Analysis of the Thymidine Kinase Genes from Acyclovir-Resistant Mutants of Varicella-Zoster Virus Isolated from Patients with AIDS

CHRISTINE L. TALARICO,* WILLIAM C. PHELPS, AND KAREN K. BIRON

*Division of Virology, Wellcome Research Laboratories, 3030 Cornwallis Road,
Research Triangle Park, North Carolina 27709*

Received 12 June 1992/Accepted 13 November 1992

Patients with AIDS often experience recurrent infections with varicella-zoster virus (VZV) requiring repeated or prolonged treatment with acyclovir (ACV), which may lead to the development of ACV resistance. The ACV resistance of isolates recovered from such patients is associated with diminished VZV thymidine kinase (TK) function. We determined the nucleotide sequences of the TK genes of 12 ACV-resistant VZV strains purified from nine patients with AIDS. Five VZV strains contained nucleotide deletions in their TK genes, introducing a premature termination codon which is expected to result in the production of a truncated protein. No detectable full-length TK protein could be immunoprecipitated from extracts of cells infected with these virus strains. These TK-deficient strains were cross resistant to the TK-dependent antiviral agents ACV, 9-(4-hydroxy-3-hydroxymethylbutyl)-guanine (penciclovir), and 1- β -D-arabinofuranosyl-5-(2-bromovinyl)uracil (BVaraU). The remaining seven strains each contained a nucleotide change that resulted in an amino acid substitution in the TK protein. These substitutions occurred throughout the TK protein, namely, in the ATP-binding site, the nucleoside-binding site, between the two binding sites, and at the carboxy terminus of the protein. We determined the effects of these mutations on the stability of TK protein expression in virus-infected cells and on the sensitivity of mutants to the TK-dependent antiviral agents ACV, BVaraU, and penciclovir.

Varicella-zoster virus (VZV), a member of the herpesvirus family, encodes a 35-kDa thymidine kinase (TK) protein that possesses both thymidine- and thymidylate-phosphorylating activities (20, 34, 51). The VZV TK protein has approximately 28% overall amino acid sequence homology with the herpes simplex virus (HSV) TK protein; however, two noncontiguous regions that are proposed to be involved in nucleotide (ATP) and nucleoside (substrate) binding show 55 and 64% homology, respectively (27, 49). Single-amino-acid substitutions in either of these conserved sites alter the enzymatic activity of both the HSV and the VZV TK proteins (10, 29, 30, 33, 49) and confer resistance to TK-dependent antiviral agents such as acyclovir (ACV) and 1- β -D-arabinofuranosyl-5-(2-bromovinyl)uracil (BVaraU; brovir; Sorivudine). VZV inhibition by these antiviral agents is due to the competitive inhibition of the viral DNA polymerase by nucleoside triphosphates. For ACV triphosphate, HSV inhibition has been shown to result from both DNA chain termination and enzyme inactivation (14, 42). ACV is selectively phosphorylated by the viral TK protein to its monophosphate derivative, which is then converted by host cellular kinases to the triphosphate form. By contrast, BVaraU is phosphorylated to the diphosphate form by the viral thymidine and thymidylate kinase activities (54). The triphosphate forms of these antiviral agents act as preferred substrates for the viral DNA polymerase and disrupt viral DNA synthesis (12, 18, 19, 36, 45, 50).

ACV is an effective treatment for mucocutaneous HSV infections (38, 53, 55) and has shown efficacy in the treatment of primary and recurrent VZV infections. Immuno-compromised patients, especially those with AIDS, often experience recurrent infections with VZV that require prolonged treatment with ACV. A lack of clinical response has

been correlated with the presence of ACV-resistant VZV in several patients with AIDS (25, 41, 47). Most of the ACV-resistant VZV isolates recovered from these patients contained either TK-deficient or TK-altered virus, although there were two documented cases in which VZV with a DNA polymerase-altered phenotype was associated with ACV resistance (41, 47).

The molecular basis of ACV resistance in VZV has been studied primarily with laboratory-derived resistant mutants. This study represents the first comprehensive analysis of the mutations in VZV associated with clinical ACV resistance. In this study, we sequenced the TK genes from 12 virus strains purified from isolates of nine AIDS patients whose VZV infections had shown poor clinical responses to chronic ACV therapy. VZV isolated from the lesions of these nine patients was resistant to ACV *in vitro* but was sensitive to the polymerase-dependent drugs foscarnet (PFA) and vidarabine (araA) (24, 32, 46). This finding is consistent with the presence of a TK mutation. We determined the nucleotide sequence changes in the TK gene associated with ACV resistance and the effect of these mutations on the level of TK protein produced during virus infection. Finally, the sensitivities of these virus strains to other TK-dependent antiviral agents were determined.

MATERIALS AND METHODS

Virus and cell strains. Clinical VZV isolates were obtained from cutaneous lesions of patients who failed to respond to ACV treatment. The detailed clinical histories and ACV therapies of five of these patients, corresponding to isolates 8807, 8808, 8811, 8812, and 9012, have been described previously (25, 47). Each isolate was assigned a number based on the year it was isolated (first 2 digits) and its order of arrival in the laboratory in that year (last 2 digits). Drug sensitivity phenotypes were determined for these mixed populations, and the viral TK activities were assessed by

* Corresponding author.

[¹²⁵I]iododeoxycytidine ([¹²⁵I]IdC) incorporation. The virus isolates were then plaque purified by three cycles in human diploid embryonic fibroblasts (MRC-5) with cell-free virus supernatants; plaque-purified strains are denoted by a hyphen followed by a number. In this article, we refer to a strain as the genetically pure population of virus derived from an isolate by plaque purification of cell-free virus. The VZV wild-type strain Ellen was obtained from the American Type Culture Collection, and the Ellen ACV⁺ 3-5-2 strain was selected by serial passage in ACV (1). Cell-associated virus stocks were prepared, maintained at -135°C, and used as infected cells in all studies. MRC-5 cells (passage level, 26 to 31) (Whittaker M. A. Bioproducts) were cultured in minimal essential medium supplemented with 8% fetal bovine serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. The 143B cells, a TK⁻ human osteosarcoma cell line, were obtained from the American Type Culture Collection and cultured in the same media as the MRC-5 cells except for the addition of 15 µg of bromodeoxyuridine per ml.

Antiviral agents. ACV was synthesized at Burroughs Wellcome Co. (Research Triangle Park, N.C.), and penciclovir was synthesized at Wellcome Laboratories (Beckenham, United Kingdom). araA was obtained from Parke-Davis Co. (Morris Plains, N.J.), PFA was obtained from Fluka AG (Buchs, Switzerland), and BVaraU was kindly provided by A. K. Field of Bristol-Myers Squibb Co. (Princeton, N.J.).

Isolation of VZV DNA. VZV DNA was extracted from nucleocapsids as previously described (52), except that 125 µg of DNase and 120 µg of RNase were added following the resuspension of cell pellets in lysis buffer. Briefly, VZV-infected cells (90 to 100% infected) were harvested in phosphate-buffered saline (PBS), freeze-thawed, and treated with DNase and RNase. Trichlorotrifluoroethane was added, and the cellular membranes were removed by centrifugation. Virions were pelleted, treated with proteinase K, and phenol extracted. DNA was pelleted, resuspended in Tris-EDTA (TE), and subsequently used for TK gene amplification by polymerase chain reaction (PCR).

Amplification, cloning, and sequencing of the TK gene. PCR primers that contained *Eco*RI and *Bam*HI sites at the 5' end of the TK-coding region and *Hind*III and *Kpn*I restriction sites at the 3' end were designed. The sequences of the primers are as follows: 5' end, GCGAATTCGGATCCC ATATCTCAACGGATAAAACCGATG, and 3' end, CCAA GCTTGGTACCTTAGGAAGTGTTCCTGAACGG. The TK DNA of each strain was amplified in three independent PCRs. One microliter (~1.0 µg) of the VZV genomic DNA sample was added to three 100-µl PCR mixtures containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (wt/vol) gelatin, 200 µM (each) deoxynucleotide, 1.0 µM (each) primer, and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). The template was denatured for 1 min at 94°C, and primers were annealed to the DNA at 65°C for 2 min and extended at 72°C for 3 min for 30 cycles.

Ninety microliters of each of the reaction mixtures was electrophoresed through a 1.0% low-melting-temperature agarose gel, and the agarose was removed from the DNA with an NACS column (Bethesda Research Laboratories). The DNA was cleaved with *Eco*RI and *Hind*III and ligated into pUC18. For each strain, the coding strand of the TK gene of one of the three clones was sequenced by using a Sequenase Version 2.0 kit (U.S. Biochemical), the universal primer, and a series of primers that spanned the coding region of the TK gene. The sequence data obtained were compared with those reported for the TK gene of the VZV

strain Dumas (11). Any sequence changes found in the clinical strains were confirmed by sequencing the remaining two clones.

Immunoprecipitation. Expression of the TK protein in VZV-infected cells was analyzed by a modified immunoprecipitation method of Harlow and Lane (23). MRC-5 cells were infected with VZV until a 90 to 100% cytopathic effect was observed. The cellular and viral proteins were labeled with 500 µCi of [³⁵S]Cys (Trans-label; ICN) in a 100-mm-diameter dish for 6 h. Cells were lysed with 600 µl of a solution (radioimmunoprecipitation assay [RIPA] buffer) containing 20 mM morpholinepropanesulfonic acid (MOPS) (pH 7.0), 150 mM NaCl, 1% (vol/vol) Nonidet P-40, 1% (vol/vol) deoxycholate, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), and 1 mM EDTA (pH 7.0) and 100 µg of each of the protease inhibitors phenylmethylsulfonyl fluoride, *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), aprotinin, and tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK). The cellular debris was removed, and the soluble proteins were immunoprecipitated with a 1:10 dilution of a polyclonal rabbit antiserum specific for the VZV TK protein expressed in *Escherichia coli* (43). The resulting immunoprecipitation complex was recovered with protein A-Sepharose, washed with RIPA buffer and disassociated with 5 M urea in RIPA buffer for 15 min at room temperature. After the addition of 1 ml of RIPA buffer (to dilute the urea), the proteins were again immunoprecipitated overnight, and the immune complex was recovered with protein A-Sepharose, washed with RIPA buffer, and denatured by boiling for 3 min in a buffer containing 62.5 mM Tris (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, 2% SDS, and 0.005% bromophenol blue. Protein was electrophoresed on an SDS-10% acrylamide gel, treated with En³Hance (DuPont), and exposed to Kodak XAR X-ray film.

Antiviral sensitivity assay. Drug sensitivities were determined by the Hybriwix DNA hybridization assay of Dankner et al. (8) modified for VZV infection (Diagnostic Hybrids, Inc., Athens, Ohio). Specifically, the virus inoculum consisted of 1,000 infected cells per well of a 24-well culture plate, and the assay incubation time was approximately 4 days. Data were analyzed by using a regression analysis program (SAS Probit, procedure 82.4; SAS Institute, Inc., Cary, N.C.).

[¹²⁵I]IdC plaque autoradiography. VZV TK activity in infected cells was assessed by a modification of the method of Martin et al. (37). TK-competent VZV phosphorylates and subsequently incorporates [¹²⁵I]IdC into replicating DNA, while TK-negative or -deficient VZV does not. Briefly, confluent MRC-5 cells were infected with approximately 75 to 100 VZV plaque-forming cells and incubated at 37°C. When plaque formation was adequate, 4 to 5 days postinfection, the cells were labeled with 4 µCi of [¹²⁵I]IdC (ICN Pharmaceuticals, Inc.) per 60-mm-diameter dish for 6 h at 37°C. After being labeled, the monolayers were washed twice with PBS, fixed with 10% formalin, and stained with 0.8% crystal violet in 50% ethanol. The rims of the plates were removed, and the cells were exposed to Kodak XAR film for 10 days at room temperature. The TK⁺ virus was visualized by the presence of a dark ring surrounding the virus plaques. All of the VZV plaques were counted, and the percentage of TK⁺-versus-TK⁻ virus was determined.

[¹⁴C]TdR plaque autoradiography. To assess the ability of the VZV TK protein to phosphorylate the natural substrate thymidine, we measured the incorporation of [¹⁴C]thymidine ([¹⁴C]TdR) into the DNA of VZV-infected cells with the TK-negative cell line 143B. Subconfluent 143B cells were

TABLE 1. Sensitivities to antiviral agents, TK protein immunoprecipitation status, and [¹²⁵I]IdC plaque autoradiography of patient VZV isolates

Patient isolate or strain	ED ₅₀ (μM) with:		Result of:	
	ACV ^a	PFA	Protein immunoprecipitation ^b	Plaque autoradiography (% TK incorporating [¹²⁵ I]IdC) ^c
8807	69 ^d	119 ^d	—	0
8808	121 ^d	116 ^d	—	0
8811	59 ^d	106 ^d	—	10 (wild type)
8812	90 ^d	152 ^d	+	50 (reduced)
8901	380	83	+	0
8919	99	116	+	100 (reduced)
9005	33	84	—	0
9006	73	127	±	100 (reduced)
9007	24	117	+	0
9012	89	176	—	4.5 (wild type)
9014	66	109	—	0
9018	50	100	—	0
9020	146	92	—	0
Oka (control)	6.5 ^e	108 ^e	ND	100
Ellen (control)	3.8 ^f	146 ^f	+	100

^a The ACV assay included 4 U of thymidine phosphorylase per ml.

^b TK protein was immunoprecipitated with a VZV TK antibody (Fig. 1). +, high expression; —, no expression; ±, low expression.

^c Percent [¹²⁵I]IdC incorporation was determined by counting all VZV plaques and dividing the number of plaques that showed incorporation of [¹²⁵I]IdC, visualized by a dark ring surrounding each viral plaque, by the total number of plaques times 100. Descriptions in parentheses are of levels of incorporation. Reduced, virus-infected cells weakly incorporated [¹²⁵I]IdC.

^d Data are from Jacobson et al. (25).

^e Average of four assays.

^f Average of three assays.

infected with approximately 75 to 100 plaque-forming cells of VZV; overlaid with 0.8% agarose in minimal essential medium supplemented with 10% fetal calf serum, 1% glutamine (200 μM), and 1% penicillin-streptomycin; and incubated at 37°C. When plaque formation was optimal (2 to 4 days postinfection), the cells were labeled with 0.1 μCi of [¹⁴C]TdR (ICN Pharmaceuticals, Inc.) per 60-mm-diameter dish for 6 h at 37°C. After being labeled, the agarose overlay was removed, and the monolayers were processed as described for the [¹²⁵I]IdC assay.

RESULTS

Analysis of clinical isolates. VZV clinical isolates were obtained from cutaneous lesions of 13 patients with AIDS who had responded poorly to ACV treatment. ACV resistance in HSV and VZV may be associated with alterations in either the TK or the polymerase gene (6, 9, 17, 47, 50). Therefore, to obtain a preliminary determination of the nature of ACV resistance in the unpurified VZV isolates, 50% effective doses (ED₅₀s) were determined for the TK-dependent antiviral agent ACV and the polymerase-dependent antiviral agents PFA (Table 1) and araA (not shown). When these values were compared with the ED₅₀s of the wild-type Ellen and Oka strains, it was observed that the clinical isolates were resistant in vitro to ACV, with ED₅₀s that were 4- to 100-fold higher than the wild-type ED₅₀s. All isolates remained sensitive to the DNA polymerase inhibitors PFA (Table 1) and araA (not shown). These results suggested that these isolates contained an alteration in the TK gene rather than in the polymerase gene.

To assess the relative levels of VZV TK protein expressed during infection of MRC-5 cells, infected cells were lysed and analyzed by immunoprecipitation with a rabbit polyclonal antibody to the VZV TK protein. As shown in Fig. 1, infection with the 13 different clinical samples resulted in the expression of different amounts of TK protein. The isolates

designated 8812, 8901, 8919, and 9007 expressed levels of TK protein similar to those of the wild-type strain (Ellen). In contrast, infection with 8807, 8808, 8811, 9005, 9012, 9014, 9018, and 9020 resulted in the expression of little or no TK protein, and isolate 9006 expressed a reduced amount of VZV TK protein in infected cells. We chose a subset of these isolates to plaque purify for analysis of the TK DNA sequence, protein expression in infected cells, and sensitivity to other TK-dependent antiviral agents. The four isolates that produced wild-type levels of protein (8812, 8901, 8919, and 9007) and five of the isolates that expressed either reduced levels of TK (9006) or no detectable TK (8807, 8808, 8811, and 9012) protein were chosen for further analysis.

Characterization of purified VZV strains. Because ACV-resistant VZV clinical isolates may contain mixtures of virus with wild-type and one or more drug-resistant phenotypes (15, 41, 46), individual virus plaques were purified for further analysis. For three of the VZV isolates, 8811, 8812, and

FIG. 1. SDS-PAGE analysis of immunoprecipitation of the VZV TK protein from MRC-5 cells infected with patient isolates. Lanes: Ellen, wild-type VZV strain; 3-5-2, laboratory-derived TK-deficient VZV strain; cell, uninfected MRC-5 cells. The position of the VZV TK protein is indicated on the right.

FIG. 2. Schematic diagram of plaque purification of VZV isolates and the methods by which TK function and expression were analyzed at each step in the purification. ACV-resistant VZV isolates were plaque purified through three cycles in MRC-5 cells; the TK gene of VZV was amplified by PCR; and the PCR products were gel purified, digested with *Eco*RI and *Hind*III, and cloned into pUC18. The TK gene was then sequenced by the dideoxynucleotide chain termination method. *, VZV isolates not plaque purified or further analyzed.

9012, the [¹²⁵I]IdC plaque autoradiography data showed the presence of 10, 50, or 4.5% of virus, respectively, that incorporated [¹²⁵I]IdC into their DNA at wild-type levels or incorporated [¹²⁵I]IdC at a reduced level compared with that of wild-type virus (Table 1). Multiple viruses from two of these isolates, 8811 and 8812, were plaque purified (Fig. 2); however, on the basis of [¹²⁵I]IdC incorporation data (Table 3), the TK⁺ virus phenotype was not recovered. On the basis of TK sequence analysis, the two genetically pure populations (8811-4 and 8811-13) derived from the 8811 isolate were identical. However, more than one TK variant was represented among the three strains plaque purified from isolate 8812; two (8812-1 and 8812-10) had an identical base substitution in their TK sequences, while the third (8812-5) harbored a different mutation. Only one virus strain representative was plaque purified from each of the remaining seven isolates: 8807, 8808, 8901, 8919, 9006, 9007, and 9012.

In the case of isolate 9007, the final virus strain, designated 9007-1, which was genetically purified from the mixed isolate, did not reflect the TK phenotype of the original population in the immunoprecipitation analysis of the infected cell extracts (Fig. 1 and 4). This result provided another indication of the genetic heterogeneity that can exist in the clinical isolates.

TK sequence analysis. The TK gene of VZV (Fig. 3) is 1,023 nucleotides in length, and it encodes a protein of 341 amino acids. The proposed ATP- and nucleoside-binding sites are defined by amino acids 12 to 29 and 129 to 145, respectively. The TK genes of the 12 plaque-purified VZV

strains were sequenced, and homology greater than 99% was observed between the strains. A comparison of the TK gene sequences of these virus strains to that of the prototype Dumas strain in GenBank showed that each of the isolates had a substitution of S-288 with L that is found in all VZV strains, other than Dumas, examined to date (29, 49).

The TK genes of 5 of the 12 VZV strains contained either a single-nucleotide deletion (8811-4 and 8811-13), a 2-nucleotide deletion (8807-3 and 9012-4), or a 4-nucleotide deletion (9007-1) (Table 2). The nucleotide deletions found in 8807-3 and 9012-4 were identical, even though the strains originated from different patients. Each of these deletions resulted in a frameshift mutation in the TK gene that introduces a premature termination codon. This should result in the expression of a truncated TK protein.

The remaining seven clinical strains purified from five isolates contained nucleotide substitutions in the TK gene that would result in amino acid substitutions (Table 2). The majority of the amino acid substitutions occurred in the nucleoside-binding sites of the TK protein in the following strains: 8808-4, 8812-1, 8812-5, 8812-10, and 8919-1. One strain, 8901-1, contained an amino acid substitution in the ATP-binding site, and one strain, 9006-1, contained a substitution between the two conserved binding sites. Strain 8808-4 contained an additional amino acid substitution in the carboxy terminus of the TK protein.

Strains 8812-1, 8812-10, 8808-4, and 8919-1 all had nucleotide substitutions that would result in amino acid changes in the proposed nucleoside-binding site of the TK protein. The three individual strains purified from patient isolate 8812 contained two different mutations: R-143 to G (8812-1 and 8812-10) and D-129 to N (8812-5). Strain 8808-4 contained not only a nucleoside-binding site alteration of C-138 to R but also a substitution of S-242 with F located in the C terminus of the protein. Strain 8919-1 contained a substitution of R-143 with K. Interestingly, this arginine is the same amino acid that was substituted in the 8812-1 and 8812-10 strains.

Strain 8901-1 contained a substitution of K-25 with R in the proposed ATP-binding site of the TK protein. This is one of five highly conserved amino acids present in the ATP-binding site. When Liu and Summers (33) substituted the corresponding lysine with an isoleucine in HSV type 1 (HSV-1) by in vitro mutagenesis, this resulted in loss of the enzymatic activity of the TK protein.

Isolate 9006-1 had a substitution of E-59 with G located between the proposed nucleoside- and ATP-binding sites of the TK protein. Two other laboratory-derived VZV mutants, which were selected for resistance to 1-β-D-arabinofuranosyl)propynuracil (882C87) but also showed cross resistance

ATP-Binding Site									
MSTDK	TDVKM	GVLRI	YLDGA	YGIGK	TTAAE	EFLHH	FAITP	NRILL	IGEPL
1		12			29				
SYWRN	LAGED	AICGI	YGTQT	RRLNG	DVSPE	DAQRL	TAHFQ	SLFCS	PHAIM
Nucleoside-Binding Site									
HAKIS	ALMDT	STSDL	VQVKN	EPYKI	MLSDR	HPIAS	TICFP	LSRYL	VGDM
					129			145	
PAALP	GLLFT	LPAEP	PGTNL	VVCTV	SLPSH	LSRVS	KRARP	GETVN	LPFVM
VLRNV	YIMLI	NTIIF	LKTNN	WHAGW	NTLSF	CNDVF	KQKLQ	KSECI	KLREV
PGIED	TLFAV	LKLPE	LCGEF	GNILP	LWAWG	METLS	NCSRS	MSPFV	LSLEQ
TPQHA	AQELK	TLLPQ	MTPAN	MSSGA	WNILK	ELVNA	VQDNT	S	
								341	

FIG. 3. Amino acid sequence of the VZV TK protein. The VZV TK open reading frame encodes a protein of 341 amino acids, shown here in single-letter code. The underlined amino acids, amino acids 12 to 29 and 129 to 145, are the proposed ATP- and nucleoside-binding sites, respectively.

TABLE 2. Sequence changes found in the TK genes of ACV-resistant mutants of VZV^a

Virus mutant	Type of change		Comment(s)
	Nucleotide	Amino acid	
V8807-3	Deletion of A-681	C-231→stop	
V8811-4	Deletion of C-682		
V8811-13	Deletion of A-76	I-38→stop	
V9007-1	Deletion of ATTT-47→-50	A-37→stop	
V9012-4	Deletion of A-681	C-231→stop	
	Deletion of C-682		
V8808-4	T-412→C; C-725→T	C-138→R; S-242→F	Nucleoside-binding site, C terminus
V8812-1	A-427→G	R-143→G	Nucleoside-binding site, altered substrate
V8812-10			
V8812-5	G-385→A	D-129→N	Nucleoside-binding site
V8901-1	A-74→G	K-25→R	ATP-binding site, altered substrate
V8919-1	G-428→A	R-143→K	Nucleoside-binding site, altered substrate
V9006-1	A-176→G	E-59→G	Between nucleoside- and ATP-binding sites, altered substrate

^a Virus strains were plaque purified from patient isolates. Original isolates from individual patients were designated by a 4-digit number as described in Materials and Methods. Virus strain sequences were compared with the GenBank Dumas strain sequence. All of the virus strains also contained an S-288→L substitution that is present in all strains other than the Dumas strain that have been sequenced to date.

to BVaraU, contained amino acid substitutions (H-97 to R and L-92 to P, respectively) in this same region (29).

Protein analysis. The expression of VZV TK protein in the 12 strains was analyzed by the immunoprecipitation of VZV-infected cell extracts with a VZV TK antibody (Fig. 4). We were interested in determining which amino acid substitutions directly affected TK function and which substitutions simply affected the intracellular stability of the TK protein.

VZV encodes a TK polypeptide of 341 amino acids with a predicted molecular mass of 35 kDa. Immunoprecipitation of cells infected with the wild-type laboratory strain Ellen identified a 35-kDa protein. A 35-kDa protein was not immunoprecipitated from uninfected cells; cells infected with a TK-deficient laboratory strain, Ellen ACV^r 3-5-2; or cells infected with the ACV^r clinical strains 8807-3, 8811-4, 8811-13, 9007-1, and 9012-1, whose TK DNA sequence predicted the production of a truncated protein (49). However, immunoprecipitation of cells infected with strains Ellen ACV^r 3-5-2, 8807-3, and 9012-1, which were each predicted to express a truncated TK polypeptide of significant size, 225 (49), 231, and 231 amino acids, respectively, showed evidence of a lower-molecular-weight protein which is unique to these strains. This protein may be a truncated TK. The nucleotide sequences of the strains 8811-4, 8811-13, and 9007-1 do not predict the expression of a TK polypeptide of any significant size, and the immunoprecipitation of cells

infected with these strains showed no evidence of a TK protein.

The introduction of amino acid substitutions had a range of effects on intracellular TK stability. Strains 8808-4, 8812-1, 8812-5, 8812-10, and 8919-1 contained amino acid substitutions present in the nucleoside-binding site of the TK enzyme. Strains 8812-1 and 8812-10, which had identical amino acid substitutions (R-143 to G), led to the production of TK protein that seemed to be expressed at levels somewhat less than those of the wild type. Strain 8812-5, which was isolated from the same patient as strains 8812-1 and 8812-10 but had an amino acid substitution present in the nucleoside-binding site different from those of those two strains, produced essentially wild-type levels of TK protein. Strain 8919-1, which, like 8812-1 and 8812-10, had an amino acid substitution at R-143 (R to K), expressed TK protein at a level that was also equivalent to that of the wild type. Strain 8808-4, which contained substitutions in the nucleoside-binding site at C-138 and in the C terminus of the protein at S-242, appeared to lead to the production of a less stable TK protein, as little TK was immunoprecipitated.

Strain 8901-1, with a predicted ATP-binding site substitution (K-25 to R), expressed a TK protein that appeared to be produced at wild-type levels. Immunoprecipitation of cells infected with 9006-1, which contains an amino acid substitution between the two proposed binding sites (E-59 to G), identified a TK protein that also appeared to be expressed at the same level as that of the wild type.

Therefore, in general, amino acid substitutions in VZV TK proteins that arose in patients during ACV treatment appeared to functionally inactivate the enzyme without substantially altering the intracellular stability of the protein. In contrast, deletions in the nucleotide sequence that are predicted to induce a translational frameshift resulted in a reduction in the stable levels of TK protein expressed in infected cells.

TK functional analysis by plaque autoradiography. The virus TK function of the purified strains was assessed in infected cells by measuring the phosphorylation and the subsequent incorporation of [¹²⁵I]IdC and [¹⁴C]TdR into the virus DNA. The ability of HSV-infected cells to phosphorylate and subsequently incorporate [¹²⁵I]IdC has been corre-

FIG. 4. SDS-PAGE analysis of the VZV TK protein immunoprecipitated from MRC-5 cells infected with plaque-purified VZV strains. Lanes: Ellen, wild-type VZV strain; 3-5-2, laboratory-derived TK-deficient VZV strain; cell, uninfected MRC-5 cells. The position of the VZV TK protein is shown on the right.

TABLE 3. Antiviral sensitivities, mutation positions, protein immunoprecipitation, and plaque autoradiography of VZV strains

Virus strain	ED ₅₀ (μM)			Mutation	Result of ^a :		
	ACV	BVaraU	Penciclovir		Immunoprecipitation of TK protein ^b	Plaque autoradiography (incorporation)	
						[¹²⁵ I]IdC	[¹⁴ C]TdR
8807-3	≥150	≥150	≥150	Deletion	— ^c	—	—
8808-4	≥150	≥150	≥150	Nucleoside BS ^d -C terminus	±	—	—
8812-1	≥150	0.45	≥150	Nucleoside BS	+	—	+
8812-5	78	20	≥150	Nucleoside BS	+	—	—
8901-1	≥150	≥150	≥150	ATP BS	+	—	±
8919-1	≥150	0.02	14	Nucleoside BS	+	±	+
9006-1	90	0.006	102	Between BS	+	±	+
3-5-2	≥150	≥150 ^e	≥150	C terminus	—	—	—
Ellen (control)	9	0.006 ^f	9.5	None	+	+	+

^a Pluses and minuses are defined in Table 1, footnote b.^b TK protein was immunoprecipitated with a VZV TK antibody (Fig. 4).^c A truncated TK protein may be immunoprecipitated.^d BS, binding site(s).^e Average of two assays.^f Average of three assays.

lated with the ability of the HSV TK to phosphorylate ACV (15, 21, 24). With these two substrates ([¹²⁵I]IdC and [¹⁴C]TdR) used as probes of VZV TK function, the VZV clinical strains showed two distinct patterns of label uptake. Strains 8812-1, 8901-1, 8919-1, and 9006-1 were able to incorporate [¹⁴C]TdR but either did not incorporate or incorporated reduced amounts of [¹²⁵I]IdC into their DNA, and they therefore were classified as TK altered. Strains 8807-3, 8808-4, and 8812-5 were unable to incorporate either [¹⁴C]TdR or [¹²⁵I]IdC and were classified as TK deficient.

Susceptibilities to other TK-dependent antiviral agents. Because in vitro resistance to one TK-dependent antiviral agent for HSV isolates does not always preclude sensitivity to other TK-dependent antiviral agents (16), we compared the in vitro susceptibilities of the VZV strains to three TK-dependent antiviral agents: ACV, BVaraU, and penciclovir [9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine]. BVaraU is an antiherpetic agent (35) that is currently in clinical trials in the United States for VZV infections (Bristol-Myers Squibb), and penciclovir has shown selective activities against HSV-1 and -2 and VZV in vitro (2, 3). Famciclovir, the oral prodrug of penciclovir, is also in clinical trials (SmithKline Beecham Pharmaceuticals). The activity of each compound is dependent on conversion to the monophosphate derivative by the VZV TK protein.

The in vitro susceptibilities of the VZV strains to ACV, BVaraU, and penciclovir were compared with those of the wild-type virus, Ellen, by a DNA hybridization assay (DNA Hybriwix method) (Table 3). Strain 8807-3, which was predicted to express a truncated TK protein of 231 amino acids, which does not appear to be very stable, was cross resistant to ACV, penciclovir, and BVaraU. Strains 8812-1 and 8812-5, with different amino acid substitutions present in the nucleoside-binding site of the TK protein, were clearly resistant to ACV and penciclovir. These strains, however, showed differential sensitivities to BVaraU, with 8812-1 being 75-fold (ED₅₀ = 0.45 μM) and 8812-5 being 3,350-fold (ED₅₀ = 20 μM) more resistant to BVaraU than the control strain, Ellen (ED₅₀ = 0.006 μM). This degree of in vitro susceptibility may reflect the reduced levels of TK polypeptide detected in virus-infected cells (Fig. 3). Strain 8919-1, which, like strain 8812-1, has an amino acid substitution at residue 143 (R to K), was resistant to ACV but was sensitive

to BVaraU and penciclovir. The conservative amino acid substitution (R-143 to K) in the TK protein of strain 8919-1 did not appreciably affect the sensitivities of the virus to BVaraU (ED₅₀ = 0.02 μM) and penciclovir (ED₅₀ = 14 μM), whereas the nonconservative substitution (R-143 to G) in the TK protein of strain 8812-1 somewhat shifted the sensitivity of the virus to BVaraU (ED₅₀ = 0.45 μM) and severely shifted its sensitivity to penciclovir (ED₅₀ ≥ 150 μM).

The nucleotide-binding site mutant, 8901-1, was resistant to all three antiviral agents. This strain was unusual because it produced wild-type levels of TK protein and still phosphorylated deoxy-TdR, although with reduced efficiency, yet it was highly resistant to all three TK-dependent antiviral agents. Strain 9006-1, which contained the mutation between the two binding sites, was resistant to ACV and penciclovir but remained sensitive to BVaraU (ED₅₀ = 0.006 μM).

DISCUSSION

The TK genes of 12 clinical VZV strains that were resistant to ACV in vitro were examined by nucleotide sequence analysis and protein expression with virus-infected cells. In both HSV and VZV, antiviral resistance can be due to either a lesion in the TK gene (28, 29, 39, 49) or, less frequently, to an alteration in the polymerase gene (7, 46). All 12 strains examined in this study contained mutations in their TK genes, which should account for the ACV resistance observed in vitro. These strains remained sensitive to the DNA polymerase inhibitor PFA, indicating the absence of DNA polymerase mutations which would confer cross-resistance or hypersensitivity to PFA. Other alterations in the VZV DNA polymerase genes in these strains could not be ruled out.

Five of the 12 strains were predicted by nucleotide sequence analysis to produce a truncated and presumably nonfunctional TK protein. A truncated TK protein was presumably immunoprecipitated from cells infected with strains 8807-3 and 9012-4, which were predicted by nucleotide sequence analysis to express a truncated TK protein of 231 amino acids. Immunoprecipitation of cells infected with the remaining three strains showed no evidence of a truncated TK protein. It is noteworthy that these two strains, 8807-3 and 9012-4, had identical nucleotide deletions (posi-

In summary, the ACV resistance found in clinical VZV strains examined in this study was associated with either deletions in the TK gene that resulted in the expression of a truncated TK protein (five strains from four patients) or

nucleotide changes that resulted in amino acid substitutions in the TK protein (seven strains from five patients). Substitutions found in the ATP- and nucleoside-binding sites and between these two binding sites all conferred in vitro resistance to ACV. Darby et al. (10) proposed that the ATP- and nucleoside-binding sites and C-336 located in the C terminus of the HSV TK enzyme formed the active center of the HSV TK enzyme. Our data confirm that the ATP- and nucleoside-binding sites are important for the normal function of the TK enzyme; no substitutions were found in the corresponding C terminus cysteine.

The phosphorylation of ACV to its monophosphate form initially depends on the binding of the ACV to the TK enzyme. Further enzymological studies of the ACV-resistant mutants will be necessary to determine the affinity of the enzyme for ACV and the rate of phosphorylation of ACV by the TK enzyme. Roberts et al. (43) observed that the TK enzyme of 7-1-3, an ACV-resistant VZV mutant that contains a substitution of R-130 with Q in the conserved region of the nucleoside-binding site of the TK protein (49), bound ACV more efficiently than did wild-type virus but phosphorylated the antiviral agent at a much slower rate than did wild-type TK enzyme. Enzymatic studies with these ACV-resistant mutants should be able to distinguish between the mutations that affect substrate binding and those that affect the phosphorylation rate of the enzyme.

From the original, non-plaque-purified patient isolates, we found that 38% of the clinical ACV-resistant VZV isolates expressed full-length TK protein, a relatively high percentage compared with the 10% of ACV-resistant HSV isolates from 10 patients that expressed a full-length TK polypeptide in a previous study (4). Interestingly, the one TK variant which induced the synthesis of a full-length HSV TK polypeptide was unable to induce the intracellular phosphorylation of thymidine (4). In this VZV study, four of six purified strains which synthesized full-length polypeptide were also capable of phosphorylating thymidine. Studies to date indicate that most clinical ACV resistance found in HSV-1 and -2 results from mutations in the viral TK gene, although alterations of the HSV polymerase have been documented previously (4, 7, 46). The role of the VZV TK protein in the pathogenesis of virus infection has not been studied, because suitable animal models for VZV infection do not exist. A variety of animal models of HSV infection have been utilized to demonstrate the importance of the viral TK protein for virulence and its requirement for reactivation from the latent state (13). The relatively high percentage of VZV isolates that expressed a full-length functional TK protein may suggest that the TK protein plays an important role in the growth of VZV in vivo. This will be difficult to assess until an appropriate VZV animal model becomes available.

As the number of patients with AIDS increases, there is likely to be a corresponding increase in the number of VZV isolates resistant to ACV and the polymerase inhibitors PFA and araA. Many patients who failed to respond to ACV treatment have subsequently responded to PFA therapy (4, 5, 47, 48). The resistance of VZV to one TK-dependent antiviral agent may not preclude the use of other TK-dependent antiviral agents for management of VZV infections. In this study, some of the strains that were resistant to ACV were not cross resistant to BVaraU or, in one instance, to either BVaraU or penciclovir. Furthermore, patient isolates may contain a mixture of TK variants, as was observed in the patient 8812 isolate, from which two strains that contained different mutations in the nucleoside-binding site

of the TK protein were purified. As another example of this heterogeneity, the original 9007 isolate produced a full-length TK protein that was expressed at wild-type levels, while the one strain plaque purified from the isolate mixture did not express any detectable TK protein in infected cells. Screening of the ACV-resistant isolates against a range of antiviral agents may be important for the successful management of patients who do not respond to polymerase-dependent antiviral agents.

ACKNOWLEDGMENTS

We thank Sylvia Stanat and Melissa Gaillard for the antiviral screening of the VZV isolates and Donna Hollowell for the deoxy-TdR incorporation data. We gratefully acknowledge the critical review of the manuscript by Nick Ellis, Grace Roberts, Dorothy Purifoy, and Graham Darby.

REFERENCES

1. Biron, K. K., J. A. Fyfe, J. E. Noblin, and G. B. Elion. 1982. Selection and preliminary characterization of acyclovir-resistant mutants of varicella zoster virus. *Am. J. Med.* 73:383-386.
2. Boyd, M. R., T. H. Bacon, and D. Sutton. 1988. Antiherpes activity of 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine (BRL 39123) in animals. *Antimicrob. Agents Chemother.* 32:358-363.
3. Boyd, M. R., T. H. Bacon, D. Sutton, and M. Cole. 1987. Antiherpesvirus activity of 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine (BRL 39123) in cell culture. *Antimicrob. Agents Chemother.* 31:1238-1242.
4. Chatis, P. A., and C. S. Crumpacker. 1991. Analysis of the thymidine kinase gene from clinically isolated acyclovir-resistant herpes simplex viruses. *Virology* 180:793-797.
5. Chatis, P. A., C. H. Miller, L. E. Schrager, and C. S. Crumpacker. 1989. Successful treatment with foscarnet of an acyclovir-resistant mucocutaneous infection with herpes simplex virus in a patient with acquired immunodeficiency syndrome. *N. Engl. J. Med.* 320:297-300.
6. Coen, D. M., and P. A. Schaffer. 1980. Two distinct loci confer resistance to acycloguanosine in herpes simplex virus type 1. *Proc. Natl. Acad. Sci. USA* 77:2265-2269.
7. Collins, P., B. A. Larder, N. M. Oliver, S. Kemp, I. W. Smith, and G. Darby. 1989. Characterization of a DNA polymerase mutant of herpes simplex virus from a severely immunocompromised patient receiving acyclovir. *J. Gen. Virol.* 70:375-382.
8. Dankner, W. M., D. Scholl, S. C. Stanat, M. Martin, R. L. Sonke, and S. A. Spector. 1990. Rapid antiviral DNA-DNA hybridization assay for human cytomegalovirus. *J. Virol. Methods* 28:293-298.
9. Darby, G., M. J. Churcher, and B. A. Larder. 1984. Cooperative effects between two acyclovir resistance loci in herpes simplex virus. *J. Virol.* 50:838-846.
10. Darby, G., B. A. Larder, and M. M. Inglis. 1986. Evidence that the 'active centre' of the herpes simplex virus thymidine kinase involves an interaction between three distinct regions of the polypeptide. *J. Gen. Virol.* 67:753-758.
11. Davidson, A. J., and J. E. Scott. 1986. The complete DNA sequence of varicella-zoster virus. *J. Gen. Virol.* 67:1759-1816.
12. Derse, D., Y.-C. Cheng, P. A. Furman, M. H. St. Clair, and G. B. Elion. 1981. Inhibition of purified human and herpes simplex virus-induced DNA polymerases by 9-(2-hydroxyethoxymethyl)guanine triphosphate: effects on primer-template function. *J. Biol. Chem.* 256:11447-11451.
13. Efsthathiou, S., S. Kemp, G. Darby, and A. C. Minson. 1989. The role of herpes simplex virus type 1 thymidine kinase in pathogenesis. *J. Gen. Virol.* 70:869-879.
14. Elion, G. B., P. A. Furman, J. A. Fyfe, P. de Miranda, L. Beauchamp, and H. J. Schaeffer. 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. *Proc. Natl. Acad. Sci. USA* 74:5716-5720.
15. Ellis, M. N., P. M. Keller, J. A. Fyfe, J. L. Martin, J. F. Rooney, S. E. Straus, S. Nusinoff-Lehrman, and D. W. Barry. 1987. Clinical isolate of herpes simplex virus type 2 that induces a

- thymidine kinase with altered substrate specificity. *Antimicrob. Agents Chemother.* **31**:1117-1125.
16. Field, H., A. McMillan, and G. Darby. 1981. The sensitivity of acyclovir-resistant mutants of herpes simplex virus to other antiviral drugs. *J. Infect. Dis.* **143**:281-285.
 17. Field, H. J., and D. M. Coen. 1986. Pathogenicity of herpes simplex virus mutants containing drug resistance mutations in the viral DNA polymerase gene. *J. Virol.* **60**:286-289.
 18. Furman, P. A., M. H. St. Clair, J. A. Fyfe, J. L. Rideout, P. M. Keller, and G. B. Elion. 1979. Inhibition of herpes simplex virus-induced DNA polymerase activity and viral DNA replication by 9-(2-hydroxyethoxymethyl)guanine and its triphosphate. *J. Virol.* **32**:72-77.
 19. Furman, P. A., M. H. St. Clair, and T. Spector. 1984. Acyclovir triphosphate is a suicide inactivator of the herpes simplex virus DNA polymerase. *J. Biol. Chem.* **259**:9575-9579.
 20. Fyfe, J. A. 1982. Differential phosphorylation of (E)-5-(2-bromovinyl)-2'-deoxyuridine monophosphate by thymidylate kinases from herpes simplex viruses types 1 and 2 and varicella zoster virus. *Mol. Pharmacol.* **21**:432-437.
 21. Fyfe, J. A., S. A. McKee, and P. M. Keller. 1983. Altered thymidine-thymidylate kinases from strains of herpes simplex virus with modified drug sensitivities to acyclovir and (E)-5-(2-bromovinyl)-2'-deoxyuridine. *Mol. Pharmacol.* **24**:316-323.
 22. Hannink, M., and D. J. Donoghue. 1985. Lysine residue 121 in the proposed ATP-binding site of the v-mos protein is required for transformation. *Proc. Natl. Acad. Sci. USA* **82**:7894-7898.
 23. Harlow, E., and D. Lane. 1988. *Antibodies: a laboratory manual*, p. 421-470. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 24. Hill, E. L., G. A. Hunter, and M. N. Ellis. 1991. In vitro and in vivo characterization of herpes simplex virus clinical isolates recovered from patients infected with human immunodeficiency virus. *Antimicrob. Agents Chemother.* **35**:2322-2328.
 25. Jacobson, M. A., T. G. Berger, S. Fikrig, P. Becherer, J. W. Moehr, S. C. Stanat, and K. K. Biron. 1990. Acyclovir-resistant varicella zoster virus infection after chronic oral acyclovir therapy in patients with the acquired immunodeficiency syndrome (AIDS). *Ann. Intern. Med.* **112**:187-191.
 26. Kamps, M. P., and B. M. Sefton. 1986. Neither arginine nor histidine can carry out the function of lysine-295 in the ATP-binding site of p60^{src}. *Mol. Cell. Biol.* **6**:751-757.
 27. Kit, S. 1985. Thymidine kinase. *Microbiol. Sci.* **2**:369-375.
 28. Kit, S., M. Sheppard, H. Ichimura, S. Nusinoff-Lehrman, M. N. Ellis, J. A. Fyfe, and H. Otsuka. 1987. Nucleotide sequence changes in thymidine kinase gene of herpes simplex virus type 2 clones from an isolate of a patient treated with acyclovir. *Antimicrob. Agents Chemother.* **31**:1483-1490.
 29. Lacey, S. F., T. Suzutani, K. L. Powell, D. J. M. Purifoy, and R. W. Honess. 1991. Analysis of mutations in the thymidine kinase genes of drug-resistant varicella-zoster virus populations using the polymerase chain reaction. *J. Gen. Virol.* **72**:623-630.
 30. Larder, B. A., Y.-C. Cheng, and G. Darby. 1983. Characterization of abnormal thymidine kinases induced by drug-resistant strains of herpes simplex virus type 1. *J. Gen. Virol.* **64**:523-532.
 31. Larder, B. A., D. Derse, Y.-C. Cheng, and G. Darby. 1983. Properties of purified enzymes induced by pathogenic drug-resistant mutants of herpes simplex virus. Evidence for virus variants expressing normal DNA polymerase and altered thymidine kinase. *J. Biol. Chem.* **258**:2027-2033.
 32. Larder, B. A., S. D. Kemp, and G. Darby. 1987. Related functional domains in virus DNA polymerases. *EMBO J.* **6**:169-175.
 33. Liu, Q., and W. C. Summers. 1988. Site-directed mutagenesis of a nucleotide-binding domain in HSV-1 thymidine kinase: effects on catalytic activity. *Virology* **163**:638-642.
 34. Lopetegui, P., Y. Matsunaga, T. Okuno, T. Ogino, and K. Yamanishi. 1983. Expression of varicella-zoster virus-related antigens in biochemically transformed cells. *J. Gen. Virol.* **64**:1181-1186.
 35. Machida, H., and S. Sakata. 1984. In vitro and in vivo antiviral activity of 1-β-D-arabinofuranosyl-E-5-(2-bromovinyl)uracil (BV-araU) and related compounds. *Antiviral Res.* **4**:135-141.
 36. Machida, H., and Y. Watanabe. 1991. Inhibition of DNA synthesis in varicella-zoster virus-infected cells by BV-araU. *Microbiol. Immunol.* **35**:139-145.
 37. Martin, J. L., M. N. Ellis, P. M. Keller, K. K. Biron, S. N. Lehrman, D. W. Barry, and P. A. Furman. 1985. Plaque autoradiography assay for the detection and quantitation of thymidine kinase-deficient and thymidine kinase-altered mutants of herpes simplex virus in clinical isolates. *Antimicrob. Agents Chemother.* **28**:181-187.
 38. Mitchell, C. D., B. Bean, S. R. Gentry, K. E. Groth, J. R. Boen, and H. H. Balfour, Jr. 1981. Acyclovir therapy for mucocutaneous herpes simplex infections in immunocompromised patients. *Lancet* **i**:1389-1391.
 39. Mori, H., K. Shiraki, T. Kato, Y. Hayakawa, K. Yamanishi, and M. Takahashi. 1988. Molecular analysis of the thymidine kinase gene of thymidine kinase-deficient mutants of varicella-zoster virus. *Intervirology* **29**:301-310.
 40. Nugier, F., P. Collins, B. A. Larder, M. Langlois, M. Aymard, and G. Darby. 1991. Herpes simplex virus isolates from an immunocompromised patient who failed to respond to acyclovir treatment express thymidine kinase with altered substrate specificity. *Antiviral Chem. Chemother.* **2**:295-302.
 41. Pahwa, S., K. Biron, W. Lim, P. Swenson, M. H. Kaplan, N. Sadick, and R. Pahwa. 1988. Continuous varicella-zoster infection associated with acyclovir resistance in a child with AIDS. *JAMA* **260**:2879-2882.
 42. Reardon, J. E., and T. Spector. 1989. Herpes simplex virus type 1 DNA polymerase. Mechanism of inhibition by acyclovir triphosphate. *J. Biol. Chem.* **264**:7405-7411.
 43. Roberts, G. B., J. A. Fyfe, R. K. Gaillard, and S. A. Short. 1991. Mutant varicella-zoster virus thymidine kinase: correlation of clinical resistance and enzyme impairment. *J. Virol.* **65**:6407-6413.
 44. Robertson, G. R., and J. M. Whalley. 1988. Evolution of the herpes thymidine kinase: identification and comparison of the equine herpesvirus 1 thymidine kinase gene reveals similarity to a cell-encoded thymidylate kinase. *Nucleic Acids Res.* **16**:11303-11317.
 45. Ruth, J. L., and Y.-C. Cheng. 1981. Nucleoside analogues with clinical potential in antiviral chemotherapy. The effect of several thymidine and 2'-deoxycytidine analogue 5'-triphosphates on purified human (α, β) and herpes simplex virus (types 1, 2) DNA polymerases. *Mol. Pharmacol.* **20**:415-422.
 46. Sacks, S. L., R. J. Wanklin, D. E. Reece, K. A. Hicks, K. L. Tyler, and D. M. Coen. 1989. Progressive esophagitis from acyclovir-resistant herpes simplex. Clinical roles for DNA polymerase mutants and viral heterogeneity? *Ann. Intern. Med.* **111**:893-899.
 47. Safrin, S., T. G. Berger, I. Gilson, P. R. Wolfe, C. B. Wofsy, J. Mills, and K. K. Biron. 1991. Foscarnet therapy in five patients with AIDS and acyclovir-resistant varicella-zoster virus infection. *Ann. Intern. Med.* **115**:19-21.
 48. Safrin, S. C., P. Crumpacker, R. Chatis, R. Davis, R. Hafner, J. Rush, H. A. Kessler, B. Landry, J. Mills, and the AIDS Clinical Trials Group. 1991. A controlled trial comparing foscarnet with vidarabine for acyclovir-resistant mucocutaneous herpes simplex in the acquired immunodeficiency syndrome. *N. Engl. J. Med.* **325**:551-555.
 49. Sawyer, M. H., G. Inchauspe, K. K. Biron, D. J. Waters, S. E. Straus, and J. M. Ostrove. 1988. Molecular analysis of the pyrimidine deoxyribonucleoside kinase gene of wild-type and acyclovir-resistant strains of varicella-zoster virus. *J. Gen. Virol.* **69**:2585-2593.
 50. Schnipper, L. E., and C. S. Crumpacker. 1980. Resistance of herpes simplex virus to acycloguanosine: role of viral thymidine kinase and DNA polymerase loci. *Proc. Natl. Acad. Sci. USA* **77**:2270-2273.
 51. Shiraki, K., T. Ogino, K. Yamanishi, and M. Takahashi. 1985. Immunohistochemical characterization of pyrimidine kinase induced by varicella-zoster virus. *J. Gen. Virol.* **66**:221-229.
 52. Straus, S. E., J. Owens, W. T. Ruyechan, H. E. Takiff, T. A. Casey, G. F. Vande Woude, and J. Hay. 1982. Molecular cloning and physical mapping of varicella-zoster virus DNA. *Proc. Natl.*

- Acad. Sci. USA **79**:993-997.
53. **Straus, S. E., H. A. Smith, C. Brickman, P. de Miranda, C. McLaren, and R. E. Keeney.** 1982. Acyclovir for chronic mucocutaneous herpes simplex virus infection in immunosuppressed patients. *Ann. Intern. Med.* **96**:270-277.
54. **Suzutani, T., H. Machida, T. Sakuma, and M. Azuma.** 1988. Effects of various nucleosides on antiviral activity and metabolism of 1- β -D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil against herpes simplex virus types 1 and 2. *Antimicrob. Agents Chemother.* **32**:1547-1551.
55. **Wade, J. C., B. Newton, C. McLaren, N. Flournoy, R. E. Keeney, and J. D. Meyers.** 1982. Intravenous acyclovir to treat mucocutaneous herpes simplex virus infection after marrow transplantation: a double-blind trial. *Ann. Intern. Med.* **96**:265-269.
56. **Weinmaster, G., M. J. Zoller, and T. Pawson.** 1986. A lysine in the ATP-binding site of p130^{gag-fps} is essential for protein-tyrosine kinase activity. *EMBO J.* **5**:69-76.