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Murine Gammaherpesvirus 68 Lacking Thymidine Kinase Shows Severe Attenuation of Lytic Cycle Replication In Vivo but Still Establishes Latency

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The lytic cycle functions of gammaherpesviruses have received relatively little attention to date, at least in part due to the lack of a convenient experimental model. The murine gammaherpesvirus 68 (MHV-68) now provides such a model and allows the roles of individual lytic cycle gammaherpesvirus proteins to be evaluated in vivo. We have used MHV-68 to determine the contribution of a gammaherpesvirus thymidine kinase (TK) to viral lytic replication and latency establishment. MHV-68 mutants with a disrupted TK gene grew normally in vitro but showed a severe attenuation of replication in the lungs after intranasal inoculation, with lytic titers at least 1,000-fold lower than those of wild-type and revertant viruses. Nevertheless, the establishment of latency by the TK-deficient mutants, while delayed, was not prevented by their lytic replication deficit. The viral TK clearly plays a crucial role in the capacity of MHV-68 to replicate efficiently in its natural host but does not seem to be essential to establish a persistent infection. The potential of TK-deficient mutants as gammaherpesvirus vaccines is discussed.

Any rational approach to controlling herpesvirus infections depends on understanding how individual viral genes contribute to pathogenesis. This can be predicted to only a very limited degree from biochemical analysis in vitro. Neither of the human gammaherpesviruses, Epstein-Barr virus (EBV) and the Kaposi's sarcoma-associated herpesvirus (KSHV), readily infects nonprimates. However, murine gammaherpesvirus-68 (MHV-68) is a natural parasite of small rodents that is genetically related to the human gammaherpesviruses (10, 37) and affords a direct means to approach the in vivo complexities of gammaherpesvirus biology in conventional mice. After intranasal inoculation, MHV-68 replicates lytically in the respiratory tract. It then spreads to lymphoid tissue, where there is a massive expansion of latently infected B cells, associated with a virus-driven immune activation syndrome reminiscent of the infectious mononucleosis illness induced by EBV (8, 22). Non-antigen-specific B-cell activation and an expansion of the $CD8^+$ $V\beta4M^+$ T-cell subset are prominent features (25, 26, 34). Latent virus declines to a lower level after the infectious mononucleosis illness but persists for life.

Herpesviruses usually infect cells that are not cycling, and therefore they must cope with an intracellular milieu deficient in deoxyribonucleotides. Since dTTP cannot be derived from intracellular ribonucleotide pools by ribonucleotide reductase, its supply presents a special problem. Cellular dTMP either is synthesized de novo, culminating in dUMP methylation by thymidylate synthase, or is salvaged from dT by thymidine kinase (TK). In most cell types the salvage pathway predominates, and TK is the rate-limiting enzyme in dTMP supply. The major (nonmitochondrial) cellular TK turns over rapidly and is induced only at the time of DNA synthesis (7). Cellular thymidylate synthase activity is similarly regulated. The supply of dTMP is thus limited to the S phase of the cell cycle. To compensate for the lack of dTMP in infected cells, all herpesviruses increase intracellular TK activity and most encode a TK homolog. The sequence conservation of the genes encoding herpesvirus TKs is fairly low, and none is closely related to the cellular gene—they may originate instead from the cellular deoxycytidine kinase (17). The herpes simplex virus (HSV) TK has been studied most intensively, and its relatively broad specificity for nucleoside analogues has made it the archetype for succesful antiviral chemotherapy. Since TK-deficient viral mutants are not uncommon, the basis for this approach has been the failure of these mutants to replicate in neurons (9, 12, 13). TK-deficient HSV also shows reduced replication in epithelial sites (9).

While the alpha- and betaherpesviruses cause disease by lytic replication, the human gammaherpesviruses cause disease chiefly when latent. However, two factors have focused attention on gammaherpesvirus TKs. First, the presence of replicating KSHV in tumors associated with this virus suggests that lytic cycle viral genes contribute to disease progression (19) and are thus potential therapeutic targets; and second, the induction and simultaneous inhibition of lytic cycle EBV replication is being developed as a strategy to clear latent genomes (11). How the gammaherpesvirus TKs function is not entirely clear. They have in common a large N-terminal extension, not present in the HSV TK, which, with EBV at least, is not required for TK activity (20). Also, the KSHV TK has, outside the context of viral infection, a suprisingly high K_m for thymidine (16). Its activity may be higher, or even qualitatively different, in virus-infected cells. Interestingly, the pathogenicity of herpesvirus saimiri, another gammaherpesvirus, is increased by expression of the HSV TK (18).

The MHV-68 TK gene has early and late kinetics and en-

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codes a protein with TK activity in vitro (24). The MHV-68 TK has already been exploited in antiviral chemotherapy: treating mice with nucleoside analogues (3, 23, 33) inhibits lytic MHV-68 replication but does not prevent the establishment of a latency. However, the TK status of the viruses establishing latency was not determined. Consequently, it is not clear whether the limited capacity of antiviral chemotherapy to control host colonization reflected a limited potency and delivery of the nucleoside analogs tested or reflected the capacity of naturally occurring TK mutants to replicate in vivo. Since viral TK mutants are likely to arise on a regular basis, a knowledge of the importance of TK in pathogenesis should help to define the theoretical limit of efficacy of TK-based antiviral chemotherapy. In addition, the basic biological role of TK in gammaherpesvirus infections is unknown. To address these questions, we have generated and characterized TK-deficient MHV-68 mutants.

MATERIALS AND METHODS

Mice. Female BALB/c and C57BL/6 mice were purchased from Harlan U.K. Ltd. (Bicester, United Kingdom) and housed at the Biological Services Unit of the Cambridge University School of Veterinary Sciences. All procedures were carried out in accordance with Home Office Project Licence 80/1579. Mice were infected intranasally at 6 to 8 weeks of age with 2×10^4 PFU of virus in a volume of 30 μl.

Cell lines. Baby hamster kidney (BHK-21) cells (American Type Culture Collection, CCL-10), a TK-deficient derivative (European Collection of Animal Cell Cultures, catalogue number 85011423), and murine embryonic fibroblasts harvested at 13 to 14 days of gestation were all grown in Dulbecco modified Eagle medium (Invitrogen, Paisley, United Kingdom) supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal calf serum (PAA Laboratories, Linz, Austria) (complete medium). The phenotype of TK^- and TK^+ BHK-21 cells was confirmed by selection in medium supplemented with 50 μg of 5-bromo-2-deoxyuridine per ml and 100 μM hypoxanthine–0.4 μM aminopterin–16 μM thymidine medium, respectively, prior to use. To make NIH 3T3 CRE cells, the *cre* gene of bacteriophage P1 was cloned into the *Eco*RI-*Xho*I sites of pMSCV-NEO (28) and *cre*-expressing retrovirus was generated by transient transfection of Phoenix-Eco cells (www.stanford.edu/group/nolan) with pMSCV-CRE-NEO. NIH 3T3 cells (American Type Culture Collection, CRL-1658) were transduced with retrovirus in the presence of 6 μg of polybrene per ml and selected in 1 mg of G418 per ml.

Virus titers. Lungs were homogenized in complete medium, frozen, thawed, and sonicated. Tissue debris was pelleted by brief centrifugation ($1,000 \times g$ for 1 min). Infectious virus in homogenate supernatants was measured by a plaque assay of 10-fold dilutions of lung homogenates on TK^+ BHK-21 cells as previously described (31). After 5 days, monolayers were fixed in 10% formaldehyde and stained with 0.1% toluidine blue and plaques were counted under a plate microscope. Latent virus was measured by explant culture of single-cell suspensions of spleens on murine embryonic fibroblast monolayers, which were fixed and stained after 6 days.

Viral mutagenesis. A kanamycin resistance gene flanked by Flp recombinase target (FRT) sites was amplified from *Bsa*I-linearized pCP15 (1) by PCR (Expand Hi Fidelity; Roche, Lewes, United Kingdom), using primers with 50-bp 5′ extensions corresponding to nucleotides 33251 to 33300 and 33451 to 33500 of the MHV-68 genome (37). The purified PCR product was electroporated into the SbcA RecBC *Escherichia coli* strain JC8679 containing the MHV-68 bacterial artificial chromosome (BAC). This allowed incorporation of the kanamycin resistance gene into the MHV-68 BAC TK locus by RecE/T-mediated recombination (1). BAC DNA (TK^- Kan^+) was isolated from chloramphenicol- and kanamycin-resistant colonies and transformed into E. coli DH10B. The integrity of the BAC and correct incorporation of the kanamycin resistance gene were confirmed by restriction enzyme mapping. *E. coli* DH10B containing the TK^- Kan^+ BAC were then transformed with a temperature-sensitive Flp recombinase expression plasmid, pCP20, as previously described (1). This resulted in excision of the kanamycin resistance gene, leaving a 167-bp insert in the TK locus, comprising a single FRT site and short flanking plasmid sequences, in place of 150 bp of viral genome (TK^- Kan^-). The bacteria were cured of pCP20 by growth at the nonpermissive temperature (43°C). Successful Flp recombination was established by replica plating of bacteria onto chloramphenicol or chloramphenicol plus kanamycin and confirmed by restriction enzyme mapping of BAC DNA. To make a revertant virus (TK^+ R), a *Bam*HI-*Sac*I unmutated MHV-68 genomic fragment (genomic coordinates 29616 to 35045) was ligated into the $RecA^+$ $SacB^+$ temperature-sensitive shuttle vector pST76K-SR (1) and transformed into *E. coli* DH10B containing the TK^- Kan^- BAC. Cointegrants were selected by growth in chloramphenicol plus kanamycin at 43°C. Cointegrant resolution was selected by growth in chloramphenicol–5% sucrose at 30°C; under these conditions, the induced expression of SacB is lethal to *E. coli*. Colonies were then screened by restriction enzyme digestion for resolution to a wild-type (WT) genomic pattern. An independent TK-deficient mutant was also made by the pST76K-SR shuttle method. An *Eco*RI-restricted MHV-68 genomic fragment (genomic coordinates 30798 to 38212) was cloned into pUC8. The central portion (33293 to 34300) of the TK open reading frame (ORF) (32879 to 34813) was excised by digestion with *Avr*II and *Pme*I, blunting with T4 DNA polymerase (New England Biolabs, Hitchin, United Kingdom), and religation with T4 DNA ligase (New England Biolabs). This modified genomic fragment was then excised with *Eco*RI, blunted with T4 DNA polymerase, and subcloned into *Sma*I-cut pST76K-SR. This plasmid was transformed into *E. coli* DH10B containing the WT MHV-68 BAC. RecA-mediated plasmid integration into the BAC and cointegrant resolution proceeded as described above, except that colonies were screened for retention of the mutant TK locus in the BAC rather than for the WT pattern. Each BAC was reconstituted into MHV-68 virus by transfecting 10 μg of BAC DNA into NIH 3T3 cells by the calcium phosphate method (Invitrogen). The *loxP*-flanked BAC-green fluorescent protein cassette was then removed by the passage of virus through NIH 3T3- CRE cells until green fluorescent protein-positive cells were no longer visible.

TK assay. TK^- BHK-21 cells were infected with MHV-68 (5 PFU per cell). After 20 h, the cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), pelleted, resuspended in 50 mM NaCl, and sonicated for 45 s on ice. Cell debris was removed by ultracentrifugation at $25,000 \times g$ for 30 min at 4°C. Supernatants were stored at −70°C. To assay thymidine phosphorylation, supernatants were incubated at 37°C for 20 min in 0.1 M phosphate buffer (pH 6.4)–4.8 mM $MgCl_2$–4.8 mM ATP–11 μM [^{14}C]thymidine (58 Ci/mol; Amersham Biosciences, Little Chalfont, United Kingdom). Samples were then boiled for 2 min, spotted in duplicate onto DEAE paper, and allowed to dry. Free thymidine was removed by washing three times for 1 h each in 5 mM ammonium formate. Bound phosphorylated thymidine was quantified by scintillation counting.

Flow cytometry. Spleens were disrupted into single-cell suspensions, washed in PBS–0.1% bovine serum albumin (BSA)–0.01% azide, and incubated for 30 min on ice with 5% mouse serum, 5% rat serum, and anti-CD16/32 monoclonal antibody (BD-Pharmingen, San Diego, Calif.). Specific staining was done with phycoerythrin-conjugated anti-CD8 and fluorescein isothiocyanate-coupled anti-Vβ4 (BD-Pharmingen). After a 1-h incubation on ice, cells were washed twice in PBS–0.1% BSA–0.01% azide and analyzed on a FACSCalibur instrument using Cellquest software (Becton-Dickinson, Oxford, United Kingdom). Data were analyzed with FCSPress v1.3.

ELISA. Maxisorp enzyme-linked immunosorbent assay (ELISA) plates (Nalge Nunc, Rochester, N.Y.) were coated overnight with goat anti-mouse immunoglobulin G (IgG) for total IgG measurements and with 0.05% Triton X-100-disrupted MHV-68 virions for virus-specific IgG as previously described (26). The plates were washed three times with PBS–0.1% Tween 20, blocked with PBS–0.1% Tween 20–1% BSA, and incubated with threefold serum dilutions for 1 h at room temperature. They were then washed four times, incubated with alkaline phosphatase-conjugated goat anti-mouse IgG, washed five times, and developed with nitrophenyl phosphate substrate (Sigma Chemical Co., Poole, United Kingdom). The reaction was terminated with an excess of NaOH, and the absorbance was read at 405 nm.

Analysis of viral transcripts. RNA was extracted from MHV-68-infected cells using RNAzolB (Tel-Test, Friendswood, Tex.), and contaminating DNA was removed with RNase-free DNase (Promega UK, Southampton, United Kingdom). cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (RT) (Promega) and amplified with *Taq* polymerase (Amersham Biosciences) and primers specific for the MHV-68 ORF20 (genomic coordinates 32117 to 32871), ORF22 (34883 to 35396), and M3 (6061 to 7278). The PCR products were analyzed over a range of cycle numbers to ensure a more quantitative comparison of transcript levels.

RESULTS

Generation of MHV-68 TK mutants. The translation start site for the MHV-68 TK is uncertain. It probably corresponds

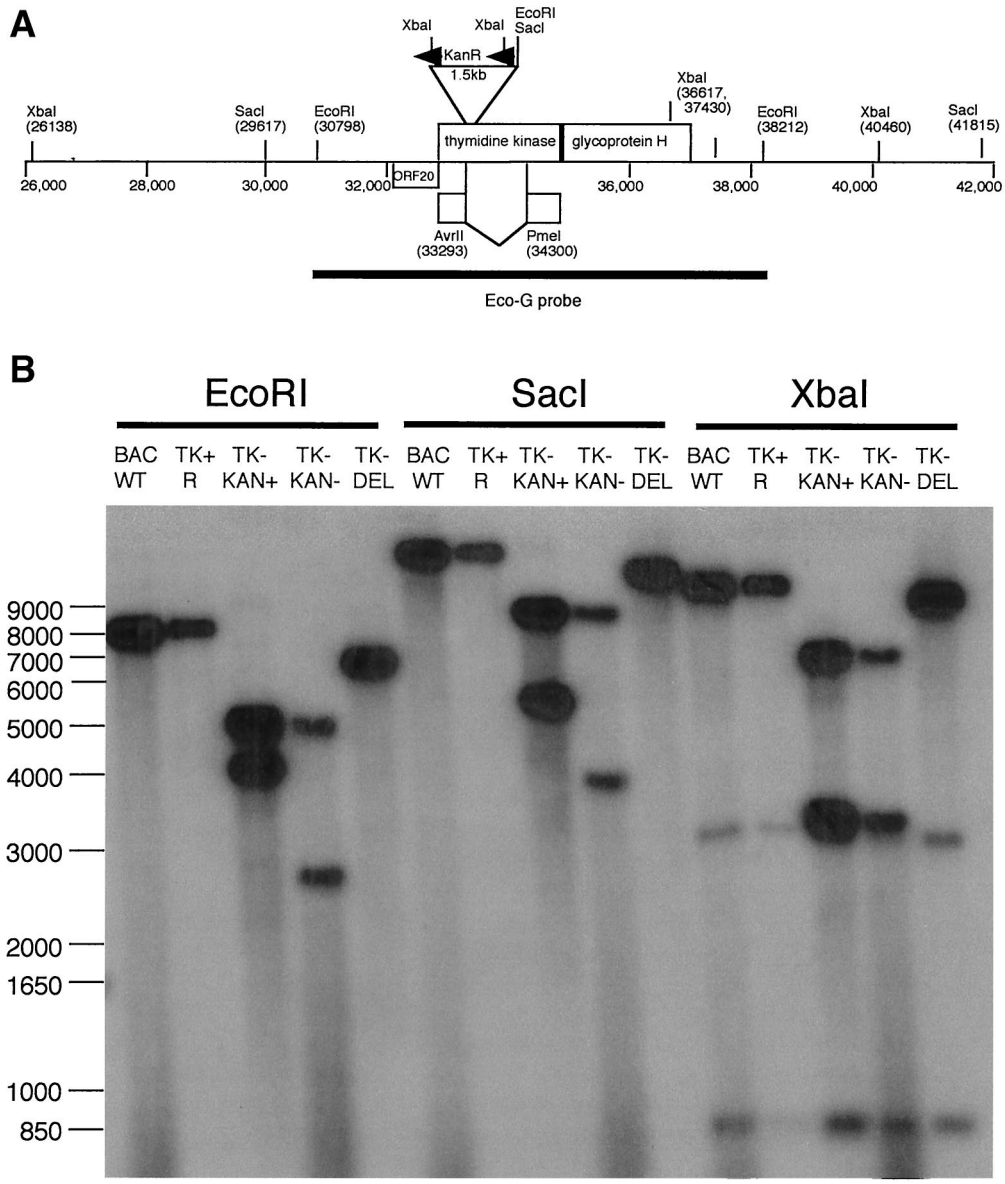

FIG. 1. Genomic structure of TK^+ and TK^- viruses. (A) The TK^- Kan^+ virus was generated by inserting a FRT-flanked kanamycin resistance gene at genomic coordinates 33301 to 33450. The kanamycin resistance coding sequence was removed by Flp recombination to leave an inset of 167 bp (TK^- Kan^-). This virus was reverted by homologous recombination with an unmutated genomic segment (TK^+ R). An independent mutant (TK^- Del) was generated by RecA-mediated recombination of BAC DNA with a genomic plasmid clone in which genomic coordinates 33293 to 34300 are deleted by digestion with *Avr*II and *Pme*I. (B) Viral DNA was digested with *Xba*I, *Eco*RI, or *Sac*I, electrophoresed, blotted, and probed with a ^{32}P-labeled genomic *Eco*RI fragment spanning the TK locus.

to the start of the TK ORF (genomic coordinate 32879) but could alternatively be at one of two downstream ATG codons (32984 and 33128). These lie within the relatively poorly conserved N-terminal extension that is characteristic of gammaherpesvirus TKs and are some distance from the conserved nucleoside and nucleotide binding sites (33860 onward). For this reason, the TK ORF was disrupted at genomic coordinate 33300, thereby minimizing the chance of internal initiation of translation to create a functional TK protein (Fig. 1A). A deletion mutant (TK-DEL) lacking the bulk of the TK ORF, including the conserved motifs related to TK function, was also generated for comparison. In each case the N-terminal and C-terminal ends of the TK ORF were preserved, since these probably contain promoter elements for the neighboring genes (ORF20 and the glycoprotein H gene, respectively).

The genomic structures of all TK mutants, as well as a revertant of the TK^- Kan^- mutant, were checked by restriction enzyme mapping of BAC DNA (data not shown) and by Southern blot analysis of viral DNA (Fig. 1B). This confirmed the expected pattern of genomic changes. The precise insertion site of the kanamycin resistance cassette was further checked by DNA sequencing of PCR products amplified from viral DNA and was found to replace nucleotides 33301 to 33450 of the viral genome as predicted. DNA sequencing also estab-

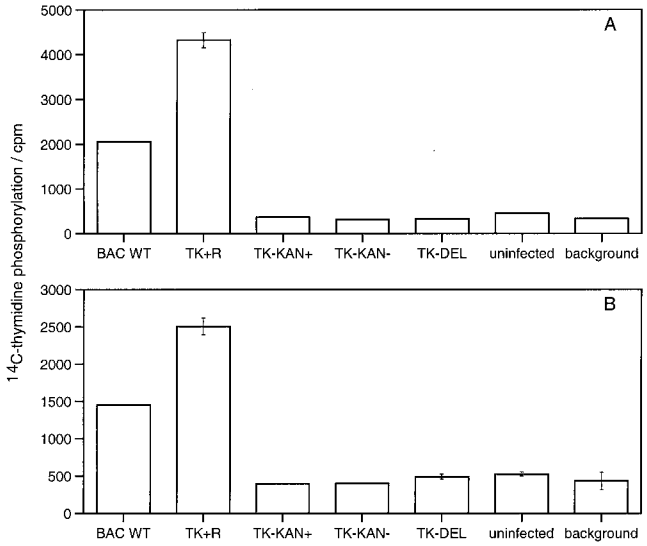

FIG. 2. TK activity of TK^+ and TK^- viruses. TK^- BHK-21 cells were infected overnight with MHV-68 (5 PFU/cell) either in the absence of inhibitors (A) or with 100 μg of phosphonoacetic acid per ml (B), to inhibit progression to late viral gene expression. dT phosphorylation was assayed by the addition of [^{14}C]thymidine to cell lysates followed by binding to DEAE paper, washing, and scintillation counting. "background" represents counts with substrate but no cell lysate. Means and standard deviations for triplicate assays are shown. Similar results were obtained in two further experiments.

lished the presence of multiple in-frame stop codons in the TK ORF of the TK^- Kan^- virus and accurate reconstitution of the TK coding sequence in the TK^+ R revertant virus.

Characterization of MHV-68 TK mutants in vitro. TK assays (Fig. 2) were used to confirm that mutagenesis had disrupted the viral TK gene and that reversion had successfully restored it. The MHV-68 TK is expressed at early times (24) and is presumably most necessary prior to viral DNA replication. Therefore, we wished to check particularly whether there was TK activity in cells at early times after infection with the TK mutants. To do this, TK^- BHK cells were infected with TK^+ and TK^- viruses (5 PFU/cell) in either the presence or absence of phosphonoacetic acid, which inhibits viral DNA replication and thus limits gene expression to the early lytic cycle. Cultures were harvested after 18 h and assayed for TK activity. No activity was seen in cells infected with the TK mutants, either early or late in lytic cycle replication, whereas activity was fully restored in the revertant virus.

The capacity of TK^- viruses to replicate in vitro was assayed after low-multiplicity infection (0.01 PFU/cell) of TK^- BHK-21 cells (Fig. 3A). No significant deficit in the growth of the TK mutant viruses was observed. A similar pattern of growth was seen in TK^+ BHK-21 cells (data not shown). In each case, the TK-deficient viruses were probably exploiting preexisting deoxyribonucleotide pools. (The de novo synthesis pathway provides dTMP in TK^- BHK-21 cells.) In an attempt to reduce these pools, MEFs were grown to passage 9 prior to low-multiplicity infection with TK^+ and TK^- viruses. At this time, their growth was considerably slowed, indicated impending senescence and thus cessation of DNA synthesis. Surprisingly, the TK^- viruses showed only a minor growth deficit compared to the TK^+ viruses in the aged MEFs (Fig. 3B), and this result was confirmed in a repeat experiment. The residual dTMP pools in senescent MEFs were presumably still sufficient for viral replication, although MHV-68 may also stimulate senescent MEFs to reenter the cell cycle.

Growth of MHV-68 TK mutants in vivo. We initially compared the capacity of BAC WT and TK^- Kan^+ MHV-68 to grow in vivo after intranasal infection of BALB/c mice. Infectious and latent viral loads in lungs and spleens were assayed over the subsequent 3 weeks (Fig. 4A). The TK^- Kan^+ virus was severely impaired in lytic growth in the lungs. It also showed reduced levels of latency at early time points (Fig. 4B), and the splenomegaly and lymphadenopathy that normally accompany the establishment of MHV-68 latency were greatly reduced (data not shown). However, despite the severe reduc-

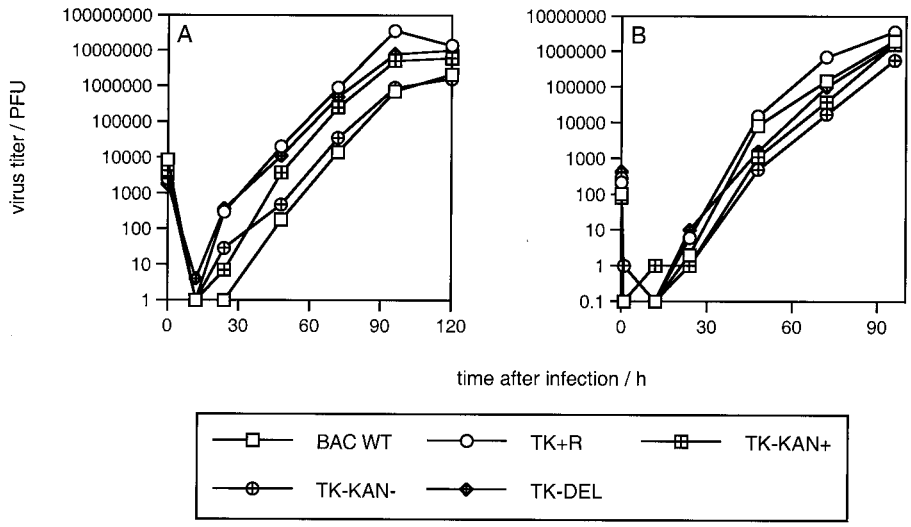

FIG. 3. Growth of TK^+ and TK^- viruses in vitro. TK^- BHK-21 cells (A) or passage 9 MEFs (B) were infected with MHV-68 (0.01 PFU/cell). Cultures were subjected to titer determination for infectious virus by plaque assay on TK^+ BHK-21 cells.

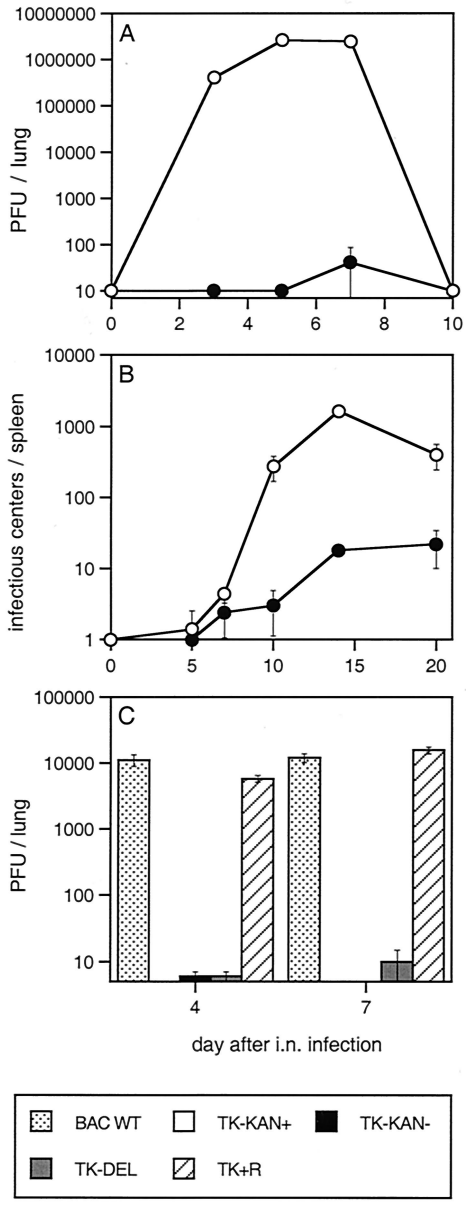

FIG. 4. Growth of TK^+ and TK^- viruses in vivo. BALB/c (A and B) or C57BL/6 (C) mice were infected intranasally with 2×10^4 PFU of MHV-68. (A and C) Infectious virus in lung homogenates was subjected to titer determination by a plaque assay on TK^+ BHK-21 cells. (B) Latent virus in spleens was subjected to titer determination by infectious center assay on passage 4-5 MEFs. In panels A and B, BAC WT virus (○) was compared with TK^- Kan^+ (●). In panel C, all five viruses were compared. Means and standard errors of the mean (n = 6) are shown.

tion in lytic replication at the site of primary infection, some TK-deficient virus was still able to seed to lymphoid tissue and establish latency. Further infections of C57BL/6 mice revealed a very similar lytic replication deficit in all the TK^- mutants and full reversion of the TK^+ R virus (Fig. 4C). Thus, the lytic replication deficit was firmly linked to the TK locus.

Transcription of viral genes adjacent to TK. ORF20, ORF21, and ORF22 are tightly packed in the viral genome, and it is likely that the promoters for ORF20 and ORF22, which are both orientated away from ORF21 (the MHV-68 TK gene), have their promoter elements within the TK coding sequence. For this reason, each TK mutation was made at least 400 bp away from the start of either neighboring ORF. The fact that no significant in vitro growth deficit was observed with the MHV-68 TK mutants implied that normal transcription of ORF20 and ORF22 was maintained, since such a deficit is seen with a disruption of either HSV homolog. Furthermore, there was no difference in phenotype between the small-insertion (TK^- Kan^-), large-insertion (TK^- Kan^+), and large-deletion (TK^- Del) TK mutants.

To compare ORF20 and ORF22 transcripts with the TK^+ and TK^- viruses, cDNA was generated from BHK-21 cells infected overnight with each mutant (5 PFU/cell) and amplified by PCR with ORF-specific primers. The reaction products were analyzed over a range of 24 to 30 cycles. This semiquantitative RT-PCR analysis showed no evidence of an effect of TK mutagenesis on the transcription of either ORF20 or ORF22 (Fig. 5).

Antiviral immunity elicited by TK mutants. The decreased lytic replication observed with the TK mutants suggested that these viruses—or others deficient in lytic replication—might provide the basis for gammaherpesvirus vaccines. To explore this possibility further, we analyzed the TK mutants for a desirable characteristic, their immunogenicity, and an undesirable one, their capacity to establish latency. The course of TK^- Kan^+ virus replication in BALB/c mice (Fig. 4) suggested that although early-latency titers were reduced, they might eventually reach those of the BAC WT virus. This proved to be the case, and 1 month after infection, splenic latency titers were equivalent in TK^- and TK^+ viruses (Fig. 6A). Measurements of latency-associated immune activation–non-antigen-specific B-cell activation and expansion of the $CD8^+$ $V\beta4^+$ T-cell subset—showed a reduction associated with the TK mutants compared to the BAC WT and TK^+ R viruses but showed considerable variation between individual mice (Fig. 6B and C). This supported the idea that latency establishment, with its accompanying infectious mononucleosis-like illness, was delayed but not prevented by the deficit in lytic virus replication. There was a similar spread in virus-specific antibody responses between mice infected with TK^- viruses, which generally correlated with the level of the nonspecific response. Thus, it appeared that defective lytic replication led to a low antigen load and consequently to poor immunogenicity; latency amplification increased the viral load and improved immunogenicity, but at the cost of viral persistence.

DISCUSSION

Disruption of the MHV-68 TK had little effect on viral growth in vitro but caused a marked attenuation of lytic replication in vivo. This delayed but did not prevent the establishment of latency. Thus, viruses largely defective in lytic replication were still able to persist and to drive an infectious mononucleosis-like illness. The implication is that disrupting lytic viral genes in human gammaherpesviruses, which cause mainly latency-associated disease, is unlikely to prove a good strategy in the generation of attenuated vaccine strains. Similarly, the capacity of latency amplification to compensate for a

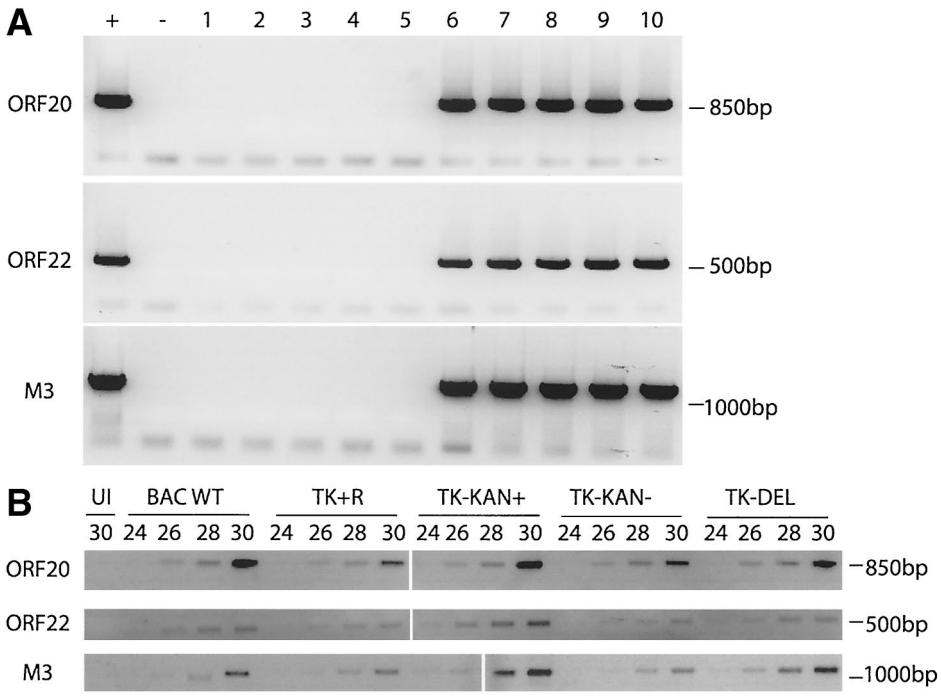

FIG. 5. Viral transcription in TK^+ and TK^- viruses. TK^+ BHK-21 cells were infected with MHV-68 (5 PFU/cell), and 18 h later RNA was extracted, reverse transcribed, and assayed for viral transcripts by RT-PCR amplification of part of each gene. Ethidium bromide-stained electrophoresed PCR products are shown. ORF20 and ORF22 (encoding glycoprotein H) flank the TK gene of MHV-68. M3 is a control early-late viral gene located >25 kb from the TK locus. (A) The specificity of each PCR amplification was confirmed by 30 cycles of amplification using BAC DNA (lane +) and cDNA from uninfected cells (lane −). Lanes 1 to 5 are RT^- controls to exclude the presence of residual viral DNA, and lanes 6 to 10 are RT^+ cDNA samples from cells infected with BAC WT (lanes 1 and 6), TK^+ R (lanes 2 and 7), TK^- Kan^+ (lanes 3 and 8), TK^- Kan^- (lanes 4 and 9), and TK^- Del (lanes 5 and 10) viruses. (B) To gain a more quantitative idea of ORF20 and ORF22 transcription, the same samples were amplified over a limiting number of cycles (24 to 30 cycles, as indicated).

defect in lytic replication probably limits the potential of TK-dependent chemotherapy in gammaherpesvirus infections.

TK-deficient HSV was initially thought to have an in vitro growth deficit; only later was this attributed to a deletion of the overlapping U_L24 gene (9). Clearly there is potential for confusion when mutating this tightly packed region of the herpesvirus genome. To allow for this, we generated three mutants, using sites well away from the neighboring ORFs. The mutants had similar phenotypes involving a severe in vivo lytic replication deficit with a relative preservation of latency establishment. None showed decreased transcription of ORF20 (the MHV-68 homolog of U_L24) or of ORF22 (which encodes glycoprotein H). Furthermore, there was no evidence of a replication deficit in BHK-21 cells, as would be expected with a disruption of either flanking gene.

TK-deficient MHV-68 showed a more severe epithelial replication deficit in vivo than did TK-deficient HSV (9). This may reflect a difference in tropism or a difference in experimental conditions. Epithelial surfaces encompass a wide range of cell phenotypes, which differ in their progress through the cell cycle, most obviously from replicating basal cells to terminally differentiated superficial cells. Experimental HSV inoculation typically involves skin scarification or subcutaneous injection, both of which expose basal epithelial cells and, by causing injury, stimulate cell division. After intranasal inoculation, MHV-68 encounters only the most superficial and thus the most fully differentiated cells of the intact epithelium. These cells have generally exited from the cell cycle and presumably contain little dTMP. TK is thus likely to be of particular importance in the first cycles of virus replication. The almost complete absence of lytic replication of TK^- viruses in the lungs indicated that the availability of dTMP limits MHV-68 replication in vivo and that other viral functions, such as an induction of cellular enzymes, cannot compensate for a lack of TK.

In contrast to HSV, which cannot reactivate from latency in the absence of TK, there was no obvious deficit in MHV-68 reactivation. Latency titers showed less TK-associated deficit than did lytic titers, and TK^- viruses reached WT latency levels by 1 month after infection. This presumably reflects differences in cell tropism between alpha- and gammaherpesviruses: while HSV is latent in terminally differentiated neurons, MHV-68 is latent in B cells, macrophages, and dendritic cells (14, 32, 38), all of which can proliferate in vivo. After intranasal MHV-68 infection of immunocompetent mice, B cells constitute the major reservoir of recoverable latent virus (32). The amplification of MHV-68 in germinal-center B cells (5) suggests that, as with EBV (2), latent MHV-68 resides in the memory B-cell pool. However, murine memory B cells normally proliferate at a low rate (15) and so should rarely contain significant amounts of dTMP. It may be that MHV-68 induced latently infected B cells to enter the S phase during viral reactivation, at least under the conditions of the infectious assay, such that a TK deficit was not important.

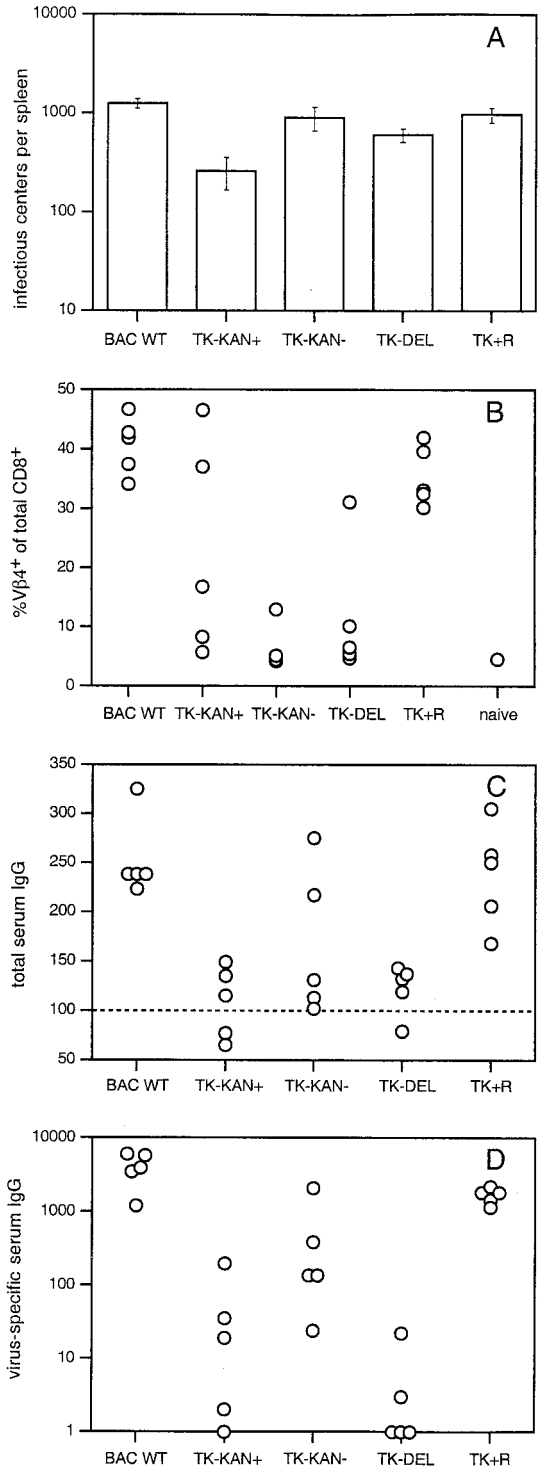

FIG. 6. Latency establishment and immune activation by TK^+ and TK^- viruses. C57BL/6 mice were infected intranasally with 2×10^4 PFU of MHV-68, and spleens and serum were harvested 1 month later. (A) Infectious-center assay for latent virus. Mean and standard error of the mean ($n = 5$) are shown. (B to D) Each point represents one mouse. (B) Virus-driven expansion of the splenic $CD8^+$ $V\beta4^+$ T-cell subset. "naive" represents age-matched, uninfected controls. (C) ELISA of total serum IgG, reflecting virus-driven B-cell activation. Units are arbitary, with naive serum being set at 100 (dashed line). (D) ELISA of virion-specific serum IgG. Units are arbitary, with naive serum being set at 1.

The capacity of TK-deficient viruses to drive latency amplification, with its attendant infectious mononucleosis-like illness, implied that this process is independent of lytic replication. This is consistent with data showing an independence of lytic and latent virus amplification with drug treatment (3), $CD8^+$ T-cell vaccination (21, 27), and antibody-mediated protection (30). $CD8^+$ T-cell vaccination with a latency epitope has also so far proved ineffective in preventing the establishment of latency (35), perhaps reflecting the action of viral evasion genes such M3 and K3 (4, 6, 29, 36). It seems that successful antiviral therapy must tackle both lytic and latent replication in order to be effective in reducing host colonization.

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