Analysis of the Bovine Herpesvirus Type 1 Thymidine Kinase (TK) Gene
from Wild-type Virus and TK-deficient Mutants

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SUMMARY

Five thymidine kinase (TK)-deficient mutants (B1 to B5) of bovine herpesvirus type 1 (BHV-1) were isolated by selection for resistance to the nucleoside analogue bromovinyldeoxyuridine. The genetic lesion in mutant B1 was localized in a 2.7 kb SalI-SalI subfragment (ITK2.7) which maps between 0.456 and 0.475 within the HindIII A fragment of the BHV-1 genome. The tk genes from wild-type and the TK− mutants B1 to B5 were cloned and sequenced using eight unique synthetic primers designed from a published sequence. The BHV-1 tk gene sequence for the strain 6660 contained some differences compared with that published previously for strain LA. Alignment of the predicted amino acid sequence of the BHV-1 TK polypeptide with different herpesvirus TKs revealed five strongly conserved regions and also identified putative functional relationships with other enzymes. Several interesting features were apparent in the tk gene sequences from the TK− mutants. The TK mutant B1 was a typical frameshift and chain termination mutant due to the deletion of a single base. The tk gene sequence of mutant B2 revealed the deletion of three bases resulting in the loss of valine at amino acid residue 174 of the TK polypeptide. The tk genes of mutants B3 to B5 contained an identical change of a single base addition resulting in frameshift and premature chain termination. In contrast to wild-type BHV-1, the TK-defective mutants were incapable of adsorbing TK-neutralizing antibodies from serum.

INTRODUCTION

Bovine herpesvirus type 1 (BHV-1) is known to cause a variety of clinical conditions in cattle world-wide. The infection produces disease ranging from mild to severe rhinotracheitis, commonly known as infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), conjunctivitis, abortion and fatal systemic infection in newborn calves (Gibbs & Rweyemamu, 1977; Ludwig, 1983).

BHV-1 is considered to be a member of the alphaherpesvirus group (Ludwig, 1983) and contains a 135 kb, double-stranded, linear DNA genome. The genome is divided into unique long (100 kb) and unique short (13 kb) segments; the latter is bracketed by inverted repeat sequences (11 kb each) (Hammerschmidt et al., 1986). Thus DNA isolated from virions is found in two isomeric forms in more or less equimolar amounts (Mayfield et al., 1983; Hammerschmidt et al., 1986; Engels et al., 1986/1987). Restriction endonuclease maps of a number of BHV-1 strains are known (Mayfield et al., 1983; Engels et al., 1986/1987).

From the study of herpes simplex virus-1 (HSV-1) which is considered to be the prototype for alphaherpesviruses, it is well established that following infection a number of virus-induced enzymes are produced within host cells (Roizman & Batterson, 1985). Like HSV, BHV-1 codes for a viral thymidine kinase (TK) (Weinmaster et al., 1982; Kit & Qavi, 1983). Studies with HSV indicate that virus-induced TK is not essential for virus replication in cell cultures (Dubbs & Kit,
Virus-induced TKs also have an important role in the mode of action of several nucleoside analogues which are potent inhibitors of virus replication. Initial phosphorylation by the virus-induced TK is the first step in the activation of several such compounds in virus-infected cells (Larder & Darby, 1984).

Published results have indicated that BHV-1 TK has different features (e.g. kinetic properties in relation to different nucleoside analogues) from HSV TK which has been better characterized (Kit, 1985). However, TK-deficient mutants of both HSV and BHV-1 can be readily isolated in the presence of nucleoside analogue inhibitors (Kit & Qavi, 1983; Bello et al., 1987). The genetic lesions in BHV-1 TK-deficient mutants have been mapped (Kit & Kit, 1986; Bello et al., 1987).

TK- mutants have been widely described among herpesviruses and may be biologically important; hence the precise nature of mutational events leading to the TK- phenotype has been the subject of interest for many years. In a much earlier study Summers et al. (1975) examined a series of bromodeoxyuridine (BVD Ur) selected HSV mutants and found that the TK polypeptide was absent or, in some cases, of lower Mr. These workers hypothesized that point mutations resulted in frameshifts in the structural gene leading to premature chain termination.

In the present study we have isolated five TK- mutants of BHV-1 in the presence of bromovinyldeoxyuridine (BVD U) and by marker rescue have confirmed the site of the genetic lesion in one mutant to be within the tk structural gene. The nucleotide sequences of the tk genes both from the wt (6660) and the five mutants (B1 to B5) were determined and compared with that of the tk gene of strain LA. The TK- mutants were further characterized by the use of anti-BHV-1 TK-specific serum.

**METHODS**

**Cells.** Bovine turbinate (BT) and Madin–Darby bovine kidney (MDBK) cells were propagated in Eagle’s minimal essential medium (Flow Laboratories) supplemented with 200 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 1% non-essential amino acids, 25 mM-sodium bicarbonate and 10% foetal bovine serum. Rabbit kidney cells (RK13) and the TK-defective cell line (M143(TK-)) were grown in the same medium as used for the propagation of MDBK cells. The M143(TK-) cells from Upjohn were kindly supplied by Dr W. P. H. Duffus.

**Virus.** BHV-1 strain 6660 (kindly supplied by Dr E. A. Rollinson, Coopers Animal Health) was used in this study. Virus stocks were prepared in MDBK or RK cells by passage at low multiplicity.

**Isolation of TK-defective mutants of BHV-1.** E-5-(2-bromovinyl)-2'-deoxyuridine (BVD U) was supplied by Dr E. De Clercq, Rega Institute, Leuven, Belgium. BHV-1 (6660) was passaged twice in MDBK cells first in the presence of 1 followed by 10 μg/ml BVD U (using multiplicities of 1 and 0.1 p.f.u./cell respectively). Plaque purification was accomplished after the second passage by selecting a single isolated plaque and re-inoculating MDBK cells in the absence of BVD U. The plaque purification step was repeated for a total of five times for each mutant. The whole isolation procedure was repeated five times such that five mutants were independently derived from the same original virus working stock and were named B1 to B5.

**Plaque reduction assay for measurement of viral drug sensitivity.** The sensitivity to BVDU of the wt and mutant viruses was measured by determining the 50% effective dose (ED50) in MDBK cells using methods similar to those described previously for HSV in BHK cells (Field & Neden, 1982).

**Assay for TK activity.** TK activity was assayed essentially as described by Klemperer et al. (1967). [3H]Thymidine (sp. act. 22 Ci/mmol, Amersham) at a concentration of 20 μCi/ml or [14C]thymidine (sp. act. 51-2 mc Ci/mmol, Amersham) at a concentration of 1.6 μCi/ml was used as substrate.

**Raising anti-BHV-1 TK serum.** Confluent monolayers of RK13 cells were infected (10 p.f.u./cell) with BHV-1 (6660) and incubated at 37°C for 24 h. The cells were harvested and the infected cell pellet was dissolved in 10 mM-Tris-HCl pH 7.6, briefly sonicated and centrifuged at 100 000 × g for 30 min at 4°C. Streptomycin sulphate and apramycin sulphate fractionations were carried out on the supernatant as described (Lee & Cheng, 1976). The last precipitate so obtained was dissolved in 10 mM Tris-HCl pH 7.6, 0.5 mM-EDTA, 2 mM-dithiothreitol and 10% glycerol. This partially purified TK was dialysed against the same buffer overnight at 4°C before assaying. To raise antiserum, two rabbits were injected intramuscularly with 5 mg/rabbit of partially purified TK which had been thoroughly mixed with an equal volume of Freund’s complete adjuvant. Four weeks later half the initial dose...
BHV-1 TK mutants

Fig. 1. Location of tk gene in the BHV-1 genome. The direction of the open reading frame is shown by the arrow (~). L refers to the long unique sequence and S to the short unique sequence containing the inverted repeats shown as boxes. The HindIII map of BHV-1 (strain Cooper; 136.9 kb) is taken from Mayfield et al. (1983).

was mixed with an equal volume of Freund's incomplete adjuvant and was given by intramuscular injection. The last step was repeated three times at 10 to 14 day intervals. The level of BHV-1 TK neutralizing antibody was monitored and the rabbits were bled 10 days after the last booster.

**TK neutralization.** The supernatant collected after centrifugation at the stage of partial purification as mentioned above was used as the source of TK enzyme extract. The method for neutralization was adapted from that described by Field et al. (1981). Equal volumes of serially diluted preimmune and hyperimmune (anti-BHV-1 TK) sera were mixed with equal volumes of appropriately diluted TK extract. The mixture was kept on ice for 2 h and then centrifuged at 13000 r.p.m. for 5 min at 4 °C. The enzyme assay of each sample was carried out in the usual way.

**Extraction of DNA from purified virions.** Virus DNA was extracted from a potassium tartrate gradient-purified preparation of BHV-1 virions using the method described by Owen & Field (1988).

**Cloning of BHV-1 restriction fragments into a plasmid.** BHV-1 (6660) DNA restriction fragments HindIII A, G and I were each cloned into plasmid pEMBL8 (Dente et al., 1983) using standard procedures (Maniatis et al., 1982) and the recombinant plasmids thus generated were named pSKM1, pSKM7 and pSKM9 respectively. pEMBL8 containing BHV-1 HindIII B, E and L fragments were kindly supplied by L. J. Owen of this laboratory.

Similarly the tk gene containing the 2.7 kb SalI–SalI fragment (TK2.7) which lies within HindIII A fragment of the BHV-1 genome (Fig. 1) was cloned in pEMBL8 and was named pTK2.7.

**Extraction of virus DNA for marker rescue.** MDBK cells grown in roller flasks were infected with mutant virus (B1) at a multiplicity of 0.1 p.f.u./cell and incubated at 37 °C for 48 h. The cell-associated virus was released by suspending the cell pellet in lysis buffer (10 mM-Tris–HCl pH 7.4, 10 mM-KCl, 1.5 mM MgCl₂, 0.5% NP40). It was centrifuged at 1000 r.p.m. for 5 min at 4 °C to pellet cell debris. The supernatant was mixed with virus-infected cell supernatant and was centrifuged at 20000 r.p.m. for 2 h at 4 °C and the virus pellet was resuspended in NTE (10 mM-Tris–HCl pH 7.4, 100 mM-NaCl, 1 mM-EDTA). From this point the procedure was as described by Stow & Wilkie (1976). To the virus suspension EDTA and SDS were added to final concentrations of 10 mM and 2% respectively. The mixture was kept on ice for 5 min. The DNA was obtained by phenol and chloroform extraction. The final aqueous phase was dialysed against 0.1 × SSC (1 × SSC is 150 mM-sodium chloride, 15 mM-sodium citrate, pH 7.4). The DNA concentration was measured spectrophotometrically at 260 nm.

**Thymidine plaque autoradiography.** The method was essentially as described by Tenser et al. (1983). Confluent monolayers of MDBK cells in 60 mm plastic Petri dishes were infected with virus samples obtained after marker rescue and after passage in the presence of HATG medium (Eagle's medium containing 15 µg/ml hypoxanthine, 1 µg/ml aminopterin, 5 µg/ml thymidine, 15 µg/ml glycine). After adsorption the dishes were overlaid with maintenance medium containing 0.5% methyl cellulose and 2% foetal calf serum and incubated for 4 days at 37 °C. The methyl cellulose overlay was removed and the medium containing [³H]thymidine (0.5 µCi/ml, 51.2 mCi/mmol; Amersham) was added and incubation was continued for a further 6 h. The monolayers were
stained with 0·1% crystal violet, dried overnight and plaques were counted. The rims of the dishes were removed, and monolayers were placed in contact with X-ray film for 5 to 7 days at room temperature. The TK+ plaques were distinguished by the heavily labelled rim.

**Marker rescue.** The marker rescue technique was adapted from Stow *et al.* (1976) and Lawrence *et al.* (1986). Briefly, BT cells at 60 to 70% confluence were cotransfected with mutant B1 DNA and wt BHV-1 HindIII DNA fragments (extracted from recombinant plasmids). This was carried out using the Ca3(PO4)2 precipitation technique (Graham & Van der Eb, 1973). At 4 h post-infection the cells were treated with 15% glucose, 10% DMSO in HEPES buffer (Stow & Wilkie, 1976). The virus-infected cells were harvested at 3 to 4 days post-infection when the cytopathic effect was confluent.

For selective increase of TK+ virus progeny, confluent M143(TK-) cells were infected (m.o.i. 1 p.f.u./cell) with the virus collected after the marker rescue stage. The infected cells were maintained in HATG medium and harvested at 36 h post-infection.

**Cloning in M13 and DNA sequencing.** Bacteriophage M13mp8 (Messing & Vieira, 1982) was grown in *Escherichia coli* (TG1). The appropriate fragment (tk2.7) from wt BHV-1 and mutants B1 to B5 were obtained either from the recombinant plasmids or after digestion of the whole virus DNA with *SalI* and cloned into M13mp8.

The dideoxynucleotide chain termination technique of Sanger *et al.* (1977) as modified by Bankier & Barrell (1983) was used to determine the nucleotide sequence of the tk genes of wt and mutant virus DNAs cloned in M13. Recombinant M13 templates were used for sequencing. A set of eight unique oligonucleotide primers (obtained from Institute of Animal Physiology, Babraham, Cambridge) designed to hybridize to the tk gene insert were derived from the nucleotide sequence of the BHV-1 tk gene published in a patent application (Kit & Kit, 1986).

**Computer analysis of nucleotide sequence data.** The program FIND from the University of Wisconsin Genetics Computer Group nucleotide sequence analysis package (Devereaux *et al.*, 1984) was used to select unique primers for sequencing the BHV-1 (6660) tk gene with the help of the published BHV-1 tk gene sequence (Kit & Kit, 1986). Analysis of the open reading frames was carried out using the program ANALYSEQ (Staden, 1986). The predicted amino acid sequence of BHV-1 TK was screened against the library of protein sequences for the presence of homology using the program FASTP (Lipman & Pearson, 1985).

**RESULTS**

*Initial characterization of BVdU-resistant mutants*

The mutants of BHV-1 (6660) which were isolated independently by selection for growth in the presence of BVdU were tested by plaque reduction in MDBK cells. This confirmed the resistant phenotype (Table 1) since the ED50 concentrations for the plaque-purified mutants B1 to B5 were found to have increased by nine- to 12-fold compared with the wt from which they were derived. All five mutants were found to have DNA restriction endonuclease patterns identical to the wt virus in respect to *BamHI, EcoRI, HindIII* and *HpaI* (data not shown).

Conventional TK enzyme assays were performed on extracts of mutant- and wt-infected MDBK or M143(TK-) cells. These tests revealed no detectable virus-specific TK activity in the mutant-infected cells in which the TK levels were similar to the uninfected cell controls (Table 1). Thymidine plaque autoradiography was performed and none of the plaques produced by the mutant viruses incorporated labelled thymidine. For each mutant approximately 1000 individual plaques were examined proving the mutant virus stocks to be essentially homogeneous, containing <0·1% TK-inducing virions. The wt virus induced plaques which incorporated label; however, owing to the difficulty with background incorporation present in the MDBK cells it was not possible to determine whether or not the parental virus stock itself contained a small proportion of TK-defective virions (data not shown). The results of these tests therefore confirmed that BVdU selection resulted in the isolation of a series of TK-defective mutants derived from BHV-1 (6660).

*Location of tk gene in the BHV-1 genome*

Previous studies in this laboratory have shown that the restriction endonuclease pattern of BHV-1 strain 6660 is similar to the Cooper strain (Owen & Field, 1988; Mayfield *et al.*, 1983). This was confirmed in the present study and 6660 was shown also to be identical to the LA strain (Engels *et al.*, 1986/1987) which was included for comparison using *BamHI, EcoRI, HindIII,* and *HpaI* restriction enzymes (data not shown).
Table 1. BVdU sensitivity and TK activity of wt and BVdU-selected mutants of BHV-1

<table>
<thead>
<tr>
<th>Virus</th>
<th>ED_{so}</th>
<th>MDBK</th>
<th>M143(TK-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHV-1 (6660)</td>
<td>0.02</td>
<td>6.10</td>
<td>3.16</td>
</tr>
<tr>
<td>B1</td>
<td>0.18</td>
<td>0.38</td>
<td>0.04</td>
</tr>
<tr>
<td>B2</td>
<td>0.21</td>
<td>0.48</td>
<td>0.06</td>
</tr>
<tr>
<td>B3</td>
<td>0.16</td>
<td>0.40</td>
<td>0.06</td>
</tr>
<tr>
<td>B4</td>
<td>0.18</td>
<td>0.42</td>
<td>0.04</td>
</tr>
<tr>
<td>B5</td>
<td>0.15</td>
<td>0.45</td>
<td>0.05</td>
</tr>
<tr>
<td>Mock-infected MDBK</td>
<td>NA</td>
<td>0.5</td>
<td>NA</td>
</tr>
<tr>
<td>Mock-infected M143(TK-)</td>
<td>NA</td>
<td>NA</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* Expressed in terms of pmol dTMP/μg protein formed in 20 min at 37 °C using infected cell extracts 24 h after infection at a multiplicity of 10 p.f.u./cell.
† Expressed as μM BVdU for 50% plaque reduction in cell monolayers infected with approx. 100 p.f.u. in duplicate.
‡ NA, Not applicable.

Table 2. Marker rescue of mutant B1 with cloned BHV-1 HindIII fragments

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Virus titre (p.f.u./ml)</th>
<th>No. of plaques (MDBK)</th>
<th>No. of plaques [M143(TK-)]</th>
<th>Rescue (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDBK†</td>
<td>M143(TK-)‡</td>
<td>TK- TK+ Total</td>
<td>TK- TK+ Total</td>
</tr>
<tr>
<td>None</td>
<td>3.2 × 10^6</td>
<td>3.2 × 10^5</td>
<td>135 0 135</td>
<td>298 0 298</td>
</tr>
<tr>
<td>A</td>
<td>3.6 × 10^6</td>
<td>4.0 × 10^5</td>
<td>590 0 590</td>
<td>476 0 476</td>
</tr>
<tr>
<td>B</td>
<td>3.6 × 10^6</td>
<td>2.4 × 10^5</td>
<td>590 0 590</td>
<td>476 0 476</td>
</tr>
<tr>
<td>G</td>
<td>3.7 × 10^6</td>
<td>3.2 × 10^5</td>
<td>605 0 605</td>
<td>236 0 236</td>
</tr>
<tr>
<td>I</td>
<td>3.6 × 10^6</td>
<td>2.4 × 10^5</td>
<td>288 0 288</td>
<td>691 0 691</td>
</tr>
<tr>
<td>pTK2.7§</td>
<td>3.6 × 10^6</td>
<td>2.4 × 10^5</td>
<td>380 53 433</td>
<td>286 140 426</td>
</tr>
</tbody>
</table>

* Marker rescue was done in BT cells.
† The virus titration and thymidine plaque autoradiography to determine virus phenotype were done in MDBK cells.
‡ M143(TK-) cells were infected with the virus progeny obtained after marker rescue and were grown in the presence of HATG for 36 h. The virus titration and thymidine plaque autoradiography to determine virus phenotype were done in MDBK cells.
§ Cloned 2.7 kb SalI–SalI subfragment which lies within HindIII A fragment of the BHV-1 genome.

Cloned BHV-1 HindIII A, B, E, G, I and L DNA fragments which represent approx. 82% of the long unique portion of the genome were used in marker rescue experiments to localize the tk gene. Of these HindIII DNA fragments only HindIII A and none other yielded TK-positive progeny on cotransfection of BT cells with mutant B1 DNA (Table 2). This localized the genetic lesion in the TK- mutant B1 to a 22.4 kb fragment which according to Mayfield et al. (1983) maps between 0.381 and 0.537 map units on the BHV-1 genome (Fig. 1). Using similar techniques Bello et al. (1987) further localized the lesion in a TK- mutant to a 2.7 kb SalI–SalI subfragment of the HindIII A fragment. The results of Bello et al. (1987) were confirmed in the present study by marker rescue (Table 2). The proportion of rescued virus was selectively amplified by growing the virus yield from the marker rescue experiment in M143(TK-) cells in the presence of HATG (Table 2). Although these cells are poorly permissive for wt BHV-1 and do not readily form plaques, a 'blind' passage gave the desired amplification. The TK-positive plaques were then readily identified in monolayers of MDBK cells by means of plaque autoradiography (Fig. 2).
Fig. 2. Rescue of B1 mutant to TK-positive phenotype by wt BHV-1 HindIII A fragment or subfragment (fTK2.7) DNA. Monolayers of BT cells were transfected with intact DNA from mutant B1 (a) or cotransfected with intact DNA from B1 plus wt BHV-1 HindIII A fragment DNA (b) or intact DNA from B1 plus fTK2.7 (c). Thymidine plaque autoradiography was carried out as described in Methods. The figure shows the X-ray film after 5 days exposure. The images of the virus plaques are clearly visible and those with labelled rims are of TK+ phenotype. There is a background labelling because normal (TK-inducing) MDBK cells were used.

Nucleotide sequence analysis of the BHV-1 tk gene

The fTK2.7 subfragment (Fig. 1) from wt BHV-1 which successfully rescued mutant B1 yielding TK-positive progeny was introduced into phage M13mp8 for sequencing. Eight synthetic oligonucleotide primers were prepared on the basis of the published BHV-1 (LA) tk gene sequence (Kit & Kit, 1986). These primers were designed to hybridize specifically to particular sequences in the predicted tk gene. Using template from single-stranded phage M13mp8 containing fTK2.7 the sequence of the coding region of BHV-1 (6660) tk gene and its flanking sequences were determined for one strand (Fig. 3).

The predicted amino acid sequence for BHV-1 TK was then compared with those of five other herpesvirus TKs (Fig. 4). An overall identity of approx. 25 to 35% was observed with different herpesvirus TKs. Short stretches of similar amino acids occurred throughout the protein and in particular five areas (I to V) having the greatest similarity were noted. In addition the regions 'a' and 'b' showed appreciable homology among alphaherpesviruses. The sequence of the proposed nucleotide-binding site of TK and other related enzymes was aligned with a similar sequence in BHV-1 TK (Table 3). In addition, the sequence of part of the thymidine-binding site (as proposed by Darby et al., 1986) of different herpesvirus TKs was aligned with a similar sequence in BHV-1 TK (Table 4).

The tk gene sequence obtained in the present study was compared with that previously published by Kit & Kit (1986) for strain LA and a number of differences were observed. Most of these differences were located within amino acid residues 112 to 137 where there were five deletions and two insertions (Fig. 5). These differences were confirmed by sequencing the opposite strand in the area of interest using another unique synthetic primer (data not shown). The result of these differences is a stretch of 26 amino acid residues which differs from the published data; furthermore, the resultant polypeptide sequence was one amino acid shorter. Unlike the tk gene sequence from strain LA our sequence for the strain 6660 contained the conserved amino acid motif forming part of the thymidine-binding site proposed by Darby et al. (1986) (Table 4). Other differences between the tk gene sequences from BHV-1 6660 and LA which give rise to amino acid substitutions are shown in Table 5.

Nucleotide sequence analysis of the TK gene of BVdU-resistant mutants

Fragment fTK2.7 from the BHV-1 TK-defective mutants B1 to B5 was cloned into M13mp8 and the nucleotide sequence of the tk genes was determined using the same strategy as described above for the wt virus.
Table 3. Alignment of homologous amino acid sequences to show the proposed nucleotide-binding sites of TK and other enzymes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Residues</th>
<th>Sequence*</th>
<th>Reference†</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHV-1 TK</td>
<td>11-27</td>
<td>V R I Y L D</td>
<td></td>
</tr>
<tr>
<td>PRV† TK</td>
<td>4-20</td>
<td>L R I Y L D</td>
<td></td>
</tr>
<tr>
<td>HSV-1 TK</td>
<td>50-66</td>
<td>L R V Y I D</td>
<td></td>
</tr>
<tr>
<td>HSV-2 TK</td>
<td>50-66</td>
<td>L R V Y I D</td>
<td></td>
</tr>
<tr>
<td>VZV TK</td>
<td>14-30</td>
<td>L R I Y L D</td>
<td></td>
</tr>
<tr>
<td>MarHV TK</td>
<td>11-27</td>
<td>L R V Y L D</td>
<td></td>
</tr>
<tr>
<td>EBV TK</td>
<td>285-301</td>
<td>C S L F L E</td>
<td></td>
</tr>
<tr>
<td>HVS TK</td>
<td>215-331</td>
<td>F F I F L E</td>
<td></td>
</tr>
<tr>
<td>Vaccinia virus TK</td>
<td>9-21</td>
<td>- - I - I -</td>
<td></td>
</tr>
<tr>
<td>Fowlpox virus TK</td>
<td>9-21</td>
<td>- - I - T -</td>
<td></td>
</tr>
<tr>
<td>Human TK</td>
<td>24-36</td>
<td>- - I - L -</td>
<td></td>
</tr>
<tr>
<td>Chicken TK</td>
<td>24-36</td>
<td>- - I F - -</td>
<td></td>
</tr>
<tr>
<td>Bovine ATPase β</td>
<td>150-166</td>
<td>G K I G L F</td>
<td></td>
</tr>
<tr>
<td>E. coli ATPase β</td>
<td>144-160</td>
<td>G K V G L F</td>
<td></td>
</tr>
<tr>
<td>Rabbit myosin</td>
<td>172-188</td>
<td>Q S I L I T</td>
<td></td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>9-26</td>
<td>S K I I F V</td>
<td></td>
</tr>
<tr>
<td>RecA protein</td>
<td>59-76</td>
<td>G R I V E I Y</td>
<td></td>
</tr>
<tr>
<td>Mouse v-p21 has</td>
<td>4-20</td>
<td>Y K L V V V</td>
<td></td>
</tr>
<tr>
<td>Human c-has/bas</td>
<td>4-20</td>
<td>Y K L V V V</td>
<td></td>
</tr>
</tbody>
</table>

* The highly conserved amino acid residues are shown in boxes.
‡ PRV, Pseudorabies virus. For other abbreviations see Fig. 4 legend.
Fig. 3. The nucleotide sequence of the coding region of the BHV-1 (6660) tk gene and its flanking sequences. The predicted amino acid is indicated above each codon. The numbering of the nucleotides (shown beneath the sequence) is arbitrary but relates to the numbers referred to in the text. The right-hand column indicates the amino acid residue numbered from the start of the open reading frame.
Table 4. Alignment of homologous amino acid sequences to show part of the proposed thymidine-binding site* of different herpesvirus TKs

<table>
<thead>
<tr>
<th>TK</th>
<th>Residues</th>
<th>Sequence†</th>
<th>Reference‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHV-1</td>
<td>128-147</td>
<td>T L V F</td>
<td>DRH - - - - PV A A C L C Y P F A R Y 1</td>
</tr>
<tr>
<td>PRV</td>
<td>108-127</td>
<td>T V V F</td>
<td>DRH - - - - PV A A T V C F P L A R F 2</td>
</tr>
<tr>
<td>HSV-1</td>
<td>158-177</td>
<td>T L L F</td>
<td>DRH - - - - PI A A L L C Y P A A R Y 3</td>
</tr>
<tr>
<td>HSV-2</td>
<td>159-178</td>
<td>T L V F</td>
<td>DRH - - - - PI A S L L C Y P A A R Y 4</td>
</tr>
<tr>
<td>VZV</td>
<td>125-144</td>
<td>I M L S</td>
<td>DRH - - - - PI A S T I C F P L S R Y 5</td>
</tr>
<tr>
<td>MarHV</td>
<td>126-145</td>
<td>V L V V</td>
<td>DRH - - - - AV G P W C A T R W R V Y 6</td>
</tr>
<tr>
<td>EBV</td>
<td>388-414</td>
<td>W I L H</td>
<td>DRH L L S A S V V F P L M L L R S Q L L S Y 7</td>
</tr>
<tr>
<td>HVS</td>
<td>316-342</td>
<td>W V M F</td>
<td>DRH P L S A T V V F P Y M H F Q N G F L S F 8</td>
</tr>
<tr>
<td>BHV-1</td>
<td>129-148</td>
<td>P S C S</td>
<td>T A T - - - - PW R A C L C Y P F A R Y 9</td>
</tr>
</tbody>
</table>

* Proposed by Darby et al. (1986).
† The conserved triplet is shown in the box.
Fig. 4. The amino acid sequence of BHV-1 TK and comparison with TKs of five other herpesviruses. The amino acids are aligned to maximize homology. The boxes outline regions of identical matching. Regions of strong conservation are indicated by roman numerals I to V, a and b. Data obtained as follows: HSV-1 [C1(101)], Wagner et al. (1981); HSV-2 (333), Swain & Galloway (1983); varicella-zoster virus (VZV), Davison & Scott (1986); marmoset herpesvirus (MarHV), Otsuka & Kit (1984); Epstein-Barr virus (EBV), Baer et al. (1984) EMBL Data Base.

The sequences flanking the coding region of the \( tk \) genes for the five mutants were identical to the corresponding sequences from wt BHV-1. However, for mutant B1 the sequence of five thymines (in the non-coding strand) at nucleotides 366 to 370 of the wt \( tk \) gene was reduced to four in the mutant. This resulted in a frameshift at amino acid residue 43 and therefore a premature termination at amino acid residue 58 of the TK polypeptide (Fig. 6 and Table 6). In mutant B2 the wt \( tk \) gene sequence TCGTCG at nucleotides 760 to 765 was reduced to TCG probably by deletion of a triplet TCG, CGT or GTC from the mutant gene. Any of these...
Fig. 5. Part of BHV-1 strain 6660 tk gene sequence to show differences from the same sequence of strain LA. The arrows indicate the five G residues which are absent from strain 6660; the box indicates two additional bases in 6660 which are not contained in the sequence published for BHV-1 LA (Kit & Kit, 1986).

Table 5. Further differences between the BHV-1 tk gene sequences from the present study (strain 6660) and the published sequence* (strain LA)

<table>
<thead>
<tr>
<th>Nucleotide position</th>
<th>Nucleotide</th>
<th>Amino acid residue in TK polypeptide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6660</td>
<td>LA</td>
</tr>
<tr>
<td>304</td>
<td>T</td>
<td>Leu (21)</td>
</tr>
<tr>
<td>913–915</td>
<td>GCG</td>
<td>Gly (223), Asp (224)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gln (21)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gly (223), Asp (224)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ala (224), His (225)</td>
</tr>
</tbody>
</table>

*Kit & Kit (1986).
† Numbered according to the sequence presented in this study. Other differences between the sequences are shown in Fig. 5.

Table 6. Changes in tk gene sequences of TK-deficient mutants*

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Change</th>
<th>Base sequence at site of mutation</th>
<th>Amino acid residue no.</th>
<th>Position of stop codon</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>Deletion of a T</td>
<td>TTTTT</td>
<td>43</td>
<td>58</td>
</tr>
<tr>
<td>B2</td>
<td>Deletion of TCG</td>
<td>TCGTCG</td>
<td>174</td>
<td>356†</td>
</tr>
<tr>
<td>B3</td>
<td>Insertion of a G</td>
<td>GGGG</td>
<td>330</td>
<td>354</td>
</tr>
<tr>
<td>B4</td>
<td>Insertion of a G</td>
<td>GGGG</td>
<td>330</td>
<td>354</td>
</tr>
<tr>
<td>B5</td>
<td>Insertion of a G</td>
<td>GGGG</td>
<td>330</td>
<td>354</td>
</tr>
</tbody>
</table>

* Nucleotide changes in the non-coding strands are shown.
† Normal stop codon in wt.

possibilities would result in the deletion of valine at amino acid residue 174 of the TK polypeptide (Fig. 6, Table 6).

The tk genes of the remaining three mutants B3 to B5 were found to contain identical mutations. In each of the mutants there was an addition of one guanine to an existing sequence of four guanines at nucleotides 1227 to 1230. The predicted consequence of this change is a frameshift at amino acid residue 330 and chain termination at 354 of the TK polypeptide (Fig. 6 and Table 6) making the polypeptide slightly smaller than the normal product which contains 357 residues.

**TK neutralization by anti-TK serum**

Anti-BHV-1 TK serum was raised against partially purified BHV-1 TK by a series of inoculations into rabbits. The TK-neutralizing activity of this serum was specific to BHV-1 TK
and had no detectable activity against EHV-1 or HSV-1 TK or TK from mock-infected cells (data not shown).

On the basis of the DNA sequence of the tk genes, mutant B1 encodes a truncated peptide (58 amino acid residues compared with 357 in the wt). However, the remaining mutants, B2 to B5 potentially code for almost full-length (albeit altered) TK polypeptides.

In order to obtain more information on the altered TKs a series of further tests was carried out. As already discussed none of the mutants B1 to B5 induced detectable virus-specific TK activity in an enzyme assay. In a preliminary study it was difficult to identify with certainty the TK polypeptide by means of polyacrylamide gel electrophoresis. By means of immunoprecipitation using the anti-BHV-1 TK-specific serum, mutants B1 and B2 failed to reveal TK products of the predicted Mr, although a product was visible at 37K with wt (data not shown).

To test the ability of the predicted TK polypeptides of the BVdU-selected mutants to react with anti-BHV-1 TK antibodies, the antiserum was adsorbed with wt- or mutant-infected cell extracts. Mutants B1 to B5 were each tested individually. Following the adsorption the serum was tested for its remaining ability to neutralize the TK induced by the wt virus. The wt-infected cell extract successfully adsorbed the TK-neutralizing activity, but none of the five mutants was able to reduce the TK-neutralizing activity of the serum (Fig. 7). The presence of enzyme activity in the samples was proven by diluting the serum (Fig. 7) indicating that TK activity was restored to the normal levels with mutant-adsorbed serum dilutions of 1/32. This experiment indicated that despite the fact that mutant B2 to B5 tk genes had the potential to code for almost full-length TK polypeptides the resultant products (if any) were incapable of adsorbing TK-neutralizing antibodies by this test.
The main findings in this study are as follows. (i) The map location of the BHV-1 \( tk \) gene was confirmed. (ii) The comparison of the nucleotide sequence of the BHV-1 \( tk \) gene with the published sequence (Kit & Kit, 1986) revealed a number of differences. (iii) The comparison of the new predicted amino acid sequence of the BHV-1 TK polypeptide with different herpesvirus TKs revealed five strongly conserved regions and several novel relationships. (iv) The sequences of three TK-defective mutant \( tk \) genes displayed a novel mutation leading to a frameshift. (v) It was observed that, in contrast to wt BHV-1, the TK-deficient mutants were incapable of adsorbing TK-neutralizing antibodies from serum.

Using a marker rescue technique the BHV-1 \( tk \) gene was localized to a 2.7 kb DNA fragment which maps between 0.456 and 0.475 in the long unique region of the genome. This confirms the location proposed in two previous independent reports using similar techniques (Bello et al., 1987; Kit & Kit, 1986).

The published \( tk \) gene sequence enabled the design of eight unique oligonucleotide primers to facilitate the sequencing of the BHV-1 strain 6660 \( tk \) gene. When this sequence was compared with that previously published for the LA strain (Kit & Kit, 1986) several differences became apparent. The substitution of three amino acid residues at two different locations in our sequence compared to that published could be due to strain variation. For example, 19 single base alterations were found between two strains of HSV-1 [CI(101) and MP] seven of which
resulted in different amino acids (Kit, 1985). However, in addition to the minor differences between the sequences for strain 6660 and that published for LA, a major difference was observed where a stretch of 26 amino acids from residues 112 to 137 of the predicted polypeptide in our sequence was at variance with the published sequence and this is unlikely to be explained by strain variation. The divergent sequence contains a portion of the region which was suggested by Darby et al. (1986) to correspond to part of a thymidine binding site. This region retains strong homology with other herpesvirus TKs, and in particular three amino acids (Asp Arg His) are found to be conserved in all eight herpesvirus TK sequences so far reported (Table 4).

The sequence data were used to compare the BHV-1 TK polypeptide with other herpesvirus TKs and a variety of other enzymes. There were five regions of strong homology which were arbitrarily numbered I to V for the purpose of this study. Region I has been reported to have homology with the proposed nucleotide-binding sites of other herpesvirus TKs (Otsuka & Kit, 1984; Gentry, 1985), other TKs and other enzymes as diverse as adenylate kinase (Kuby et al., 1984) and myosin (Walker et al., 1982). Moreover, recently it has been shown by means of site-directed mutagenesis that the five highly conserved amino acids GXXGXGKT (where X represents various amino acids), which occur at residues 56 to 63 of the HSV-1 TK polypeptide, are essential for enzyme function and appear to be involved in the substrate-binding domains of the enzyme (Liu & Summers, 1988). The regions II and IV have a high degree of conservation among different herpesvirus TKs but similar sequences were not seen in poxvirus or mammalian TKs or adenylate kinases (Boyle et al., 1987; Bradshaw & Deininger, 1984; Kwoh & Engler, 1984; Lewis, 1986; Lin et al., 1985; Kuby et al., 1984). The functional roles of these two regions and of regions 'a' and 'b' of the polypeptide are still unknown.

Region III is a conserved sequence which in HSV-1 has been implicated in the determination of substrate specificity of the enzyme (Darby et al., 1986). In BHV-1 TK this sequence occurs at residues 128 to 147 and our comparison of the sequences in this region leads us to suggest that part of the nucleoside-binding site proposed by Darby et al. (1986) extends several residues further towards the N-terminal end of the herpesvirus TK. Moreover a site-directed mutagenesis study in the Asp Arg His motifs of HSV-1 TK (E. M. Rushton, personal communication) and Epstein–Barr virus TK (N. Davis-Poynter, personal communication) which are within the region III as mentioned above had a marked effect on enzyme activity.

Region V is a region of homology which has not yet been widely reported but it is conserved among all the herpesvirus TKs so far examined. In the BHV-1 TK sequence this is an arginine-rich region and a similar region has been reported in porcine adenylate kinase (Heil et al., 1974; Sachsenheimer & Schulz, 1977; Pai et al. 1977) and it has been suggested that an arginine-rich region moves towards the nucleoside-binding site (region I) in the presence of substrate (Pai et al., 1977). It is of interest that a TK substrate specificity mutant of HSV-2 which was resistant to acyclovir (Kit et al., 1987) was found to contain a single amino acid substitution in the TK polypeptide (His for Arg at residue 223) in this same arginine-rich region.

The sequence analysis of the tk genes of BVdU-resistant mutants revealed the individual mutations leading to the TK-defective phenotype. In the original studies of Summers et al. (1975) a series of BUdR-resistant mutants of HSV-1 were shown to induce truncated TK polypeptides which led these authors to propose that a frameshift mutation led to premature termination. This phenomenon was confirmed more recently in two TK-deficient mutants of HSV-2 for which the tk gene sequences were reported (Kit et al., 1987). In the present study the first of the BHV-1 TK-defective mutants (B1) was found to be of this type, with a single base deletion at amino acid residue 43 leading to termination at amino acid residue 58 resulting in a much shortened polypeptide. The mutant B2 appeared to have the deletion of a single amino acid (valine at residue 174, contained in the conserved region IV described above). This mutant therefore has the potential to code for an almost full-length polypeptide. It was interesting to note that mutants B3 to B5 had the identical mutation which took the form of single base addition. This also gave rise to a frameshift at residue 330 and chain termination at 354. Thus the potential polypeptide was much altered in its amino acid composition and slightly shortened. To our knowledge this kind of mutation involving addition of a base has not been previously reported in relation to herpesvirus TK. Recently, a frameshift and chain termination due to the
addition of adenine to an existing sequence of seven adenines has been discovered in the envelope glycoprotein gene of the human immunodeficiency virus (R. Daniels, personal communication).

The fact that three mutants were identical could be because the parental virus stock already contained a subpopulation corresponding to this variant. Analysis of HSV-1 isolates has revealed the presence of low proportions of TK- virions (Martin et al., 1985; Coen, 1986; Field & Owen, 1988) or virions with mutations in DNA polymerase (Parris & Harrington, 1982).

On the question of altered or truncated TK peptides which would be anticipated in extracts of cells infected with mutant viruses however, such extracts failed to adsorb TK-neutralizing antibodies from serum. This was expected for mutant B1 which is predicted to encode a truncated TK polypeptide. However, it was surprising that B2 and especially B3 to B5 failed to adsorb the neutralizing antibodies given their expected almost full-length products. The explanation may be that the active site epitopes are no longer exposed to the antibodies important in neutralization; this would be more likely if the mutations in the TK polypeptide result in the enzyme being unstable or possibly defective in dimerization or having a different structure.

When the amino acid sequences of BHV-1 and HSV-1 TKs were compared it was noted that the structural alignment at the N terminus commenced after 39 amino acids in HSV-1 TK (which is a larger protein). Furthermore deletion of 45 N-terminal amino acids of HSV-1 TK gave rise to a 39K product which (although it retained enzyme activity) was shown to be less stable than wt (Haarr & Flatmark, 1987). These workers suggested that this could be either because the truncated enzyme itself or TK-specific mRNA was more rapidly degraded. The complete absence of a comparable N-terminal amino acid sequence in BHV-1 TK is of unknown significance. The amino acid changes in mutants B2 to B5 may also be affecting the stability of the TK gene products and this would therefore explain their inability to adsorb TK-neutralizing antibody.

The structural analysis of TKs from different herpesviruses and from substrate specificity mutants should eventually provide thorough elucidation of this virus-coded enzyme and give a fuller explanation of biochemical data such as we report here for this enzyme which is important both in pathogenesis and in the design of specific inhibitors.

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REFERENCES


BHV-1 TK mutants


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