Physical Mapping of Drug Resistance Mutations Defines an Active Center of the Herpes Simplex Virus DNA Polymerase Enzyme

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The genome structures of herpes simplex virus type 1 (HSV-1)/HSV-2 intertypic recombinants have been previously determined by restriction endonuclease analysis, and these recombinants and their parental strains have been employed to demonstrate that mutations within the HSV DNA polymerase locus induce an altered HSV DNA polymerase activity, exhibiting resistance to three inhibitors of DNA polymerase. The viral DNA polymerases induced by two recombinants and their parental strains were purified and shown to possess similar molecular weights (142,000 to 144,000) and similar sensitivity to compounds which distinguish viral and cellular DNA polymerases. The HSV DNA polymerases induced by the resistant recombinant and the resistant parental strain were resistant to inhibition by phosphonoacetic acid, acycloguanosine triphosphate, and the 2',3'-dideoxynucleoside triphosphates. The resistant recombinant (R6-34) induced as much acycloguanosine triphosphate as did the sensitive recombinant (R6-26), but viral DNA synthesis in infected cells and the viral DNA polymerase activity were not inhibited. The 2',3'-dideoxynucleoside-triphosphates were effective competitive inhibitors for the HSV DNA polymerase, and the K_i values for the four 2',3'-dideoxynucleoside triphosphates were determined for the four viral DNA polymerases. The polymerases of the resistant recombinant and the resistant parent possessed a much higher K_i for the 2',3'-dideoxynucleoside triphosphates and for phosphonoacetic acid than did the sensitive strains. A 1.3-kilobase-pair region of HSV-1 DNA within the HSV DNA polymerase locus contained mutations which conferred resistance to three DNA polymerase inhibitors. This region of DNA sequences encoded for an amino acid sequence of 42,000 molecular weight and defined an active center of the HSV DNA polymerase enzyme.

The growth of herpes simplex virus (HSV) in the presence of antiviral agents such as phosphonoacetic acid (PAA) or acycloguanosine (ACG) leads to the selection of drug-resistant mutants (13, 25). This resistance is genetically stable and provides a distinct phenotype in HSV designated PAA^r and ACG^r. The mutations conferring resistance provide two markers for viral gene functions and the physical locations of these mutations, *paa*^r and *acg*^r, have been defined in the herpes genome (5, 6, 8). The selection of HSV type 1 (HSV-1) and HSV-2 mutant viruses which are resistant to PAA readily occurs after passage of the virus in the presence of the drug, is the result of a single mutation (14),

and is due to an altered HSV DNA polymerase activity (5, 13). The guanosine analog ACG [9-(2-hydroxyethoxymethyl)guanine; acyclovir] requires phosphorylation to exert its antiviral effect (9, 11). The virus-coded deoxypyrimidine kinase produces phosphorylation to ACG monophosphate, and a subsequent conversion to di- and triphosphates presumably occurs by cellular pathways (11). The ACG triphosphate (ACG-TP) is a main metabolite of infected cells and is an effective inhibitor of wild-type (WT) HSV DNA polymerase (11, 12, 26). Growth of HSV in the presence of ACG selects for resistant mutants; this resistance is controlled by two genetic loci in the HSV genome, the viral deoxypyrimi-

dine kinase and the viral DNA polymerase loci (7, 25).

Restriction endonuclease analysis of the genomes of plaque-purified HSV-1/HSV-2 intertypic recombinants has permitted the physical map limits for two distinct regions controlling ACG to be defined within the HSV DNA polymerase locus (8). One of these regions contains the mutation for *acg*^r closely linked to the mutation for *paa*^r, and these mutations are found within a 1.3-kilobase-pair region of HSV DNA for HSV-2 and within a 2.6-kilobase-pair region for HSV-1. The other region is found within adjacent sequences of HSV DNA and defines the type-specific sensitivity of HSV-1 and HSV-2 ACG. These regions are found in equivalent positions within the HSV-1 and HSV-2 genome in the area defined as the HSV DNA polymerase locus. Two of these intertypic recombinants contain an HSV-1 DNA insertion in an otherwise HSV-2 genome and differ significantly in their sensitivity to ACG and PAA. These recombinants were derived by marker rescue of temperature-sensitive (*ts*) mutations and resulted from a cross between the DNA of HSV-2 *ts*6 (HG-52) and HSV-1 strain 17 (*ts*⁺, *paa*^r-1) (R6 series) (5). The R6-26 recombinant is sensitive to plaque inhibition by PAA and ACG, whereas the R6-34 recombinant exhibits PAA^r and ACG^r. In this report the viral DNA polymerases induced by these recombinants and their parental strains have been partially purified and tested for sensitivity and resistance to ACG-TP, PAA, and the known chain-terminating compounds, the 2',3'-dideoxynucleoside triphosphates (didNTP's). This report correlates genome structures and the presence of the gene markers for *paa*^r and *acg*^r with the function of purified HSV DNA polymerase in the presence of three compounds of dissimilar structure. A 1.3-kilobase-pair region of HSV DNA within the viral DNA polymerase locus contains a mutation or mutations conferring resistance to three DNA polymerase inhibitors. The DNA sequences within this region encode for an active center of the herpes simplex virus DNA polymerase.

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MATERIALS AND METHODS

Materials. Acyclovir was a generous gift from John Beale of Burroughs Wellcome Ltd., Beckenham, United Kingdom. PAA was a gift from Lacey Overby of Abbott Laboratories, Inc., North Chicago, Ill. Activated salmon sperm DNA prepared by mild pancreatic

DNase digestion as previously described (29) was a kind gift of Helen Moss-Rixon, MRC Virlogy Unit, Glasgow. Deoxynucleoside triphosphates (dNTP's), didNTP's, and oligodeoxyguanydic acid-polydeoxycytidylic acid [oligo(dG)₁₂₋₁₈·poly(dC)] were from P. L. Biochemicals. Radiochemicals were from Radiochemical Center, Amersham. [³H]ACG was prepared by New England Nuclear Corp.

Cells and viruses. Baby hamster kidney (BHK 21-C13) and Vero cells were grown in Eagle medium supplemented with 10% tryptose broth and 10% bovine calf serum (21). Cell monolayers (10⁷ cells) in 90-mm plastic petri dishes (Falcon Plastics) were used throughout. Virus infection was carried out with 10 PFU/cell in medium without tryptose. All virus strains used were derived from stocks prepared in Glasgow by low-multiplicity passage in BHK 21-C13 cells. The temperature-sensitive mutant of HSV-2, *ts*6 (strain HG-52), has been previously described (27). The PAA^r mutant of HSV-1 strain 17 (*ts*⁺, *paa*^r-1) was isolated by passage in the presence of PAA (100 mg/ml) and was characterized previously (13). The HSV-1/HSV-2 intertypic recombinants R6-26 and R6-34 were derived by crosses between HSV-2 *ts*6 (strain HG-52) DNA and restriction endonuclease-cleaved DNA from HSV-1 strain 17 (*ts*⁺, *paa*^r-1) DNA, isolated by marker rescue, and plaque purified at 38.5°C (6).

Preparation of cell extracts and purification of HSV DNA polymerase. All infected cells were harvested at 18 h postinfection to prepare cytoplasmic extracts. To demonstrate that the resistant recombinant R6-34 induces DNA polymerase activity which is resistant to ACG-mediated inhibition, cytoplasmic extracts were prepared from confluent baby hamster kidney cells (BHK-C13) infected with WT HSV-2 (strain HG-52), R6-26, or R6-34 (multiplicity of infection, 10 PFU/cell), and at 7 h postinfection 20 μM ACG in water was added to the infected cells, or water alone was added to control dishes. The infected cells were incubated in the presence or absence of ACG at 37°C, and cultures were harvested at 18 h postinfection. Cell extracts were prepared by lysing cells in 0.25 M potassium phosphate buffer (pH 7.5) containing 1 mM each of dithiothreitol, EDTA, ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid, and 0.5% Triton X-100. After centrifugation to remove nuclei (15,000 × *g* for 1 h at 4°C), aliquots of cytosol extracts were kept in 35% ethylene glycol at -20°C until use. These extracts were employed directly to assay HSV-induced DNA polymerase activity by measuring the incorporation of [³²P]dGTP into the synthetic DNA template primer, oligo(dG)₁₂₋₁₈·poly(dC).

For purification of HSV DNA polymerase, 200-μl samples of fresh cell extracts (600 μg of protein) were layered on linear glycerol density gradients (20 to 50% glycerol in 0.25 M potassium phosphate buffer [pH 7.5] containing 1 mM dithiothreitol and 0.1 mM EDTA), velocity sedimentation was carried out in a Sorvall TV 885 rotor (45,000 × *g* for 15 h at 2°C) with appropriate markers for molecular weight, 0.25-ml fractions were collected, and 10 μl of each fraction was assayed for DNA polymerase activity (17).

DNA synthesis. Infected cells were grown in the absence or presence of 5 μM ACG in 10 ml of medium

containing 87.5 μCi of carrier-free $^{32}\text{P}_i$. Cells were harvested 18 h postinfection by scraping them into cold reticulocyte standard buffer. After being pelleted, cells were suspended in reticulocyte standard buffer containing 10 mM EDTA, 1% sodium dodecyl sulfate, and 0.5 mg of proteinase K per ml and incubated at 50°C for 3 h. Then DNA was phenol extracted twice and precipitated with alcohol at -20°C overnight. The washed and dried DNA pellets were suspended in 10 mM Tris-hydrochloride (pH 8)-10 mM EDTA containing 100 μg of RNase A per ml and 1,000 U of T1 RNase (Worthington Diagnostics) per ml and incubated for 3 h at 37°C. The DNA was alcohol precipitated, washed, and dissolved in 50 mM Tris-hydrochloride (pH 8)-10 mM EDTA, and CsCl was added to give a final density of 1.715 g/cm³. Equilibrium density CsCl gradient centrifugation was carried out in a Sorvall TV865 rotor at 43,000 rpm for 18 h at 20°C. Gradients were dripped from the bottom, and fractions were collected directly onto Whatman 3M paper slips. Determination of trichloroacetic acid-precipitable radioactivity was performed by the Bollum technique (4).

Measurement of ACG-TP. To determine the amount of ACG-TP formed in infected cells, [^3H]ACG (50 $\mu\text{Ci}/\text{ml}$; 200 $\mu\text{Ci}/\text{mmol}$; New England Nuclear Corp.) was added at 7 h after infection, cells were incubated at 37°C until 18 h postinfection and then harvested, and extracts were prepared. Medium was removed, the dishes were washed three times in 0.5 ml of ice-cold 0.85% NaCl, and soluble nucleotides were extracted in 0.5 ml of 0.4 M HClO₄ for 30 min at 4°C. The extracts were neutralized with KOH and concentrated by lyophilization. The ACG-TP was separated on polyethyleneimine cellulose thin-layer chromatography sheets developed with 2.0 M LiCl-2.0 N HCOOH (1:1, vol/vol). The chromatography sheets were fractionated, and the triphosphate was eluted from the adsorbent with 1 ml of 0.1 N HCl and counted in a liquid scintillation counter. The protein in the cellular extracts was determined by the method of Lowry et al. (20), and the results are expressed as picomoles of nucleotide formed per microgram of cell protein.

The [^3H]ACG was obtained by tritiation of ACG, and then [^3H]ACG was purified by ascending chromatography on Whatman 3M paper in *N*-propanol-water (7:3). The *R_f* for ACG in this system is 0.53 (11); this corresponded to the only UV-absorbing material observed on the chromatogram. The [^3H]ACG was eluted and rechromatographed before use.

HSV DNA polymerase assay. When the synthetic template primer, oligo(dG)₁₂₋₁₈·poly(dC), was employed, the standard reaction mixture contained (in a final volume of 100 μl): 50 mM Tris-hydrochloride (pH 8.0), 50 μg of bovine serum albumin, 0.5 mM dithiothreitol, 7.5 mM MgCl₂, 100 mM ammonium sulfate, 0.01 mM [^{32}P]dGTP (1,450 cpm/pmol), and 50 μg of oligo(dG)₁₂₋₁₈·poly(dC) per ml. With activated DNA as the template primer, the standard reaction mixture contained (in a final volume of 100 μl): 50 mM Tris-hydrochloride (pH 8.0), 50 μg of bovine serum albumin, 0.5 mM dATP, dCTP, dGTP, and [^{32}P]dTTP (200 cpm/pmol), and 100 μg of activated salmon sperm DNA. Under these conditions, all DNA polymerase

assays exhibited linear kinetics of incorporation for 20 min. Incubation was performed at 37°C for the indicated time, and samples of the reaction mixture were spotted onto Whatman GF/C glass filters of Whatman no. 1 filter paper, precipitated by 10% trichloroacetic acid, and treated to determine acid-insoluble radioactivity as previously described (17, 24). One unit of HSV DNA polymerase activity is defined as the amount of enzyme catalyzing the polymerization of 1 nmol of deoxynucleotides per h under standard assay conditions.

Alkaline DNase assay. Alkaline DNase was monitored as described by Morrison and Deir (23) by following the release of acid-soluble products from [^3H]thymidine-labeled DNA. The reaction mixture contained (in a final volume 100 μl): 50 M Tris-hydrochloride (pH 9), 5 mM MgCl₂, 10 nM dithiothreitol, and 10 μg of double-stranded, [^3H]thymidine-labeled BHK 21-C13 DNA (6,250 cpm/ μg) and either 5-, 10-, or 20- μl samples of cell extract in 200 μl of a suspension of Hyflo-SuperCel (20 mg/ml; Koch-Light Labs) in 1 M perchloric acid. After mixing and centrifugation at 10,000 $\times g$ for 2.5 min, 150- μl samples of the supernatants were counted in 10 ml of Aquasol cocktail. Under the given conditions, 1 U of alkaline DNase activity is defined as the release of 1 nmol of deoxynucleotides per h at 37°C.

Thymidine kinase assay. Thymidine kinase activity was assayed by measuring the amount of [^3H]thymidine phosphorylated by an extract of infected cells by the method of Jamieson et al. (15, 16). Briefly, the reaction mixture contained (in a final volume of 100 μl): 20 mM potassium phosphate (pH 7.5), 10 mM MgCl₂, 10 μM [^3H]thymidine (10 $\mu\text{Ci}/\mu\text{mol}$), and cell extract at a concentration of 50 to 100 μg of protein per ml. After incubation for 30 min at 37°C, radioactivity bound to Whatman DE81 filter disks was determined. One unit of thymidine kinase activity is defined as the conversion of 1 nmol of thymidine into a phosphorylated form within 1 h at 37°C under the stated assay conditions. Protein concentrations were determined by the method of Lowry et al. (20).

RESULTS

Development of HSV-1/HSV-2 intertypic recombinants and determination of the HSV DNA polymerase locus. Seventy-four HSV-1/HSV-2 intertypic recombinants have been generated by marker rescue of temperature-sensitive mutants exhibiting defective DNA synthesis at the nonpermissive temperature (5, 6). The genome structures of these recombinants have been determined by restriction endonuclease analysis of the genomes after plaque purification and have been employed in determining the physical map limits of five temperature-sensitive mutations (*ts*) and mutations conferring resistance to PAA (*paa*^r) and ACG (*acg*^r) in both HSV-1 and HSV-2 (5, 6, 8). The genome structures for the recombinants resulting from crosses between HSV-2 *ts*6 (HG-52) and HSV-1 strain 17 (*ts*⁺, *paa*^r-1) (R6 series) were analyzed

with seven restriction endonucleases in the region from 30 to 50 map units, enabling the physical map limits of the HSV-2 *ts6* mutation and the *paa-1* and *acg-1* mutations of HSV-1 (strain 17) to be established (6, 8). Two of these R6 recombinants, R6-26 and R6-34, were selected for further analysis to determine whether mutations within the HSV DNA polymerase locus which confer resistance to plaque inhibition by ACG and PAA induce an altered HSV DNA polymerase activity. These two recombinants were selected because they possess similar small HSV-1 DNA insertions in an otherwise HSV-2 genome. This HSV-1 DNA corrects the *ts6* mutation permitting both recombinants to replicate at the nonpermissive temperature, but these two recombinants differ widely in their sensitivity to PAA and ACG. The R6-34 recombinant has an HSV-1 DNA insertion extending from the HSV-1 *Bgl*III i-d site to the HSV-1 *Eco*RI m-o site (map units 39.6 to 42.8, including the region of uncertainty), is PAA^r (efficiency of plaqueing, 0.6 in the presence and absence of 100 μ g of PAA per ml) and markedly ACG^r (50% inhibitory dose, 17.5 μ M). The R6-26 recombinant, on the other hand, contains HSV-1 DNA sequences extending from the HSV-1 *Bgl*III i-d site to the HSV-2 *Bam* h'-j' site (map units 39.6 to 41.0, including the region of uncertainty), but is still PAA^s (efficiency of plaqueing, 0.4×10^{-4} in the presence and absence of 100 μ g of PAA per ml) and ACG^s (50% inhibitory dose, 0.12 μ M) (8). The genome structures of these two recombinants and their restriction endonuclease sites have been previously published (6, 8). The recombinants and their parental strains were employed in all infections, and their polymerases were partially purified and compared.

Effect of ACG on viral and cellular DNA synthesis in infected cells. The recombinant and parental strains differed markedly in their sensitivity to PAA and ACG when analyzed in a plaque reduction assay. This efficiency of plaqueing in the presence of 5 μ M ACG compared with that in the absence of inhibitor was 82% for R6-34, <0.5% for R6-26, 45% for PAA^r-1, and 9% for HSV-2 *ts6* (8). Since the effect of PAA on DNA synthesis of parental strains and PAA^r mutants has been previously determined (13), it was of interest to also measure the effects of ACG on viral and cellular DNA synthesis in cells infected with the recombinants and their parental strains. To show that the presence of the *acg*^r-1 mutation affects the ability of infected cells to synthesize cellular and viral DNA in the presence of ACG, the DNA profiles from infected cells in the presence and absence of ACG were compared. Confluent cells (BHK) were infected in the absence or presence of ACG (5

μ M), and the incorporation of ³²P into DNA was determined at 18 h postinfection by analysis of total cellular DNA on CsCl density gradients (Fig. 1). In the presence of the drug, viral DNA synthesis of the *ts6* parent and the R6-26 recombinant was hardly detectable. Viral DNA synthesis of the PAA^r-1 parent and the R6-34 recombinant were resistant to ACG, with 20 and 23%, respectively, of viral DNA being present in the absence of the drug. Cellular DNA synthesis was decreased by ACG in all four infections, but 40% of cellular DNA synthesis remained in the presence of 5 μ M ACG. For both the PAA^r-1 and the R6-34 recombinant, viral DNA synthesis is inhibited to a greater extent by ACG (5 μ M) than is plaque formation, indicating that the ACG^r virus may be more effective in utilizing viral DNA in the formation of infectious virus. Little of the newly synthesized viral DNA appears to be required for effective packaging into infectious virus. A direct comparison of the efficiency of plaqueing and viral DNA synthesis in the presence of ACG is not possible from these two experiments because the cells were infected at different multiplicities and analyzed at differ-

FIG. 1. Equilibrium density gradients of DNA from infected cells in the presence and absence of ACG. BHK cells were infected with HSV-2 (HG-52) *ts6* (A), HSV-1 PAA^r-1 (B), R6-26 (C), or R6-34 (D) virus and grown in the presence (○) or absence (●) of 5 μ M ACG and ³²P. At 18 h postinfection cells were harvested, and DNA was extracted and analyzed by CsCl density gradient centrifugation. Fractions were collected and precipitated in 5% trichloroacetic acid, and radioactivity was determined. The DNA profiles obtained in the presence and absence of ACG are plotted on the same figure for comparison. Arrows indicate the density of HSV DNA (1.729 g/cm³) or cellular DNA (1.705 g/cm³).

ent times after viral infection. To radiolabel with ^{32}P , the cells had to be grown in PO_4 -free medium as well. However, the R6-34 recombinant and the PAA⁻1 parental virus clearly show the highest resistance of viral DNA synthesis and the highest efficiency of plaqueing in the presence of ACG.

An altered viral DNA polymerase confers resistance to ACG-mediated inhibition. The extracts from HSV-2 WT (HG-52)-, R6-26-, and R6-34-infected cells incubated in the presence and absence of ACG (20 μM) were prepared at 18 h postinfection when ACG had been present (7 to 18 h). These cytoplasmic extracts were employed as an enzyme source in the assay of HSV DNA polymerase activity by using oligo(dG)₁₂₋₁₈·poly(dC) as a template primer and measuring the incorporation of [^{32}P]dGTP into acid-insoluble material after 10 min at 37°C. With each of the cellular extracts obtained without ACG treatment, the kinetics of polymerization proceeded linearly for 20 min. When increasing amounts of cellular extract were employed in the DNA polymerase assay a linear increase in incorporation of [^{32}P]dGTP into the synthetic template was also observed; this increase was almost completely inhibited by extracts prepared from ACG-treated HSV-2 WT- or R6-26-infected cells (Fig. 2A and B). The extract prepared from ACG-treated, R6-34-infected cells, however, exhibited linear kinetics of DNA polymerase activity similar to those of the extract of R6-34-infected cells prepared in the absence of ACG (Fig. 2C).

To exclude the possibility that the ACG-resistant DNA polymerase activity induced by R6-34 was merely due to the failure of R6-34-infected cells to make an active inhibitor, the amount of ACG-TP formed in cells infected with R6-26 and R6-34 in the presence of [^3H]ACG was measured directly by polyethyleneimine cellulose chromatography. It has been previously shown that the ACG-TP is the active inhibitor of HSV DNA polymerase (11, 12, 26). The amount of ACG-TP formed in R6-26- and R6-34-infected cells per microgram of cellular protein was very similar and was much greater than that formed in uninfected Vero cells (Table 1).

To confirm that the R6-34-infected cells induce an active inhibitor of DNA polymerase activity, the ability of heat-inactivated extracts from ACG-treated, infected cells to inhibit HSV DNA polymerase was compared. When 10 μl of an infected cell extract was heated at 60°C for 10 min and then added to an active cell extract, little inhibition was observed (97.9% of DNA polymerase activity remaining). An extract, prepared in the presence of ACG (20 μM , 7 to 18 h

FIG. 2. HSV DNA polymerase activity of extracts of BHK cells infected with recombinant viruses in the presence and absence of ACG. Cells were infected with HSV-2 (HG-52) WT (A), R6-26 (B), or R6-34 (C) in the absence (●) or presence (○) of ACG (20 μM), and extracts prepared at 18 h postinfection. HSV DNA polymerase activity was determined by measuring [^{32}P]dGMP incorporated with an oligo(dG)₁₂₋₁₈·poly(dC) template primer in standard assay for 10 min at 37°C as described in Table 3. Protein concentration was determined by method of Lowry et al. (20).

TABLE 1. Formation of ACG-TP in cells infected with recombinant viruses^a

Virus	Total ACG-TP formed (pmol)	ACG-TP formed (pmol/ μg of cell protein)
R6-26	100.5	5.0
R6-34	81.0	4.9
Vero alone	2.0	0.14

^a Confluent Vero cells in 90-mm dishes were infected at a multiplicity of infection 10, and at 6 h [^3H]ACG was added (200 $\mu\text{Ci}/\text{mmol}$). Cells were harvested at 18 h and extracted with HClO_4 , and ACG-TP was determined by polyethyleneimine cellulose chromatography. Protein in cellular extracts determined by method of Lowry et al. (20).

postinfection) and inactivated, produced 59.9% inhibition of the DNA polymerase activity induced by HSV-2 WT-infected cells. This same level of inhibition was observed after the addition of inactivated extracts prepared from ACG-treated, R6-26- or R6-34-infected cells (Table 2). Thus, infection with the WT HSV-2 or the two recombinants containing a single HSV-1 DNA

TABLE 2. Effect of ACG on HSV DNA polymerase activity of extracts of BHK cells infected with HG52, R6-26, and R6-34 virus

Cell extract ^a	Additions ^b	HSV DNA polymerase activity (% of control)	Relative inhibition (%) ^c
HG52		100.0	
	HG-52	97.9	
	HG-52 plus ACG	38.0	59.9
	R6-26	91.3	
	R6-26 plus ACG	37.5	53.8
	R6-34	99.4	
	R6-34 plus ACG	35.3	64.1
R6-26		100.0	
	HG-52	115.3	
	HG-52 plus ACG	30.6	84.7
	R6-26	138.3	
	R6-26 plus ACG	40.0	98.3
	R6-34	119.6	
	R6-34 plus ACG	30.2	89.4
R6-34		100.0	
	HG-52	105.0	
	HG-52 plus ACG	88.4	16.6
	R6-26	95.0	
	R6-26 plus ACG	85.1	9.9
	R6-34	66.1	
	R6-34 plus ACG	79.3	-13.2

^a BHK cells were infected with the indicated virus and grown in the absence or presence of ACG (20 μ M) from 7 h postinfection, and cell extract was prepared at 18 h postinfection. Heat-inactivated extract was prepared by heating at 60°C for 30 min to inactivate DNA polymerase. Samples (10 μ l) of extract alone or with 10 μ l of inactivated extract were incubated for 10 min at 37°C under HSV DNA polymerase assay conditions using 50 μ g of oligo(dG)₁₂₋₁₈·poly(dC) as template and 0.01 mM [³²P]dGTP (1,450 cpm/pmol). The values for 100% represent the incorporation of 71.4, 23.5, and 12.1 pmol of dGMP per μ g of cell protein for HG52; R6-26; and R6-34-infected cells, respectively.

^b Inactivated cell extract and ACG-treated cell extract were added as indicated.

^c Relative inhibition by ACG-treated inactivated extracts is the difference obtained in polymerase activity of a given extract assayed with mock-treated or ACG-treated inactivated cell extracts.

insertion in an otherwise HSV-2 genome induced similar amounts of an active inhibitor of HSV DNA polymerase activity. The DNA polymerase activity induced by the R6-26 recombinant was extremely sensitive to inhibition by the heat-activated extracts, revealing an 84 to 98% decrease in DNA polymerase activity. The DNA polymerase activity of the HSV-2 *ts6* mutant was inhibited by 58.9% by inactivated extracts,

a figure very similar to the inhibition observed for HSV-2 WT DNA polymerase. Extracts from infected cells alone produced no inhibition.

The DNA polymerase activity induced by the ACG-resistant recombinant R6-34 or the PAA^r-1 HSV-1 parent virus was markedly resistant to inhibition by inactivated extracts from cells infected in the presence of ACG. In the absence of any inhibitor, 12.1 pmol of [³²P]dGTP per μ g of protein was incorporated into DNA by the extract from R6-34 infected cells; this was decreased by only 16.6 to 9.9% after the addition of extracts from ACG-treated HSV-2 WT-, or R6-26-infected cells (Table 2). In the presence of an inactivated extract from ACG-treated, R6-34-infected cells, the R6-34-induced DNA polymerase was actually stimulated by 13%. The PAA^r-1 HSV-1 parent DNA polymerase was inhibited by only 11.7%. Thus, the R6-34 recombinant and its PAA^r-1 parent induced HSV DNA polymerase activity which was resistant to the ACG-induced inhibitor present in infected cell cytoplasm. This cytoplasmic inhibitor is ACG-TP, and the resistance of the R6-34-induced DNA polymerase to ACG-TP is due to an altered viral DNA polymerase.

The altered viral DNA polymerase is resistant to inhibition by didNTP's. The HSV DNA polymerase was purified from BHK cells infected with the recombinants and their parental strains by sedimentation in glycerol density gradients (Fig. 3). All four sedimentation experiments revealed a major peak of DNA polymerase activity sedimenting only slightly slower than the sedimentation standard, glyceraldehyde-3-phosphate dehydrogenase (molecular weight, 144,000). The peak has a molecular weight of approximately 142,000 to 144,000, the estimated molecular weight of the HSV DNA polymerase (17, 24). The major alkaline DNase activity and the dNTP's were clearly separated. The peak fractions possessing the DNA polymerase activity were further examined for their properties in high salt and with inhibitors of DNA polymerase activity, *N*-ethylmaleimide and PAA, two inhibitors previously shown to provide sensitive criteria for discriminating HSV DNA polymerases from cellular α and β DNA polymerases (28). All four of the DNA polymerases were clearly stimulated in the presence of 100 μ M ammonium sulfate by 5- to 10-fold when activated salmon sperm DNA was employed as a template (Table 3). A concentration of 2.5 μ M *N*-ethylmaleimide inhibited the observed DNA polymerase activity obtained from all four infections by greater than 95% (Table 3). By the criteria of molecular weight, stimulation by high salt, and inhibition by *N*-ethylmaleimide, therefore, the observed DNA polymerase activity pos-

FIG. 3. Purification of HSV DNA polymerase from parental and recombinant virus-infected cells. Extracts from cells infected with HSV-2 (HG52) *ts6* (A), HSV-1 PAA'-1 (B), R6-26 (C), and R6-34 (D) were layered on linear glycerol gradients (20 to 50%) and centrifuged (Sorvall TV 885 rotor, 45,000 rpm, 2°C, 15 h). Fractions were collected, and 10- μ l samples of each fraction were assayed for HSV DNA polymerase with activated salmon sperm DNA as a template (●). Alkaline DNase activity (○) was also determined under standard incubation conditions in 10- μ l samples of each fraction. Sedimentation markers were included in each gradient and are indicated as follows: 1, catalase; 2, glyceraldehyde-3-phosphate dehydrogenase; 3, bovine serum albumin; 4, cytochrome c; and 5, a mixture of [32 P]dATP and [32 P]dGTP.

essed the properties of the HSV DNA polymerase enzyme, and the DNA polymerase activity obtained after all four infections appeared to have a similar molecular weight.

The structure of ACG-TP is similar to that of the didNTP's in that these nucleoside analogs lack a 3'-OH group, preventing chain elongation (2). The didNTP's are well-established inhibitors of DNA synthesis which become incorporated into the primer and produce chain termination. The four didNTP's were compared for the ability to inhibit the viral DNA polymerase from the two recombinants and their parental strains. Initial experiments employing oligo-(dG) $_{12-18}$ ·poly(dC) as a template primer to measure the incorporation of [32 P]dGTP indicated that the R6-34-induced DNA polymerase in cytosol extracts was resistant to inhibition by didGTP. Viral DNA polymerase activity in extracts of HSV-2 WT (strain HG-52)-, R6-26-, and R6-34-infected cells was compared, and the concentration of didGTP required to inhibit 50% of the DNA polymerase activity (50% inhibitory dose) was determined. The 50% inhibitory dose values for the viral DNA polymerase induced by HSV-2, R6-26, and R6-34 were 5, 1, and 74 μ M of didGTP, respectively (data not shown). Thus, the R6-34 DNA polymerase activity required 74 times more didGTP for 50% inhibition than did the DNA polymerase induced by R6-26.

The inhibition constants (K_i) for all four didNTP's with activated DNA were determined with the purified viral DNA polymerase from

TABLE 3. Template characteristics and apparent kinetic constants of the partially purified HSV DNA polymerase enzymes of parental and recombinant viruses

Virus	HSV DNA polymerase activity (pmol incorporated) under the following conditions ^a :				K_m (μ M) ^b		K_i (μ M) ^b	
	SS DNA	SS DNA plus AS	SS DNA plus AS plus NEM	Oligo(dG) $_{12-18}$ ·poly(dC)	dNTP's	dGTP	didNTP's	PAA
<i>ts6</i>	11.2	57.1	1.4	27.5	10.8	22.3 $\pm$ 5%	22.6	8.2
PAA'-1	13.5	128.2	6.1	57.0	11.4	22.3 $\pm$ 5%	122.5	37.5
R6-26	9.1	85.2	1.0	25.8	11.9	22.3 $\pm$ 5%	14.7	4.5
R6-34	14.6	142.9	2.4	69.9	10.9	22.3 $\pm$ 5%	135.1	39.0

^a HSV DNA polymerase activity was determined under standard incubation conditions (20 min at 37°C) with either 100 μ g of activated salmon sperm (SS) DNA per ml or 25 μ g of oligo(dG) $_{12-18}$ ·poly(dC) per ml with or without 100 mM ammonium sulfate (AS) and 2.5 mM *N*-ethylmaleimide (NEM). The source of viral DNA polymerase was the peak fraction of enzyme activity from the glycerol gradient shown in Fig. 3.

^b The K_m data for dGTP and dNTP's and the K_i data for didNTP's were derived from Fig. 4 and 5. The amounts of protein used were 6.2, 6.4, 5.9, and 6.9 g for *ts6*, PAA'-1, R6-26, and R6-34, respectively. For the K_m and K_i determination, one-half the amount of enzyme was used. The K_i for PAA was calculated from graphs by plotting PAA concentration versus V_{max}/v on a Dixon plot. The K_m for dGTP was derived from Fig. 5 by using 25 μ g of oligo(dG) $_{12-18}$ ·poly(dC) per ml as a template.

the recombinants and the parental strains (Fig. 4 and Table 3). The units of initial enzyme velocity (v) were expressed as picomoles of dTMP incorporated per microgram of protein during a 20-min assay; enzyme activity was linear over 20 min. In the Lineweaver-Burke double-reciprocal plot of $1/v$ versus $1/(\text{dNTP})$, the competitive inhibition-type kinetics expected for a chain-terminating compound were observed (Fig. 4). All four of the didNTP's had the same K_i for activated DNA. The K_i for each of the didNTP's with viral DNA polymerase was lowest for R6-26 (K_i , 14.5 μM) followed by HSV-2 *ts6* (K_i , 22.6 μM) and was highest with the purified DNA polymerases from HSV-1, PAA⁻-1 (K_i , 122.5 μM) and R6-34 (K_i , 135.1 μM).

FIG. 4. Determination of K_m for dNTP's and K_i for didNTP's of parental and recombinant HSV DNA polymerases. HSV DNA polymerase activity was assayed on the purified peak fractions from Fig. 3 under standard incubation conditions (20 min at 37°C) with 100 μg of salmon sperm DNA per ml by using all four dNTP's at equivalent concentrations. The rate of incorporation of [^{32}P]dTTP in percent in the absence (●) or presence (○) of 50 mM of each of the four didNTP's was used as the initial velocity (v) in the double-reciprocal Lineweaver-Burke plot. The enzyme activity was linear over 20 min. In the absence of inhibitor, 100% represents specific activity of 9.2, 18.4, 15.4, and 22.8 pmol of dTMP per μg of protein for *ts6* (A), PAA⁻-1 (B), R6-26 (C), and R6-34 (D), respectively.

The peak fractions of purified DNA polymerase were also employed to test for the sensitivity of the enzyme activity to inhibition by PAA. Activated salmon sperm DNA was employed as a template; the reactions were linear over 20 min, and the K_i for PAA was determined from the Dixon plot of V_{max}/v versus PAA. The K_i for PAA was again lowest for R6-26 (K_i , 4.5 μM) followed by HSV-2 *ts6* (K_i , 8.2 μM) and was highest with the purified polymerases from HSV-1 PAA⁻-1 (K_i , 37.5 μM) and R6-34 (K_i , 39.0 μM) (Table 3). The comparative K_i values for PAA and for the four didNTP's with the parental and recombinant DNA polymerases correlated extremely well with the resistance of these enzymes to ACG-TP, to the resistance of viral DNA synthesis in the presence of ACG, and to the resistance of HSV plaque formation to ACG (8).

To exclude the possibility that resistance of viral DNA polymerase to ACG-TP, PAA, and didNTP's is due to an altered affinity of the resistant polymerase for dNTP's, the K_m values for each of the dNTP's were determined by using activated salmon sperm DNA or oligo-(dG)₁₂₋₁₈·poly(dC) as a template. The K_m values were essentially identical for all four dNTP's or for dGTP with the synthetic template for all four viral polymerases (Table 3 and Fig. 5). The altered viral DNA polymerase activity was not due to an altered K_m .

FIG. 5. Determination of K_m for dGTP. The peak fractions from Fig. 3 were employed to measure the HSV DNA polymerase activity in the parental and recombinant viruses. HSV DNA polymerase was assayed under standard conditions (10 min at 37°C) with oligo(dG)₁₂₋₁₈·poly(dC) (25 $\mu\text{g}/\text{ml}$) as a template, and the rate of incorporation of [^{32}P]dGTP in percentage of complete incorporation was used to calculate initial velocities in the double-reciprocal plot. The specific activities for the DNA polymerase of HSV-2 *ts6* (○), HSV- PAA⁻-1 (●), R6-26 (Δ), and R6-34 (□) were 0.23, 0.78, 0.33, and 0.19 pmol/ μg of protein per min, respectively.

To exclude the possibility that the recombinants differed in the expression of viral deoxypyrimidine kinase, the enzyme required for the phosphorylation of ACG (11, 25), this enzyme activity was measured directly in the extracts which were employed in the DNA polymerase assay. Viral deoxypyrimidine kinase activity (units per microgram of protein) was 4.4, 4.0, and 3.2 for the HSV-2 WT-, R6-26-, and R6-34-infected cell extracts, respectively, in the absence of ACG. In the presence of ACG (20 μ M), 7.2, 6.8, and 8.0 U/ μ g of protein were detected for the HSV-2 WT-, R6-26-, and R6-34-infected cell extracts, respectively. The ACG-treated cellular extracts exhibited a twofold increase in thymidine kinase activity in all cases. Since alkaline DNase activity could interfere with the template in the DNA polymerase assay and perhaps account for apparent differences in viral DNA polymerase activity induced by different viruses, the alkaline DNase activity induced by the recombinant viruses was also determined. It had previously been shown, however, that under the assay conditions employed here an oligo-(dG)₁₂₋₁₈·poly(dC) template was not hydrolyzed by alkaline DNase (16). Alkaline DNase activity was similar in all extracts, and the extracts prepared with R6-26 and R6-34-infected cells in the presence and absence of ACG exhibited identical specific activities. The activities of these two viral specific enzymes appeared to be identical in cells infected with these recombinant viruses, and it is unlikely that they play any significant role in the differences observed in the viral DNA polymerase activities induced by these recombinants.

DISCUSSION

In this study we have employed HSV-1/HSV-2 intertypic recombinants with well-characterized genome structures to demonstrate that the mutations which confer resistance of HSV to ACG or PAA produce an altered HSV DNA polymerase activity which exhibits resistance to three inhibitors of DNA polymerase.

Two recombinants, R6-34 and R6-26, which contain HSV-1 DNA insertions within the DNA polymerase locus of an otherwise HSV-2 genome were selected for further analysis and purification of the viral DNA polymerase. R6-34 has an HSV-1 DNA insertion extending from map units 39.6 to 42.8 including the region of uncertainty and is ACG^r and PAA^r. The R6-26 recombinant contains an HSV-1 DNA insertion starting from the same HSV-1 *Bg*III i-d site on the left extending from map units 39.6 to 41.0, including the region of uncertainty, and is PAA^s, and ACG^s. The mutations in HSV-1 DNA which

confer resistance to PAA and ACG in the R6-34 recombinant must be contained in the DNA sequences defined by map units 41.0 to 42.8. By analysis of two other recombinants, R6-19 and R6-30, these limits can be further narrowed to the DNA sequences defined by the HSV-2 *Bam*HI h'-j' site to the HSV-1 *Kpn* x-c site (map units 41.0 to 41.8) or 1.3 kilobase pairs (5, 6, 8). This is sufficient genetic information to encode for an amino acid sequence of 42,000 daltons either as a separate polypeptide or part of a larger protein. This region of DNA sequences is within the mapping limits of the HSV-1 (Kos) *ts*D9 mutation defining the structural gene for HSV DNA polymerase (5) and is likely to include the DNA sequence defining the active center of the HSV DNA polymerase.

These two recombinants induce identical amounts of thymidine kinase and alkaline DNase activity in the presence and absence of ACG (8), and the differences in sensitivity to ACG and PAA cannot be explained by a difference in the activities of these two enzymes. These two recombinants induce identical amounts of ACG-TP, the active cytoplasmic inhibitor of viral DNA polymerase, in the presence of ACG. The R6-34 recombinant induces a viral polymerase which is resistant to ACG-TP, whereas the R6-26 recombinant and the HSV-2 WT induce DNA polymerase activities which are sensitive to ACG-TP.

The purified DNA polymerase induced by these two recombinants and the parental strains, HSV-2 *ts*6 (strain HG52) and HSV-1 strain 17 (*ts*⁺, *paa*⁻¹), possess a similar molecular weight of 142,000–144,000 on sedimentation in glycerol density gradients. The polymerase activities were stimulated by high salt, inhibited by *N*-ethylmaleimide, and possessed the properties of HSV DNA polymerase. The peak fractions of the purified viral DNA polymerase were employed to test for the sensitivity of HSV DNA polymerase to PAA, and the viral DNA polymerases induced by R6-34 and the PAA⁻¹ parent were resistant to inhibition by PAA, possessing a high *K_i*.

Partially purified viral polymerases were also tested for sensitivity to inhibition by the four didNTP's. These dideoxy analogs have been instructive regarding the mechanism of polymerization by the *Escherichia coli* DNA polymerase, Pol I (2). The didNTP serves as polymerase substrate to elongate a DNA chain by one residue. A chain terminated with such a dideoxynucleoside is inert to further elongation and relatively inert to exonuclease action at the primer terminus or to attack by PP_i. The didNTP, lacking a 3'-OH group vital for chain growth, is still bound to the dNTP binding site

on the enzyme with the same affinity as the natural triphosphate and forms a polymerization complex protecting the primer terminus from nucleolytic attack. With the HSV DNA polymerases induced by the R6-26 recombinant and the HSV-2 *ts6* parent, the didNTP's act as effective competitive inhibitors, revealing low K_i values (Table 3). It is of interest that the apparent K_i of the human leucocyte polymerase for didTTP is 9.6 μ M (1), a value in general agreement for the K_i values of the sensitive HSV DNA polymerases for didNTP's (14.7 to 22.6 μ M).

The viral DNA polymerase of the R6-34 recombinant or the HSV-1 PAA'-1 parent, however, was markedly resistant to inhibition by all four didNTP's. This suggests that the mutation which confers resistance of these viral polymerases to ACG-TP may confer resistance to all four didNTP's as well. The data presented in Fig. 2 indicating the effect of ACG-TP in the cellular extract on inhibiting dGTP polymerization by the viral polymerases of R6-26 and R6-34 with the oligo(dG)₁₂₋₁₈·poly(dC) template is instructive concerning the role of ACG-TP in binding to the viral polymerase. ACG-TP has previously been shown to bind to the HSV DNA polymerase and act as a competitive inhibitor (11, 26). The R6-34 DNA polymerase is not affected by the presence of ACG-TP and is probably not bound by the inhibitor, whereas the DNA polymerase of R6-26 is markedly inhibited by the presence of ACG-TP, presumably by binding to the viral polymerase (Fig. 2B and C). Since the didNTP binds to the triphosphate binding site on the *E. coli* Pol I enzyme, and the HSV DNA polymerase presumably also possesses such a site, a mutation in the DNA polymerase locus on HSV which results in preventing any analog lacking a 3'-OH group from binding with the same affinity as the natural triphosphate to the triphosphate binding site may allow polymerization to proceed. This mutation could result in a more stringent recognition by the triphosphate binding site of the enzyme and could result in a failure to incorporate compounds lacking a 3'-OH group. Since the proposed site of action of PAA on the HSV DNA polymerase is at the pyrophosphate exchange site, a mutation which confers resistance to PAA and ACG-TP and didNTP suggests that the PP_i exchange site is very closely adjacent to the triphosphate binding site in the enzyme. These results provide additional support for the proposal that HSV DNA polymerase conforms to the model of DNA polymerase advanced by Kornberg, i.e., that DNA polymerase possesses an active center containing multiple sites, each

with a different function (18). A mutation producing an alteration in one function of the active center may affect the others as well. It has been shown that these functions are closely linked in the *E. coli* Pol I enzyme, and a consequence of chain termination by didNTP's is the inhibition of PP_i exchange and exonuclease action (2). In HSV DNA polymerase, the 3'→5' exonuclease and PP_i exchange functions may be closely associated because PAA, an inhibitor of pyrophosphate exchange, inhibits 3'→5' exonuclease function to a similar degree (17). Unlike didNTP's or ACG-TP, however, PAA does not get incorporated into DNA, and it does not serve as a substrate for HSV DNA polymerase (3, 22).

An alternative explanation for the observed resistance of the R6-34 and PAA'-1 DNA polymerases to inhibition by didNTP is that these altered polymerases possess an enhanced ability to excise these analogs by the 3'→5' exonuclease, or "editing," function of HSV DNA polymerase. After incorporation of an altered analog, the 3'→5' exonuclease function catalyzes the removal of 3'-terminal nucleosides from the primer template as well as the template-dependent conversion of dNTP's to monophosphates (17). A DNA polymerase does not require such an exonuclease function to be resistant to the inhibitory effects of didNTP's as both eucaryotic polymerases of CV-1 cells (10) and leukocytes from patients with human myelogenous leukemia (1) are resistant to inhibition by the analog didTTP. The cellular α polymerase from myelogenous leukemia cells is sensitive to inhibition by didTTP, however (1). Since neither the eucaryotic α nor β polymerase possesses exonuclease function, and since they differ in their sensitivity to didTTP, the absence of the exonuclease function does not explain resistance. The mechanism of resistance to didTTP exhibited by α polymerase most likely is due to its ability to discriminate between didTTP and dTTP, preventing incorporation of the didTTP.

A potential objection to the use of hybrid genomes for the purpose of genetic analysis of herpes viral functions is that the recombinant genome will produce new recombinant polypeptides which are so altered from parental proteins that conclusions about parental functions are not valid. This appears to be an unlikely possibility in the present case, however, because the altered resistance of the DNA polymerase of the R6-34 recombinant is observed in the parental HSV-1 PAA'-1 virus as well. The apparent K_i values for the didNTP's are very similar for the DNA polymerase of the R6-34 recombinant and the HSV-1 PAA'-1 parent (K_i , 135.1 and 122.5 μ M, respectively). In addition, the K_i values for

the DNA polymerase of the R6-26 recombinant and the HSV-2 *ts6* parent are also very similar, and these two strains are sensitive to PAA and ACG. The molecular weights of all four viral DNA polymerases are identical in the glycerol gradient analysis and are the same as the molecular weight of the HSV-1 DNA polymerase (17, 24).

The HSV-1 sequences which define the mutations for *acg*^r and *paa*^r map in equivalent positions in both HSV-1 and HSV-2 (5, 6, 8). The type-specific sensitivity to ACG in HSV-1 and HSV-2 can be transferred separately from the *acg*^r mutation (8). These arguments suggest that the viral functions in the parental strains are represented faithfully in the recombinants and that the recombinant genomes provide useful probes for the separation and analysis of viral gene functions.

This report illustrates the utility of combining fine structure mapping of the HSV DNA polymerase locus with the use of specific inhibitors of DNA polymerase functions to separate the functions of the HSV DNA polymerase. This is a goal which has previously been realized only with procaryotic polymerases. In defining a 1.3-kilobase-pair region of DNA sufficient to code for an amino acid sequence of 42,000 daltons, which contains mutations to three DNA polymerase inhibitors of dissimilar structure, we have defined the region coding for the active center of the HSV DNA polymerase molecule. PAA, a PP_i analog inhibiting PP_i exchange (19); didNTP analogs lacking a 3'-OH group and shown to bind to the triphosphate binding site on *E. coli* Pol I (2); and ACG-TP, a compound which serves as a substrate for the HSV DNA polymerase, is a competitive inhibitor of dGTP (11, 12, 26), and has many features in common with the didNTP's, act on separate functions in the HSV DNA polymerase; all inhibit the polymerization reaction. Whether one mutation or three separate ones account for the HSV DNA polymerase resistance to these compounds, the fact that the resistance mutations map in such a small region of the DNA provides support that this region defines an active center for the HSV DNA polymerase. Kornberg has described an active center as a specially adapted polypeptide surface which recognizes and accommodates several nucleoside structures (18), and these studies define such a region on the HSV DNA polymerase polypeptide. If the HSV DNA polymerase has a molecular weight of 140,000 to 150,000, the largest limits of this active center would be molecular weight 42,000, about a third of the enzyme molecule. Further definition and subdivision of this region will require similar studies on mu-

tants and recombinants which separate resistance mutations to inhibitors of HSV DNA polymerase.

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