
Anne-Kathrin Brunnenmann, a, b Kathrin Bohn-Wippert, b Roland Zell, b Andreas Henke, b Martin Walther, b Oliver Braum, a, ** Gregor Maschkowitz, a Helmut Fickenscher, a Andreas Sauerbrei, b Andi Krumbholz a

Institute for Infection Medicine, Christian-Albrecht University and University Medical Center Schleswig-Holstein, Kiel, Germany; Institute of Virology and Antiviral Therapy, German Consulting Laboratory for HSV and VZV, Jena University Hospital, Friedrich Schiller University, Jena, Germany

In this study, approaches were developed to examine the phenotypes of nonviable clinical varicella-zoster virus (VZV) strains with amino acid substitutions in the thymidine kinase (TK) (open reading frame 36 [ORF36]) and/or DNA polymerase (Pol) (ORF28) suspected to cause resistance to antivirals. Initially, recombinant TK proteins containing amino acid substitutions described as known or suspected causes of antiviral resistance were analyzed by measuring the TK activity by applying a modified commercial enzyme immunoassay. To examine the effects of these TK and Pol substitutions on the replication of recombinant virus strains, the method of en passant mutagenesis was used. Targeted mutations within ORF36 and/or ORF28 and an autonomously expressed gene of the monomeric red fluorescent protein for plaque identification were introduced into the European wild-type VZV strain HJO. Plaque reduction assays revealed that the amino acid substitutions with unknown functions in TK, Q303stop, N334stop, A163stop, and the deletion of amino acids 7 to 74 aa (Aaa 7 to 74), were associated with resistance against acyclovir (ACV), penciclovir, or brivudine, whereas the L73I substitution and the Pol substitutions T237K and A955T revealed sensitive viral phenotypes. The results were confirmed by quantitative PCR by measuring the viral load under increasing ACV concentrations. In conclusion, analyzing the enzymatic activities of recombinant TK proteins represent a useful tool for evaluating the significance of amino acid substitutions in the antiviral resistance of clinical VZV strains. However, direct testing of replication-competent viruses by the introduction of nonsynonymous mutations in a VZV bacterial artificial chromosome using en passant mutagenesis led to reliable phenotypic characterization results.

Herpes zoster is caused by endogenous varicella-zoster virus (VZV) reactivation, especially in the elderly or in patients with immunodeficiencies. The disease occurs in 5 to 32% of transplant patients (1) and is related to significant morbidity and mortality, with fatality rates of up to 28% (1–3). The efficacy of antiviral therapy was demonstrated by multiple randomized controlled studies. Acyclovir (ACV), its prodrug valacyclovir, famciclovir (the prodrug of penciclovir [PCV]), and (E)-5-(2-bromovinyl)-2'-deoxyuridine (brivudine [BVDU]) are approved as therapeutics in many countries to treat VZV (4). These nucleoside analogues are phosphorylated by the viral thymidine kinase (TK) (open reading frame 36 [ORF36]) and cellular kinases to form a triphosphate that blocks viral DNA polymerase (Pol) (ORF28) by acting as competitive inhibitors and/or DNA chain terminators. Acyclovir resistance has been reported in immunocompromised, but not in immunocompetent, patients (5). If a patient shows therapeutic failure within 7 to 10 days, drug susceptibility should be evaluated (4, 6, 7), and alternative drugs are required. Foscarnet (FOS) and cidofovir (CDV), both inhibitors of Pol (8, 9), act independently of the viral TK and are recommended in such cases. Resistance testing of VZV is mainly based on a sequence analysis of the viral TK and Pol genes, since virus cultivation often fails (5, 7). However, in the absence of viable strains, the role of novel nonsynonymous mutations in VZV resistance cannot be clarified by the correlation of the resistance phenotypes and genotypes. Consequently, reliable information about resistance-related amino acid substitutions of VZV is limited. Furthermore, colony reduction assays have not gained broad acceptance for use in the phenotypic detection of ACV-resistant VZV variants (10). Therefore, alternative methods are essential for verifying the significance of nonsynonymous mutations in the TK and Pol genes of VZV for resistance. Thus, herpesvirus genomes can be manipulated with recombinant techniques based on the entire viral genome cloned as a bacterial artificial chromosome (BAC) (11–15). One strategy for targeted recombination is the Red recombination-based en passant mutagenesis technique for the seamless manipulation of BACs (16).

In this study, approaches were developed to examine the TK activities and resistance phenotypes of VZV variants, which are impossible to isolate from patients in cell culture. To this end,
several recently described amino acid substitutions in the TK that are known or suspected to cause resistance of clinical VZV strains to antivirals (5) were analyzed initially by the determination of the enzymatic activities of recombinant TK proteins using an enzyme immunoassay (ELA). Thereafter, en passant mutagenesis of a VZV BAC clone on the basis of the European HJO strain was employed to generate targeted mutations of the TK and DNA Pol genes (3, 15). Thus, a valuable system for studying the genotypes and phenotypes of clinical VZV isolates known to be refractory to antiviral treatment was developed, and the causative role of specific VZV sequence variants in phenotypic drug resistance was proven.

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MATERIALS AND METHODS

Virus strains. This study included seven VZV strains from patients with zoster and one obtained from a patient with encephalitis (5) (Table 1). The phenotypes of three strains with the following TK substitutions were described previously: first, the amino acid exchange Q303stop, observed in two patients with AIDS developing persistent zoster under ACV treatment (17, 18); second, the T256M substitution found after in vitro adaptation to ACV (19); and third, the W225R substitution in one strain cultured from the BAC pHJOpac (15) using Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The fragment was inserted into the Xhol- and XbaI-digested transfer vector plasmid pEPkan-S (provided by Karsten Tischer). The integrity of the BAC mutagenesis procedure (16, 24).

Expression of recombinant TK proteins and determination of enzymatic activity. The expression of recombinant TK proteins was determined and the enzyme activity assay carried out as described previously (22), with some modifications. The amplified TK genes (EasyXpress Plus linear template kit; Qiagen, Hilden, Germany) of the wild-type vOka were inserted into the Xhol- and EcoRI (Roche Diagnostics, Mannheim, Germany)-digested pIX 3.0 vector (Qiagen). Thymidine kinase mutants were generated by site-directed mutagenesis using the GeneArt site-directed mutagenesis system kit (Life Technologies, Darmstadt, Germany). Proteins were transcribed and translated in vitro from plasmids using the EasyXpress protein synthesis kit (Qiagen). The visualization of proteins was carried out as described previously (22). The anti-VZV TK goat antobody (1:200 dilution; Santa-Cruz, Heidelberg, Germany) and the alkaline phosphatase-conjugated rabbit anti-goat antibody (1:500 dilution; Acris, Herford, Germany) were applied as primary and secondary antibodies. The oligonucleotides used for mutagenesis and cloning are listed in Table S1 in the supplemental material.

The DiviTum test system (Biovica International AB, Uppsala, Sweden) was used for TK activity determination, and 1% bromo-2-deoxyuridine (BrdU) served as a TK substrate. This compound is phosphorylated to its monophosphate form by the functional TK and further to triphosphate by cellular kinases. After the immobilization of triphosphate by DNA synthesis, BrdU is detected according to the indirect EIA technique by an anti-BrdU antibody conjugated to alkaline phosphatase. The amount of binding corresponds to the degree of TK activity. The reaction was evaluated after 30 min of incubation of alkaline phosphatase with the chromogenic substrate, and the activity of each recombinant protein was given in DiviTum units (DU) per 0.2 ng of protein. The vector pIX 3.0 without TK was used as a negative control, the recombinant protein of wild-type vOka was used as a positive control, and that with the resistance-related amino acid substitution W225R was used as a resistance control. The cutoff values for TK activity were calculated as five times the mean values, along with the standard deviation (SD) of the negative control. Variants with activities within a range of 20% higher than the cutoff value were considered drug resistant. The results within a range of 20% higher than the cutoff value and the lower 10% of the positive control were interpreted as weakly resistant, and findings that were >10% of the positive control were considered drug sensitive.

Generation of TK and Pol recombinant VZV isolates using two-step en passant mutagenesis. The ORF36 and flanking sequences were amplified from the BAC pHJOpac (15) using Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The fragment was inserted into the Xhol- and XbaI-digested transfer vector plasmid pCeu2 (23). Further, a selection marker was amplified from the plasmid pEPkan-S and inserted according to the principle of en passant mutagenesis. Single mutations resulting in L73I, A163stop, W225R, T256M, and the deletion of nucleotides nt 19 to 223 (deletion of amino acids [aa] 7 to 74 [Aaa 7 to 74]) was not verified (5). The genotyping of virus strains revealed the Pol substitutions A95ST or T237K, which were not characterized phenotypically (5). The VZV Oka vaccine strain (vOka) isolated from the varicella vaccine Varilrix (GlaxoSmithKline, Munich, Germany) was used as a phenotypically drug-sensitive wild-type strain.

The intact decanucleotide sequence of interest was amplified from the BAC pHJOpac (15) using Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The fragment was inserted into the Xhol- and XbaI-digested transfer vector plasmid pCeu2 (23). Further, a selection marker was amplified from the plasmid pEPkan-S and inserted according to the principle of en passant mutagenesis. Single mutations resulting in L73I, A163stop, W225R, T237K, and N334stop were generated using the GeneArt site-directed mutagenesis system kit.

An ORF36 deletion mutant of pHJOpac was generated in Escherichia coli strain GS1783 using the en passant mutagenesis procedure (16, 24). After PCR amplification, the mutated ORF36 variants originating from the transfer constructs were inserted into the ΔORF36 mutant. The TK mutant with Aaa 7 to 74 and the Pol mutants with the A95ST and T237K substitutions were generated directly by en passant mutagenesis. All BACs of the mutants were additionally equipped with the gene for the monoclonal red fluorescent protein (mRFP) generated from the plasmid vector pEP,mRFP-in (provided by Karsten Tischer). The integrity of the BAC constructs was tested by restriction fragment length polymorphism analysis and sequencing of the regions of interest. The cloning strategy is described previously (22).
demonstrated in Fig. S1 in the supplemental material, and the oligonucleotides used for en passant mutagenesis or site-directed mutagenesis are described in Table S1 in the supplemental material. Virtual cloning and sequence analyses were performed with the Vector NTI Advance 11.1 software (Life Technologies).

The BAC DNA was transfected into permissive MeWo melanoma cells using Lipofectamine 2000 (Life Technologies). The reconstituted virus strains were passaged up to six times by cocultivation of the infected with uninfected MeWo cells to allow the excision of the mini-F vector by internal homologous recombination events (15, 16). Comparative multistep replication kinetics were performed in quadruplicate per three independent experiments, and viral plaques were counted under a fluorescence microscope (Olympus, Hamburg, Germany). To verify TK gene expression from the functional virus strains, the total RNA from the infected MeWo cells was extracted using the RNeasy minikit (Qiagen) and DNase I (Qiagen). Next, RNA was reverse transcribed into cDNA using the RT2 First Strand kit (Qiagen). The viral TK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplificates were generated with Taq DNA polymerase (Thermo Fisher Scientific).

The protein lysates of the infected and noninfected MeWo cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Darmstadt, Germany). The membranes were incubated with either a primary anti-TK polyclonal antibody (vC-20, Santa Cruz, CA), an anti-gI monoclonal antibody (catalog no. 8612; Millipore), or an anti-/H9252-actin monoclonal antibody (catalog no. 4970; Cell Signaling Technologies, Frankfurt, Germany). The secondary alkaline phosphatase-conjugated antibody (Jackson ImmunoResearch, Suffolk, United Kingdom) was detected with the SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific). For indirect immunofluorescence, the infected cells were fixed with 2% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 10% fetal calf serum. Viral TK was stained with a primary anti-TK polyclonal antibody (vC-20) and an Alexa-488 conjugated secondary antibody (catalog no. A-11001; Life Technologies). The plaques were analyzed under the IX80 inverted fluorescence microscope (Olympus).

Phenotypic testing of recombinant VZV TK and Pol mutants for drug susceptibility. For the plaque reduction assays, recombinant VZV strains with mRFP were grown and titrated in the human Caucasian fetal lung fibroblast WI38 cell line (European Collection of Cell Cultures, Salis-

**FIG 1** Recombinant TK variants. (A) Detection of recombinant VZV TK variants with full-length proteins (37 kDa; wild-type vOka, and L73I, W225R, and T256M substitutions) or truncated TK proteins (27 kDa; Δaa 7 to 74) migrating with an apparent molecular weight that is lower than calculated. The truncated TK proteins with premature translation stops (A163stop and N334stop) were not detected. The negative control was an empty plX3.0 vector, the positive control was the wild-type enzyme of vOka; and the resistance control was the mutant resulting in W225R. (B) Mean activities plus SD of recombinant TK (in DU per 0.2 ng of protein) containing various amino acid substitutions associated with the positions of substitutions studied, on the basis of three replicates per two independent preparations. Vector plX3.0 without TK revealed the activity value of 38 (± SD, 54). On the basis of this negative control, a cutoff value of 244 was calculated for resistance (cutoff, bold dark gray line; limit for weak resistance, dotted dark gray line). The positive control of wild-type vOka resulted in the highly positive value of 38,258 (± SD, 2,535). (C) Schematic map of the TK protein with conserved regions (black boxes), a homopolymer region consisting of 6 cytosine (C) bases, and the positions of the respective amino acid substitutions shown (arrows).
bury, United Kingdom) or in human embryonic lung fibroblasts (HELF), as described previously (25). Next, HELF were used for phenotypic resistance testing of recombinant VZV strains against ACV (GlaxoSmithKline, Uxbridge, United Kingdom), BVDU (Berlin-Chemie AG, Berlin, Germany), FOS (AstraZeneca, Wilmslow, United Kingdom), and CDV (Visistide; Gilead Sciences, Inc., Foster City, CA, USA) (5). For resistance testing against PCV, human thyroid cells grown in Opti-MEM plus GlutaMAX and 5% fetal calf serum (FCS) (Life Technologies) were used by analogy with HELF. The cells were infected with a multiplicity of infection of 0.12. The antivirals were applied at a final half-log dilution over a previously described range (26). After incubation for 3 days, viral plaques were counted under a fluorescence microscope (Diaphot; Nikon, Japan). The experiments were performed twice. The 50% effective concentration (EC_{50}) was presented as the arithmetic mean and the SD (5). vOka and the recombinant wild-type HJO (HJO wt) served as the drug-sensitive strains. The recombinant VZV strains were considered ACV, BVDU, PCV, and CDV resistant if the mean EC_{50}s were computed as at least four times the mean value plus the SD of the that of the susceptible reference strain vOka (5, 27). The EC_{50} of >330 μM were considered FOS resistant (28). The recombinant viruses were used for phenotypic testing after 5 to 16 cell culture passages. Resequencing was not performed, since VZV isolates have been shown to be stable for up to >20 passages (29).

For the quantitative real-time PCR (qPCR), MeWo cells were infected with similar numbers of PFU of each virus mutant (passage number 5) and incubated with either 35.2 μM or without ACV in the case of resistant recombinant virus strains or with increasing ACV concentrations ranging from 0.28 to 35.2 μM in the case of sensitive virus strains. Three days postinfection (p.i.), total DNA was extracted from cells using the DNeasy blood and tissue kit (Qiagen). Next, the viral genomes were quantified using oligonucleotides and a TaqMan probe targeting ORF38 (30). The primer-probe sequences are provided in Table S1 in the supplemental material. The PCR was performed with the QuantiTect probe kit (Qiagen) on a 7500 real-time PCR system (Life Technologies), according to the manufacturer’s recommendations. The concentration of pHJOpac BAC DNA was measured spectrophotometrically. DNA of a known size was used in serial 10-fold dilutions to generate standard curves by plotting the cycle thresholds (C_{T}) against the logarithm of the starting amount. The DNA samples were measured in triplicate and used for the calculation of the mean ± SD values.

FIG 2  Multistep replication kinetics of virus recombinants. The multistep replication kinetics of the recombinant TK mutants resulting in W225R, Q303stop/A955T(Pol), T256M, N334stop, A163stop, Δaa 7 to 74, and L73I and the Pol mutants resulting in A955T and T237K (dotted lines) in comparison to the respective red fluorescent counterpart (dashed lines) and the recombinant wild-type HJO (solid line) are shown. The data are plotted as the mean and SD of the results determined with four samples from three independent experiments.
RESULTS

Expression and activity of recombinant TK proteins. Western blotting was performed to identify the recombinant TK proteins. The proteins with the amino acid substitutions L73I, W225R, and T256M were detected at approximately 37 kDa, compared to the positive control of vOka (Fig. 1A). The recombinant protein H9004 aa 7 to 74 was visible at 27 kDa. In comparison, the pIX3.0 vector without TK did not show any signal. In contrast, the truncated TK proteins with premature stops in translation (A163stop, Q303stop [not shown], and N334stop) were not visualized, most likely due to the lack of the TK C terminus for binding the anti-VZV TK antibody. The recombinant TK proteins with the amino acid changes W225R, T256M, A163stop, Q303stop, N334stop, or H9004 aa 7 to 74 exhibited activities below the cutoff value of 244 DU/0.2 ng (Fig. 1B) and were considered resistant. In addition, the recombinant TK with the L73I substitution revealed low activity (1,838 DU/0.2 ng) equivalent to <10% of the positive control. The positions of the amino acid substitutions studied are summarized in Fig. 1C.

Generation of recombinant viruses and comparison of replicative capacity. To obtain reliable phenotypic results, replication-competent viruses were generated by en passant mutagenesis, a method suitable for the targeted insertion of only a single TK and/or Pol substitution in an otherwise wild-type backbone. All recombinant virus strains were characterized by the autonomously expressed mRFP reporter protein. The BAC vector release occurred during the fourth to sixth passages of virus cultivation by internal recombination events. The replicative capacity of each
TABLE 2 Drug resistance profiles of the specific targeted VZV mutants

<table>
<thead>
<tr>
<th>Amino acid substitutions by virus</th>
<th>EC50 (mean ± SD) (µM) for:&lt;br&gt;ACV</th>
<th>PCV</th>
<th>BVDU</th>
<th>FOS</th>
<th>CDV</th>
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<td>TK (ORF36) variants</td>
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<tr>
<td>L73I</td>
<td>7.92 ± 0.44</td>
<td>2.80 ± 0.34</td>
<td>&lt;0.18</td>
<td>211.2 ± 75.9</td>
<td>1.34 ± 0.10</td>
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<td>A163stop</td>
<td>&gt;35.2</td>
<td>&gt;31.6</td>
<td>&gt;24.0</td>
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<tr>
<td>W225R</td>
<td>&gt;35.2</td>
<td>&gt;31.6</td>
<td>&gt;24.0</td>
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<tr>
<td>T256M</td>
<td>&gt;35.2</td>
<td>&gt;31.6</td>
<td>&gt;24.0</td>
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<tr>
<td>Q303stop/A955T (Pol)</td>
<td>&gt;35.2</td>
<td>&gt;31.6</td>
<td>&gt;24.0</td>
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<tr>
<td>N334stop</td>
<td>&gt;35.2</td>
<td>&gt;31.6</td>
<td>&gt;24.0</td>
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<td>Δaa 7–74</td>
<td>&gt;35.2</td>
<td>&gt;31.6</td>
<td>&gt;24.0</td>
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<td>Pol (ORF28) variants</td>
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<td>T237K</td>
<td>7.04 ± 1.11</td>
<td>3.50 ± 0.82</td>
<td>&lt;0.18</td>
<td>221.0 ± 81.8</td>
<td>6.90 ± 2.73</td>
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<tr>
<td>A955T</td>
<td>5.72 ± 0.44</td>
<td>2.40 ± 0.22</td>
<td>&lt;0.18</td>
<td>58.7 ± 0.66</td>
<td>1.20 ± 0.54</td>
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<tr>
<td>HJO wt</td>
<td>6.23 ± 1.33</td>
<td>3.16 ± 0.73</td>
<td>&lt;0.18</td>
<td>77.1 ± 19.9</td>
<td>2.05 ± 0.59</td>
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<tr>
<td>vOka</td>
<td>6.17 ± 3.02</td>
<td>3.88 ± 2.03</td>
<td>&lt;0.18</td>
<td>41.3 ± 19.7</td>
<td>3.28 ± 0.99</td>
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a ACV, acyclovir; PCV, penciclovir; BVDU, bromovinyldeoxyuridine; FOS, foscarnet; CDV, cidofovir. The values are calculated on the basis of two replicates, and those indicating resistance are in bold type. Human embryonic lung fibroblasts were used for ACV, BVDU, FOS, and CDV, and human thyroid cells were used for PCV. The cutoff values for resistance for the various drugs are as follows: ACV, 27.70 M; PCV, 17.55 µM; BVDU, 0.72 µM; FOS, 330 µM; and CDV, 14.11 µM.

Discussion

The objective of this study was to develop approaches to study the phenotypes of nonviable clinical VZV strains with TK and/or DNA Pol substitutions suspected to cause antiviral resistance. In order to define the impact of nonsynonymous mutations on VZV TK activity, a functional TK test on the basis of a commercial EIA was established. The ORF36 was cloned into an expression vector, and the substitutions were introduced by site-directed mutagenesis. The mean TK activity of the sensitive reference strain vOka revealed high values far beyond the cutoff, whereas the activities of the mutated TK variants with the W225R, T256M, Q303stop/A955T (Pol), and N334stop and Δaa 7 to 74 replicated at 35.2 µM ACV, 31.6 µM PCV, and 24.0 µM BVDU and were classified as resistant, whereas CDV and FOS decreased virus replication (Table 2). The ACV resistance was confirmed by qPCR, since comparable amounts of viral copies were detected despite a high ACV concentration of 35.2 µM (Fig. 4B). The comparison of the mean VZV copies at high ACV concentrations (35.2 µM) revealed differences by a factor of 100 between the groups of the sensitive strains and the resistant strains (Fig. 4C).

Susceptibility to antivirals determined by plaque reduction assay and quantitative PCR. All strains, including HJO with wild-type configuration and vOka as references, were tested against ACV, PCV, BVDU, FOS, and CDV by counting plaques via the mRFP reporter gene. The TK variant with the L73I substitution was classified as sensitive, with the EC50s of all tested antivirals being similar to the values of the reference strains (Table 2). DNA Pol mutants were tested with antivirals affecting TK and Pol, demonstrating a sensitive phenotype, with EC50s below the cutoff. In strains exhibiting a sensitive phenotype against ACV, the qPCR results demonstrated diminished viral replication under increasing ACV concentrations by a factor of 1,000. Moreover, under this condition, the TK variant with the L73I substitution, as well as both Pol variants with the T237K and A955T substitutions, showed replicative properties comparable to those of the recombinant HJO strain (Fig. 4A). The observed sensitive phenotype of the Pol variant with the A955T substitution led to the decision to further insert the Q303stop TK substitution into this construct in order to obtain a virus carrying both substitutions, as this was previously observed in a patient’s specimen (5, 18). The TK variants with the substitutions A163stop, W225R, T256M, Q303stop/A955T (Pol), and N334stop, and Δaa 7 to 74 replicated at 35.2 µM ACV, 31.6 µM PCV, and 24.0 µM BVDU and were classified as resistant, whereas CDV and FOS decreased virus replication (Table 2). The ACV resistance was confirmed by qPCR, since comparable amounts of viral copies were detected despite a high ACV concentration of 35.2 µM (Fig. 4B). The comparison of the mean VZV copies at high ACV concentrations (35.2 µM) revealed differences by a factor of 100 between the groups of the sensitive strains and the resistant strains (Fig. 4C).
of replication-competent viruses is necessary to overcome these problems.

Recombinant technologies have become important and versatile tools for manipulating herpesvirus genomes, which were cloned as BACs (14, 31, 32). One specific approach is \textit{en passant} mutagenesis, which was used previously in the generation of single-nucleotide substitutions in the phosphotransferase or Pol genes of human cytomegalovirus (33, 34). In contrast, an intermediate transfer construct was generated in this report, into which the desired nucleotide substitutions were introduced by site-directed mutagenesis. Short oligonucleotides of approximately 30 nt facilitated the introduction of multiple substitutions in close proximity. \textit{En passant} mutagenesis was used to insert the mRFP reporter gene into a short noncoding region between the poly(A) sites of ORF17 and ORF18, in which \textit{cis}-acting regulatory elements are unlikely to be present. Multistep replication kinetics were performed to exclude alterations in viral replication caused by sequence insertions. Each VZV mutant was then compared with its red fluorescent counterpart, as well as the recombinant wild-type HJO, and relevant differences were not detected. Since

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**FIG 4** Quantification of viral genomes by quantitative PCR. MeWo cells were infected with comparable PFU/ml per strain, and qPCR was performed from the extracted DNA of cell lysates obtained 3 days p.i. (A) The wild-type HJO virus strain and those with the L73I, A955T, and T237K substitutions were tested using ACV concentrations ranging from 0 to 35.2 μM and classified as sensitive. (B) The resistant strains were tested using the high ACV concentration of 35.2 μM or in the absence of ACV (0 μM). (C) Mean values of genome copies at 35.2 μM ACV for all mutants (TK and Pol) were compared to the mean of the HJO wild-type strain. The data are plotted as the mean and SD of three measurements.
TK activity is dispensable in cell culture (35), ORF36 expression was confirmed at the RNA and protein levels. Specific RNA was detected in the infected cells, as indicated by RT-PCR, and the TK protein was demonstrated by Western blotting, except for the truncated TK mutants (those resulting in Q303stop, A163stop, and N334stop). However, the expression of those variants in their native form was demonstrated by indirect immunofluorescence.

For drug susceptibility testing, red fluorescent virus strains were cultivated on different cell lines suitable for the respective antivirals. A modified conventional plaque-reduction assay was used in which red fluorescent plaques were counted at a broad range of drug concentrations. In all but one TK mutant virus, the resistance phenotype was clearly defined. While the W225R (5), T256M (19), and Q303stop (17, 18) substitutions were confirmed to be related to resistance, A163stop, N334stop, and Δaa 7 to 74 were identified as the results of novel resistance mutations. The hitherto unclear substitution L73I led to reduced TK activity, which was considered a sensitive phenotype. Thus, this substitution can be attributed to a natural polymorphism of the VZV TK gene. Two Pol substitutions with unclear significance with respect to ACV resistance were identified as the results of novel resistance mutations. The native form was demonstrated by indirect immunofluorescence.

Viral load was measured by applying qPCR changes (15). The insertion of a reporter gene facilitates specific detection of the desired point mutations, without any secondary genomic termini of the VZV genome, the BAC vector is released within the context of the National Reference Laboratory Network.

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