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# Identification of the Thymidine Kinase Gene of Feline Herpesvirus: Use of Degenerate Oligonucleotides in the Polymerase Chain Reaction To Isolate Herpesvirus Gene Homologs

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**Feline herpesvirus 1 (FHV) is the causative agent of viral rhinotracheitis in cats. Current vaccination programs employing attenuated live and killed FHV vaccines have been effective in reducing the incidence of this disease. As an initial step in the development of recombinant FHVs for use in the vaccination of cats, we have identified the thymidine kinase (TK) gene of this feline-specific alphaherpesvirus. Comparisons of the amino acid sequences of other herpesvirus TK proteins have shown that these proteins are highly divergent, sharing only short regions of imperfect amino acid identity. We have used the polymerase chain reaction method of DNA amplification to increase the specificity associated with the use of short, highly degenerate oligonucleotide probes derived from regions of imperfect amino acid conservation. These methods were used to isolate the TK gene of FHV and should prove to be useful in the identification of new members of other viral and cellular gene families. A recombinant FHV bearing a deletion in the identified TK gene was constructed and shown to possess the expected TK$^-$ phenotype. The FHV TK gene is located at a position of approximately 40% in the long unique component of the FHV genome. The location of the TK gene and the location and orientation of flanking FHV genes, homologs of herpes simplex virus type 1 UL24 and UL22, are conserved among alphaherpesviruses.**

Feline herpesvirus 1 (FHV) is the causative agent of feline viral rhinotracheitis in cats (4, 12). Before the advent of current modified live and killed feline viral rhinotracheitis vaccines (1, 7, 40, 51), FHV infection accounted for a large portion of upper respiratory disease in cats. The success of current feline viral rhinotracheitis vaccination procedures has in part limited research interest in this virus. Recently, the development of live recombinant herpesvirus vaccines, such as those to protect swine against pseudorabies disease (19, 29), has rekindled interest in the molecular characterization of this feline herpesvirus.

FHV is a typical member of the alphaherpesvirus subfamily. The FHV genome comprises approximately 134 kilobase pairs (kb) of DNA and is subdivided into long and short components of 104 and 30 kb, respectively (43). The short region contains inverted repeat sequences which mediate inversion of this region, relative to the long region, to generate two isomers of the FHV genome. A restriction endonuclease map of the genome has been reported (43), and this cleavage pattern appears to be extremely well conserved among FHV isolates (16). FHV grows readily in feline cells in culture; growth in vitro and in vivo is limited to Felidae.

We were interested in studying this virus further, as a member of the family of clinically significant alphaherpesviruses and as a potential recombinant virus for use in the vaccination of cats. Live recombinant herpesviruses can be constructed by taking advantage of homologous recombination to introduce into the viral genome DNA sequences that have been modified by using recombinant DNA techniques (19, 25, 33, 39, 41, 52, 55). In some cases, viral virulence factors have been deleted in recombinant viruses to yield attenuated live virus vaccine strains. For example, inactivation of the thymidine kinase (TK) gene has been shown to reduce the virulence of vaccinia virus and herpesviruses (2, 11, 19, 21, 54). This observation has provided the basis for the development of several TK$^-$ herpesvirus vaccines (19, 29; M. Kit and S. Kit, European patent 0226029, June 1987).

In addition, relevant genes from heterologous pathogens can be inserted into the genome of recombinant viruses to express foreign immunogens in vivo during vaccination. This approach has been developed extensively using recombinant vaccinia viruses (27, 34, 37). Among herpesviruses, several have been engineered to function as vectors for the expression of heterologous proteins, including herpes simplex virus type 1 (HSV-1) (50), pseudorabies virus (56, 60), and varicella-zoster virus (VZV) (25). In much of this work, insertion within the viral TK gene has provided a convenient method to select for recombinant viruses by virtue of the resulting TK$^-$ phenotype (25, 27, 33). Here we report the isolation of the TK gene of FHV, as an initial step in the development of this feline-specific herpesvirus as a vector for vaccination in cats.

## MATERIALS AND METHODS

**Cells and viruses.** FHV strains UC-D and UT88-1729 were kindly provided by Niels Pedersen (University of California School of Veterinary Medicine, Davis) and Malcolm McCracken (University of Tennessee Veterinary Teaching Hospital, Knoxville), respectively. FHV was grown on CRFK feline kidney cells (5) in Dulbecco modified essential medium containing 10% fetal bovine serum. Plaques were obtained by growth under 0.35% Noble agar, and TK$^-$ plaques were selected for in the presence of 100 μg of thymidine arabinoside (Raylo Chemicals, Edmonton, Alberta, Canada) per ml. Viral DNA was prepared from sodium dodecyl sulfate-proteinase K-treated cytoplasmic nucleocapsids by sodium

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iodide density gradient centrifugation as described by Walboomers and Schegget (59). HSV-1 DNA was similarly prepared from infected Vero cells. Phenol extraction was substituted for density gradient centrifugation in small-scale FHV DNA preparations used for the screening of recombinant viruses.

**Oligonucleotides.** Oligonucleotides were prepared by an automated solid-support-based method, using a model 510 prototype DNA synthesizer (Perkin-Elmer Cetus Instruments, Norwalk, Conn.). Cyanoethyl diisopropylaminophosphoramidates and long-chain alkylamine controlled-pore glass supports were obtained from American Bionetics, Hayward, Calif. Mixed-base sites in degenerate oligonucleotides were created during individual syntheses by using the simultaneous multiple-amidate addition capability of the synthesizer. Oligonucleotides were purified by polyacrylamide gel electrophoresis and reverse-phase high-pressure liquid chromatography.

**PCR.** Polymerase chain reactions (PCRs), using the thermostable DNA polymerase of *Thermus aquaticus*, were as described previously (44) except that degenerate oligonucleotide primers were used at a concentration of 25 to 100 pm per 50-μl reaction. Temperature cycling of the PCRs was accomplished using a DNA thermal cycler (Perkin-Elmer Cetus Instruments). PCR cycles were as described previously (44) except that the annealing temperature was reduced to 37°C in the first five cycles to facilitate the annealing of short oligonucleotides. The subsequent 35 cycles utilized an annealing temperature of 50°C.

**Nucleic acid techniques.** PCR products were analyzed by NuSieve agarose gel electrophoresis as previously described (45). PCR products were molecularly cloned in BlueScript plasmids (Stratagene, La Jolla, Calif.) after restriction endonuclease digestion of cleavage sites contained within the primers (26) and excision from polyacrylamide gels (30). *T. aquaticus* DNA polymerase was obtained from Perkin-Elmer Cetus Instruments. Other nucleic acid enzymes, including restriction endonucleases, were from New England BioLabs, Inc., Beverly, Mass., or Bethesda Research Laboratories, Inc., Gaithersburg, Md., and were used according to the instructions of the manufacturers. Molecular cloning techniques were as described by Maniatis et al. (28). Radiolabeled RNA probes for DNA blot hybridization were produced from BlueScript plasmids and were used according to protocols provided by Stratagene.

DNA sequence analysis (47) utilized sequencing primers appropriate to the BlueScript plasmids as well as internal oligonucleotide primers. Double-stranded plasmid templates were sequenced by using Sequenase T7 DNA polymerase (U.S. Biochemicals Corp., Cleveland, Ohio).

DNA and protein sequence analysis was performed by using the sequence analysis software package from the University of Wisconsin (9).

**Recombinant virus construction.** A bacterial plasmid containing a deletion in the identified FHV TK gene was constructed using standard molecular cloning techniques. This plasmid and FHV strain UT88-1729 DNA were cotransfected into CRFK cells by using the calcium phosphate precipitation method (14). Progeny virus was harvested when full cytopathic effect was evident, and recombinant FHV plaques were isolated in the presence of thymidine arabinoside (araT). The desired recombinant virus was identified by restriction endonuclease analysis.

**TK assay.** CRFK cells were infected by using a high multiplicity of FHV, and cells were harvested at various times up to 12 h postinfection. Cell extracts were prepared and assayed for TK enzymatic activity as described by Post et al. (38). Cellular TK activity was inhibited by the incorporation of 100 μM TTP in the reaction (3).

## RESULTS

**Isolation of FHV TK.** DNA sequence analysis of the TK genes of alphaherpesviruses shows that these proteins are highly divergent, with only short, scattered regions of amino acid similarity (17). Thus, simple methods of DNA hybridization, using heterologous TK probes, would not be productive in the isolation of the FHV TK gene. The divergence among TK genes is so extensive that the use of oligonucleotide hybridization probes is also problematic. A comparison of the TK proteins of HSV-1 (32, 58) and VZV (8) reveals only seven collinear regions in which four amino acids are identically conserved. A similar sequence comparison of the TK proteins of pseudorabies virus (M. Kit and S. Kit, U.S. patent 4514497, 1985) and HSV-1 also identifies seven collinear regions in which four amino acids are conserved, but only three of these regions are conserved among the three herpesviruses. In comparing all known alphaherpesvirus TK proteins (including HSV-2 [20, 53], marmoset herpesvirus [36], equine herpesvirus 1 [42], and bovine herpesvirus 1 [32a; Kit and Kit, European patent]), we are unable to find any region of four amino acids that is identically conserved among all TK proteins.

To improve the specificity associated with the use of short and highly degenerate oligonucleotide probes, we chose to couple their use to the PCR DNA amplification method (35, 44, 45). PCR is a method wherein a region of DNA flanked by two oligonucleotide primers can be amplified by as much as $10^6$-fold through repeated cycles of primer annealing, DNA polymerase extension, and thermal denaturation of the DNA product. The increase in specificity contributed by PCR to the use of short, highly degenerate primers derives from the requirement that two oligonucleotides must anneal to opposite DNA strands, in the proper relative orientation, for amplification to occur. Other sites of annealing generate configurations that do not give rise to amplified products. Furthermore, from the predicted location of the oligonucleotide primers on the DNA template, one can determine the expected size of the discrete PCR product.

The amino acid sequences of several alphaherpesvirus TK proteins were compared to reveal regions that are relatively conserved. Dot matrix analysis was performed using the University of Wisconsin sequence analysis software package. In these analyses, evolutionarily related amino acids were scored by using the comparison tables of Dayhoff (15, 49). Several moderately conserved regions were identified, and visual inspection of these resulted in the choice of the five oligonucleotide primer sites shown in Table 1. Three of the sites chosen fall within regions of the TK protein that have been previously identified and implicated in enzymatic function. Sites 1 and 2 are located in a region of TK involved in ATP binding (6, 18, 24), and site 3 is located in a region implicated in nucleoside binding (6). Primer sites 4 and 5 are in regions without known function but which are conserved among TK proteins. As anticipated from the discussion above, no site is identically conserved among all of the known TK proteins.

The oligonucleotide primers used are shown in Table 2. The TK-specific primer sequences range from 11 to 14 nucleotides in length, and the primers additionally include, at the 5′ end, a 9-nucleotide extension that contains a restriction endonuclease cleavage site. Primers equivalent to

TABLE 1. Moderately conserved TK regions used to generate oligonucleotide primers for PCR

| Virus[a] | Primer site | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| | 55[b] | 61 | 162 | 222 | 286 |
| HSV-1 | DGPHG | GKTT | DRHP | RPGE | DTLF |
| | 18 | 24 | 129 | 190 | 255 |
| VZV | DGAYG | GKTT | DRHP | RPGE | DTLF |
| | 83 | 89 | 182 | 241 | 308 |
| PRV | DGAYG | GKST | DRHP | RAGE | DTLF |
| | 16 | 22 | 130 | 188 | |
| MarHV | DGPHG | GKST | DRHA | RPGE | —[c] |

[a] PRV, Pseudorabies virus; MarHV, marmoset herpesvirus.
[b] Amino acid positions are from the following references: HSV-1, 31; VZV, 8; pseudorabies virus, deduced from Kit and Kit, U.S. patent; and marmoset herpesvirus, 36.
[c] —, A homologous region is not found in the published sequence.

the coding strand of DNA (+ primers) contain *Hin*dIII sites; primers complementary to the coding strand (− primers) contain *Eco*RI sites. These exogenous sequences become incorporated into the PCR product and facilitate subsequent molecular cloning of the product. Furthermore, the extensions contribute to the reannealing of the primers to PCR products generated after the initial PCR cycle (26). The primers used incorporate all of the coding degeneracy and all of the amino acid variations shown in Table 1. The total degeneracy of the primers varies from 48- to 384-fold.

Primers were used in PCR experiments in the following pairwise combinations: +1 and −3, +2 and −3, +3 and −4, +3 and −5, and +4 and −5. PCR was essentially as previously described (44) except that the stringency of annealing was relaxed in the initial five cycles. This modification was intended to facilitate the annealing of short oligonucleotide primers. We have subsequently found that this modification is not essential for amplification with the current oligonucleotide primers.

Preliminary experiments were performed using the molecularly cloned HSV-1 TK gene as a template for PCR. Under the conditions used, only primer pairs +1 and −3 and +2 and −3 generated sufficient PCR product of the expected size to be visible by ethidium bromide staining after agarose gel electrophoresis of the reaction mixture.

Those primer pairs that were able to amplify were then used in PCR experiments with HSV-1 and FHV strain UC-D genomic DNA as templates. Only primer pair +1 and −3 generated a visible band of the expected size with either the HSV-1 or FHV template (Fig. 1); primer pair +2 and −3 yielded the expected band with HSV-1 DNA but not with FHV DNA (data not shown).

The 350-base-pair (bp) PCR product of the FHV template was molecularly cloned in a BlueScript plasmid by taking advantage of the primer-derived restriction endonuclease sites. DNA sequence analysis of this product revealed significant amino acid similarity with the expected region of

TABLE 2. Oligonucleotide primers used in PCR

| Primer | Nucleotide sequence[a] (amino acid sequence[b]) | TK-specific length (nt[c]) | Degeneracy (fold) |
|---|---|---|---|
| | *D G P H G* | | |
| | *A Y* | | |
| +1 | 5′tcaaagcttGAYGGNSCNYAYGG | 14 | 256 |
| | *G K T T* | | |
| | *S* | | |
| +2[d] | 5′tcaaagcttGGNAARWCNAC | 11 | 80 |
| | 5′tcaaagcttGGNAARAGYAC | | |
| | *D R H P* | | |
| | *A* | | |
| +3 | 5′tcaaagcttGAYCGNCAYSC | 11 | 48 |
| | 5′tcaaagcttGAYAGRCAYSC | | |
| −3 | CTRGCNGTRSGcttaagctc5′ | 11 | 48 |
| | CTRTCYGTRSGcttaagctc5′ | | |
| | *R P G E* | | |
| | *A* | | |
| +4 | 5′tcaaagcttCGNSCNGGNGA | 11 | 192 |
| | 5′tcaaagcttAGRSCNGGNGA | | |
| −4 | GCNSGNCCNCTYcttaagctc5′ | 12 | 384 |
| | TCYSGNCCNCTYcttaagctc5′ | | |
| | *D T L F* | | |
| −5 | CTRTGNGANAARcttaagctc5′ | 12 | 96 |
| | CTRTGNAAYAARcttaagctc5′ | | |

[a] The International Union of Pure and Applied Chemistry symbols used to denote multiple nucleotides are as follows: Y = C +T; R = A + G; S = C + G; W = A + T; N = A + C + G + T. Nucleotides encoding TK-specific sequences are in uppercase letters; those encoding endonuclease restriction site extensions onto the primers are in lowercase letters.
[b] Amino acids are shown in italics.
[c] nt, Nucleotides.
[d] In most cases, two degenerate oligonucleotides were required to encompass all codons desired.

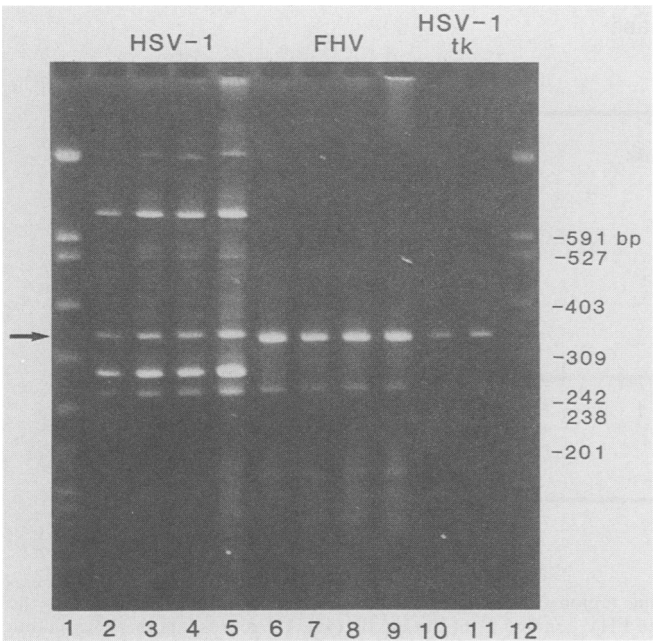



FIG. 1. PCR amplification of FHV TK. Genomic DNA of HSV-1 (30 to 1,000 ng, lanes 2 to 5) and FHV-1 (30 to 1,000 ng, lanes 6 to 9) was subjected to PCR amplification, using primer pair +1 and −3 as described in the text. A plasmid containing the HSV-1 TK gene (pHSV-106; Bethesda Research Laboratories) provided an additional control for TK-specific amplification (10 ng of linearized plasmid, lanes 10 and 11). The expected TK-specific 350-bp DNA fragment is indicated by an arrow. Molecular size markers are indicated in lanes 1 and 12.

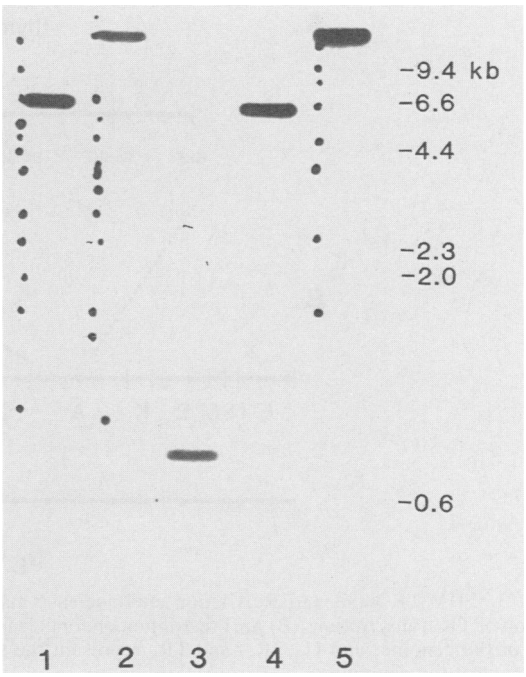



FIG. 2. Genomic localization of the FHV TK gene. FHV DNA was digested to completion with the following restriction endonucleases: lane 1, *Eco*RI; lane 2, *Hin*dIII; lane 3, *Eco*RI plus *Hin*dIII; lane 4, *Eco*RI plus *Sal*I; and lane 5, *Sal*I. Fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose as described by Maniatis et al. (28). Filters were probed using radiolabeled RNA prepared from the BlueScript plasmid containing the 350-bp PCR product. Molecular size markers (*Hin*dIII-λ DNA) are indicated. Selected FHV fragments were marked to permit the accurate identification of hybridizing bands and appear as dots on the autoradiogram.

known herpesvirus TK sequences. The FHV TK sequence obtained from four independently isolated molecular clones was identical, although the clones differed in the particular oligonucleotide primer sequence used.

Southern blot analysis of the FHV genome was performed by using a radiolabeled RNA probe generated from the BlueScript plasmid containing the 350-bp PCR product (Fig. 2). The 6.6-kb *Eco*RI fragment containing this portion of the FHV TK gene was isolated and subcloned as 5.8- and 0.8-kb *Eco*RI-*Hin*dIII fragments (pFHVtk5.8 and pFHVtk0.8, respectively). Further Southern blot analysis, using pFHV tk0.8 as a probe, localized additional upstream sequences to a 3.8-kb *Sal*I-*Hin*dIII fragment (data not shown). This fragment was molecularly cloned from FHV genomic DNA as pFHVtk3.8. A map of the entire 9.6-kb region of FHV containing the TK gene is shown in Fig. 3A. This region is located within the largest *Sal*I fragment of the FHV genome (Fig. 2), which has been mapped to a position of approximately 40% in the FHV long unique component (*Sal*I-A [43]). From additional mapping of the FHV genome (unpublished data), we deduce that the TK gene is oriented from right to left as shown in Fig. 3. This location and orientation within the long unique region are conserved among other alphaherpesviruses (8, 31).

**FHV TK DNA sequence.** The DNA sequence of the FHV TK gene is shown in Fig. 4. The G+C base composition agrees with that of total FHV DNA as determined by Herrmann et al. (16) (46 and 50%, respectively). The TK coding region was deduced by homology to known herpesvirus TK proteins and is indicated. The initiating ATG (defined as position +1) is the first ATG after an in-frame termination codon at position −88 bp and occurs within a context consistent with that noted by Kozak (22). An in-frame translation termination codon is at position +1029 bp. The predicted FHV TK protein comprises 343 amino acids.

**Construction of recombinant TK$^-$ FHV.** To obtain genetic and biochemical confirmation that the identified TK gene encodes FHV TK, we constructed a recombinant FHV in which the TK coding sequence had been modified to delete the nucleoside-binding domain of the deduced TK protein. Bacterial plasmid ptkΔ*Eco*RV-*Hin*dIII (pGC113) contains the entire FHV TK gene and flanking regions (from the *Sal*I site to the proximal *Bam*HI site, as indicated in Fig. 3A) but lacks TK coding sequences between the *Eco*RV and *Hin*dIII sites (indicated in Fig. 4). A synthetic oligonucleotide polylinker was used to join these sites in the plasmid construction. The resulting protein is predicted to contain a novel serine residue inserted at the site of the glycine 117-to-lysine 233 deletion.

This mutation was introduced into FHV by using calcium phosphate coprecipitation techniques to obtain homologous recombination between plasmid and herpesvirus genomic sequences. Plasmid ptkΔ*Eco*RV-*Hin*dIII and FHV strain UT88-1729 genomic DNA were cotransfected into CRFK cells, and progeny virus was harvested and plaqued onto CRFK cells in the presence of 100 μg of araT per ml to select for recombinant TK$^-$ virus. Previous studies had shown that this thymidine analog provides stringent selection against the replication of TK$^+$ FHV (48), and we have used this selection method to isolate spontaneous TK$^-$ FHV (unpublished data). AraT-resistant viruses were screened by restriction

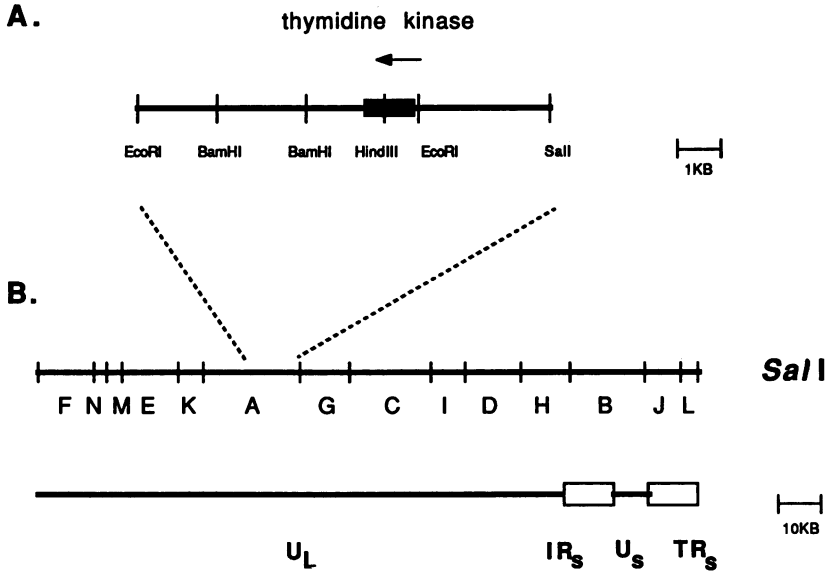



FIG. 3. FHV TK gene. (A) Restriction endonuclease map of genomic region containing the FHV TK gene. The arrow indicates the direction of TK transcription. (B) *Sal*I restriction endonuclease map of the FHV genome from Rota et al. (43). $U_L$ represents the long unique region of the genome, and $U_S$, $IR_S$, and $TR_S$ represent the short unique, inverted, and terminal repeat regions, respectively, of the short component of the genome.

endonuclease analysis for the presence of the *Eco*RV-*Hin*dIII deletion. All araT-resistant viruses examined contained the expected deletion. One virus was further plaque purified and was designated FHV-113. As expected, the 6.6-kb *Eco*RI fragment containing the FHV TK gene is reduced in size by approximately 345 bp in FHV-113 (Fig. 5).

The araT-resistant phenotype of FHV-113 was shown to be attributable to a defect in TK by direct enzymatic assay of TK activity in extracts of infected cells. Results of these assays (Fig. 6) confirm that the araT-resistant FHV-113 is deficient in TK enzymatic activity. Thus, genetic and biochemical analyses support the assignment based on the deduced amino acid sequence and establish that the identified gene encodes FHV TK.

## DISCUSSION

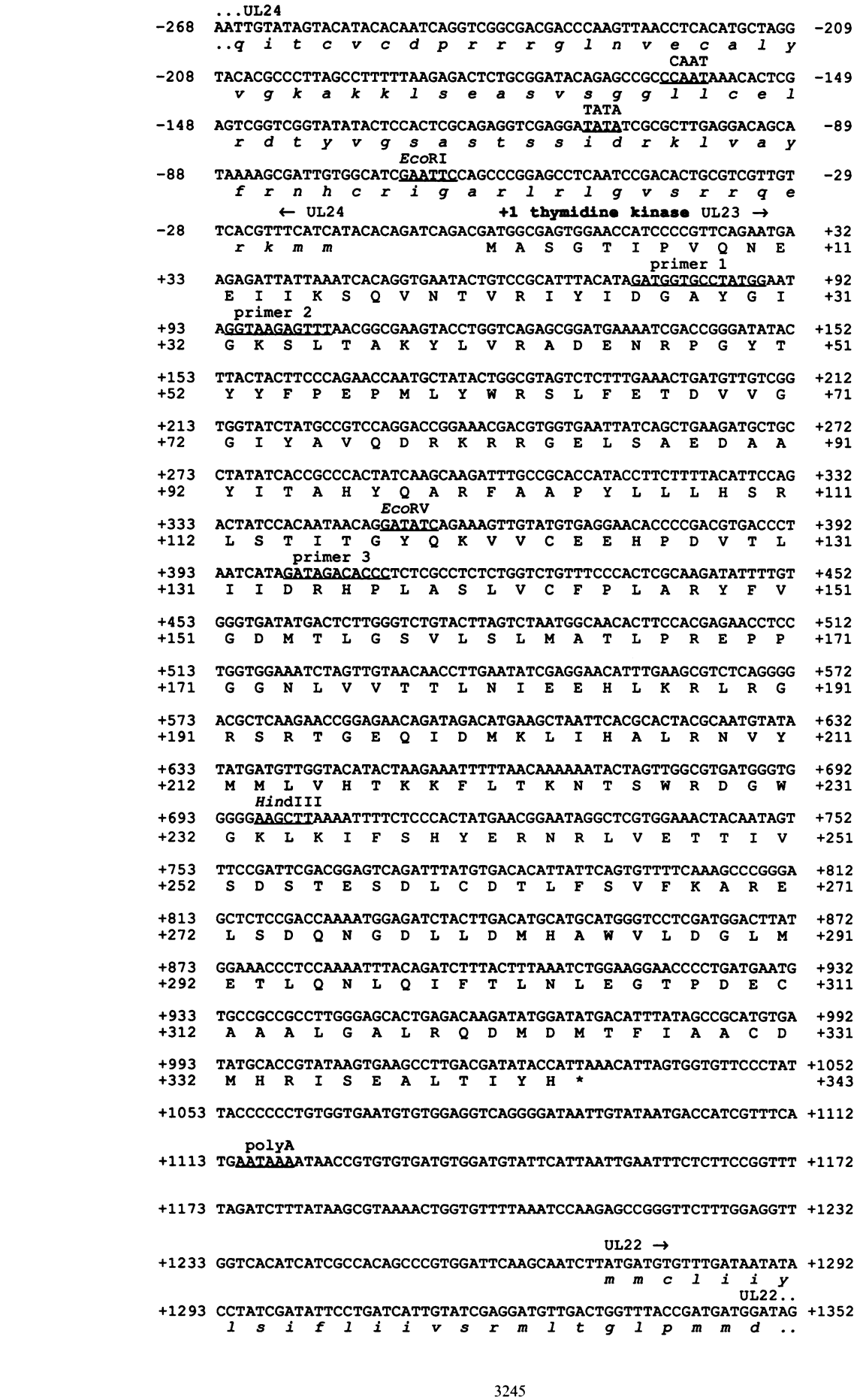
The TK proteins of alphaherpesviruses are highly divergent in amino acid sequence, showing only short, scattered regions of amino acid similarity (17). Efforts to identify the FHV TK gene by conventional DNA hybridization methods would therefore not have been productive. The specificity of oligonucleotide probes can be enhanced through the use of the PCR method. The structural requirements for PCR amplification impose stringent constraints on the relative location and orientation of the two oligonucleotide primers; the knowledge of the size of the specific PCR product allows for the further exclusion of irrelevant products. Thus, PCR might be expected to permit the use of very short and highly degenerate oligonucleotides in the identification of novel genes.

In this report, we describe the use of PCR to identify the TK gene of FHV. A sequence comparison of alphaherpesvirus TK proteins had suggested five moderately conserved regions in TK proteins. Although none of these regions is identically conserved among TK proteins, the similarity is sufficient to allow the design of a series of short, degenerate oligonucleotide primers. Degenerate primers have been used in PCR to encompass the degeneracy of the genetic code for a specific amino acid sequence (23, 26). Here, we extended the degree of degeneracy to include amino acid sequences that differ but are presumed to be related.

One pair of primers (primers +1 and −3) was able to amplify a product of the expected size. These primer sites are located in two moderately conserved regions of TK that are believed to be involved, respectively, in ATP and nucleoside binding (6). DNA sequence analysis of the molecularly cloned PCR product revealed homology to the expected region of alphaherpesvirus TK. This PCR product was then used as a hybridization probe to isolate the entire FHV TK gene.

The use of PCR to enhance the specificity of short, degenerate oligonucleotide probes should facilitate the isolation of gene homologs and evolutionarily related sequences which share only short and imperfect regions of amino acid identity. The divergence seen among herpesvirus TK proteins is typical of that seen in other families of homologous proteins among herpesviruses (31), and this method should enable the rapid isolation of new members of these gene families. To date, we have used these methods to isolate the

FIG. 4. Nucleotide and amino acid sequence of FHV TK. The DNA sequence of the FHV TK gene and flanking regions is shown. Numbering is from the initiating methionine residue of the TK protein. The deduced amino acid sequence of the TK protein is shown in uppercase letters; the amino acid sequences of the presumptive HSV-1 glycoprotein H (UL22) and basic protein (UL24) homologs are shown in lowercase letters. Sites used to generate oligonucleotide probes 1, 2, and 3 are indicated, as are presumptive CAAT, TATA, and polyadenylation sequences within the TK gene. Restriction endonuclease sites referred to in the text are also underlined.


...UL24
CAAT
TATA
EcoRI
← UL24
+1 thymidine kinase UL23 →
primer 1
primer 2
EcoRV
primer 3
HindIII
polyA
UL22 →
UL22..

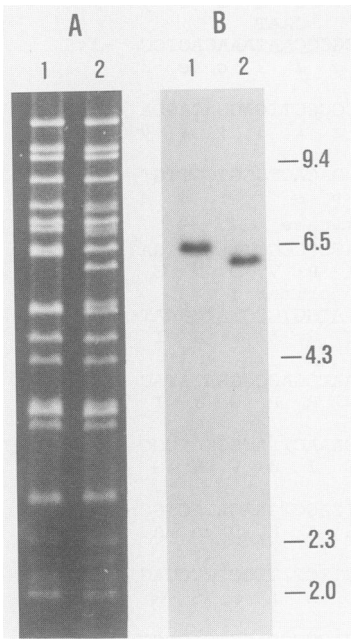



FIG. 5. Restriction endonuclease analysis of recombinant FHV. (A) *Eco*RI restriction endonuclease digestion of parental FHV UT88-1729 (lane 1) and recombinant araT-resistant FHV-113 (lane 2). (B) The *Eco*RI-digested DNA visualized in panel A was transferred to nitrocellulose, and TK sequences were identified by using a nick-translated hybridization probe comprising the 3′ portion of the FHV TK gene (*Hin*dIII to proximal *Bam*HI; Fig. 3). The TK-containing 6.6-kb *Eco*RI fragment in the parental virus (lane 1) is reduced by approximately 345 bp in the deletion-containing virus FHV-113 (lane 2). Molecular size markers (*Hin*dIII-λ DNA) are indicated. Several other *Eco*RI fragments which also do not comigrate in both viral DNAs map to the repeat regions of FHV (43); a variation in the molecular size of these regions has been noted in unrelated experiments in which FHV is plaque purified from a population (unpublished data).

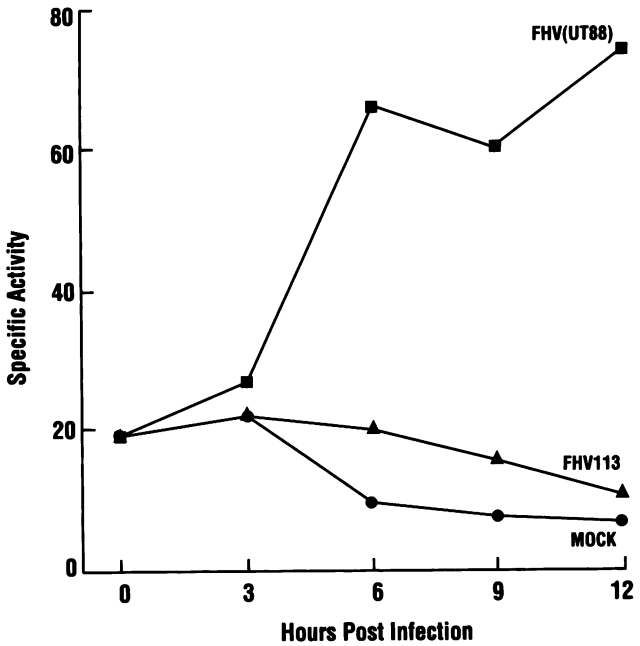



FIG. 6. TK activity in CRFK cells infected with recombinant FHV. CRFK cells were infected with parental FHV UT88-1729 or recombinant araT-resistant FHV-113 or were mock infected. Cells were harvested at the indicated times postinfection, and the virus-specific TK enzymatic activity ([$^3$H]thymidine conversion per unit of protein) was determined as described in Materials and Methods.

major capsid protein and glycoprotein B gene homologs of FHV (unpublished data).

This method is not without pitfalls. Three of the five primer pairs tested were not able to mediate PCR amplification with a known HSV-1 template. We have not explored alternate conditions that might permit amplification by these primers. In other cases the amino acid divergence within the new homolog may fall beyond that encoded by the degenerate oligonucleotide primers. In the FHV TK gene, a threonine-to-leucine change in the final position at primer site 2 prevented PCR amplification with this primer. As expected from the positive PCR results, primer sites 1 and 3 are conserved in FHV TK.

DNA sequence analysis of the identified FHV TK gene revealed an open reading frame of 343 amino acids. This compared with 376 amino acids in HSV-1 TK and 341 amino acids in VZV TK. A comparison of the amino acid sequences of these TK proteins is shown in Fig. 7. The FHV protein is clearly a member of the family of herpesvirus TK proteins. Although regions of amino acid identity are short and scattered, herpesvirus TK proteins are discernibly similar and evolutionarily related.

An examination of the DNA sequence upstream from the FHV TK coding region reveals canonical TATA and CAAT sequences at −110 and −161 bp, respectively. Downstream from the termination codon TAA, a canonical polyadenylation signal, AATAAA, is found at position +1115 bp. Further definition of the FHV TK promoter and polyadenylation site awaits mapping of the termini of the FHV TK mRNA.

The genomic location and orientation of the TK gene with the long unique region of FHV are consistent with those determined in other alphaherpesviruses (8, 31). Downstream from the presumed FHV TK polyadenylation signal is an open reading frame that continues beyond the sequence presented. This open reading frame encodes a region that is reminiscent of signal peptide sequences (57) and may encode a protein that is homologous to the similarly located glycoprotein H (UL22) of HSV-1 (31). At the 5′ end of the FHV TK gene, starting at position −15 bp on the strand opposite that encoding TK, is an open reading frame whose position and amino acid sequence are also conserved among alphaherpesviruses (e.g., HSV-1 UL24). This basic protein may play a role in viral growth, and mutations that affect expression of this protein may mediate attenuation phenotypes previously attributed to TK mutations (17, 46).

Genetic and biochemical confirmation of the identity of the FHV TK gene was obtained through the construction of a live recombinant FHV in which the TK coding sequence had been modified to delete the nucleoside-binding domain of the deduced TK protein. This virus, FHV-113, was isolated by selection in the presence of the thymidine analog araT. Direct TK enzymatic assay of CRFK cells infected with this virus confirmed the $TK^-$ phenotype.

A functional TK gene is not essential for the in vitro growth of herpesviruses (10). In our studies we have observed no growth differences between the recombinant $TK^-$ FHV and the parental $TK^+$ virus. Viral plaques and cytopathic effect develop in parallel and are morphologically similar; virus yields are comparable. Loss of TK activity has been associated with attenuation of other herpesviruses in

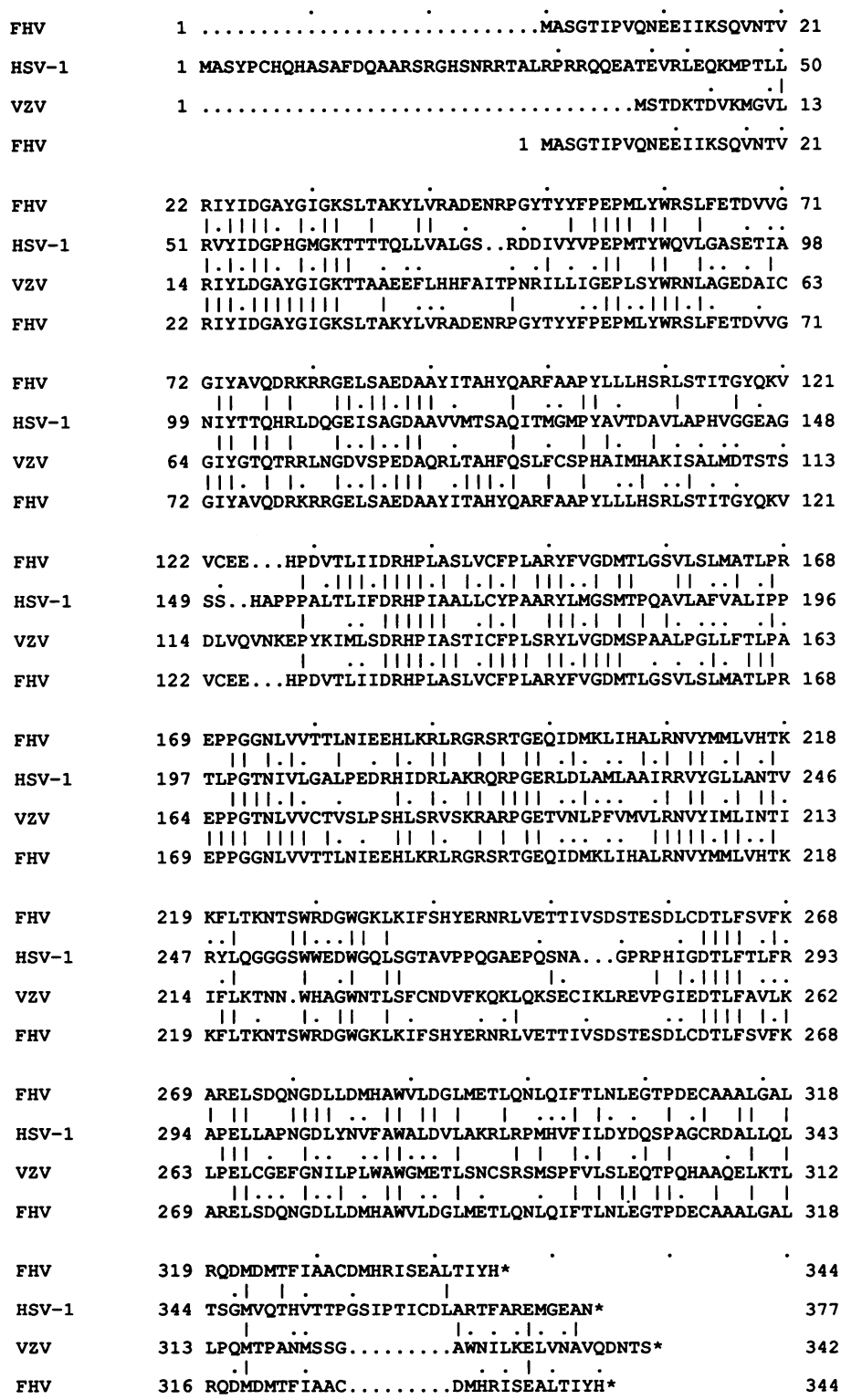



FIG. 7. Amino acid sequence comparison of FHV, HSV-1, and VZV TK proteins. Amino acid sequences were aligned for maximum homology. Amino acid identities are shown as |; evolutionarily related changes (comparison values of $\geq +0.5$ in the University of Wisconsin sequence analysis software package comparison tables [15]) are indicated as dots.

vivo, and studies to assess the virulence of the recombinant $TK^-$ FHV are planned. If sufficiently attenuated as a result of the deletion in TK, this virus may constitute a safe and nonreverting vaccine strain for feline viral rhinotracheitis vaccination.

Recombinant FHVs may also prove to be useful as vectors for the expression of heterologous vaccine immunogens in cats. Previous attempts to immunize cats against feline leukemia virus infection by using a recombinant vaccinia virus expressing the envelope gene of this retrovirus have been unsuccessful (13). This may in part reflect the limited replication potential of vaccinia virus in cats. Recombinant FHVs encoding the expression of heterologous proteins may be useful in delineating protective immunogens and may provide an effective means of vaccinating cats against feline leukemia virus and other infectious diseases.

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