACAACAAT
D1/3/4    ------------------------------------------------------------
D6/3/1    ------------------------------------------------------------
D10/3/2   ------------------------------------------------------------

                                                                    2120
AD169     CCGGGCAGCACGGCCGCCACGAAGGTGTATATTGCGGGTTCGGTGGTTATCGACATGTAC
D1/3/4    ------------------------------------------------------------
D6/3/1    ------------------------------------------------------------
D10/3/2   ----------------------------------------------------A*--------

                                                                    2180
AD169     CCTGTATGCATGGCCAAGACTAACTCGCCCAACTATAAGCTCAACACTATGGCCGAGCTT
D1/3/4    ----------------------------------------A-------------------
D6/3/1    ----------------------------------------A-------------------
D10/3/2   ------------------------------------------------------------
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FIG. 2. Nucleotide changes in the DNA polymerase sequences of HCMV AD169 mutants. *, no change in amino acid. Numbers at the right correspond to the nucleotide positions of the HCMV AD169 *Eco*RI-M fragment (20).

coworkers (12) and designated A. Recently, Hwang et al. reported a seventh region of homology (17). All of these regions are listed in Table 2 in the order in which they appear in the DNA polymerases relative to the N terminus. The linear order which is conserved among the polymerases is IV-A-II-VI-III-I-VII-V (17, 37). The mutation in D10/3/2 lies within region IV. The mutation in D1/3/4 and D6/3/1 is found between regions IV and A. The homology among herpesvirus DNA polymerases sequences remains high between these two regions, although the sequence conservation is not found outside this virus group (12, 34).

DISCUSSION

In this report, we describe the isolation of three GCV-resistant mutants of HCMV. This high-level GCV resistance has been shown to be the combined effect of changes in two viral functions: the DNA polymerase and the kinase required

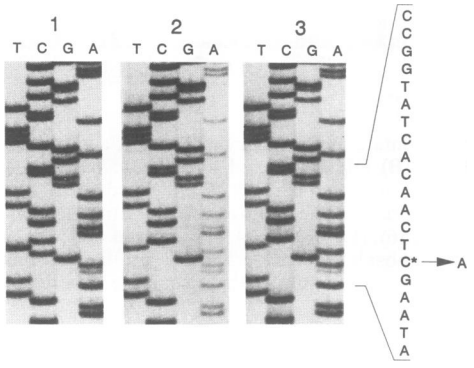

FIG. 3. Autoradiogram showing partial nucleotide sequence of the DNA polymerase gene from (panel 1) the wild-type AD169, (panel 2) the mutant D1/3/4, and (panel 3) the recombinant HP/A1B/4. The base change from C in strain AD169 to A in D1/3/4 and HP/A1B/4 at position 2160 is indicated.

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HCMV  AD169      405  T G Y N I N S F D L K Y I L T R
      D10/3/2    405  T G Y N I N S V D L K Y I L T R
HHV6             361  T G Y N I N N F D L K Y L L I R
EBV              376  T G Y N V A N F D W P Y I L D R
HSV-1            463  T G Y N I I N F D W P F L L A K
HSV-2            464  T G Y N I I N F D W P F V L T K
VZV              444  T G Y N I V N F D W A F I M E K
                                    *

HCMV  AD169      493  K T N S P N Y K L N T M A E L Y
      D1/3/4     493  K T N S P N Y K I N T M A E L Y
      D6/3/1     493  K T N S P N Y K I N T M A E L Y
HHV6             433  K I T A Q N Y K L D T I A K I C
EBV              448  K L S L S D Y K L D T V A R H L
HSV-1            532  K I K L S S Y K L N A V A E A V
HSV-2            533  K V K L S S Y K L N A V A E A V
VZV              513  K L K L S S Y K L D S V A R E A
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FIG. 4. Amino acid sequences in regions surrounding the site of substitutions in the HCMV mutants D1/3/4, D6/3/1, and D10/3/2. Residues substituted in these mutants are marked with an asterisk (*). The homologous sequences of the other herpesviruses are shown for comparison. The amino acid residue numbers are from Teo et al. (34). HHV6, human herpesvirus 6; EBV, Epstein-Barr virus; VZV, varicella-zoster virus.

for GCV phosphorylation (2, 22, 32). Unlike HSV, few drug-resistant mutants of HCMV have been studied further to determine the mechanism of resistance, and no mutations have been mapped to specific nucleotides of the DNA polymerase gene.

All three of our HCMV mutants demonstrate marked reductions in infected-cell anabolism of GCV compared with the wild-type AD169 (Fig. 1). Others have reported similar decreased GCV anabolism in GCV-resistant mutants from both laboratory HCMV strains (2) and clinical HCMV isolates (30). In cells infected with HSV, GCV is monophosphorylated by the virus-encoded thymidine kinase (29); however, HCMV does not encode a thymidine kinase homolog (4). Instead, GCV appears to be phosphorylated by a recently identified protein kinase homolog encoded by the HCMV *UL97* open reading frame (22, 32). We have identified a point mutation in the *UL97* gene of one of our mutants (unpublished data), and therefore, we postulate that the reduced GCV anabolism in cells infected with our mutants is the result of changes in this enzyme.

The initial evidence that the polymerase genes of the GCV-resistant strains might also contain mutations was the observed cross-resistance to the nucleoside monophosphate

TABLE 2. Conserved regions of the DNA polymerases of HCMV and HSV[a]

HCMV residues	HSV residues	Conserved region
379–421	437–479	IV
538–598	577–637	A
696–742	694–736	II
771–790	772–791	VI
805–845	805–845	III
905–919	881–896	I
962–970	938–946	VII
978–988	953–963	V

[a] The regions are listed in order from the N terminus of the polymerase. Regions I to VI are from Wong et al. (37); region A is from Gibbs et al. (12); region VII is from Hwang et al. (17). The amino acid substitution in D10/3/2 is at residue 412 in region IV; the amino acid substitutions in D1/3/4 and D6/3/1 are at residue 501, which lies between regions IV and A.

analogs HPMPA and HPMPC. Sequencing of the DNA polymerase genes of the three mutants revealed that each encodes a polymerase containing a single nucleotide base change which produces an amino acid substitution. The same base substitution resulting in a conservative amino acid change from leucine to isoleucine at residue 501 is present in both D1/3/4 and D6/3/1. Since these two strains were isolated independently from the same AD169 stock, the presence of the same mutation may reflect both the limited number of functional mutations conferring GCV resistance and the frequency with which these mutations occur in the wild-type population. Similar results have been reported by Larder et al. (21) for a series of DNA polymerase mutants of HSV-1, of which three have identical point mutations.

In order to demonstrate that a conservative substitution could result in drug resistance, the DNA polymerase gene of D1/3/4 was transferred to the wild-type background. Nucleotide sequence analysis demonstrated that the same base substitution found in the parental strain D1/3/4 was present in the recombinant HP/A1B/4 (Fig. 4). When cells were infected with HP/A1B/4, wild-type GCV anabolic activity was observed (Fig. 1). The HPMPA and HPMPC resistance of the recombinant remains the same as that of the parental mutant, but GCV resistance is reduced by 75% compared with that of the parental strain. This is not surprising, since the high-level GCV resistance of D1/3/4 is apparently the result of two mutations. Transfer of only the polymerase mutation would be expected to lead to a lower level of GCV resistance. The fact that the recombinant HP/A1B/4 remains hypersensitive to AraT suggests that the altered DNA polymerase may play a role in this hypersensitivity as well. It appears that even a conservative amino acid change can have a significant effect on drug susceptibility.

The base substitution found in D10/3/2 is 267 bases in the 5′ direction from that of the mutation found in D1/3/4 and D6/3/1. This mutation results in an amino acid change from phenylalanine to valine at residue 412 in the polypeptide. Marker transfer experiments are under way to confirm that this mutation also confers resistance to HPMPA, HPMPC, and GCV as well as hypersensitivity to AraT.

Both phenylalanine 412 and leucine 501 are conserved in the DNA polymerases of all the other human herpesviruses for which sequence data are available: HSV-1 and HSV-2, Epstein-Barr virus, varicella-zoster virus, and human herpesvirus 6 (20, 34). Phenylalanine 412 is also conserved in other prokaryotic and eukaryotic DNA polymerases, including human DNA polymerase α, ϕ29, vaccinia virus, and *Saccharomyces cerevisiae* (1, 3, 24, 37) (Fig. 4). Although these amino acid residues are highly conserved among DNA polymerases, the viability of these three HCMV mutants is not significantly affected by a single conservative substitution.

Of the large number of drug resistance mutations which have been assigned to the polymerase locus of HSV (5, 6, 12, 13, 16, 17, 21, 36), the majority have been mapped within the C-terminal conserved regions, which encode amino acid residues 690 to 1100 (12, 37). Specifically, these residues comprise conserved regions II, III, and V described by Wong et al. (37) and the recently identified region VII (17) (Table 2). The HCMV mutants that we have isolated have base changes toward the 5′ end of the polymerase gene. The substitution in D10/3/2 lies in conserved region IV, while the substitution found in D6/3/1 and D1/3/4 lies between regions IV and A.

Conserved regions IV and A have been predicted to be components of a possible 3′→5′ exonuclease domain (1, 3, 15, 37). The evidence for this functional domain is based on site-directed mutagenesis studies of the DNA polymerase of ϕ29 by Bernad and co-workers (1). Conservation of these sequences among DNA polymerases supports the hypothesis that this region of the enzyme has 3′→5′ exonuclease activity. In addition, 3′→5′ exonuclease function has been found to be tightly associated with the DNA polymerases of Epstein-Barr virus (35), HSV (19), and HCMV (25).

More recent evidence, however, suggests that it may not be possible to define the functional domains of the HSV polymerase at the level of the primary amino acid sequence. Knopf and Weisshart (19) were able to neutralize both the 3′→5′ exonuclease and polymerizing functions of the HSV DNA polymerase by using antibodies specific for each of the N-terminal, central, and C-terminal peptide regions. Gibbs and coworkers (14) constructed HSV mutants by site-specific mutagenesis in region IV. These were base substitutions resulting in conservative amino acid changes. Two of the mutants were nonviable. Thus, the polymerization function of the enzyme was apparently inhibited by a mutation in the region thought to have 3′→5′ exonuclease function.

Other evidence suggests that the amino acid residues that contribute to the nucleotide and pyrophosphate binding sites are not confined to the C-terminal portion of the polypeptide, as has been postulated previously (13). Crumpacker et al. (6) described a GCV-resistant mutant of HSV-2 which was sensitive to ACV and PAA. Wang et al. (36) isolated suppressor mutants of HSV from a parental aphidicolin-resistant, PAA-hypersensitive strain. The suppressor mutations which conferred resistance to PAA were mapped to the N-terminal portion of the polymerase gene.

Our data support the growing body of evidence that the nucleotide-binding site of the HSV polymerase may be altered by changes at sites other than the C-terminal region of the polypeptide. Thus, the amino acid substitutions in the polymerases of these HCMV mutants could be involved directly in nucleotide binding or indirectly in determination of the tertiary structure of the binding site. It is also possible that the drug resistance of the mutants could result directly from altered 3′→5′ exonuclease function. Further analysis of the polymerases encoded by these mutants should contribute to the determination of the functional domains of this enzyme.

ACKNOWLEDGMENTS

Grant support was provided by Potts Foundation grant LU#3043-RCC/Potts.

We thank Lois Spafford and Lynn Hilger for their technical assistance.

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