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SHORT COMMUNICATION

Murine Gammaherpesvirus-68 Encodes Homologues of Thymidine Kinase and Glycoprotein H: Sequence, Expression, and Characterization of Pyrimidine Kinase Activity

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We have sequenced a 4.5-kb fragment of DNA spanning the junction of the *Bam*HI D and E fragments of murine gammaherpesvirus-68 (MHV-68). This sequence was found to code for two major open reading frames (orfs) of 1934 and 2192 bp which showed significant homology to the thymidine kinase (TK) and glycoprotein H (gH) sequences of other gammaherpesviruses. Upstream from the TK gene another orf was found which showed amino acid sequence homology to the HSV1 UL24 gene. Analysis of the 1934-bp orf revealed the presence of all six of the recognized sites that are conserved between herpesvirus TKs although, uniquely among sequenced herpesvirus TK enzymes, MHV-68 lacks the consensus nucleotide binding site GXXGXGK, the second glycine being replaced by alanine. The MHV-68 TK has a predicted M_r of 68,443, while the gH is predicted to have a M_r of 82,890. Northern blot analysis showed an early TK message of 2.6 kb and a late gH-specific message of 2.5 kb. Both TK and gH probes detected a 4.3-kb late message, implying that this late message spans gH and TK. The TK coding sequence was expressed using an *in vitro* transcription translation system and was shown to encode functional TK activity. © 1996 Academic Press, Inc.

Murine gammaherpesvirus-68 (MHV-68) is the first natural pathogen of murid rodents to be classified as a gammaherpesvirus (*17, 6*). The study of gammaherpesviruses, particularly EBV, has been hampered by the lack of a fully permissive cell culture system and because there is no small animal model for EBV infection. MHV-68 is permissive in a number of cell lines and will infect laboratory mice and is therefore of value in the study of gammaherpesvirus acute and latent infection in a natural system. In order to develop MHV-68 as a model for gammaherpesvirus infection it is necessary to identify and characterize homologues of proteins that are known to have a role in the infectious cycle of other gammaherpesviruses.

The herpesvirus thymidine kinase (TK) has been of interest for a variety reasons. Herpesvirus TKs have a much broader substrate specificity than cellular TK, being more accurately termed pyrimidine kinases, and are able to phosphorylate a variety of nucleoside analogues. This difference in the specificity of cellular and viral TK activities has been exploited by the use of antiviral drugs, such as acyclovir, which are selectively phosphorylated by herpesvirus TK. The TK locus is also a widely used, nonessential site for the generation of recombinants, particularly as there are well-established procedures that can select for either the presence or the absence of TK (*12*). TK is located within a block of genes that are conserved between herpesvirus subgroups, being flanked upstream by a homologue of HSV1 UL24 and downstream by the glycoprotein H (gH) gene (*1*). The gH protein molecule is found on the envelope of herpesviruses and has a role in entry of the virus into a target cell. The exact role of gH in cell entry is unclear. Monoclonal antibodies to EBV gH have been shown to be virus neutralizing (*15*) and there is evidence that lack of gH abrogates the ability of the virus to fuse with target cells (*7*). Although homologues of UL24 are found in all sequenced herpesviruses, the gene product is poorly characterized and its function in the viral life cycle is not known.

Previous data have mapped the approximate locations of some MHV-68 genes and shown that the genomic organization of MHV-68 is similar to that of other gammaherpesviruses (*6*). Based on these data we predicted that the MHV-68 TK and gH genes would be located near to the junction of the *Bam*HI D and E fragments. Subclones of the *Bam*HI D and E clones, obtained from Professor A. A. Nash, University of Cambridge, UK, were made using *Pst*I and *Bam*HI to give 5.3- and 2.7-kb fragments, respectively, as shown in Fig. 1. A series of nested deletions were constructed for each clone using S1 nuclease (Pharmacia). A total of 4556 bp extending from a *Pst*I site 2566 bp inside *Bam*HI E to a position 1985 bp into *Bam*HI

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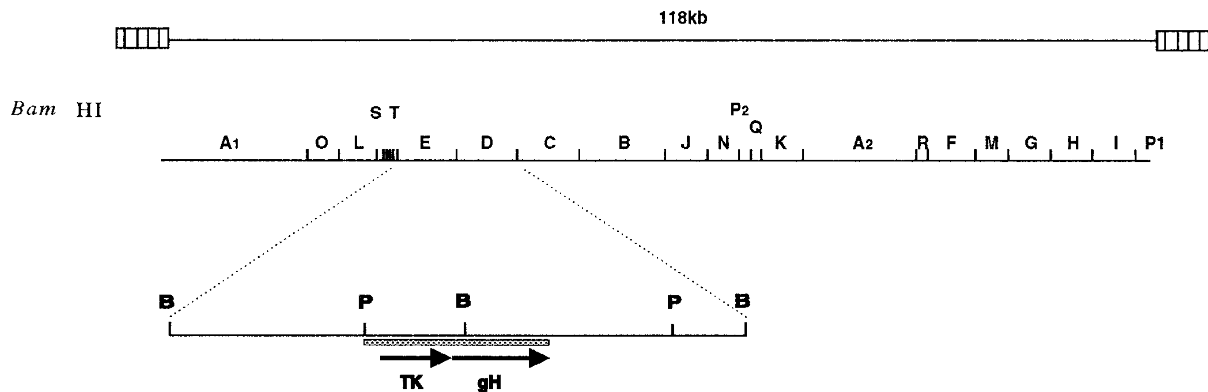

FIG. 1. *Bam*HI restriction map of MHV-68 (*5*) showing the genomic location of MHV-68 TK and gH genes. The expanded region shows the *Bam*HI (B) and *Pst*I (P) sites used for cloning; the region sequenced is indicated by a gray bar. The location and direction of translation of the TK and gH orfs are shown.

D (Fig. 1) was sequenced on an ABI373A sequencer using fluorescent dideoxy chain termination chemistry (PRISM kit; Perkin–Elmer). The sequence was confirmed by analysis of the complementary strand using custom-made oligonucleotide primers. A PCR product was generated using full length viral DNA as template to allow sequencing of the junction between the *Bam*HI D and E fragments. Sequence assembly and subsequent analysis using the University of Wisconsin Genetics Computer Group suite of programs (*4*) revealed two major open reading frames (orf), the approximate positions of which are shown in Fig. 1.

The first orf is 1935 bp long starting 406 bp from the *Pst*I site in *Bam*HI E, the translated amino acid sequence having a predicted molecular weight of 72,255. However, the native protein may be smaller as there are two subsequent ATGs 104 and 248 bp into the orf which are also potential start points for translation. These would produce proteins with predicted M_rs of 68,444 and 62,874, respectively. The level of both DNA and protein sequence homology between herpesvirus TKs is very low. Six conserved sites have been identified (*2*) which are present in all known herpesvirus TKs and the first four sites are shown in Fig. 2. Site 1 consists of the consensus sequence GXXGXGK which is a recognized nucleotide binding site while the nucleoside binding site encompasses the DRH conserved within site 3. The MHV-68 TK amino acid sequence is unique among a total of 19 herpesvirus TKs that have been sequenced in that the middle glycine in the nucleotide binding site is replaced by alanine. To ensure that a glycine to alanine mutation had not been introduced during cloning procedures, direct sequencing of a PCR product generated from viral DNA was performed and confirmed the presence of an alanine-coding triplet. It has been suggested that herpesvirus TKs have evolved from cellular deoxycytidine kinase, rather than thymidine kinase (*8*), and in this context it is interesting to note that the although the change to alanine is unique among herpesvirus TKs, it is present in the cellular deoxycytidine kinase. The MHV-68 TK has a long amino-terminal segment of 235 residues before the first conserved site, a feature that is consistently present in gammaherpesviruses (Fig. 2). Previous work with the EBV TK has shown that this region is highly antigenic but is not required for TK activity (*14*).

The second coding sequence was 2193 bp long, starting 20 bp downstream from the end of the TK orf. The methionine at the start of the orf is in a good context for initiating transcription, having a G at +1 bp and an A at −3 bp (*3*). When translated, the orf gave a protein of 730 amino acids with two hydrophobic domains each of approximately 20 amino acids. The first of these was reminiscent of a signal peptide, and a signal peptidase site was predicted, using the method of von Heijne (*19*), to lie after a glycine 26 amino acids into the protein. Cleavage at this point would yield a mature peptide of 704 amino acids in length. The second hydrophobic domain was close to the end of the molecule and was of sufficient length to act as a membrane anchor sequence. This fits the pattern seen in other gHs with a signal leader sequence at the amino terminus and a membrane anchor sequence at the carboxy terminus of the protein.

A third orf was detected on the strand complementary to the TK and gH coding sequences running beyond the *Pst*I site used for cloning and which on further analysis was found to be a homologue of HSV UL24. There is a conserved overlap between the EBV and the HVS TK and UL24 homologues of 2 bp, and the same overlap was found between the MHV-68 TK and UL24 orfs. Any attempt to remove the entire MHV-68 TK orf is likely to disrupt expression of the UL24 gene and although the function of the UL24 homologue in EBV and HVS has not been studied, there is evidence to show that lack of a functional UL24 gene product reduces the ability of HSV1 to grow in culture (*11*). In contrast work with BHV1 has found that lack of UL24 has no effect on viral growth in culture (*20*).

The nucleotide sequence presented here has been

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                          Site 1                Site 2    Site 3    Site 4
                          *******               ******     ***       ***
HVT    NH2- 11-  RVYLDGPFGIGKTSILNAM -14- EPMKYW -70- DRHPVAAILCYP -43-COOH
MDV    NH2- 20-  RVYLDGSMGIGKTSMLNEI -14- EPMKYW -70- DRHPISATVCYP -43-COOH
HSV1   NH2- 50-  RVYIDGPHGMGKTTTTQLL -13- EPMTYW -73- DRHPIAAILCYP -42-COOH
HSV2   NH2- 50-  RVYIDGPHGVGKTTTSAQL -14- EPMTYW -73- DRHPIASILCYP -42-COOH
VZV    NH2- 13-  RIYLDGAYGIGKTTAAEEF -15- EPLSYW -75- DRHPIASTICFP -42-COOH
EHV4   NH2- 26-  RIYLDGVYGIGKSTTGRVM -14- EPMAYW -72- DRHPVASTVCFP -42-COOH
PRV    NH2- 04-  RIYLDGAYGTGKSTTARVM -09- EPMAYW -69- DRHPVAATVCFP -42-COOH
FHV    NH2- 21-  RIYIDGAYGIGKSLTAKYL -15- EPMLYW -72- DRHPVAATVCFP -42-COOH
BHV1   NH2- 11-  RIYLDGAHGLGKTTTGRAL -14- EPMAYW -82- DRHPVAACLCYP -42-COOH
BHV4   NH2-126-  FIFFEGVMGVGKTTLLKTL -12- EAMNYW -70- DRHALSACLVFP -41-COOH
EHV2   NH2-296-  FLYLEGSMGVGKTTLIRHM -12- EPMFYW -69- DRHPLSATLVFP -41-COOH
HVS    NH2-210-  FIFLEGSIGVGKTTLLKSM -13- EPIAYW -66- DRHPLSATVVFP -41-COOH
EBV    NH2-285-  SLFLEGAPGVGKTTMLNHL -12- EPMRYW -69- DRHLLSASVVFP -41-COOH
MHV68  NH2-328-  VLYFDGGIAVGKTTALEAA -11- EPIPYW -67- DRHMVSPTVIFP -41-COOH
KSV    NH2-255-  LLYLEGVMGVGKSTLVNAV -13- EPMVYW -70- DRHLLSPAVVFP -41-COOH
dCK    NH2- 22-  KISIEGNIAAGKSTFVNIL -11- EPVARW -74- DRYIFASNL.YE -44-COOH
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FIG. 2. Alignment of the amino acid sequences of four conserved sites in 15 herpesvirus TKs and human dCK. *, The conserved sites proposed by (*2*). Dots represent characters introduced to produce the alignment. BHV1 and 4, bovine herpesvirus types 1 and 4; dCK, human deoxycytidine kinase; EBV, Epstein–Barr virus; EHV2 and 4, equine herpesvirus types 2 and 4; FHV, feline herpesvirus; HSV1 and 2, herpes simplex types 1 and 2; HVS, herpesvirus saimiri; HVT, turkey herpesvirus; KSV, Kaposi's sarcoma virus; MDV, Marek's disease virus; MHV68, murine herpesvirus 68; PRV, pseudorabiesvirus; and VZV, varicella zoster virus.

submitted to the GenBank Database and has been assigned the Accession No. X93468.

Analysis of the messages encoding TK and gH orfs was undertaken using Northern blotting to study the temporal regulation and control of mRNA production. C127 cells were infected with MHV-68 at 10 PFU per cell and harvested at 4, 8, and 24 hr. Infections were also carried out for 24 hr in the presence of phosphonoacetic acid (PAA) at 100 μg/ml or acyclovir (ACV) at 100 μM, both of which block herpesvirus DNA replication. Total cytoplasmic RNA was extracted and analyzed as described previously (*16*). Probes were generated by PCR, amplifying DNA located entirely within the reading frames of MHV-68 TK and gH genes. The TK gene probe was 335 bp long, starting 1173 bp into the TK orf, and the gH gene probe was 1576 bp, starting 480 bp into the gH orf. Following amplification, "PCR Prep" purification columns (Promega) were used to remove unincorporated primers and nucleotides from PCR products which were then radioactively labeled using a random priming kit (Boehringer–Mannheim). Following hybridization with a TK-specific probe the Northern blot was stripped and reprobed with a gH-specific probe. This made it possible to make accurate comparisons of the size of messages seen on each blot. The TK specific probe detected a message of approximately 2.6 kb. A message of this size would be large enough to allow for transcription of the entire TK orf, terminating at a poly(A) site identified immediately after the end of the orf. There is, however, a promoter element at 208 bp and an ATG at 249 bp into the TK orf. Transcription could start at this point and terminate at an alternative poly(A) site 684 bp downstream from the TK orf thereby reducing the length of the protein product by 83 amino acids. Expression of the 2.6 kb message was seen 4 hr after infection and was not blocked by the presence of PAA or ACV, indicating that MHV-68, like other herpesviruses, expresses TK as an early gene.

The gH-gene-specific probe detected a message that was smaller than the 2.6 kb band specific to TK. In Fig. 3 this is labeled as 2.5 kb. This message was the correct size to start at a promoter element identified 87 bp upstream from the start of the gH orf and to terminate after a poly(A) signal 19 bp from the end of the gH orf. The MHV-68 gH gene was found to be expressed as a late message; the 2.6-kb-specific band was seen 8 hr after infection and this message was blocked by PAA and ACV.

EBV has an early bicistronic message that encompasses both TK and gH genes. Figure 3 shows that both the probes used detected a message of approximately 4.3 kb, expression of which is strongest after 8 hr and is blocked by PAA and ACV. It is possible therefore that MHV-68 also has an mRNA that spans both TK and gH orfs but which is expressed as a late message.

In order to demonstrate that the MHV-68 TK orf coded for a functional TK, it was expressed using an *in vitro* transcription translation system. Primers were designed which would amplify the TK and leave a *Bam*HI restriction enzyme site at the start and a *Pst*I site at the end of the orf: primer 1, 5′ ACGG**GGATCC**GT<u>ATG</u>GCTTCTGGAGGTAAA 3′; primer 2, 5′ CGA**CTGCAG**<u>TTA</u>CTACTGAGGGTCTCC 3′. Restriction enzyme sites are shown in bold and the start and stop codons of the TK ORF are underlined. PCR was carried out on the *Bam*HI E fragment using the primers shown. Products were cleaved with *Bam*HI and *Pst*I and subsequently cloned into pBluescript (KS) (Stratagene). Coupled transcription translation reactions were performed using a rabbit reticulocyte lysate system (Promega). Reactions were carried out as per manufacturer's instructions based on 1 μg of substrate DNA with

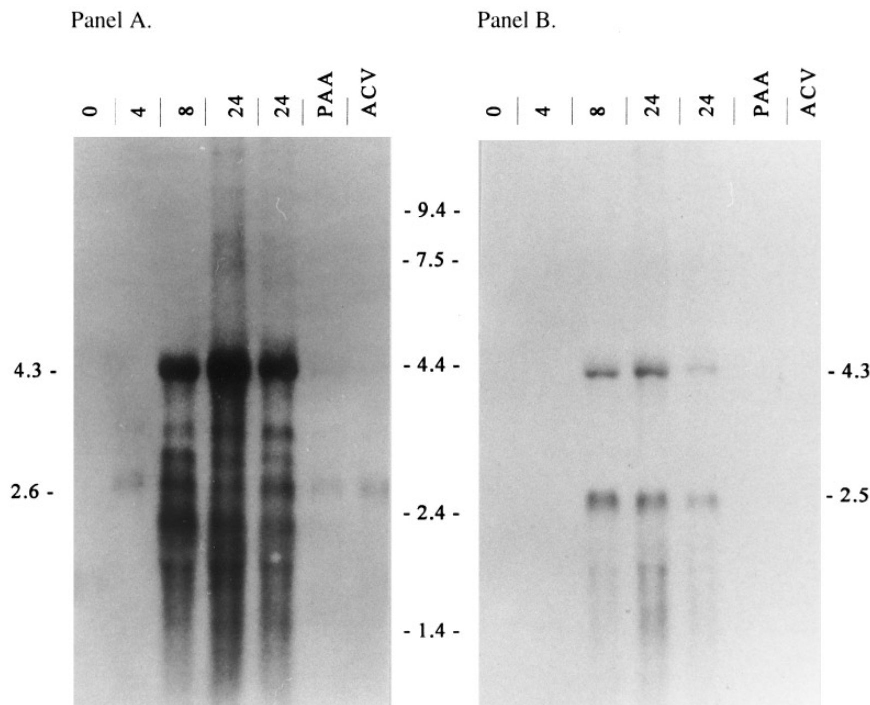

FIG. 3. Northern blot analysis of MHV-68 TK and gH. Mouse epithelial (C127) cells were infected with 10 PFU of MHV-68 per cell and harvested at 0, 4, 8, and 24 hr postinfection. Cells infected in the presence of PAA or ACV were harvested at 24 hr postinfection. Cytoplasmic RNA was extracted from the cells and analyzed by Northern blotting using the (A) TK- or (B) gH-specific probes described in the text. An autoradiogram of each blot is shown with the positions of an RNA ladder shown (in kilobases) in the center. The sizes of major TK- and gH-specific RNAs (in kilobases) are indicated.

50 μM thymidine added to the reaction to stabilize nascent thymidine kinase (*10*). Protein products were detected by the incorporation of radioactive label into the reaction mixture and subsequent analysis on polyacrylamide gels. No products were seen when vector alone was added to the transcription translation reaction. When the MHV-68 TK clone was added a protein band of the predicted size was seen (data not shown). Products were assayed for thymidine kinase activity essentially as described by Littler and Arrand (*13*) using 50 μM [^{3}H]thymidine at a specific activity of 20 Ci/mmol and 7 m*M* ATP (Pharmacia). Typically, 20 μl of transcription translation reaction products were assayed in a total volume of 200 μl. Aliquots of 50 μl were removed to give zero time controls and immediately boiled for 5 min to denature any active TK present. Further aliquots were boiled after 30- and 60-min incubations at 37°. Samples were then spotted onto DE81 filter circles (Whatman) and washed three times in 2 m*M* ammonium formate. The filters were rinsed twice in 95% ethanol and air dried prior to scintillation counting. Table 1 shows that transcription translation reactions which had either no DNA, or only vector DNA, gave only background activity; however, when the MHV-68 TK orf was included in the vector a high level of TK activity was seen.

MHV-68 is sensitive to anti-herpetic drugs including acyclovir (*18*). Although we have not determined the ability of the TK to phosphorylate these nucleoside analogues, it seems likely that their mode of action upon MHV-68 is through this protein. In terms of sequence comparison, our results show that the MHV-68 TK gene is more closely related to that of HVS than that of EBV. However, since EBV but not HVS is sensitive to acyclovir (*9*), it would appear that the MHV-68 TK is more functionally related to that of EBV. The sensitivity of MHV-68 to acyclovir underlines its usefulness as a model for the study of antiviral drugs directed against TK *in vivo.*

TABLE 1

TK Activity in *in Vitro* Transcription Translation Reactions

DNA sample	Incubation time (min) 0	30	60
None	608	460	520
pKS	477	490	684
pKS + MHV-68 TK orf	429	4791	8172

Note. In vitro transcription translation reaction products were incubated with 50 μM [^{3}H]thymidine (specific activity 20 Ci/mmol) and 7 m*M* ATP for the times shown. The numbers shown are the counts obtained when the assay mixture was spotted onto DE81, which specifically binds phosphorylated thymidine, and counted on a scintillation counter.

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