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Nucleotide Sequence Changes in Thymidine Kinase Gene of Herpes Simplex Virus Type 2 Clones from an Isolate of a Patient Treated with Acyclovir

SAUL KIT,[1]* MICHAEL SHEPPARD,[1] HIROSHI ICHIMURA,[1] SANDRA NUSINOFF-LEHRMAN,[2] M. NIXON ELLIS,[2] JAMES A. FYFE,[2] AND HARUKI OTSUKA[1]

Division of Biochemical Virology, Baylor College of Medicine, Houston, Texas 77030,[1] and Burroughs Wellcome Co., Research Triangle Park, North Carolina 27709[2]

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To identify the nucleotide changes that occur in drug-induced thymidine kinase (TK) mutants of herpes simplex virus type 2 (HSV-2), we compared the nucleotide sequences of the *tk* genes of two mutant HSV-2 clones isolated from a patient who had been treated with acyclovir [9-(2-hydroxyethoxymethyl)guanine; ACV] with the nucleotide sequence of the parental TK^+ HSV-2(8703) strain isolated from the same patient. One of the mutants, TK-altered (TK^A) HSV-2(9637), was ACV resistant but induced the incorporation of [^{14}C]thymidine into the DNA of infected rabbit skin cells. The nucleotide sequence of the *tk* gene of mutant TK^A HSV-2(9637) had a single change (G to A) at nucleotide 668, which would cause an arginine-to-histidine substitution at amino acid residue 223 of the TK polypeptide. The second ACV-resistant mutant, TK^- HSV-2(8710), did not induce detectable incorporation of [^{14}C]thymidine into the DNA of infected rabbit skin cells. This mutant exhibited a deletion of a single base at nucleotide 217 of its nucleotide sequence. This deletion would cause a frameshift mutation at amino acid residue 73 and chain termination at amino acid residue 86 of the TK polypeptide. The nucleotide sequence of TK^+ HSV-2(8703) was the same as that of the laboratory strain, TK^+ HSV-2(333). The nucleotide sequence of a bromodeoxyuridine-resistant TK^- HSV-2(333) mutant of TK^+ HSV-2(333) also exhibited a single-base deletion, but at nucleotide 439. This deletion would cause a frameshift mutation at amino acid residue 147 and chain termination at amino acid residue 182. The frameshift mutations of TK^- HSV(8710) and TK^- HSV-2(333), respectively, occurred in sequences in which C was repeated three times and G was repeated seven times. The results raise the possibility that TK^- frameshift mutations of HSV-2 may be common.

The clinical management of herpes simplex virus (HSV) infections has become more effective in recent years with the introduction of selective anti-HSV drugs, such as acyclovir [9-(2-hydroxyethoxymethyl)guanine; ACV], *E*-5-(2-bromovinyl)-2′-deoxyuridine (BVDU), and 2′-fluoro-5-iodo-1-β-D-arabinofuranosylcytosine (FIAC) (11, 14, 35). These drugs inhibit the replication of HSV in vitro and in vivo, while manifesting little or no toxicity. The selectivity of ACV, BVDU, and FIAC as anti-HSV agents is based on the specific interaction with one or more virus-encoded enzymes, e.g., the HSV thymidine kinase (TK) and the viral DNA polymerase. HSV TK is also associated with significant thymidylate kinase activity (19, 21) and, unlike its cellular counterpart, recognizes ACV, BVDU, FIAC, and other nucleoside analogs as substrates, thereby restricting their phosphorylation (activation) to HSV-infected cells (11, 14, 25, 26, 35). HSV DNA polymerase is more vulnerable to the triphosphate forms of ACV, BVDU, and FIAC than are cellular DNA polymerases α, β, and γ (6, 11). However, recent reports describing the isolation of ACV-resistant HSV variants from patients undergoing therapy provide a reminder that the emergence of drug-resistant strains may pose a potential threat to future treatments (5, 7, 8, 12, 53).

The best-known mechanism for the development of HSV mutants resistant to nucleoside analogs is through mutations that produce nonfunctional TK enzymes (13, 16, 20). Although the growth of TK^- HSV mutants in exponentially growing cultured cells under normal conditions is unimpaired, TK^- mutants may replicate poorly in quiescent cells in the G1 phase of the cell cycle, so that their virulence in animals would be markedly reduced (17, 46, 51).

There are two strategies by which HSV may develop resistance to ACV, BVDU, and FIAC and, at the same time, retain pathogenicity. Both strategies entail mutations which lead to the retention of a TK^+ phenotype. The first mechanism of drug resistance involves mutations which alter the substrate specificity of the viral DNA polymerase, so that replication becomes insensitive to inhibition by the analog triphosphates (5–7, 16, 18). The second mechanism is a mutation in the *tk* gene resulting in the induction of an enzyme with altered substrate specificity, so that its ability to phosphorylate the drug or its monophosphate or both is severely impaired while at the same time its ability to phosphorylate thymidine is retained (9, 19, 21, 31).

Two TK^+ ACV-resistant mutants and one TK^+ BVDU-resistant mutant of HSV type 1 (HSV-1) which induce abnormal TK enzymes (TK^A mutants) with impaired ability to phosphorylate the drugs used in their isolation have been described (16, 17, 31). The nucleotide sequences of these TK^A HSV-1 mutants have also been compared with the nucleotide sequence of the parental TK^+ HSV-1 strain (9, 23). One aim of this research was to define some of the features of the putative nucleoside-binding site of the TK enzyme. An understanding of the structure of this site and the nature of the interactions between the amino acid side chains of the enzyme and elements of the substrate would be invaluable in the design of further anti-HSV nucleoside analog drugs. However, at this writing, there have been no

* Corresponding author.

nucleotide sequencing studies reported for the *tk* genes of TK^A HSV type 2 (HSV-2) mutants.

Recently, clinical isolates of HSV-2 that do not appreciably phosphorylate ACV but can detectably phosphorylate thymidine have been obtained (15, 36). From one of the clinical isolates of an ACV-treated patient with recurrent HSV-2, three plaque-purified clones were isolated that induced normal levels of TK activity (TK^+), no detectable TK activity (TK^-), and TK activity with altered substrate specificity (TK^A) (15, 36). To elucidate the kinds of mutations within the *tk* gene that caused the loss or alteration of TK activity, we determined the nucleotide sequences of the coding regions of the *tk* genes of these three clones of HSV-2 as well as the nucleotide sequence of a TK^- HSV-2(333) mutant previously isolated by 5-bromodeoxyuridine selection.

MATERIALS AND METHODS

Cells. Rabbit skin fibroblasts (RAB-9; ATCC 1414) were propagated in Eagle minimum essential medium (Flow Laboratories, Inc., McLean, Va.) supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.005% neomycin, 20 mM bicarbonate, and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] buffer (pH 7.3). Bromodeoxyuridine-resistant mutants [TK^- RAB(BU)] were grown in the same growth medium as parental RAB-9 cells but were supplemented with bromodeoxyuridine (25 μg/ml), except for the passage immediately preceding each experiment (28).

Viruses. The TK^- HSV-2(333) mutant was a bromodeoxyuridine-resistant variant of TK^+ HSV-2(333) (27, 29, 46). The TK^+ HSV-1(KOS) strain has been described previously (6, 29). The three strains designated TK^+ HSV-2(8703), TK^- HSV-2(8710), and TK^A HSV-2(9637) were plaque-purified clones made from a single clinical isolate, VL6015, from a male patient who had been treated with ACV for recurrent genital herpes (36). The VL6015 clinical isolate was regrown and plaque purified, and the three clones, 8288, 8279, and 8295, were isolated (36). Each of these three clones was plaque purified two more times, and then virus stocks were prepared. The stock virus pool derived from clone 8288 was designated TK^+ HSV-2(8703). From clone 8279, TK^A HSV-2(8708) was obtained (15, 36), and the stock virus pool derived from clone 8708 was designated TK^A HSV-2(9637). The stock virus pool derived from clone 8295 was designated TK^- HSV-2(8710). Virus stocks were prepared by infecting RAB-9 cells at an input multiplicity of about 0.01 PFU per cell at 34.5°C in Eagle minimum essential medium plus 10% fetal bovine serum. Plaque titrations were performed in RAB-9 cells at 34.5°C (27, 28). The DNA from TK^+, TK^-, and TK^A HSV-2 strains was prepared from infected RAB-9 cell cultures by the Triton X-100 method of Pignatti et al. (43) and purified as described previously (40).

Thymidine plaque autoradiography. Thymidine plaque autoradiography experiments were carried out as described by Tenser et al. (50), except that monolayer cultures of TK^- RAB(BU) cells in 100-mm petri dishes were used as host cells and the TK^- RAB(BU) cell cultures were infected with about 100 to 1,000 PFU of the HSV-2 strains per dish. Three days after infection, cells were labeled for 6 h with [^{14}C]thymidine (3 μCi per dish) (ICN Pharmaceuticals Inc., Irvine, Calif.), stained with crystal violet (0.5%), and exposed to X-ray film for 1 to 3 days at −70°C (46).

Plaque reduction tests in the presence of ACV. Plaque reduction tests were performed as described by Lopez et al. (35). RAB-9 cells were plated in six-well plates (Falcon tissue culture plates; Becton Dickinson Labware, Oxnard, Calif.) at 7.5×10^5 cells per well and incubated at 37°C for 2 days. Cells were infected with one of three HSV-2 isolates at 150 PFU/0.1 ml per well for 1 h and overlaid with 1% methylcellulose in Eagle minimum essential medium plus 10% fetal bovine serum and different concentrations of ACV (see Table 1). Incubations were carried out at 34.5°C for 4 days, cells were fixed and stained with 0.1% crystal violet, and the plaques were counted.

Cloning of the *tk* gene. HSV-2 DNA (10 μg) was cleaved with *Bgl*II, and the fragments were then fractionated in a Spinco SW40.1 rotor by 10 to 40% sucrose gradient centrifugation at 180,000 × *g* for 20 h at 4°C. Portions of each fraction were analyzed by agarose gel electrophoresis. The fractions with a DNA fragment corresponding in size to HSV-2 *Bgl*II-G (17.5 kilobases [kb]), which is known to encode the HSV-2 *tk* gene (27), were mixed with 2 volumes of ethanol. The precipitated DNA fragments were collected by centrifugation, dried, and ligated to pBR322 (cleaved with *Bam*HI and dephosphorylated; New England Biolabs, Inc., Beverly, Mass.). The ligation products were used to transform *Escherichia coli* K-12 strain RR1 as described previously (40), and Amp^r Tet^s colonies were isolated. The plasmid containing the *Bgl*II G fragment was identified by mapping the restriction endonuclease *Bam*HI, *Cla*I, *Eco*RI, *Hin*dIII, and *Kpn*I sites (27). In some experiments, HSV-2 DNA was cleaved with *Eco*RI and *Cla*I and fractionated by sucrose gradient centrifugation, and a 2.5-kb DNA fragment which encodes the HSV-2 *tk* gene (27) was cloned at the *Eco*RI and *Cla*I sites of pBR325.

Sequencing of the *tk* gene. The plasmid containing the HSV-2 *Bgl*II G fragment was cleaved with *Eco*RI and *Cla*I, and the 2.5-kb DNA fragment which encodes the *tk* gene was isolated by sucrose gradient centrifugation (10 to 40% sucrose, 180,000 × *g* for 20 h at 4°C in a Spinco SW40.1 rotor). The entire 2.5-kb DNA fragment or its subfragments were cloned in M13mp18 or M13mp19 phages, and single-stranded DNA of recombinant phages was prepared (27, 38, 41). DNA sequencing was carried out by the dideoxynucleotide chain termination method (45). The oligodeoxynucleotide primers (15-mer), which were designed to hybridize to HSV-2 at specific sites, were synthesized with cyanoethyl derivative phosphoramidite chemistry by using a SYSTEC automated DNA synthesizer. The universal primer which hybridizes to single-stranded phage M13 DNA 3′ to the polycloning sites was purchased from New England BioLabs.

RESULTS

Characteristics of HSV-2 isolates. Plaque reduction tests in the presence of ACV were carried out to confirm the phenotype of the HSV-2 isolates (Table 1). The wild-type TK^+ HSV-2(8703) and TK^+ HSV-2(333) strains showed approximately the same sensitivity to ACV. The TK^+ HSV-1(KOS) strain appeared to be somewhat more sensitive than the TK^+ HSV-2 strains, as expected (11). Nine-times-higher concentrations and 15-times-higher concentrations of ACV were required to obtain 50% plaque reduction for the TK^A HSV-2(9637) and TK^- HSV-2(8710) strains, respectively, as compared with the wild-type TK^+ HSV-2(8703) strain.

Thymidine plaque autoradiography experiments were carried out to assay for the capacity of the HSV-2 clones to incorporate [^{14}C]thymidine into DNA (Fig. 1). Plaques formed by inoculating TK^+ HSV-2(8703) into TK^- RAB(BU) cells incorporated labeled thymidine into DNA, but none of the plaques formed in the TK^- RAB(BU) cells

TABLE 1. Sensitivity of HSV isolates to plaque inhibition by ACV

Virus strain	ED_{50} (μM)[a]
TK^+ HSV-1(KOS)	<0.5
TK^+ HSV-2(333)	0.84
TK^+ HSV-2(8703)	0.56
TK^A HSV-2(9637)	7.1
TK^- HSV-2(8710)	12.7

[a] ED_{50}, Dose required to reduce plaquing efficiency by 50%. Values were determined by plaque reduction assays in RAB-9 cells and were read directly from plaque reduction curves. The TK^+ HSV-1(KOS) and TK^+ HSV-2 (333) strains were tested at 0, 0.5, and 5 μM ACV. The TK^+ HSV-2(8703) strain was tested at 0, 0.25, 0.5, 0.75, 1, and 2 μM ACV. The TK^A HSV-2(9637) isolate was tested at 0, 0.5, 1, 5, 10, and 20 μM ACV. The TK^- HSV-2(8710) isolate was tested at 0, 5, 10, 20, and 50 μM ACV.

by TK^- HSV-2(8710) incorporated labeled thymidine into DNA. Previous thymidine plaque autoradiography experiments had also shown that plaques formed by inoculating TK^+ HSV-2(333) into TK^- RAB(BU) cells incorporated labeled thymidine into DNA, while those formed by infecting TK^- RAB(BU) cells with TK^- HSV-2(333) did not (46).

The plaques formed by inoculating TK^A HSV-2(9637) into TK^- RAB(BU) cells were weakly positive for the incorporation of [^{14}C]thymidine into DNA, indicating that this isolate retained the capacity to induce TK activity even though it was ACV resistant. Thus, HSV-2(9637) resembles the parental HSV-2(8408) strain, and HSV-2(8710) resembles the parental HSV-2(8295) strain, which have previously been classified as TK^A and TK^-, respectively (15, 36).

Nucleotide sequence analyses of *tk* genes of HSV-2 isolates. DNA preparations from plaque-purified virus strains 8703, 8710, and 9637 were digested with restriction endonucleases *Bgl*II, *Cla*I, and *Kpn*I. The restriction endonuclease patterns of these samples closely resembled those of TK^+ HSV-2(333) but differed markedly from those of TK^+ HSV-1(KOS), thereby confirming that the isolates were HSV-2 strains (29).

The nucleotide sequences of the *tk* genes of the HSV-2 strains were determined by using the strategy shown in Fig. 2. The combination of nine synthetic oligodeoxynucleotide primers which specifically hybridize to the HSV-2 *tk* gene allowed us to sequence most of the coding region by using only two templates: single-stranded phage M13mp18 or phage M13mp19 DNAs containing inserts of the 2.5-kb *Eco*RI-*Cla*I fragment of the HSV-2 *tk* gene. Subfragments obtained by cleaving the 2.5-kb fragment with *Bam*HI or *Kpn*I were cloned in M13mp18 or M13mp19 and sequenced by using an M13 universal 17-mer primer (New England BioLabs).

The nucleotide sequence of the TK^+ HSV-2(333) *tk* gene has previously been reported (27, 49). We found no mutations in the coding region or the upstream flanking region of the *tk* gene of TK^+ HSV-2(8703), as compared with TK^+ HSV-2(333). However, a single nucleotide alteration in the coding region of the *tk* gene of TK^A HSV-2(9637) was demonstrated in the sequencing ladder (Fig. 3). The change from cytosine to thymine is shown by the arrowheads. This result indicates that a guanine-to-adenine transition occurred at nucleotide 668 in the noncoding strand of TK^A HSV-2(9637). The predicted change in the amino acid sequence is from arginine to histidine at amino acid residue 223 of the TK polypeptide sequence.

The repeat sequence of three cytosines at nucleotides 215 to 217 of the noncoding strands of the *tk* gene of the wild-type TK^+ HSV-2(333) and TK^+ HSV-2(8703) strains was reduced to two cytosines by deletion of one cytosine from the sequence of the noncoding strand of the *tk* gene of the ACV-resistant mutant TK^- HSV-2(8710) (Fig. 3). The reduction of three guanines to two guanines in the corresponding regions of the coding strands of the wild-type TK^+ HSV-2 parent and the TK^- HSV-2(8710) mutant, respectively, are shown in Fig. 4. The predicted result is a frameshift mutation at amino acid residue 73 of the TK polypeptide and chain termination at amino acid residue 85 (Fig. 5). Similarly, the repeat sequence of seven guanines at nucleotides 433 to 439 of the wild-type TK^+ HSV-2(333) *tk* gene was reduced to six guanines by deletion of one guanine from the *tk* gene of the bromodeoxyuridine-resistant mutant TK^- HSV-2(333) (Fig. 3). This would result in a frameshift

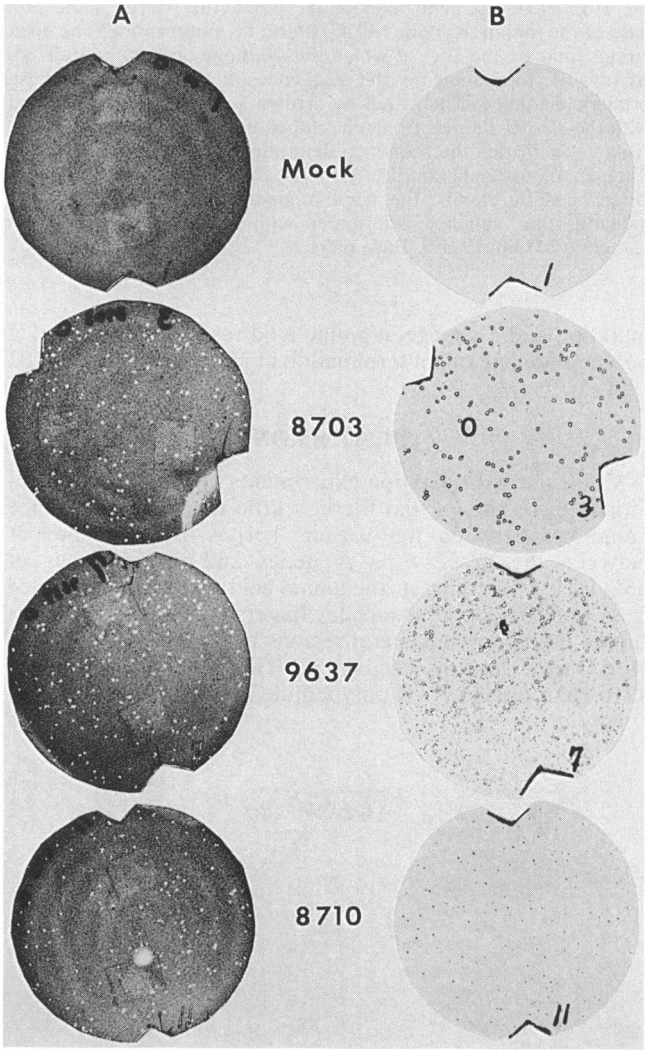

FIG. 1. Thymidine plaque autoradiography of HSV-2 clones from an ACV-treated patient. (A) Crystal violet-stained monolayers of mock-infected and HSV-2-infected TK^- RAB(BU) cells; (B) autoradiogram obtained after monolayers were incubated with [^{14}C]thymidine for 6 h. From top to bottom, the plates were from mock-infected, TK^+ HSV-2(8703)-infected, TK^A HSV-2(9637)-infected, and TK^- HSV-2(8710)-infected cells. It should be noted that the background of black dots seen on some of the X-ray films after autoradiography (B) are artifacts and are unrelated to the virus plaques seen after crystal violet staining (A).

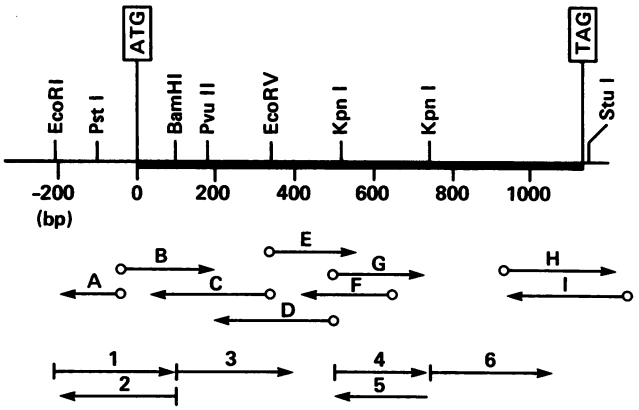

FIG. 2. Strategy for determination of the nucleotide sequence of the HSV-2(333) *tk* gene. Nucleotide numbering starts at the first base of the initiation codon (AUG) of the TK polypeptide. The open circles indicate the sites at which the synthetic primers hybridized. Arrows A to I indicate the sequences determined with these primers. Sequences indicated by arrows 1 and 2 were determined with the *Eco*RI-*Bam*HI fragment cloned in M13mp18 or M13mp19. Arrow 3 indicates the sequence determined with the *Bam*HI-*Kpn*I fragment cloned in M13mp18. Arrows 4 and 5 indicate the sequences determined by cloning the *Kpn*I fragment in M13mp19. Arrow 6 indicates the sequence determined with the *Kpn*I-*Cla*I fragment cloned in M13mp19. bp, Base pairs.

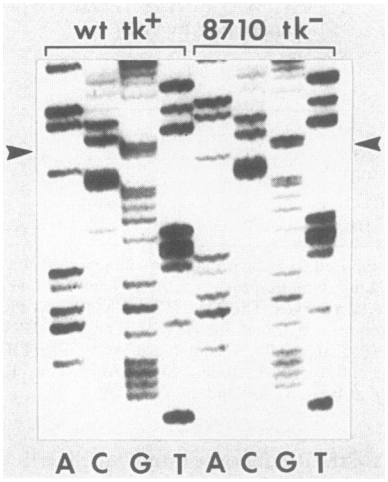

FIG. 4. Sequencing ladders around the mutated regions in the coding strands of the *tk* genes of the TK$^-$ HSV-2(8710) mutant and the wild-type (wt) TK$^+$ HSV-2 parent. Arrowheads point to the three guanines in the sequence of the wild-type TK$^+$ HSV-2 parent and the two guanines in the sequence of the TK$^-$ HSV-2(8710) mutant.

mutation and a change at amino acid residue 147 of the TK polypeptide and chain termination at amino acid residue 182 (Fig. 5).

DISCUSSION

Molecular hybridization experiments under stringent conditions have shown that there is little nucleotide sequence homology between the various herpesvirus *tk* genes or between the herpesvirus *tk* genes and the *tk* genes of poxviruses. In contrast, the amino acid sequences predicted from the nucleotide sequences have shown clear but interrupted homology in several regions of the TK polypeptides (40). One of the conserved amino acid sequences at residues 49 to 66 of the HSV TK polypeptide (Leu-Leu-Arg-Val-Tyr-Ile-Asp-Gly-Pro-His-Gly-Val-Gly-Lys-Thr-Thr-Thr-Ser) exhibited noticeable sequence homology to those of the TK polypeptides of seven herpesviruses (10, 25, 34), the TK polypeptides of five poxviruses (3, 25, 52), and the cytosol TK polypeptides of human, mouse, Chinese hamster, and chick cells (4, 30, 32, 33). This same conserved polypeptide sequence of HSV also showed sequence homology to β subunits of bovine and bacterial mitochondrial ATPase, to the p21 protein of the human c-*bas*/*bas*-1 oncogene, to the *ras* genes of the Harvey and Kirsten murine sarcoma viruses, to the *Saccharomyces cerevisiae* c-*ras* gene, to amino acid sequences of adenylate kinase, phosphofructokinase, and nematode myosin (22, 40, 55), and to the large T antigens of polyomavirus, the E1 proteins of papillomavirus, and the nonstructural protein (NS1) of parvoviruses (1). Since the aforementioned proteins bind nucleotides and use ATP or GTP or both in catalysis, Walker and co-workers (22, 55) have suggested that the indicated regions form part of a

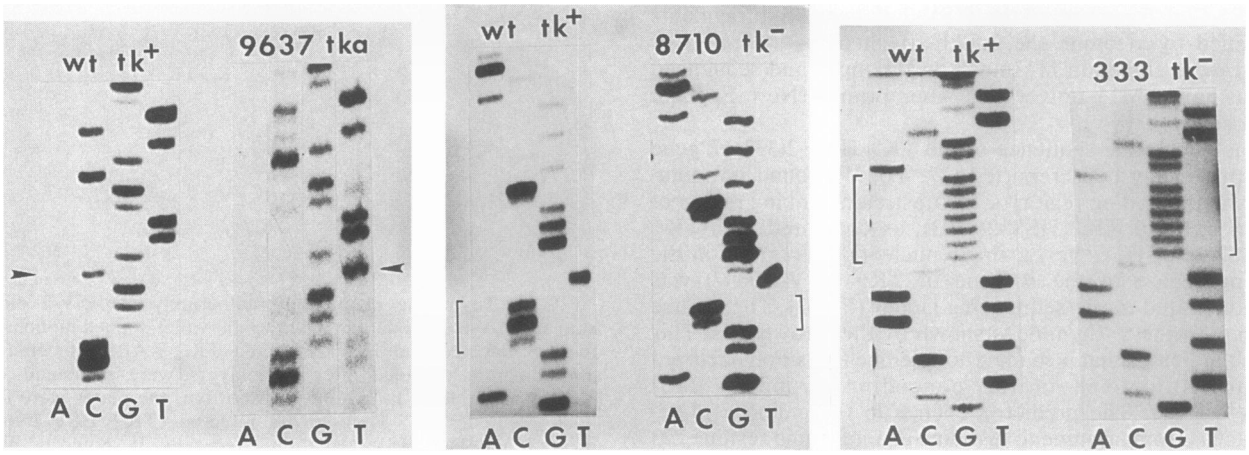

FIG. 3. Sequencing ladders around the mutated regions in the *tk* genes of HSV-2 isolates. The sequencing ladders for TK^A HSV-2(9637) and wild-type (wt) TK$^+$ HSV-2(8703) (leftmost panel) read the coding strand of the *tk* gene. The other ladders read the noncoding strands of the *tk* genes of the TK$^-$ HSV-2 mutants and their wild-type TK$^+$ HSV-2 parents.

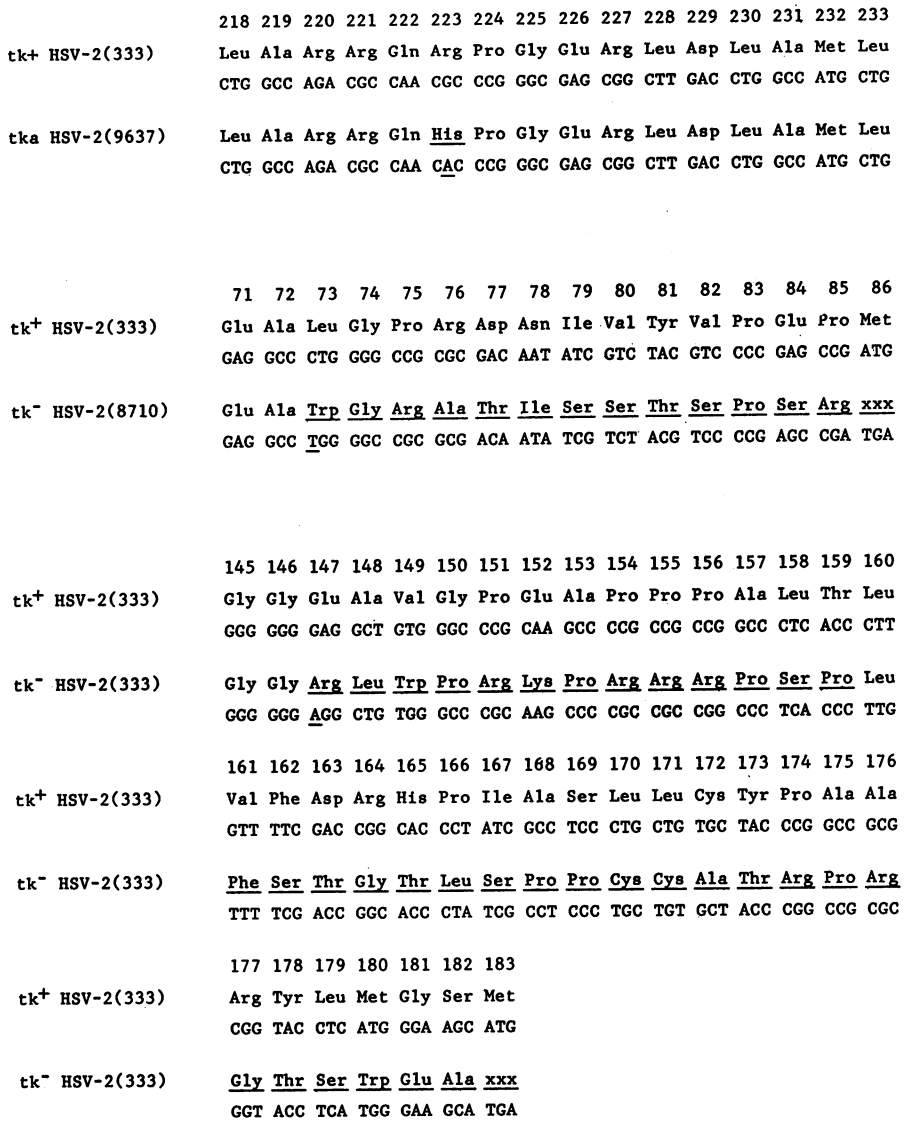

FIG. 5. Nucleotide sequences of the mutated regions of the HSV-2 *tk* genes and the deduced amino acid sequences of the TK polypeptides. The altered nucleotides and amino acid residues are underlined. The amino acid residue numbers are shown above each sequence.

nucleotide-binding pocket. This view is supported by structural evidence on adenylate kinase and by chemical and photoaffinity labeling studies of myosin. In adenylate kinase, the sequence Gly-Gly-X-Gly-X-Gly (residues 15 to 20) is involved in making a bend linking two antiparallel β-sheets that form part of the AMP-binding pocket. A similar sequence is found in the β subunit of mitochondrial ATPase, and the predicted secondary structure in this region is a β bend. Likewise, the predicted secondary structure of the corresponding sequence of the HSV TK polypeptide makes a β bend. The lysine residue that follows the predicted loop sequence has been conserved throughout the examples, suggesting that it may interact with the alpha phosphate of a nucleotide (22, 40, 55).

A second region of conserved amino acid sequences in the HSV TK polypeptide occurs at residues 168 to 178 (Ala-Leu-Leu-Cys-Tyr-Pro-Ala-Ala-Arg-Tyr-Leu) of the HSV-1 TK polypeptide (54). Darby and co-workers (9) showed that drug-resistant HSV-1 mutants with distinct alterations in apparent affinities for nucleoside substrates had changes in this region of the polypeptide sequence. Specifically, there were substitutions of threonine for alanine at residue 168 and of glutamine for arginine at residue 176, respectively, in the BVDU-resistant HSV-1 mutant B3 and in the ACV-resistant HSV-1 mutant Tr7. Darby et al. (9) also observed that the only difference between the amino acid sequences of wild-type strains of HSV-1 and HSV-2 in residues 168 to 178 involved the replacement of the alanine at residue 168 of the HSV-1 TK polypeptide with a serine residue in the HSV-2 TK polypeptide. This substitution is similar to that in BVDU-resistant HSV-1(B3). Wild-type HSV-2 strains are appreciably more resistant to BVDU than are wild-type HSV-1 strains (11). Fyfe and co-workers (19, 21) have shown that a thymidylate kinase activity is associated with the TK enzymes induced by wild-type HSV-1 and HSV-2 and by HSV-1(B3). However, the three enzyme activities differ in phosphorylating activity for bromovinyldeoxyuridine monophosphate (BVDUMP). The wild-type HSV-1 enzyme efficiently phosphorylates BVDU to BVDUMP and the diphosphate form. The wild-type HSV-2 and HSV-1(B3) enzymes

TABLE 2. Frequency of repeated bases in the coding regions of viral and eucaryotic *tk* genes

Source of *tk*	Nucleotide	No. of repeats in the *tk* coding region containing indicated no. of bases:				
		3	4	5	6	7
HSV-1[a]	G or C	38	12	6	1	1
	A or T	11	2			
HSV-2	G or C	34	15	6	2	1
	A or T	3	2	1		
Chinese hamster cells[b]	G or C	12	3	1		
	A or T	6	1	1		

[a] Data are from Wagner et al. (54) and McKnight (37).
[b] Data are from Lewis (32).

phosphorylate BVDU to BVDUMP but are inefficient in phosphorylating BVDUMP to the diphosphate form. The inefficiency of the wild-type HSV-2 and HSV-1(B3) enzymes in catalyzing the second phosphorylation step (BVDUMP to the diphosphate form) is consistent with the insensitivity of HSV-2 and HSV-1(B3) virus replication to BVDU. Thus, the sequencing data showing that residue 168 is altered in wild-type HSV-2 and HSV-1(B3) TK enzymes do not necessarily show that residue 168 has a role in thymidine binding, as suggested by Darby et al. (9). An alternative interpretation is that residue 168 has a role in dTMP and BVDUMP binding.

A third mutant, HSV-1(S1), isolated in vitro in the presence of high concentrations of ACV, had a substitution at residue 336 of the TK polypeptide of tyrosine for cysteine (9). This amino acid substitution at residue 336 had a marked effect on ATP binding as well as on nucleoside binding, suggesting that the wild-type Cys-336 residue might be situated close to or constitute part of both the ATP-binding site and the nucleoside-binding site. For example, it seemed possible that the Cys-336 residue near the C terminus of the HSV-1 TK polypeptide formed a disulfide bond with the Cys-171 residue (9). To test this hypothesis, Inglis and Darby (23) isolated recombinant-derived TK enzyme mutants in which serine or glycine replaced cysteine at residue 171. Analyses of the mutant enzymes in bacterial extracts showed, however, that these substitutions had little effect on the TK activity, indicating that the side chain of this residue was not involved in nucleoside binding and was not essential for TK catalytic activity.

In the case of the clinical isolate TK^A HSV-2(9637), the amino acid substitution of histidine for arginine occurred at residue 223 of the TK polypeptide. This mutation is outside the nucleoside-binding site proposed by Darby et al. (9). Since we do not know how the TK polypeptide folds in three dimensions or how substitution of one amino acid residue for another affects the structure and since chemical and photoaffinity labeling studies of the TK enzyme and its substrates have not been performed, it may be premature to speculate on the site of nucleoside binding from only the sequence data of *tk* mutants.

It is of interest to note that both of the TK^- HSV-2 mutants used in our study were frameshift and chain termination mutants. The mutations occurred at repeats of cytosine or guanine by the deletion of one base in each case. Summers et al. (48) examined polypeptides induced by TK^- HSV-1 mutants and found that most of the TK^- mutants had either a loss or a shortening of the 40-kilodalton TK polypeptide. Hence, it is possible that TK^- frameshift mutations of HSV-1 are common.

Genetic and protein sequencing studies of the bacteriophage T4 lysozyme gene have revealed that frameshift mutations arise preferentially in repeated DNA sequences (39, 47). In bacteriophage T4, repeating adenines and thymines are frameshift hot spots (39, 44, 47). In the *Salmonella hisD* gene, one frameshift hot spot was found to be a DNA sequence of four tandem cytosines (24). In polyomavirus, a frameshift mutation which arises from the deletion of any one of nine consecutive cytosines in the region of polyomavirus DNA encoding both the midregion of large T antigen and the C-terminal region of middle T antigen has been described (56). Consecutive rows of a single nucleotide have been found to be hot spots for frameshift mutations at the immunoglobulin heavy-chain locus of mice (2). Additions or deletions presumably arise by slippage or stuttering of the DNA polymerase at the homonucleotide run or by misalignment of the replicating and parental DNA strands (39, 44, 47).

Since the HSV *tk* gene is enriched in guanine · cytosine base pairs, there is a high frequency of guanine and cytosine repeats in the coding region of the *tk* gene. Table 2 illustrates that HSV *tk* genes contain a higher number of homonucleotide repeats than do cellular *tk* genes. If these homonucleotide repeats are potential hot spots for frameshift mutations, it would be expected that the HSV *tk* gene would have a high mutation frequency. Parris and Harrington (42) examined many clinical isolates of HSV which had never been exposed to ACV. They found in every isolate HSV variants, at a frequency of 10^{-4}, which were resistant to high concentrations of ACV. Since the majority of the ACV-resistant variants were TK^-, the mutation frequency of the HSV *tk* gene was about the same, i.e., 10^{-4}. One reason for this high mutation frequency could be the large number of guanine and cytosine repeats in the coding region of the HSV *tk* gene.

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LITERATURE CITED

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