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Homopolymer Mutational Hot Spots Mediate Herpes Simplex Virus Resistance to Acyclovir

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In the majority of cases, the mechanism underlying the resistance to acyclovir (ACV) of herpes simplex viruses (HSVs) is thymidine kinase (TK) deficiency. Plaque isolates from eight ACV-resistant (ACV^r) clinical isolates from AIDS patients, of which five reactivated, were sequenced to determine the genetic lesion within the *tk* gene conferring resistance and whether this may have correlated with reactivation potential. Mutations were clustered within two homopolymer nucleotide stretches. Three plaque isolates (1737-14, 90-150-3, and 89-650-5) had insertion mutations within a stretch of 7 guanosines, while two isolates (89-063-1 and 89-353-1) had frameshift mutations within a stretch of 6 cytosines (a deletion and an insertion, respectively). Mutations resulted in premature termination codons, and the predicted 28- and 32-kDa truncated TK products were detected by Western blot analysis of virus-infected cell extracts. The repair of one homopolymer frameshift mutation (in isolate 1737-14) restored TK activity, demonstrating that this mutation is the basis of TK deficiency. Of the five reactivated isolates, four were TK deficient and contained frameshift mutations while the fifth retained TK activity because of its altered-TK or Pol$^-$ phenotype. These data demonstrate that the majority of ACV^r clinical isolates contain frameshift mutations within two long homopolymer nucleotide stretches which function as hot spots within the HSV *tk* gene and produce nonfunctional, truncated TK proteins.

Genital herpes simplex virus (HSV) infections are a major public health problem (3, 20, 26). In the immunocompetent host, recurrences are self-limiting, but infections in untreated immunocompromised individuals can become chronic and progressive (41, 49). Acyclovir (ACV) is a potent and selective antiviral nucleoside which remains the "gold standard" of treatment and suppression in both normal and immunocompromised hosts (27, 33, 38, 40, 48). Clinically significant resistance to ACV has been almost exclusively seen in the immunocompromised population, where it occurs in up to 5% of patients (4, 8, 13, 14, 47). The mechanism responsible for the majority of ACV-resistant (ACV^r) isolates is a lack of viral thymidine kinase (TK), the enzyme required for initial phosphorylation of ACV before it undergoes further phosphorylation by cellular kinases to its active triphosphate form (16). Occasionally, ACV resistance can be due to an alteration of the TK protein such that the usually promiscuous spectrum of TK activity excludes ACV (9, 12, 16, 23, 24), or a mutation may occur in the viral DNA polymerase (*pol*) gene (2, 7, 30, 36). More recently, isolates designated as low TK producers (TK^{LP}) have been identified which retain a small but detectable amount of TK activity (arbitrarily defined as 1 to 15% by enzymatic assays) but test ACV^r by susceptibility testing (16).

TK-deficient (TK^D) mutants possess the ability to establish and maintain latency but are unable to reactivate in mouse models, suggesting that they should be unable to reactivate in patients (6, 11, 46). Recently, however, reports of reactivated TK^D clinical isolates have emerged (37, 39). Some of these isolates have been demonstrated to express TK activity at levels even below those of TK^{LP} strains. These levels are below the level of detection of conventional assays and have been termed "ultralow," demonstrating that the amounts of TK activity required for reactivation are far lower than previously appreciated (39). Finally, HSV clinical isolates have been demonstrated to exhibit high degrees of TK heterogeneity, which may result in difficulties in characterization of DNA sequences and protein products (35, 36).

The aim of this work was to sequence a collection of ACV^r clinical isolates and thus define the spectrum of TK genotypic lesions within such isolates. We found that five of eight isolates contained mutations at two homopolymer stretches, suggesting the presence of two mutational hot spots which produce nonfunctional, truncated TK proteins.

MATERIALS AND METHODS

Patients. Clinical isolates were derived from two sources. Initial work was performed on WJ clinical isolates, derived from an AIDS patient who was the subject of a previous report (39) and from whom isolates 1737 (reactivated TK^D) and 1773 (wild-type TK [TK^{WT}] control) were derived. The remaining seven clinical isolates were derived from AIDS Clinical Trials Group (ACTG) study 095, a trial of antiviral therapies in AIDS patients with ACV^r HSV (37). Clinical isolates 89-063, 89-353, 90-150, and 2370 were ACV^r reactivated isolates obtained in the absence of drug pressure, while isolates 89-390, 89-650, and 90-110 were ACV^r strains that evolved during ACV therapy and which were followed by ACV-susceptible (ACV^s) isolates at next reactivation.

Virologic studies. Clinical isolates were obtained, identified, and typed as previously described (34). Viral stocks were grown and titered, and drug susceptibility assays were performed by plaque reduction assays to determine the ACV concentrations required to inhibit plaque formation by 50% (ID_{50}s, in micrograms per milliliter) as previously described (39). Resistance to ACV or foscarnet was defined as an ID_{50} above 2.0 or 100 μg/ml, respectively. Techniques used

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TABLE 1. ACV and PFA susceptibilities and TK phenotype for HSV clinical and plaque isolates

Source	Site	Isolate type							
		Clinical[b]				Plaque[d]			
		Strain[a]	ID_{50}		Phenotype[c]	Strain	ID_{50}		Phenotype[c]
			ACV	PFA			ACV	PFA	
Control		G	0.03	62.7	TK^{WT}				
		ACGr4	27.7	53.4	TK^{D}				
		615.8	2.1	238.6	Pol^{-}				
WJ	Genital	1773	0.11	ND[e]	TK^{WT}	1773-5	0.14	ND	TK^{WT}
	Genital	1737	6.6	ND	TK^{D}	1737-14	11.8	ND	TK^{D}
ACTG-095	Perirectal	89-063	12.8	ND	TK^{D}	89-063-1	19.2	ND	TK^{D}
	Genital	89-353	6.2	ND	TK^{WT}/TK^{D}	89-353-1	21.8	ND	TK^{D}
	Genital	90-150	3.7	ND	TK^{WT}/TK^{D}	90-150-3	23.7	ND	TK^{D}/TK^{WT}[f]
	Perirectal	2370	2.1	ND	TK^{A} or Pol^{-}	2370-2	19.5	41.2	TK^{A} or Pol^{-}
	Orofacial	89-390	6.0	ND	TK^{D}	89-390-4	9.5	24.6	TK^{D}
	Perirectal	89-650	10.0	ND	TK^{D}	89-650-5	10.1	ND	TK^{D}
	Perirectal	90-110	8.7	ND	TK^{D}	90-110-4	9.5	ND	TK^{D}

[a] Strain G is TK^{WT}, ACGr4 is TK^{D}, and 615.8 is Pol^{-}. WJ isolates include the TK^{WT} isolate 1773 and the TK^{D} isolate 1737. ACTG-095 isolates were all ACV^{r} isolates from AIDS patients unresponsive to ACV.
[b] Clinical isolates were obtained directly from HSV lesions in AIDS patients.
[c] As determined by plaque autoradiography with [^{125}I]iododeoxycytidine.
[d] Plaque isolates were obtained by plaque purification of corresponding clinical isolates.
[e] ND, not determined.
[f] Heterogeneity improved to 89% TK^{D}.

for plaque purifications and plaque autoradiography with [^{125}I]iododeoxycytidine have been described elsewhere (39).

DNA sequencing. DNA sequencing of the *tk* gene was performed by cycle sequencing of PCR-generated products after viral DNA purification from Vero cells infected with plaque isolates. DNA purification was performed after inoculation of confluent Vero cells (multiplicity of infection, 0.1) and incubation until there was a greater than 80% cytopathic effect. The cell monolayer was then washed with cold phosphate-buffered saline and incubated on ice for 5 min with 1 ml of cold lysis buffer (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate). Cells were harvested and nuclei were removed by centrifugation at $800 \times g$ rpm for 5 min at 4°C, and the supernatant was incubated for 2 h at 37°C with a solution containing 50 μg of proteinase K per ml, 5 mM EDTA, and 12.5 μg of RNase A per ml. The solution was extracted three times with phenol-chloroform and then was chloroform extracted once before ethanol precipitation with 0.3 M sodium acetate and resuspension in 100 μl of distilled water.

PCR was performed with 2 μg of template and 50 pmol of each primer external to the *tk* open reading frame (JS1 [CTGATCAGCGTCAGAGCGTT] and JS8 CGCTTATGGACACACCACAC]). Amplification conditions included denaturation at 100°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min, and then an extension step at 72°C for 10 min. After electrophoretic separation and visualization with ethidium bromide in 0.8% agarose, the TK product was purified with a Sephaglas DNA purification kit (Boehringer Mannheim Biochemica, Laval, Quebec, Canada). Cycle sequencing was performed with both external primers as well as 14 overlapping sense and antisense internal primers end labeled with [γ-^{32}P]dATP (6,000 Ci/mmol; Amersham Life Sciences, Oakville, Ontario, Canada) to encompass the entire *tk* open reading frame (JS2, CTCATCAGCGTC AGAGCGTT; JS4, TCATTGTTATCTGGGCGCTG; JS5, AATGGCGGACA GCATGGCCA; JS6, TGTCTACGATCTACTCGCCAA; JS7, AATCCAGGA CAAATAGATGC; JS9, TACCTCATGGGAAGCATGAC; JS10, CTGCTGC GGGTTTATATAGA; JS11, GTAAGTCATCGGCTCGGGGA; JS12, GGGG AGGCGGCGGTGGTAAT; JS13, GGGTAGCACAGCAGGGAGGC; JS14, GGAACAGGGCAAACAGCGTG; JS15, CACATTTTTGCCTGGGTCTT; JS17, GTTCGGTCAGGCTGCTCGTG; JS18, CAAACGTGCGCGCCAGGT CG; and JS19, GTGGGGTCCGTCTATATAAA). Reactions were carried out with a dsDNA Cycle Sequencing System (Gibco BRL, Burlington, Ontario, Canada). Reaction conditions included denaturation at 95°C for 3 min, 20 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 70°C for 60 s, and then 10 cycles of denaturation at 95°C for 30 s and extension at 70°C for 60 s. Products were then denatured at 100°C for 5 min and run on a 6% polyacrylamide gel, which was subsequently dried and exposed to X-ray film. All sequences were then confirmed by automated sequencing.

Western blot analysis. Identification of TK protein was performed by Western blotting with a monoclonal antibody produced against HSV type 2 (HSV-2) TK (kindly provided by Ken Powell, Glaxo Wellcome, Beckenham, United Kingdom). Cells in culture dishes (60 by 15 mm) were inoculated with virus at a multiplicity of infection of 3, adsorbed for 1 h, and incubated with 5% minimal essential medium for 5 h. The monolayer was then washed with 2 ml of ice-cold phosphate-buffered saline, the cells were lysed with 1 ml of cold lysis buffer for 15 min on ice, and nuclei were removed by centrifugation at $800 \times g$ for 5 min at 4°C. Protein was then precipitated from the supernatant by adding 4 volumes of 100% acetone, incubating at −20°C for 1 h, centrifuging at $11,000 \times g$ for 10 min at 4°C, washing with 80% acetone, drying the pellet, and resuspending it in 50 μl of sodium dodecyl sulfate sample buffer. The samples were denatured at 100°C for 5 min, electrophoresed at 25 mA for 4 h through a 6% stacking–10% resolving acrylamide gel, and then blotted onto a nitrocellulose membrane by using an electroblotting transfer apparatus (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Antibody incubations and detection were performed with a LumiGLO Western blot kit; chemiluminescent horseradish peroxidase system (Kirkegaard & Perry Laboratories, Gaithersberg, Md.). Briefly, the membrane was blocked with 0.5% milk blocking solution for 1 h at room temperature, incubated with a 1/5,000 dilution of primary antibody for 1 h at room temperature, washed three times in wash buffer, incubated with 60 U of secondary antibody (goat anti-mouse; Boehringer Mannheim Biochemica) per ml, and washed three more times. A chemiluminescent substrate was then applied to the membrane for 1 min prior to exposure of the membrane to X-ray film.

Mutation repair. DNA from isolate 1737-14 was used in a PCR with primers JS1 and JS8 to generate a 1.6-kb fragment representing the TK gene. This was then used as a template in a PCR with primers TKF (CCCAAGCTTGGGGT GGCGTTGAAC) and TKR (CTAGTCTAGACTAGTTCGGGCTTCCGTGT TTG) to generate a 1,200-bp fragment representing the TK coding region and a short noncoding region at each end with a unique *Hin*dIII site at the 5′ end and an *Xba*I site at the 3′ end. This was then ligated into pRc/CMV after digestion with *Hin*dIII and *Xba*I, yielding plasmid 1737-14 IIA-3. To repair the mutation in the homopolymer sequence of this TK gene (bases 433 to 439 of the open reading frame), primers TK7G (CGGGGCTTGCGGGCCCACAGCCTCCCC CCCGATATGAGGAGC) and TKSC (GCGAACGCCTTGTAGAAGCGGGT ATGGCTTCTCACGCCGG) were used to generate a fragment containing 7 G residues in place of the 8 G residues of the mutant gene. The resulting fragment was then recombined into plasmid 1737-14 IIA-3 by digestion of the fragment and the plasmid with *Apa*I and pflM1, followed by ligation. Ten recombinants were selected, and the plasmid DNA was transfected into TK-deficient mouse L cells by using lipofectamine (Gibco BRL, Burlington, Ontario, Canada). After 2 days, cells were incubated with medium containing HAT supplement (Gibco BRL) to select for cells expressing TK. After 7 days of growth, the medium was removed and the monolayer was incubated with 0.1% methylene blue in 70% ethanol for 10 min to stain the cell colonies.

RESULTS

Susceptibility testing. HSV resistance to ACV has been shown to occur in the clinical setting, where it is seen almost exclusively in immunocompromised patients. Accordingly, all

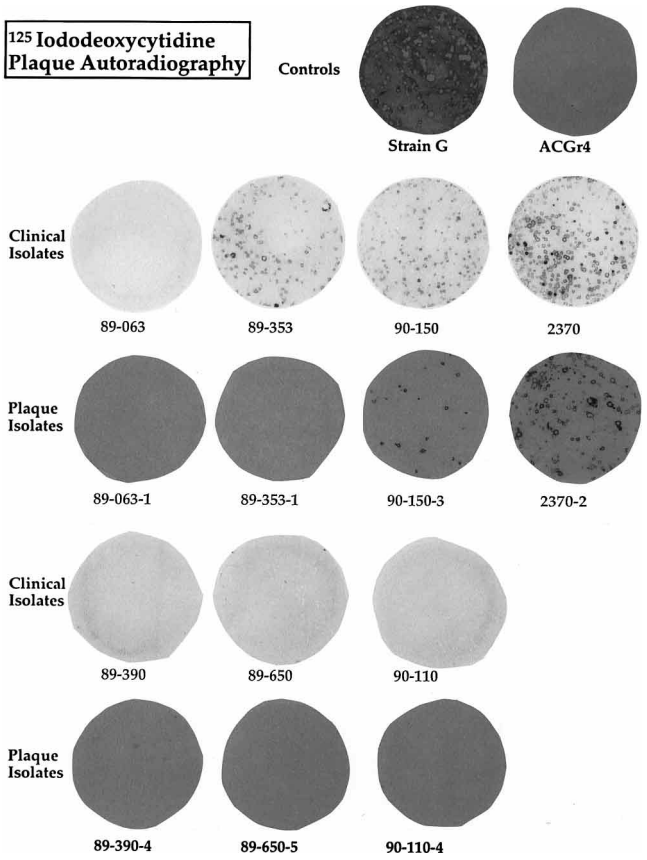

FIG. 1. Plaque autoradiography of ACTG 095-derived clinical isolates and respective plaque isolates using [^{125}I]iododeoxycytidine. Clinical isolates 89-353, 90-150, and 2370 have plaques expressing high TK activity. Plaque isolate 89-353-1 is homogeneously TK^D, while isolate 90-150-3 is 89% TK^D. All plaques from isolate 2370-2 continued to express high TK activity due to resistance on the basis of a non-TK^D phenotype. All other clinical and plaque isolates are homogeneously TK^D.

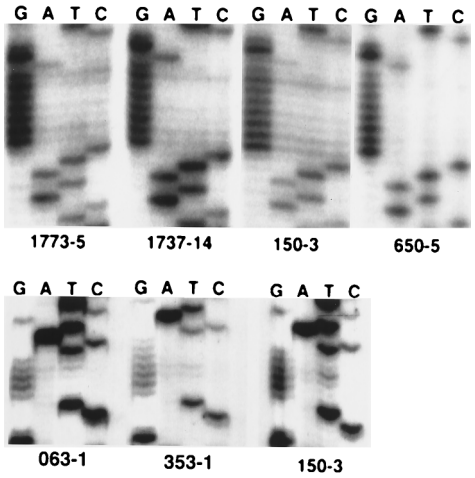

FIG. 2. Sequencing gels of plaque isolates demonstrating mutations. The top panel shows the noncoding (mRNA) strand of *tk* and demonstrates that the WJ-derived ACV^r plaque isolate, 1737-14, has a G addition in a stretch of 7 G residues compared to the ACV^s isolate 1773-5. ACTG 095-derived isolates 90-150-3 and 89-650-5 demonstrate the identical insertion mutation. The lower panel shows the strand complementary to the one above it and demonstrates a G deletion within 89-063-1 and a G insertion within 89-353-1 in a homopolymer stretch of 6 nucleotides. This would be represented as a stretch of 6 C residues in the noncoding (mRNA) strand.

clinical isolates to be studied were obtained by swabbing HSV lesions of AIDS patients which were unresponsive to ACV therapy. All clinical isolates were determined to be HSV-2 by immunofluorescence. To verify that clinical isolates were truly resistant to ACV, all isolates initially were subjected to susceptibility testing, with plaque reduction assays being performed to determine the ACV ID_{50}s. All clinical isolates were shown to have ID_{50}s above 2.0 μg/ml, demonstrating ACV resistance (Table 1).

Assessment of heterogeneity and plaque purification. It has been demonstrated previously that clinical isolates that are resistant to ACV can contain high levels of TK^{WT} virus (35, 36), which may interfere with further analysis if entire clinical-isolate populations are used. To eliminate this possibility, the clinical isolates used in this study were analyzed for evidence of TK heterogeneity by plaque autoradiography (Fig. 1). The results of these assays showed that isolates 89-353, 90-150, and 2370 consisted of populations containing 48, 59, and 100% TK^{WT} plaques, respectively (Fig. 1). Therefore, plaque purifications were performed on all clinical isolates to reduce heterogeneity so that subsequent DNA sequencing and protein studies could be performed. The resulting plaque isolates were also subjected to ACV susceptibility testing, and all were demonstrated to be resistant, with ID_{50}s above 2.0 μg/ml (Table 1). To determine whether heterogeneity had been reduced, plaque isolates were subjected to an additional round of plaque autoradiography. Of the corresponding plaque isolates, 89-063-1, 89-353-1, 89-390-4, 89-650-5, and 90-110-4 were homogeneous populations of TK^D virus whereas 90-150-3 was 89% TK^D. By contrast, all plaques of 2370-2, despite plaque purification, continued to express TK activity, suggesting that this isolate had either a Pol^- or a an altered-TK (TK^A) phenotype rather than a TK^D phenotype (Fig. 1). Isolate 1737 and its plaque isolate, 1737-14, were characterized by plaque autoradiography in a previous study and were both shown to be homogeneously TK^D (39).

TK gene sequencing. In an attempt to identify the genetic basis for the alteration of TK activity in these isolates, the TK genes of all plaque isolates were sequenced. To do this, primers were produced for amplification of the TK region of the genome and the resulting PCR products were purified and sequenced by both manual and automated methods. Sequencing of WJ-derived plaque isolates demonstrated that 1773-5 (control) differed from 1737-14 (TK^D) by a single G insertion in a homopolymer stretch of 7 G residues between nucleotides 433 and 439 of the open reading frame (Fig. 2). Of the ACTG study 095-derived plaque isolates, both 90-150-3 and 89-650-5 also contained a G insertion in the same homopolymer stretch, suggesting the presence of a mutational hot spot. Of the remaining ACTG study 095-derived plaque isolates, two (89-063-1 and 89-353-1) also contained frameshift mutations (a deletion and an insertion, respectively), but these occurred in a downstream stretch of six cytosines between nucleotides 550 and 555. These data suggested the presence of a second homopolymer hot spot (Fig. 2).

A different mutation was identified in plaque isolate 90-110-4, which contained a 17-nucleotide deletion. This deletion was flanked on either side by a TCCG repeat motif, suggesting looping out due to misalignment of complementary strands during DNA replication, beginning at either nucleotide 111 or 115. This mutation would lead to a frameshift close to the N-terminal part of the polypeptide, at either amino acid 37 or 39.

Plaque isolate 2370-2 had two nonconservative base pair substitutions when compared to the reference HSV-2 333 TK sequence (21): an A-to-G mutation at position 231 of the open reading frame and a G-to-A mutation at position 1009, resulting in the substitution of aspartic acid for asparagine and tyrosine for cysteine, respectively. Thus, 2370-2 was expected to produce a full-length TK product. The latter amino acid has been reported to be involved in ATP and nucleoside binding (10, 32) and may have mediated ACV resistance via a TK^A phenotype. Plaque isolate 89-390-4 also had two nonconservative nucleotide substitutions compared to HSV-2 333: an A-to-G mutation at position 115 of the open reading frame and a G-to-A mutation at position 649, resulting in amino acid substitutions of glycine for glutamic acid and histidine for arginine, respectively. This also predicted the production of a full-length product.

Western blot analysis. The sequencing data indicated that several of the isolates had suffered mutations that would lead to frameshifts and result in truncated proteins. To test this hypothesis, Western blot assays were performed on extracts of cells infected with plaque isolates, using a monoclonal antibody to HSV-2 TK. WJ control plaque isolate 1773 and control strain G both produced the expected full-length 40-kDa protein. By contrast, analysis of the protein products formed by plaque isolates 1737-14, 90-150-3, and 89-650-5, which contained the G insertion within the homopolymer stretch of 7 G residues, showed that a 28-kDa truncated protein product occurred, which is the size predicted for the isolates. Truncated TK proteins produced fainter bands than the wild type, requiring a larger inoculum for detection and resulting in overexposure of wild-type bands. Plaque isolates containing frameshift mutations within the downstream homopolymer stretch of 6 C residues (89-063-1 containing a C deletion and 89-353-1 containing a C insertion) also produced truncated TK products (of 32 and 28 kDa, respectively), as predicted by their mutations (Fig. 3).

Plaque isolate 90-110-4, which contained the 17-nucleotide deletion, did not produce a detectable TK product. It appeared that the early frameshift caused by this mutation rendered the translation product unrecognizable by the monoclonal antibody (Fig. 3). Plaque isolates 2370-2 and 89-390-4, which did not contain frameshift mutations, both produced the expected full-length 40-kDa protein products (Fig. 3).

Mutation repair. To determine whether the single base change could account for the loss of TK activity in these virus isolates, the extra G in the homopolymer string of 8 G residues in strain 1737-14 was repaired so that it contained 7 G's. To this end, a two-step PCR procedure was devised to generate a plasmid containing the 1737-14 *tk* gene with a targeted deletion of a single G in the homopolymer stretch. This fragment was then cloned into the mammalian expression vector pRC/CMV and introduced into TK-deficient mouse L cells by transfection. Following selection in HAT medium to kill cells that remain TK deficient, colonies of TK-expressing cells arose in the samples transfected with the repaired gene but not in control cells transfected with the mutant *tk* gene or an irrelevant control sequence (see Fig. 5). Taken together, these results indicate that the single base change introduced to repair the mutant *tk* gene restored its TK activity. Thus, the loss of TK activity in the mutant virus could be accounted for solely by this mutation.

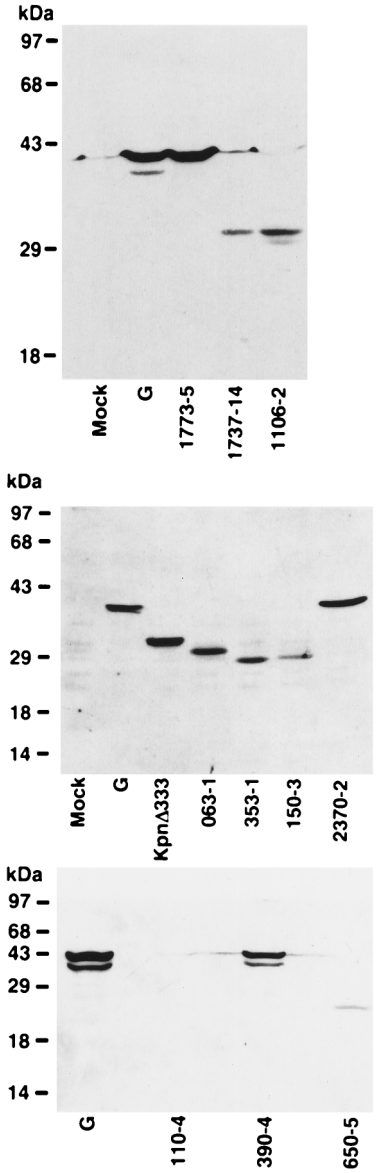

FIG. 3. Western blot analysis of TK protein products from plaque isolates. The top panel shows the blot of WJ-derived plaque isolates. Lanes: Mock, mock infected with no detectable TK protein; G, wild-type control strain G; 1773-5, TK^{WT} plaque isolate 1773-5, which produces a full-length 40-kDa product similar to that of control strain G; 1737-14, ACV^r plaque isolate 1737-14, which produces the predicted 28-kDa truncated product; 1106-2, a plaque isolate from another ACV^r clinical isolate which also produces the 28-kDa truncated product. The middle and lower panels show protein products produced by ACTG 095-derived plaque isolates. Lanes: Mock, mock infected; G, wild-type control strain G; KpnΔ333, control TK^D strain producing a truncated product of ca. 35 kDa. Plaque isolates 90-150-3 and 89-650-5 (containing the same G insertion as 1737-14) produce the predicted truncated 28-kDa protein. Plaque isolate 89-353-1 (containing the C insertion) also produces the predicted 28-kDa protein, while 89-063-1 (containing the C deletion) produces the predicted 32-kDa truncated product. Plaque isolates 2370-2 and 89-390-4 produced full-length 40-kDa proteins. Plaque isolate 90-110-4 produces no detectable protein product.

DISCUSSION

This report identifies several *tk* mutations in clinical isolates which result in resistance to ACV. Moreover, the data show that frameshift mutations at a limited number of long homopolymer nucleotide stretches underlie the majority of defects in TK activity and appear to be mutational hot spots. This is significant as the identification of a hot spot may suggest a mechanism for the appearance of ACV resistance.

The results demonstrated that of the eight ACV^r isolates

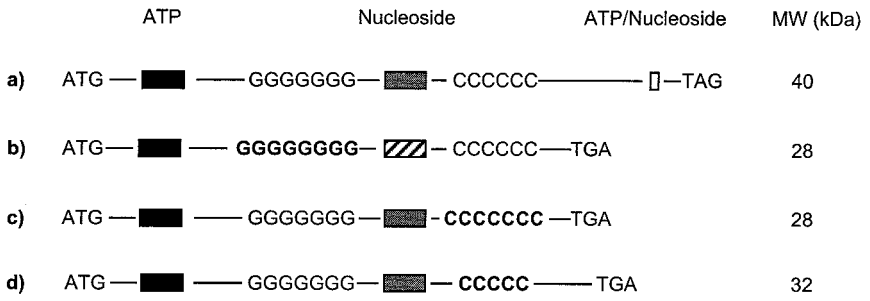

FIG. 4. Diagram depicting the locations of mutations at the two identified hot spots, their effects on protein product size, and their effects on regions of TK identified as binding to ATP, nucleoside, or both. (a) Diagram of wild-type TK, depicting sites of homopolymer runs of 7 G's and 6 C's, producing a full-length 40-kDa TK protein containing all binding sites, as seen in isolate 1773-5. (b) G insertion within the stretch of 7 G's which alters the amino acids within the nucleoside-binding site and codes for a premature stop codon which results in a truncated 28-kDa protein lacking amino acid 336, which is involved in both ATP and nucleoside binding. This mutation was detected in isolates 1737-14, 90-150-3, and 89-650-5. (c) C insertion within the stretch of 6 C's which codes for a premature stop codon at the same site as in panel b and also results in a truncated 28-kDa product lacking amino acid 336. This was detected in isolate 353-1. (d) C deletion within the same string of 6 C's depicted in panel c but coding for a premature stop codon further downstream than that of panels b and c, resulting in a 32-kDa truncated protein also lacking amino acid 336. This was detected in isolate 89-063-1. MW, molecular mass (in kilodaltons).

sequenced, three contained identical G insertions within a stretch of 7 G residues and two contained C frameshift mutations at a second downstream stretch of 6 C's. These two homopolymer stretches are the longest in the gene. The predicted consequences of such mutations would be that the frameshifts would alter every amino acid beyond the mutation and would code for a premature stop codon, resulting in a truncated protein devoid of activity (Fig. 4). The restoration of TK activity in one isolate following repair of its homopolymer mutation demonstrated that these frameshift mutations were responsible for TK deficiency (Fig. 5).

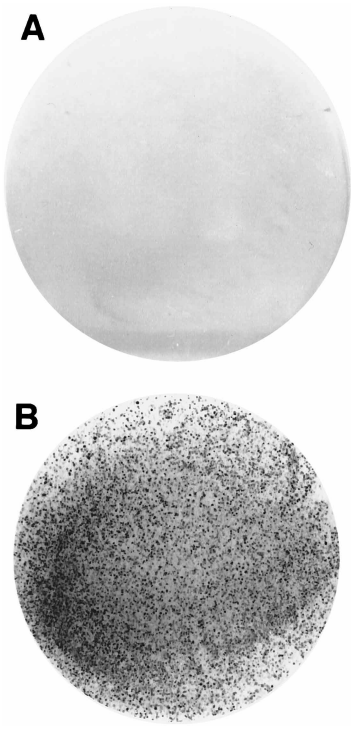

FIG. 5. Selection of TK-expressing cells in HAT medium. DNAs containing the mutant *tk* gene from 1737-14 (A) and the repaired gene (B) were transfected into TK-deficient mouse L cells growing on 10-cm plates by use of lipofectamine (see Materials and Methods). Following transfection, cells were allowed to grow for several days and then selected in HAT medium to kill cells that remained TK deficient. Dishes were stained after 7 days and photographed.

This is the largest collection of ACV-resistant clinical isolates sequenced to date. Prior work has shown that truncated TK proteins are commonly produced by ACV^r HSV-1 laboratory-derived mutants and HSV-2 clinical isolates, suggesting that chain termination mutants are common, although these isolates were not sequenced (5, 43). Two other reports have described frameshift mutations in the homopolymer stretch of 7 G residues: one was a deletion in a laboratory-derived TK^D HSV-2 isolate and the other was an addition in a plaque-purified HSV-1 clinical isolate (19, 22). In addition, laboratory-derived isolates were shown to occur more frequently at G-C homopolymers stretches (18). These data further enhance the evidence that frameshift mutations at such homopolymer sites are a major mechanism mediating ACV resistance. Other reports of TK gene sequences in TK^D clinical isolates have identified two isolates containing C deletions in strings of 3 C's (22, 29). Interestingly, there have been only two reports of base substitutions in TK^D clinical isolates, both of which retained neurovirulence (5, 45). These data are in marked contrast to varicella-zoster *tk* sequence data; this gene has a much lower G-C content with fewer long homopolymer sequences, and mutations within ACV^r clinical isolates have been shown to be widely distributed throughout the gene (25, 44).

Homopolymer nucleotide stretches have been shown to be particularly susceptible to frameshift mutations in other systems. Sites of homopolymer nucleotides have been shown to be mutational hot spots in the T4 bacteriophage (28, 31, 42) and polyomavirus (50) genomes, as well as within eucaryotic DNA, such as the mouse immunoglobulin heavy chain locus (1). Such hot spots have been shown to be susceptible to both spontaneous and mutagen-induced mutations, with the rate of mutation being proportional to the number of identical nucleotides. The putative mechanistic model of mutation involves local misalignment of base pairs within homopolymer stretches which provides multiple sites for misaligned but complementary base pairing (42). Alternatively, the viral DNA polymerase may preferentially slip or stutter at homopolymer residue stretches. Such data suggest that homopolymer nucleotide stretches may be mechanistically involved in mutational hot spots throughout diverse biological systems. Given that these are the longest nucleotide homopolymer stretches in the gene and that the gene is particularly G-C rich, it is likely that such

TABLE 2. Plaque isolates with respective mutations, TK phenotypes, and reactivation abilities

Isolate[a]	Mutation(s)[b]	Phenotype[c]	Potential for reactivation[d]
1737-14	G insertion	TK^D	Yes
89-063-1	C deletion	TK^D	Yes
89-353-1	C insertion	TK^D	Yes
90-150-3	G insertion	TK^D	Yes
2370-2	Substitutions	TK^A or *pol*	Yes
90-110-4	17-bp deletion	TK^D	No
89-390-4	Substitutions	TK^D	No
89-650-5	G insertion	TK^D	No

[a] Plaque isolates derived from ACV-resistant clinical isolates by plaque purification.

[b] Mutations within the *tk* gene open reading frame detected in plaque isolates, demonstrating whether they were frameshift mutations (additions or deletions) or base substitutions.

[c] TK phenotype of plaque isolates as determined by plaque autoradiography with [^{125}I]iododeoxycytidine.

[d] Reactivation potential of parent clinical isolate from which plaque isolate was derived.

a long stretch is composed of G-C pairs simply by chance, particularly in light of the fact that the HSV *pol* gene has been shown to have a high mutation frequency (18). Alternatively, as ACV is a guanosine analog, it may influence viral DNA polymerase to selectively slip or stutter in regions containing G-C homopolymers and induce frameshift mutations, although since ACV is an obligate chain terminator of viral DNA, it would have to do so without being incorporated into the growing DNA chain.

The sequencing approach in this work was unique in that it involved plaque purification of clinical isolates which exhibit TK heterogeneity within resistant populations. This had not been accomplished previously, and such improved TK purity allowed the gathering of more specific sequence information. It also highlights the need for an assessment of heterogeneity by techniques such as plaque autoradiography prior to sequencing. Because this approach samples only a single plaque, it is possible that different mutations may have been present in other TK-deficient plaques but does not detract from the finding of a high frequency of homopolymer mutations. For each clinical isolate, multiple ACV^r plaques need to be sequenced to assess the homogeneity of ACV^r mutations.

Such nucleotide stretches may also have a mechanistic role in reactivation of TK^D HSV isolates. Five of the ACV^r isolates reported here (1737-14, 89-063-1, 89-353-1, 90-150-3, and 2370-2) originated from reactivated clinical isolates. All four reactivated TK^D plaque isolates contained frameshift mutations (Table 2). There are two possible roles for homopolymers in reactivation of TK^D mutants. Homopolymer base stretches have been associated with increased reversion frequencies (28), which could, in turn, allow for the emergence of heterogeneity within a pure population. Such wild-type subpopulations could produce sufficient TK activity to allow reactivation in a mouse model and in the clinical situation, as demonstrated previously (39). Such reversion may also account for the TK heterogeneity of clinical isolates 89-353 and 90-150 demonstrated by plaque autoradiography (Fig. 1). Alternatively, such homopolymer stretches have been demonstrated to undergo translational frameshifting, until now a phenomenon reported only in RNA viruses but which recently has been demonstrated in vitro by an isolate containing a G insertion in the same stretch of 7 G residues. This may result in the production of a full-length TK protein in sufficient quantity to allow reactivation and may be related to the generation of unusual RNA structures within such stretches (17, 19). This would not, however, explain the presence of plaques with high TK activity. The relative contributions of both mechanisms remain to be defined. By contrast, isolate 2370 maintained endogenous TK activity despite plaque purification due to a TK^A or Pol^- phenotype and would be expected to retain its ability to reactivate (Table 2).

Putative hot spots may occur not only in reiterations of single base pairs but also within more complex repeats (15). This may also be the basis of resistance within 90-110-4, in which a 17-nucleotide deletion flanked by a common motif was looped out. An early deletion in the amino acid sequence such as this is consistent with our inability to detect the truncated peptide by using the monoclonal antibody. The mechanisms of ACV resistance in isolates 2370-2 and 89-390-4 remain undefined. Both produce full-length proteins and contain two nonconservative nucleotide substitutions within the open reading frame when compared to a reference TK^{WT} strain. Isolate 2370-2 retained TK activity, and the site of one amino acid substitution has been previously identified as being associated with ATP and nucleoside binding, suggesting it may have conferred a TK^A phenotype. Alternatively, it may be a *pol* mutant. By contrast, isolate 89-390-4 was TK deficient, as determined by plaque autoradiography, and possible mechanisms of resistance include an alteration in protein conformation and changes in regulatory sequences.

We have demonstrated that HSV *tk* mutations preferentially occur at a limited number of hot spots within long homopolymer nucleotide stretches rather than being randomly distributed throughout the gene. Two hot-spot sequences have been identified herein. In addition, such stretches may provide a mechanistic explanation for the ability of TK-deficient populations to produce sufficient TK to allow reactivation.

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