JOURNAL OF VIROLOGY, June 1989, p. 2847–2852
0022-538X/89/062847-06$02.00/0

Vol. 63, No. 6

# Genetic and Biochemical Characterization of the Thymidine Kinase Gene from Herpesvirus of Turkeys

SANDRA L. MARTIN,†* DIANE I. APARISIO, AND PRADIP K. BANDYOPADHYAY

*Synergen, Inc., 1885 33rd Street, Boulder, Colorado 80301*

Received 14 November 1988/Accepted 21 February 1989

**The thymidine kinase gene encoded by herpesvirus of turkeys has been identified and characterized. A viral mutant ($ATR^0$) resistant to 1-β-D-arabinofuranosylthymine was isolated. This mutant was also resistant to 1-(2-fluoro-2-deoxy-β-D-arabinofuronosyl)-5-methyluracil and was unable to incorporate [$^{125}$I]deoxycytidine into DNA. The mutant phenotype was rescued by a cloned region of the turkey herpesvirus genome whose DNA sequence was found to contain an open reading frame similar to that for known thymidine kinases from other viruses. When expressed in *Escherichia coli*, this open reading frame complemented a thymidine kinase-deficient strain and resulted in thymidine kinase activity in extracts assayed in vitro.**

Herpesvirus of turkeys (HVT) is a nonpathogenic, naturally occurring virus that is closely related to the oncogenic Marek's disease virus. HVT vaccination is used widely to control Marek's disease in domestic poultry. The two viruses share extensive homology across the length of their 155-kilobase (kb) genomes (8, 11, 31), and the basic structure of their genome is similar to the genomes of herpes simplex virus and varicella-zoster virus (2, 5).

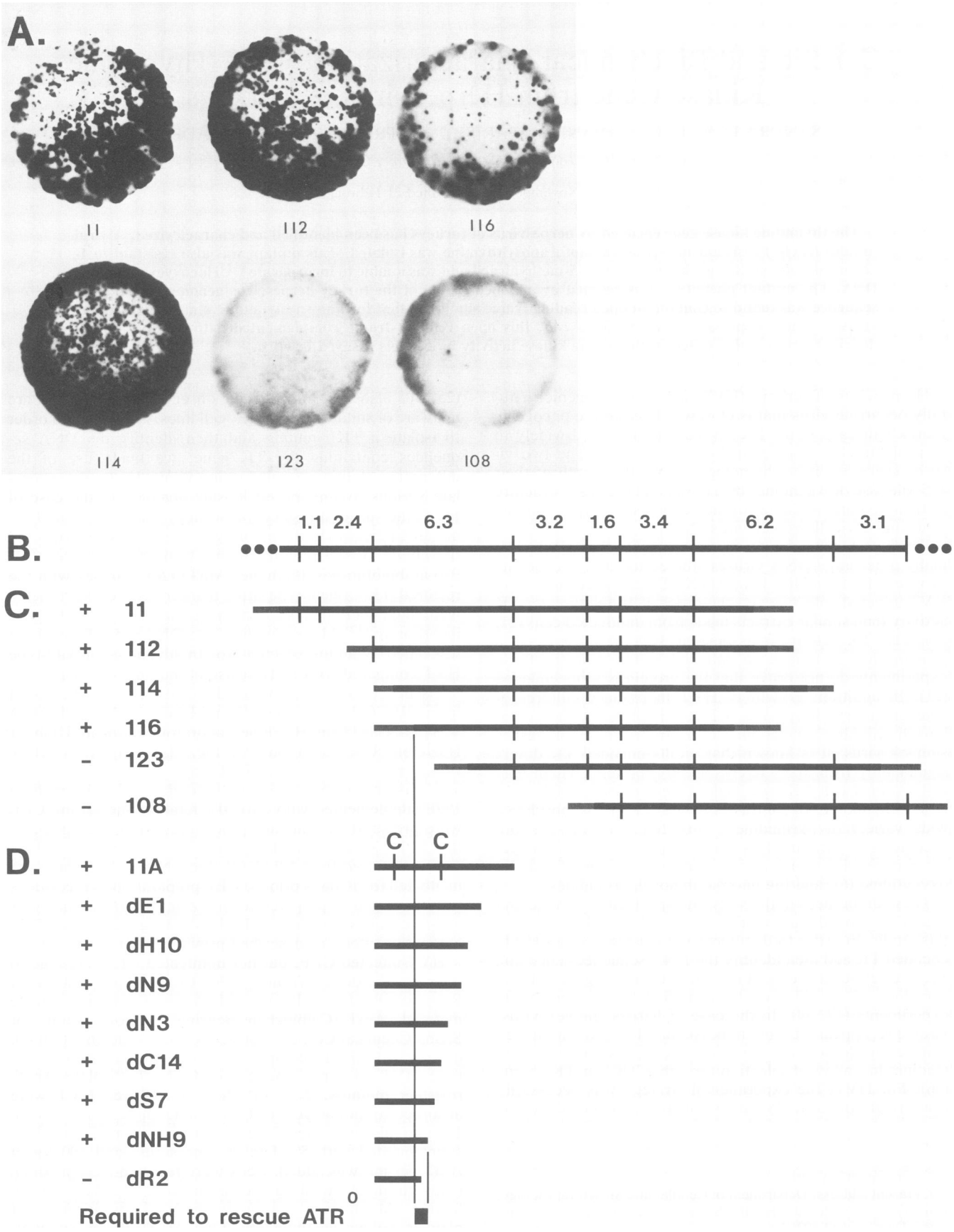
Previous biochemical evidence suggests that HVT, like many other large-DNA viruses studied to date, encodes its own gene for thymidine kinase (TK). Extracts prepared from HVT-infected cells have on the order of 10-fold-greater TK activity than similar extracts made from uninfected cells (21, 32). This TK activity is associated with the presence of a protein that is unique to infected cells, although these experiments do not prove that the protein is virus encoded (21). In addition, growth of HVT in primary cultures of chicken embryo fibroblasts (CEF) is inhibited by fluoroarabinsylpyrimidine nucleotides (32). In the case of herpes simplex virus, it is known that virally-encoded TK differs significantly from the TK encoded by the mammalian host cell. Animal cell TKs are highly specific for a single substrate, thymidine. In contrast, the viral TKs phosphorylate a wide variety of pyrimidines. This difference in substrate specificity is particularly significant in the case of halogenated pyrimidines such as 5-bromodeoxycytidine, 5-iododeoxycytidine (6), and the fluoroarabinosylpyrimidines.

These differences in the substrate specificities of viral and host TKs proved particularly valuable during the course of this study. In order to document the existence of an HVT-encoded TK and then identify the DNA sequences encoding TK, we set out to isolate $TK^-$ mutants. The region of DNA encoding TK could then be identified by marker rescue experiments (17, 34). In the case of herpes simplex virus, viral TK appears to be nonessential for growth in cell culture. In addition, the use of $TK^-$ host cells and a selective medium has allowed selection for either $TK^+$ or $TK^-$ virus (36). For HVT, the experimental strategy was necessarily less straightforward. HVT does not grow in mammalian cells (25, 30; also our unpublished observations), and we are unaware of suitable avian $TK^-$ cell lines. Therefore, in order to isolate a $TK^-$ mutant and then identify the DNA sequences containing the TK gene, we made use of the differential abilities of cellular and viral TKs to phosphorylate various pyrimidine nucleoside analogs. In the case of HVT, the successful selection strategy for isolation of $TK^-$ mutants relied upon the inability of the wild-type virus (and the ability of the mutant) to grow in the presence of 1-β-D-arabinofuranosylthymine (AraT) (26). Viruses with the restored $TK^+$ phenotype after marker rescue experiments were then identified by screening for plaques that could incorporate [$^{125}$I]deoxycytidine into DNA (35). This screen was aided by an unexpected growth advantage of wild-type HVT compared with AraT-resistant mutants of HVT.

Tissue culture medium, medium 199E, was purchased from GIBCO Laboratories, Grand Island, N.Y., and serum was purchased from HyClone Laboratories, Logan, Utah, or Hazleton Research Products, Inc., Lenexa, Kans. HVT FC126 was obtained from B. Calnek (Cornell University) and was grown in primary or secondary CEF in medium 199E supplemented with 2 to 10% fetal bovine serum. CEF were prepared by standard procedures from pathogen-free ($S^-$ SPF COFAL Marek's *gs chf*-39) chicken eggs which were purchased from SPAFAS, Inc., Norwich, Conn., and incubated for 10 days prior to CEF preparation. Virus stocks were passaged or prepared for freezing as cell-associated or cell-free stocks from infected cells showing greater than 70% cytopathic effect, as described previously (4).

HVT-infected CEF, but not uninfected CEF, are able to convert [$^{125}$I]deoxycytidine (>700 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) into acid-precipitable material. AraT (Calbiochem-Behring, La Jolla, Calif., or Sigma Chemical Co., St. Louis, Mo.) has little effect on the growth of CEF but completely inhibits the ability of wild-type HVT to form plaques on CEF. To isolate AraT-resistant mutants, $2 \times 10^4$ PFU of cell-free HVT were absorbed to monolayers of CEF containing $2 \times 10^7$ cells in 150-mm dishes. After 30 min, 30 ml of medium 199E, supplemented with 5% fetal bovine serum and 100 μg of AraT per ml, was added. Cells were fed with fresh medium (substituting 5% fetal bovine serum for 2%) 2 days postinfection. Monolayers were screened microscopically for plaques 4 days postinfection. One AraT-resistant mutant,

* Corresponding author.
† Present address: Department of Cellular and Structural Biology, University of Colorado Health Sciences Center, 4200 E. Ninth Ave., Denver, CO 80262.


A.
11
112
116
114
123
108
B.
1.1 2.4 6.3 3.2 1.6 3.4 6.2 3.1
C.
+ 11
+ 112
+ 114
+ 116
- 123
- 108
D.
C C
+ 11A
+ dE1
+ dH10
+ dN9
+ dN3
+ dC14
+ dS7
+ dNH9
- dR2
Required to rescue ATR0

$ATR^0$, was isolated in our initial screen of $2 \times 10^5$ PFU. Subsequently, two additional independent AraT-resistant mutants, $ATR^1$ and $ATR^5$, were isolated by this procedure.

These mutants all failed to incorporate [$^{125}$I]deoxycytidine into acid-precipitable material. In addition, they grew more slowly than the wild type and consistently yielded stocks with 10- to 50-fold-lower titers. Our studies have focused on $ATR^0$; however, in all aspects tested, $ATR^1$ and $ATR^5$ behaved as did $ATR^0$. $ATR^0$ is also resistant to 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-methyluracil (B. Calnek, personal communication). [$^{125}$I]deoxycytidine-positive plaques (35) were obtained when CEF were infected with wild-type HVT but not with $ATR^0$. This property was used to identify the TK gene in marker rescue experiments.

High-molecular-weight, total infected-cell DNA was made by standard procedures (24). Roughly 10% of the DNA prepared in this manner is viral (27; our unpublished results); the remainder is from the CEF. Total HVT-infected-CEF DNA is infectious when transfected into CEF by standard procedures (13) for calcium phosphate precipitation. In our hands, transfection typically resulted in an average of 10 PFU/μg of total infected-CEF DNA. Total HVT-infected-CEF DNA was rendered noninfectious by cleavage with the restriction endonucleases *Sal*I (restriction enzymes were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; Bethesda Research Laboratories, Inc., Gaithersburg, Md.; or New England BioLabs, Inc., Beverly, Mass. and used according to the recommendations of the manufacturer) and then cotransfected with infectious $ATR^0$ DNA into CEF. Plaques were obtained, and some of them were able to incorporate [$^{125}$I]deoxycytidine in the plaque assay. Our collection of 17 EMBL3 bacteriophage clones containing wild-type HVT DNA inserts was then screened by using a similar protocol to identify the segment of HVT DNA that restored wild-type function to $ATR^0$. These phage clones were representatives from an HVT library made from total infected-cell DNA which was partially cleaved with the restriction endonuclease *Sau*3AI. DNA fragments (15 to 20 kb were ligated into *Bam*HI-cleaved EMBL3 DNA (7) (Stratagene, San Diego, Calif.) and packaged in vitro (Gigapack Plus; Stratagene). The subset of clones containing HVT DNA was identified by screening plaque lifts with a nick-translated (Bethesda Research Laboratories) HVT-specific probe made with gel-purified (9) HVT DNA. Figure 1A shows autoradiograms of plaques obtained when DNA from six of the HVT-containing recombinant phage clones was cleaved with *Sal*I and cotransfected into CEF with $ATR^0$ DNA. Of the 17 tested, only 4 members from this group of 6 EMBL3 clones containing contiguous HVT DNA inserts were found to restore both the ability to incorporate [$^{125}$I]deoxycytidine and the wild-type growth rate upon cotransfection (Fig. 1B and C). These phages defined the region of DNA that contained the wild-type counterpart of $ATR^0$ as lying within a 4-kb region. The location was further delineated by making a series of 3′-end deletions of the 6.3-kb *Sal*I fragment, p11A (Fig. 1D), and assaying their ability to rescue the $ATR^0$ phenotype. These results localized the site of the mutation of $ATR^0$ to a specific region of the HVT genome less than 1 kb in length.

The DNA sequence of 2,033 base pairs, bounded by two *Cla*I restriction endonuclease cleavage sites (Fig. 1D) and encompassing the $ATR^0$ region as defined by marker rescue experiments, was determined (1, 16). These sequence data were analyzed with Pustell programs (International Biotechnologies, Inc., New Haven, Conn., and PC/GENE, IntelliGenetics, Mountain View, Calif.). Within this region there is an open reading frame of 310 amino acids (ORF1) with the potential of encoding a protein whose predicted molecular weight is 35,510 daltons (Fig. 2A). This putative protein is similar to a number of TKs in the region known to be the nucleotide-binding pocket (Fig. 2B).

In order to demonstrate that ORF1 encodes a functional TK, it was fused in-frame to the β-galactosidase gene in pUC18 to obtain pUC18-ORF1 (Fig. 3A). For this, the 2.5-kb *Xba*I fragment from p11A, encompassing most of ORF1, was inserted into *Xba*I-cut pUC18 (Fig. 3A). The 3′ *Xba*I site was then eliminated by cutting with *Cla*I and *Sal*I, filling in the ends, and religating. Next, the plasmid was cut with *Sac*I and *Xba*I, and four overlapping oligonucleotides were inserted such that the N-terminal 14 amino acids of ORF1, including the ATG, were reconstructed. pUC18-ORF1 thus constructed had 10 additional amino acids at the N terminus of ORF1; the first 6 amino acids were from the N terminus of β-galactosidase, amino acids 7 through 9 were from the pUC polylinker, and amino acid 10 was from our oligonucleotides, giving rise to a new *Nde*I site. Plasmids were transformed into *Escherichia coli* DH5 (Bethesda Research Laboratories); DNA was isolated, and its sequence was determined in order to confirm the correct reconstruction of ORF1. This plasmid was transformed into a $TK^-$ strain of *E. coli*, RS225 (14, 18, 33). RS225 transformants of pUC18-ORF1 were selected and maintained on LM agar containing 12.5 μg of 5-fluorouracil and 25 μg each of thymidine and uridine per mg, in which functional TK is required for growth (22). Extracts were made from RS225 alone, and from RS225 containing either pUC18, pUC18-ORF1, or pTdK (a plasmid carrying the *E. coli* TK gene [12]), and these extracts were assayed for TK activity (15, 35). The extracts from RS225 alone and from RS225 with pUC18 showed no significant phosphorylation of [$^{14}$C]thymidine in this assay. In contrast, the extracts made from RS225 bearing pTdK or pUC18-ORF1 were able to phosphorylate [$^{14}$C]thymidine (Fig. 3B). The results of this experiment establish that HVT ORF1 encodes a protein with TK activity. The difference in the observed TK activity between extracts containing pTdK-encoded TK and HVT ORF1-encoded TK is not necessarily due to a difference in specific activity of the enzyme. These experiments do not distinguish between a difference in the

FIG. 1. Marker rescue of $ATR^0$ phenotype and physical map of this region of DNA. (A) [$^{125}$I]deoxycytidine plaque assay following transfection with $ATR^0$ and various phage DNAs; autoradiogram of fixed, infected cells. Plaques were present on all six plates, although those on plates 108 and 123 failed to incorporate [$^{125}$I]deoxycytidine. The dark spots on 108 and 123 were examined microscopically and found to correspond to large clumps of cells rather than to plaques. (B) Location of *Sal*I restriction endonuclease cleavage sites in this region of HVT DNA; fragment sizes are given in kilobases. (C) Endpoints of overlapping phage clones from this region of DNA and results of the [$^{125}$I]deoxycytidine plaque assay, which are indicated by + (plaques incorporated [$^{125}$I]deoxycytidine) and − (plaques failed to incorporate [$^{125}$I]deoxycytidine), by using these DNAs in cotransfections with $ATR^0$ (see legend to panel A above). Stippled areas represent lambda arms. (D) Endpoints of the 6.3-kb *Sal*I fragment, 11A, and a series of deletions derived from it. Results of the [$^{125}$I]deoxycytidine plaque assay with these clones in cotransfections with $ATR^0$ are again indicated by + and −. Also shown are the *Cla*I sites, C, defining the region whose sequence was determined, and a thick bar that represents the region defined by these marker rescue experiments as having the mutation leading to the $ATR^0$ phenotype.

**A.**

```
   1 ATCGATGAAAGTCTGTACATATAGCCTTCTTTTTTAATGGTGTCGAACGTATAACTACAAGCCCGCGTCTCGCGACAAACACTAAAACGGGGCAAATGATAGTATGATCAGATCCCCTGG

 121 GGGCCATGCGCTCGATCAACGTTTTAGACTCCAGCAACTGCTTAGTCCCTGTTGCGCGTTGCTCATTCTTGCTGGCCGTCTTGAGGTTGCGGTAAAATCTGCAAGTTTTCAGTTCGATTA

 241 TGACACAGACATTGTTACAGCCTTCCACCAATAACCCGCCAGATAAGGTGAACATGCAAATGCAATCTGGCCTTCTGCGCCCCAGGTCTACTTCAAAGGCCAATCGAAACGCCGACGCCC

 361 CTTTAAGTAGTTCAGAAGGCAGGATCTTGCTTAATATCTTCTGTAATGTTCTACCAACTGTATTCCCCGCCGACGACTGGTCAAAGTTTTCAGCAGCAAGCTTTTTGTAAAAACGATTAT

 481 GACCACGGACACCCGCTTTTAGCAATCCTGCCATAAGGTGGTTTCGCCGCGTGCTTGCCTCGAAGACAATTGCCAGCTAATCCAGCATTACCATATTTCCTTGGCTTGCATTTGGATCTG

          Met Ala Leu Pro Arg Arg Pro Pro Thr Leu Thr Arg Val Tyr Leu Asp Gly Pro Phe Gly Ile Gly Lys Thr Ser Ile Leu Asn
 601  CGCGTCGATG GCA TTG CCG AGA AGA CCG CCC ACG TTA ACG CGA GTT TAT CTA GAC GGA CCG TTT GGT ATA GGC AAA ACG TCT ATA CTA AAC

      Ala Met Pro Asp His Thr Pro Asp Gly Ala Pro Ile Leu Lys Val Tyr Glu Pro Met Lys Tyr Trp Arg Cys Gln Ser Thr Asp Leu Val
 692  GCT ATG CCC GAC CAC ACG CCC GAT GGG GCT CCT ATA TTG AAA GTG TAC GAA CCA ATG AAA TAT TGG AGA TGC CAG TCT ACC GAT TTG GTG

      Val Ala Ala Asn Glu Thr Pro Glu Arg Arg Arg Gly Gly Ala Leu Ser Arg Phe Gln Ser Asp Met Ile Met Ala Ser Ile Gln Ala Arg
 782  GTA GCT GCC AAC GAA ACG CCA GAA CGT AGG CGT GGT GGA GCT TTA TCA CGA TTC CAA TCT GAC ATG ATC ATG GCA TCT ATA CAA GCC AGA

      Phe Ala Asp Pro Tyr Leu Leu Phe His Glu Arg Leu Ser Ser Lys Cys Arg Gly Lys Ile Glu Ile Cys Asp Thr Pro Ala Ile Ile Leu
 872  TTT GCC GAT CCA TAT TTG CTT TTT CAC GAA CGG TTA TCA TCT AAA TGT AGA GGA AAA ATA GAA ATA TGC GAT ACT CCA GCA ATT ATA TTA

      Met Leu Asp Arg His Pro Val Ala Ala Ile Leu Cys Phe Pro Ile Thr Arg Tyr Leu Leu Gly Glu Tyr Ser Leu Glu Met Leu Ile Ser
 962  ATG CTG GAT AGG CAC CCT GTG GCG GCG ATA TTA TGT TTC CCA ATC ACT CGC TAT TTA CTT GGA GAA TAT TCT TTG GAA ATG TTG ATT AGC

      Ser Ile Ile Arg Leu Pro Leu Glu Ser Pro Gly Cys Asn Leu Thr Val Thr Ile Leu Pro Asp Glu Lys Glu His Val Asn Arg Ile Cys
1052  TCT ATA ATA AGA CTT CCG TTG GAA TCC CCC GGA TGC AAC CTG ACA GTC ACA ATC CTT CCC GAC GAA AAG GAA CAC GTT AAT AGG ATT TGT

      Ser Arg Asp Arg Pro Gly Glu Thr Ala Asp Arg Asn Met Leu Arg Thr Leu Asn Ala Val Tyr Ala Ser Leu Val Asp Thr Val Lys Tyr
1142  TCA AGA GAT AGA CCG GGT GAA ACG GCA GAT AGA AAT ATG CTC AGA ACA CTC AAT GCC GTA TAC GCA TCT TTG GTG GAC ACG GTT AAA TAC

      Ala Asn Leu Thr Cys Pro Tyr Glu Lys Glu Ser Trp Glu Met Glu Trp Leu Gly Leu Pro Trp Phe Glu Glu Ser Leu Leu Glu Glu Phe
1232  GCA AAT CTA ACA TGC CCT TAC GAG AAA GAA AGC TGG GAA ATG GAA TGG TTG GGA CTT CCC TGG TTT GAA GAG TCA TTA CTT GAA GAA TTC

      Ile Ser Arg Pro Arg Pro Val Ile Cys Ser Arg Thr Arg Met Pro Leu Asp Arg Thr Leu Leu Ala Ile Phe Lys Arg Lys Glu Leu Cys
1322  ATC TCG CGT CCC CGC CCT GTT ATT TGT TCG AGA ACT CGA ATG CCG CTG GAC CGA ACT CTC CTG GCC ATT TTT AAA CGG AAA GAG CTG TGT

      Ser Glu Asn Gly Glu Leu Leu Thr Gln Tyr Ser Trp Ile Leu Trp Gly Leu Leu Thr Lys Leu His Thr Ile Asn Val Glu Leu Phe Asp
1412  AGC GAA AAT GGG GAG CTG TTA ACT CAG TAT TCT TGG ATA TTG TGG GGA TTA CTG ACT AAA CTA CAC ACC ATT AAT GTC GAA TTA TTT GAC

      Ile Ser Gly Met Ser Arg Arg Glu Cys Ala Thr Leu ---
1502  ATT AGC GGT ATG TCA CGT CGA GAA TGC GCC ACG CTA TAA TGCATACTATGCCGGAGAGATTGTCTACTCTCGCTAGCTGGAATGATTTATGCGAGCTTGAAGATGAT

1609  GTAATTTCCTATAATAAGGGAATGTGTAACGAGGTTGGAGCGTCTCGATAATTCTTCTTAATCTGCTGGTATTGGTTACTGCCATAACTTAATATTGGTCCATGCTAGAATAGTCATACG

1729  CTACGATCTGTTGCTATATATGACTATCGCCAAACTGTTAAACCGCGAAGAATATATTTCATATAAACCTAAGGGCCCCTCAGTCTGATTTTTTGTGAAAACGTGTATACATGAAGTTTT

1849  ACTGCATAATCCGTTTCATGATCATAGCGAATCTTTATTCATCTTACCAAATATCGCTTCCAGGCACATATCCATCGCAAATATTGCTTGACATGAAGAACTCGCCGCTCGTACGCTTTA

1969  ATATATCGACGCGTGATTATAAAGACGAGACACTCTGGATACGGAAAAATTCGACATTTGTTTATATCGAT
```

**B.**

```
HVT      (9) L T R V Y L D G P F G I G K T S I L
HSV-1   (49) L L R V Y I D G P H G M G K T T T T
EBV    (287) S L - - F L E G A P G V G K T T M L
HSV-2   (49) L L R V Y I D G P H G V G K T T T S
MaHV    (10) I L R V Y L D G P H G V G K S T T A
```

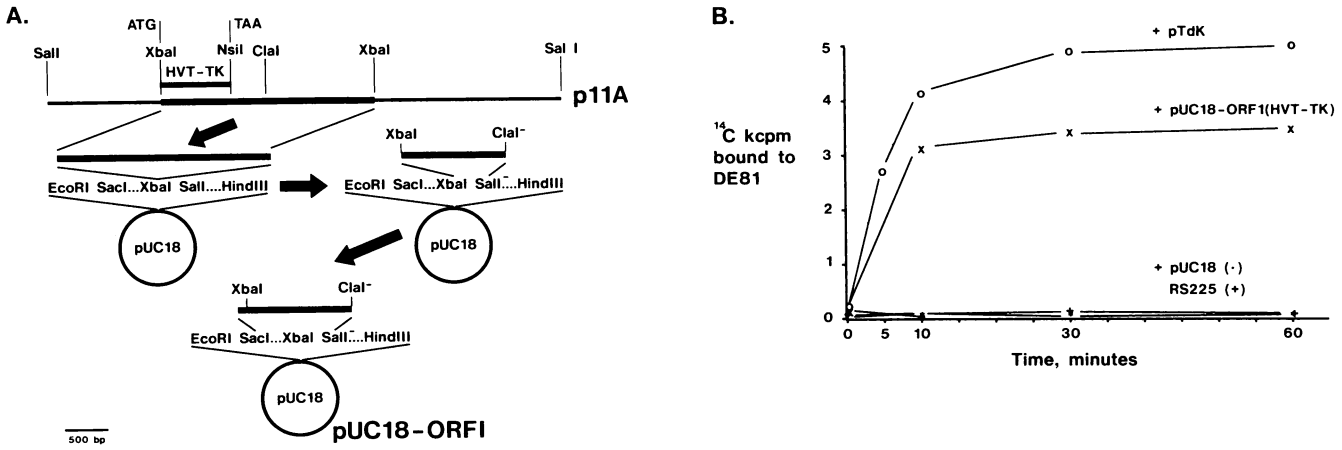



FIG. 3. Expression of HVT ORF1 in *E. coli*. (A) Construction of the ORF1 expression plasmid (see text). bp, Base pairs. (B) TK activity in bacterial lysates prepared from *E. coli* RS225 and *E. coli* RS225 containing plasmid pUC18, pUC18-ORF1, or pTdK. Lysates were made from an equivalent number of cells and were assayed for the ability to phosphorylate [$^{14}$C]thymidine (Amersham Corp., Arlington Heights, Ill.; 61 mCi/mmol). The lysate from RS225 containing pTdK was diluted 1:10 for assay. Under the conditions of the assay, [$^{14}$C]thymidine does not bind to DE81 paper, while its phosphorylated derivative is bound.

level of synthesis of the two enzymes and a difference in their enzyme activities. Also, the TK encoded by pUC18-ORF1 is a fusion protein and may thus have altered enzymatic activities.

On the basis of (i) pharmacological behavior of HVT and the AraT-resistant mutants, (ii) homology of ORF1 to other TK genes that have been characterized previously, and (iii) ability of the protein encoded by ORF1 to function as TK when assayed in vitro following expression in bacteria, we conclude that ORF1 in HVT encodes a TK and that the mutation(s) leading to the AraT resistance phenotype lies within this TK gene.

The TK gene appears to be required for efficient replication of HVT in CEF, since the growth of AraT$^0$ is compromised and no HVT-specific TK activity can be detected in CEF infected with ATR$^0$. Interestingly, vaccination of chicks with the TK-deficient mutant, ATR$^0$, is as efficient as vaccination with wild-type HVT in protecting poultry against Marek's disease (unpublished observation).

In Fig. 2A, the longest open reading frame has been designated the TK gene. Examination of the DNA sequence reveals one other long (>300-base-pair) open reading frame in this region encoded by the opposite strand of DNA. This second open reading frame begins within the TK open reading frame (although the initial methionines from the two open reading frames fall outside the overlapping region) and extends 777 base pairs, potentially encoding a protein with a molecular mass of about 29 kilodaltons. The derived protein sequence is similar to that of open reading frames found in the analogous head-to-head orientation with respect to TK in several other herpesviruses, including herpes simplex viruses types 1 and 2, herpesvirus saimiri, and varicella-zoster virus (19). In herpes simplex virus type 1, mutations in this open reading frame (designated UL24) lead to a small-plaque phenotype and reduced virus growth in cell culture (19). It is possible that our AraT-resistant mutants are growth impaired relative to the wild type, because the mutation affects the expression of this adjacent gene rather than that of TK itself. Alternatively, if HVT TK participates in DNA synthesis as a member of a multienzyme complex, a mutation in TK may be expected to impair viral replication. Whatever its precise molecular basis, this property of reduced growth of AraT-resistant mutants facilitated our marker rescue experiments by providing a selective advantage to the wild-type recombinants. There are conditions in which HSV has been found to require its TK gene for successful replication in cell culture (20). Vaccinia virus and pseudorabies virus are also found to require the TK activity for virulence in the host organism (3, 23).

The identification and characterization of the TK gene in HVT makes it possible to devise procedures for the insertion of foreign genes into HVT. This offers the long-term potential of manipulating the HVT genome such that it can be used as a viral vector for the introduction of foreign genes into poultry. Although the lack of suitable avian TK$^-$ cell lines precludes the use of standard procedures (29, 34, 36) for the

FIG. 2. DNA sequence. (A) Sequence data. The nucleotide sequence of the 2,033-base-pair *Cla*I fragment and the derived amino acid sequence of ORF1 (beginning at the first methionine codon) are given. The DNA sequence in this region is 44% G+C, in keeping with the 46% reported for the entire genome (8). The sequences CCAAT, beginning at 340, and TAATA, beginning at 392, may represent the promoter for transcription of the TK gene. No conspicuous polyadenylation consensus sequence is found 3′ of the coding sequence; perhaps it occurs downstream of the sequenced region. The restriction endonuclease cleavage sites for the *Xba*I and *Nsi*I sites shown in Fig. 3A lie at positions 649 and 1540, respectively. (B) Comparison of amino acid sequences from a region of HVT ORF1 and nucleotide binding pockets of known viral TKs. The single-letter code is used to specify amino acids, and the most conserved amino acids are shown in bold type. The nucleotide-binding pocket was identified in herpes simplex virus type 1 (HSV-1) by Gentry (10). This alignment for herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and marmoset herpesvirus (MaHV) TKs is from the work of Otsuka and Kit (28). The Epstein-Barr virus (EBV) sequence and alignment are from Littler et al. (22). The region of amino acid sequence similarity between the HVT sequence and the others was identified by using the Pustell dot matrix program (International Biotechnologies). The position of the first amino acid shown with respect to the first methionine in each sequence is given in parenthesis following the sequence name.

selection of $TK^+$ recombinants in a background of $TK^-$ HVT, the extreme growth advantage of HVT with wild-type sequences at the TK locus compared with $ATR^0$ serves as a selection. After two rounds of infection, the wild-type recombinants essentially take over the population, making it feasible to identify interesting recombinants by screening with the [$^{125}$I]deoxycytidine plaque assay.

We thank Bruce Calnek, Mike Capage, Don Coen, Dan Doherty, Larry Gold, Steve Nordeen, Jim Smiley, and Marianne Tecklenburg for experimental and intellectual contributions.

## LITERATURE CITED


1. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and $^{35}$S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80**:3963–3965.
2. **Buckmaster, A. E., S. D. Scott, M. J. Sanderson, M. G. Boursnell, N. L. J. Ross, and M. W. Binns.** 1988. Gene sequence and mapping data from Marek's disease virus and herpesvirus of turkeys: implications for herpesvirus classification. J. Gen. Virol. **69**:2034–2042.
3. **Buller, R. M. L., G. L. Smith, K. Cremer, A. L. Notkins, and B. Moss.** 1985. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. Nature (London) **317**:813–815.
4. **Calnek, B. W., C. Garrido, W. Okazaki, and I. V. Patrascu.** 1972. *In vitro* methods for the assay of turkey herpesvirus. Avian Dis. **16**:52–56.
5. **Cebrian, J., C. Kaschka-Dierich, N. Berthelot, and P. Sheldrick.** 1982. Inverted repeat nucleotide sequences in the genomes of Marek disease virus and the herpesvirus of the turkey. Proc. Natl. Acad. Sci. USA **79**:555–558.
6. **Cooper, G. M.** 1973. Phosphorylation of 5-bromodeoxycytidine in cells infected with herpes simplex virus. Proc. Natl. Acad. Sci. USA **70**:3788–3792.
7. **Frischauf, A. M., H. Lehrach, A. Poustka, and N. Murray.** 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. **170**:827–842.
8. **Fukuchi, K., M. Sudo, A. Tanaka, and M. Nonoyama.** 1985. Map location of homologous regions between Marek's disease virus and herpesvirus of turkey and the absence of detectable homology in the putative tumor-inducing gene. J. Virol. **53**:994–997.
9. **Gardella, T., P. Medveczky, T. Sairenji, and C. Mulder.** 1984. Detection of circular and linear herpesvirus DNA molecules in mammalian cells by gel electrophoresis. J. Virol. **50**:248–254.
10. **Gentry, G. A.** 1985. Locating a nucleotide-binding site in the thymidine kinase of vaccinia virus and of herpes simplex virus by scoring triply aligned protein sequences. Proc. Natl. Acad. Sci. USA **82**:6815–6819.
11. **Gibbs, C. P., K. Nazerian, L. F. Velicer, and H.-J. Kung.** 1984. Extensive homology exists between Marek disease herpesvirus and its vaccine virus, herpesvirus of turkeys. Proc. Natl. Acad. Sci. USA **81**:3365–3369.
12. **Goff, S. P., and P. Berg.** 1979. Construction, propagation and expression of simian virus 40 recombinant genomes containing the *Escherichia coli* gene for thymidine kinase and a *Saccharomyces cerevisiae* gene for tyrosine transfer RNA. J. Mol. Biol. **133**:359–383.
13. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
14. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–579.
15. **Hare, D. L., J. R. Sadler, and J. L. Betz.** 1984. Regulated high-level expression of the herpes simplex type I thymidine kinase gene in *Escherichia coli*. Gene **32**:117–128.
16. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**: 351–359.
17. **Hutchison, C. A., III, and M. H. Edgell.** 1971. Genetic assay for small fragments of bacteriophage ϕX174 deoxyribonucleic acid. J. Virol. **8**:181–189.
18. **Igarashi, K., S. Hiraga, and T. Yuna.** 1967. A deoxythymidine kinase deficient mutant of *Escherichia coli*. I. Mapping and transduction studies with phage 80. Genetics **57**:643–654.
19. **Jacobson, J. G., S. L. Martin, and D. M. Coen.** 1989. A conserved open reading frame that overlaps the herpes simplex virus thymidine kinase gene is important for viral growth in cell culture. J. Virol. **63**:1839–1843.
20. **Jamieson, A. T., G. A. Gentry, and J. H. Subak-Sharpe.** 1974. Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. J. Gen. Virol. **24**:465–480.
21. **Kit, S., G. N. Jorgensen, W.-C. Leung, D. Trkula, and D. R. Dubbs.** 1973/1974. Thymidine kinases induced by avian and human herpes viruses. Intervirology **2**:299–311.
22. **Littler, E., J. Zeuthen, A. A. McBride, E. T. Sorensen, K. L. Powell, J. E. Walsh-Arrand, and J. R. Arrand.** 1986. Identification of an Epstein-Barr virus-encoded thymidine kinase. EMBO J. **5**:1959–1966.
23. **Lomniczi, B., A. S. Kaplan, and T. Ben-Porat.** 1987. Multiple defects in the genome of pseudorabies virus can affect virulence without detectably affecting replication in cell culture. Virology **161**:181–189.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Meulemans, G., P. Halen, P. Schyns, and H. Vindevogel.** 1973. Susceptibility of mammalian and avian cell cultures to infection with cell-free turkey herpesvirus. J. Comp. Pathol. **83**:605–608.
26. **Mocarski, E. S., L. E. Post, and B. Roizman.** 1980. Molecular engineering of the herpes simplex virus genome: insertion of a second L-S junction into the genome causes additional genome inversions. Cell **22**:243–255.
27. **Mucenski, M. L., J. P. Donahoe, and M. T. Muller.** 1986. Characterization of herpesvirus of turkeys replication using recombinant DNA. Avian Dis. **30**:255–263.
28. **Otsuka, H., and S. Kit.** 1984. Nucleotide sequence of the marmoset herpesvirus thymidine kinase gene and predicted amino acid sequence of thymidine kinase polypeptide. Virology **135**:316–330.
29. **Post, L. E., S. Mackem, and B. Roizman.** 1981. Regulation of genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with gene promoters. Cell **24**:555–565.
30. **Purchase, H. G., B. R. Burmester, and C. H. Cunningham.** 1971. Responses of cell cultures from various avian species to Marek's disease virus and herpesvirus of turkeys. Am. J. Vet. Res. **32**:1811–1823.
31. **Ross, L. J. N.** 1985. Molecular biology of the virus, p. 113–150. *In* L. N. Payne (ed.), Marek's disease. Martinus-Nijhoff, The Hague, The Netherlands.
32. **Schat, K. A., R. F. Schinazi, and B. W. Calnek.** 1984. Cell-specific antiviral activity of 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) against Marek's disease herpesvirus and turkey herpesvirus. Antiviral Res. **4**:259–270.
33. **Sclafani, R. A., and J. A. Wechsler.** 1981. *dnaB125*, a *dna*B nonsense mutation. J. Bacteriol. **146**:1170–1173.
34. **Smiley, J. R.** 1980. Construction *in vitro* and rescue of a thymidine kinase-deficient deletion mutation of herpes simplex virus. Nature (London) **285**:333–334.
35. **Summers, W. C., and W. P. Summers.** 1977. [$^{125}$I]deoxycytidine used in a rapid, sensitive, and specific assay for herpes simplex virus type I thymidine kinase. J. Virol. **24**:314–318.
36. **Summers, W. P., M. Wagner, and W. C. Summers.** 1975. Possible peptide chain termination mutants in thymidine kinase gene of a mammalian virus, herpes simplex virus. Proc. Natl. Acad. Sci. USA **72**:4081–4084.