Vol. 74, No. 15

Murine Gammaherpesvirus 68 Cyclin D Homologue Is Required for Efficient Reactivation from Latency

ANN T. HOGE, SARA B. HENDRICKSON, AND WILLIAM H. BURNS*

*Departments of Medicine and Microbiology and Molecular Genetics,
Medical College of Wisconsin, Milwaukee, Wisconsin 53226*

Murine gammaherpesvirus 68 (MHV68) is a gammaherpesvirus that was first isolated from murid rodents. MHV68 establishes a latent infection in the spleen and other lymphoid organs. Several gammaherpesviruses, including herpesvirus saimiri, human herpesvirus 8, and MHV68, encode proteins with extensive homology to the D-type cyclins. To study the function of the cyclin homologue, a recombinant MHV68 has been constructed that lacks the cyclin homologue and expresses β-galactosidase as a marker (MHV68^{cy-}). MHV68^{cy-} grows in vitro with kinetics and to titers similar to those of the wild type. BALB/c mice infected with mixtures of equivalent amounts of the wild type and MHV68^{cy-} show deficient growth of the MHV68^{cy-} in an acute infection. Infection of SCID mice with virus mixtures also showed decreased MHV68^{cy-} virus growth, indicating that the deficiency is not mediated by T or B cells. Although mice infected with mixtures containing 100 times as much MHV68^{cy-} had greater splenic titers of the mutant virus than wild-type virus in acute infection, at 28 days postinfection splenocytes from these mice reactivated primarily wild-type virus. Quantitative PCR data indicate that equivalent genomes were present in the latent state. Reinsertion of the cyclin homologue into the cyclin-deleted virus restored the wild-type phenotype. These results indicate that the MHV68 cyclin D homologue mediates important functions in the acute infection and is required for efficient reactivation from latency.

The gammaherpesviruses are an important group of pathogens in humans and animals most notable for their associations with tumor formation in the host and ability to establish latent infections within lymphocytes (18, 31). Herpesvirus saimiri (HVS) causes lymphomas in primates and can transform T lymphocytes (19). Epstein-Barr virus (EBV) is a significant human pathogen causing lymphomas and nasopharyngeal carcinoma as well as transforming lymphocytes in vitro (27; D. H. Crawford, J. A. Thomas, G. Janossy, P. Sweny, O. N. Fernando, J. F. Moorhead, and J. H. Thompson, Letter, Lancet i:1355–1356, 1980). Human herpesvirus 8 (HHV8) is associated with Kaposi's sarcoma, body cavity-based lymphomas, and Castleman's disease in humans (7, 9, 39, 47; P. S. Moore and Y. Chang, Letter, Science 270:15, 1995). Mouse herpesvirus 68 (MHV68) is a gammaherpesvirus that can serve as a model system for these viruses (36, 40).

MHV68 was isolated from wild rodents and infects inbred and outbred rodents (29; D. Blaskovic, M. Stancekova, J. Svobodova, and J. Mistrikova, Letter, Acta Virol. 24:468, 1980). Infection is characterized by hematogenous spread with exudative pneumonia and splenomegaly (29, 48). MHV68 latently infects B lymphocytes as well as other cell types (41, 45, 49, 54, 56) and has also been associated with lymphoproliferative disease (43).

In addition to sharing biological characteristics with the other gammaherpesviruses, there is substantial sequence homology among the gammaherpesviruses (13, 51). Many of the gammaherpesviruses, including HHV8, HVS, and MHV68, encode a homologue to the D-type cyclins (2, 28, 32, 51; Y. Chang, P. S. Moore, S. J. Talbot, C. H. Boshoff, T. Zarkowska, K. Godden, H. Paterson, R. A. Weiss, and S. Mittnacht, Letter, Nature 382:410, 1996). The notable exception is EBV, which upregulates cellular cyclin D2 expression (3, 5, 38). Levels of the cyclin proteins fluctuate throughout the cell cycle and regulate cell cycle progression. The cyclin proteins function by binding to the cyclin-dependent kinases (CDK) which phosphorylate substrates responsible for cell cycle progression. Based on sequence homology, the gammaherpesvirus-encoded cyclins are most similar to the host D-type cyclins. The D-type cyclins (D1, D2, and D3) function in G_1 phase to regulate progression into S phase. The host D-type cyclins bind to CDK 4 and 6, which phosphorylate retinoblastoma protein releasing the E2F transcription factors which stimulate progression into S phase (1, 26, 34, 35).

The virally encoded cyclins have been shown to share features with cellular cyclin but have also acquired altered characteristics. Like host D-type cyclins, HHV8 v-cyclin binds to CDK 6 and phosphorylates and inactivates retinoblastoma protein (15). MHV68-encoded cyclin induces cell cycle progression, and transgenic mice expressing MHV68 v-cyclin produce lymphoid tumors as they age (50).

Prior to this work the role of MHV68 v-cyclin in nononcogenic infections of MHV68 had yet to be defined. To explore its role in various aspects of viral infection, we constructed a recombinant MHV68 lacking the cyclin D homologue (MHV68^{cy-}). We demonstrate here that the virus replicates efficiently in cell culture but exhibits a deficiency in replication in acute infection in BALB/c mice. We further show that the cyclin-deficient virus can establish latency but very inefficiently reactivates from the latent state. We repaired the virus by reinserting the cyclin gene and demonstrate that the wild-type phenotype is restored (MHV68^{rep}).

MATERIALS AND METHODS

Viruses and tissue culture. Our MHV68 is the same strain used by A. Nash and coworkers (44). The complete sequence of this virus is known (51). Virus stocks used for mouse infections were passaged on a previously described line of rat embryo fibroblasts (REFs) until complete cytopathic effect (CPE) was observed (4). The titer of the wild-type virus was 2.5×10^7 PFU/ml, and the recombinant virus titer ranged between 6.0×10^6 and 2.0×10^7 PFU/ml. REF

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* Corresponding author. Mailing address: Departments of Medicine and Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, WI 53226. Phone: (414) 456-4989. Fax: (414) 456-6533. E-mail: wburns@mcw.edu.
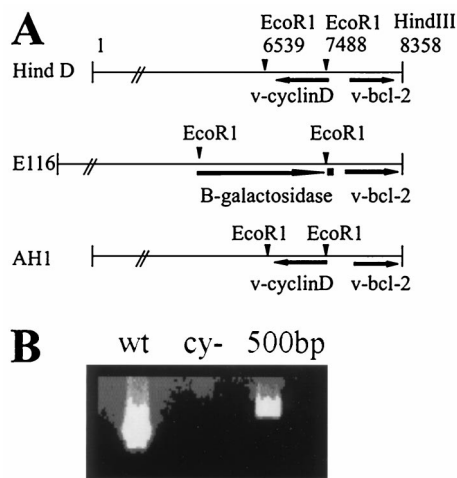
FIG. 1. Construction of recombinant MHV68[cy−] and MHV68[rep]. (A) The cyclin-encoding *Eco*RI restriction fragment was removed from the 8.35-kb *Hin*dIII fragment (Hind D) to generate the transfer vector used to delete v-cyclin (E116). To repair MHV68[rep], the *Eco*RI restriction fragment was reinserted into E116 in place of the β-Gal cassette to generate AH1. (B) PCR amplification of v-cyclin from MHV68[cy−] (cy−) was compared to MHV68[wt] (wt).

cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 30 μg of gentamicin per ml, 0.3 μg of amphoterocin B per ml, and 2 mM L-glutamine (complete MEM).

Plasmid construct. v-cyclin was deleted through homologous recombination with a transfer vector as described in Fig. 1. Virion DNA from MHV68 was digested with *Hin*dIII. The 8.35-kb fragment encoding v-cyclin (Hind D) was inserted into pSK (Stratagene, La Jolla, Calif.) lacking an *Eco*RI restriction site (13). The β-galactosidase (β-Gal) expression cassette in pSVB (Clontech, Palo Alto, Calif.) was excised with *Eco*RI and inserted into the *Eco*RI site of the transfer vector, replacing a 949-bp portion of the v-cyclin open reading frame to yield E116. The orientation of β-Gal (Fig. 1) was determined by sequencing the fusion between the 5′ end of β-Gal and the MHV68 genome. To repair the cyclin-deleted recombinant virus, the *Eco*RI restriction fragment encoding v-cyclin was inserted in place of the β-Gal cassette in the transfer vector to yield AH1.

Transfection and recombinant virus isolation. Subconfluent REFs were co-transfected with E116 (1 μg) and MHV68-infected cell DNA by the calcium phosphate precipitation method. The repaired virus was generated by cotransfection of AH1 and DNA from cells infected with MHV68[cy−]. CPE usually occurred within 6 days posttransfection. Supernatants from frozen and thawed cultures were diluted and screened for β-Gal-positive virus. Recombinant virus was isolated by limiting dilution cultures on REF cells in 96-well tissue culture plates. Supernatant from these plates were transferred to empty plates and frozen while the original plates were fixed and stained for β-Gal expression. Wells positive for β-Gal virus were again diluted onto 96-well plates for multiple rounds of limited dilution culture until the recombinant virus was isolated and verified by plaque assay, PCR, restriction analysis, and Southern blotting.

To assess purity of recombinant virus, PCR was performed with cyclin-specific primers. DNA from cells infected with the recombinant virus and wild type were amplified in 50-μl reaction mixtures with Supermix (Gibco BRL). PCR conditions were as follows: a 5-min denaturation at 94°C, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by an extension phase of 10 min at 70°C. PCR products were electrophoresed on a 1.3% agarose gel. Primer sequences were as follows: 5′-GCATAGGGCGCAGAAGATT-3′ and 5′-CGCAGCGAA AGAGAACACG-3′.

To examine the possibility that MHV68[cy−] was reactivating from latency but deleting the β-Gal marker, PCR was performed as above with a cyclin-specific primer, 5′-CCACCCAGTTGGCATACCT-3′, and a primer flanking the cyclin gene, 5′-GTAAGGGAATTCGGTAAATTC-3′.

Southern and Northern analyses of recombinant viruses. DNA for Southern blot analysis was extracted from infected REFs and digested with *Eco*RI and *Hin*dIII. Electrophoresis, blotting, and hybridization conditions were done by established procedures. A nick translation system (Gibco BRL) was used with [α-^{32}P]ATP (Amersham, Arlington Heights, Ill.) to label the 949-bp v-cyclin-encoding *Eco*RI restriction fragment. Virion DNA was extracted and was digested with restriction enzymes and electrophoresed on a 0.7% agarose gel for analysis (4). Total cell RNA was extracted from infected REF cells with TRIzol (Gibco BRL) according to manufacturer recommendations. Northern analysis was performed according to standard procedures.

In vitro infections. REFs or BALB/c 3T3 cells (approximately 5 × 10^5 per well in a six-well plate) were infected with MHV68[wt], MHV68[cy−], or MHV68[rep] at a multiplicity of infection (MOI) of 5 or 0.01. After a 1-h adsorption, the wells were washed with MEM. At various times after infection, the supernatants were frozen and thawed, and the amount of infectious virus in the supernatants was determined by plaque assay on REF cells as described below.

In vivo infections. Female BALB/c mice (age, 4 to 6 weeks) were obtained from Frederick Cancer Research Labs (Frederick, Md.). Mice were inoculated intraperitoneally with virus in 1 ml of complete MEM. At various times after infection, mice were sacrificed and assayed for infectious virus in spleens by mincing the spleens or isolating splenocytes as described below and sonicating in 1 ml of complete MEM with 2 pulses of 180 W for 3 s/pulse at 4°C using the microprobe from a Branson sonicator. Sonicates were assayed on REF monolayers. After infection for 1 h at 37°C, cells were overlaid with 0.9% methylcellulose in MEM supplemented with 5% fetal bovine serum, 25 mM HEPES, 2 mM L-glutamine, and 30 μg of gentamicin per ml. Six days after infection, monolayers were fixed and stained with methylene blue or X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and plaques were counted. For X-Gal staining, cells were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M Na$_2$PO$_4$ for 10 min. After rinsing in phosphate-buffered saline, plaques were stained with X-Gal (2 mg/ml) in 1.3 mM MgCl$_2$, 3 mM K$_4$Fe(CN)$_6$, 3 mM K$_3$Fe(CN)$_6$, and 100 mM Na$_2$PO$_4$. For methylene blue staining, cells were fixed in ethanol and then stained with 0.5% methylene blue.

To determine the sensitivity of this assay, an uninfected spleen in 1 ml of complete MEM was spiked with virus of known titer and the spleen was treated as described above to determine the sensitivity of the assay. The titer of the sonicated virus decreased by 80% from the titer of the unsonicated virus which was determined in parallel, reducing the sensitivity of the assay from approximately 2 to 10 PFU/spleen. To assay for latent virus, splenocytes were isolated 28 days after infection. Spleen cells were separated from stromal tissue by teasing with needles. Stroma and spleen cells were resuspended in MEM and allowed to settle for 10 min. Cells remaining in suspension were pelleted and resuspended in 1 ml of distilled water to lyse red blood cells. Ten milliliters of complete MEM was added quickly, and splenocytes were washed in complete MEM. Thirty to 100 times more cells were sonicated and assayed for infectious virus than were explanted to recover reactivated virus.

For treatment of splenocytes with lipopolysaccharide (LPS), splenocytes were isolated as above and resuspended in complete MEM with or without 5 μg of LPS per ml (VWR, Batavia, Ill.). After 48 h, splenocytes were transferred to monolayers of REFs and observed for 2 to 4 weeks for CPE.

Real-time PCR analysis. Quantitation of viral genomes was performed with real-time PCR using the 7700 Sequence Detection System (PE Biosystems, Foster City, Calif.). This method is based on continuous optical monitoring of a fluorogenic PCR (14, 17). In addition to the two amplification primers, an oligonucleotide homologous to the amplified region is included in the reaction mixture. The oligonucleotide is bound to a fluorescent tag that serves as a reporter and a quencher that suppresses fluorescence. During the extension phase of PCR, the 5′-to-3′ exonuclease activity of *Taq* cleaves the reporter from the probe, releasing it from the quencher and resulting in an increase in fluorescence emission which was detected by the laser detector of the ABI Prism 7700. After crossing a sequence detection threshold, the PCR amplification results in a fluorescent signal proportional to the amount of PCR product generated. Initial template concentration is derived from the cycle number (C_T) at which the fluorescent signal crosses a threshold in the exponential phase of the PCR. Standard graphs of C_T values obtained from serially diluted positive controls were used to derive values for unknowns.

Three primer-probe concentrations were designed using Primer Express software (PE Biosystems). We utilized a primer and probe set specific to v-cyclin to detect MHV68[wt], a set spanning the β-Gal insertion site to detect MHV68[cy−] and a set specific to the double-copy murine RNase P gene, to which viral genomes were normalized. To generate standard curves for v-cyclin, the *Eco*RI fragment encoding v-cyclin was cloned into pBluescript SK, and for β-Gal, the previously described plasmid E116 was used. Mouse DNA extracted from spleen cells was used to generate the standard curve for the RNase P control. Standard curves were generated from the C_T values from serial dilutions of each template. Copy number for infected and uninfected spleens were calculated from the C_T values of samples using standard curves of C_T values corresponding to known copy numbers. These calculations were performed utilizing sequence detection systems software (PE Biosystems). Each sample was tested in duplicate, and the mean of the two values was shown as the copy number of the sample. Calculated copy numbers were normalized to RNase P and then adjusted to represent 1 ng of genomic DNA. Correlation coefficients calculated by Sequence Detection Systems software were as follows: 0.984 for cyclin, 0.996 for β-Gal, and 0.966 for RNase P.

Reactions were performed with universal master mix and universal cycling conditions (PE Biosystems). PCR primers were synthesized by PE Biosystems or Life Technologies, Inc. (Gaithersburg, Md). Primers were used at a concentration of 900 nM for each primer and 200 nM of probe in a 25-μl reaction mixture. Primers sequences were as follows: for v-cyclin, 5′-CCTGTCAGCTACCCAC GAGAG-3′ and 5′-CCACCCAGTTGGCATACCT-3′; for B-Gal, 5′-CACATT CCACAGCCAAGCTGTA-3′ and 5′-CAACATTCCACCTTCAACAAACA-3′; and for RNase P, 5′-GATGCCTCCCTCGCCG-3′ and 5′-CTCAGCCATT GAACTCGCAC-3′. Probe sequences were as follows: for cyclin, VIC-TTCCA GAGTCAATAGTTTGTCAGCTGTTGTTG-TAMRA; for β-Gal, VIC-CGAG

TCGAATTCTTGACTGGCCATG-TAMRA; and for RNaseP, 6FAM-AGCTT
GGAACAGACTCACGGCCAGC-TAMRA.

RNA dot blot. RNA dot blot assays were performed by adding 3 volumes of
denaturing solution (66% formamide, 8% formaldehyde, 0.6× MOPS [morpho-
linepropanesulfonic acid] buffer, pH 7.0) to 1.5 μg of RNA. The samples were
heated for 15 min at 65°C followed by the addition of 2 volumes of cold 20× SSC
(1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Equivalent amounts of
RNA were applied under vacuum (96-well minifold; Schleicher & Schuul, Inc.,
Keene, N.H.) to BIOTRANS nylon membrane (ICN Biomedicals Inc., East
Hills, N.Y.) prewetted with 2× SSC. The filter was then washed three times with
2× SSC, baked at 80°C for 2 h, and prehybridized for 2 h at 42°C in 4 ml of RNA
hybridization solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine
serum albumin, 5× SSC, 0.1% sodium dodecyl sulfate, 50 mM sodium phosphate
[pH 9.5], and 50% deionized formamide). Hybridization was carried out over-
night at 42°C in 4 ml of RNA hybridization solution with the addition of 500 ng
of cyclin D2 probe (a gift from C. Sherr) labeled as described previously.

RESULTS

Recombinant virus design and purification. The v-cyclin
gene was deleted from the MHV68 genome by homologous
recombination with the E116 transfer vector shown in Fig. 1A.
To generate the transfer vector used for repair of MHV68^{cy-},
the EcoRI restriction fragment encoding v-cyclin was rein-
serted in place of the β-Gal cassette (AH1).

To create MHV68^{cy-}, E116 and DNA from cells infected
with MHV68^{wt} were cotransfected into REFs. After CPE was
detected, viral supernatant was screened for β-Gal expression
to detect recombinant virus. The recombinant virus was then
isolated from wild-type virus by multiple rounds of limiting-
dilution cultures. When dilution cultures yielded all β-Gal-
expressing virus, DNA was extracted for analysis. PCR am-
plification for the cyclin gene from REFs infected with
MHV68^{cy-} was negative for cyclin amplification, demonstrat-
ing that the v-cyclin gene had been deleted from the
MHV68^{cy-} genome (Fig. 1B). DNA isolated from REFs in-
fected with purified MHV68^{cy-} was used in a cotransfection
with AH1, and MHV68^{rep} was isolated by limiting dilution for
β-Gal-negative virus.

Analysis of recombinant viruses. Purified MHV68^{cy-} and
MHV68^{rep} were analyzed by restriction digestion (Fig. 2A),
Southern hybridization (Fig. 2B), and Northern analysis (Fig.
3C). Virion DNA was digested with EcoRI and HindIII and
was electrophoresed on an agarose gel (Fig. 2A). No rear-
rangements outside of Hind D in the MHV68^{cy-} genome are
apparent. The arrow on the right indicates the Hind D restric-
tion fragment that is not visible in MHV68^{cy-} due to the
insertion of the β-Gal cassette that adds approximately 3 kb to
the size of the restriction fragment. This is more apparent upon
hybridization with the Hind D restriction fragment (Fig. 2B).
The upper panel shows the altered migration pattern of the
HindIII restriction fragment in MHV68^{cy-}. In the lower panel
of Fig. 2B, the approximately 1-kb v-cyclin-encoding restriction
fragment is absent in MHV68^{cy-} and present in MHV68^{wt} and
MHV68^{rep}. To ensure that transcription of v-cyclin from
MHV68^{rep} was normal, and that transcription of neighboring
bcl-2 homologue has not been altered by deletion of v-cyclin,
Northern blot analysis was performed (Fig. 2C). Hybridization
with v-cyclin (upper panel) showed an identical expression
pattern of two different-sized messages from MHV68^{wt} and
MHV68^{rep} and no cyclin expression from MHV68^{cy-}. The
same Northern blot was stripped and probed with v-bcl-2.
v-bcl-2 expression was similar in all samples, indicating that its
transcription was not altered in MHV68^{cy-} and MHV68^{rep}.

Cyclin-deleted MHV68 grows efficiently in cell culture. The
efficiency of replication of MHV68^{cy-} in cell culture was com-
pared to MHV68^{wt} and MHV68^{rep} at MOIs of 5 and 0.01. In
the single-step growth curve, in which cells were infected at an
MOI of 5, MHV68^{cy-} grew with kinetics and to titers similar to

those of MHV68^{wt} and MHV68^{rep} in both REF and BALB/c
3T3 cells (Fig. 3A). In the multistep growth curve, in which
cells were infected at an MOI of 0.01, MHV68^{cy-} grew simi-
larly to MHV68^{wt} and MHV68^{rep} (Fig. 3B). All three viruses
replicated at about a log higher titer in REF cells than BALB/c
3T3 cells after infection at a low MOI. However, there was no
significant difference between MHV68^{cy-} and MHV68^{wt} and
MHV68^{rep} in any experiment, indicating that v-cyclin appears
to be dispensable for replication in two cell lines.

Cyclin-deleted virus is deficient in acute infections. The use
of MHV68 as a model system for other gammaherpesviruses
allows analysis of the phenotype of the cyclin-deleted virus in
vivo. BALB/c mice were infected with 10^6 PFU of MHV68^{wt},
MHV68^{cy-}, and MHV68^{rep}. Splenic titers of virus were deter-
mined at 3, 5, and 7 days after intraperitoneal injection.
Splenocytes were disrupted by sonication to release intracellu-
lar virus. Sonicates were then assayed on monolayers of REFs,
and plaques were stained with X-Gal 6 days after infection.
Titers of MHV68^{cy-} drop more quickly than MHV68^{wt} levels,
becoming undetectable by 7 days postinfection (Fig. 4A). Re-
pair of the cyclin deletion restores titers to those of MHV68^{wt},
indicating that v-cyclin is required for efficient acute infection.

One explanation for the deficiency of MHV68^{cy-} in acute
infection in light of the efficient replication in cell culture is
that the deficiency is immunologically mediated. To address
this possibility, we infected SCID mice with MHV68^{cy-} in a
mixture of 10^4 PFU MHV68^{wt} and 10^4 PFU MHV68^{cy-} vi-
ruses (Fig. 4B). After 7 days, splenic titers of MHV68^{cy-} were
13% of titers of MHV68^{wt}. The titer of MHV68^{cy-} in other
organs was consistently lower than that of MHV68^{wt}. This
provides a qualitative assessment of the nature of the replica-
tion deficiency, which supports the observation that MHV68^{cy-}
is deficient in acute infections and that this deficiency is inher-
ent to the virus and is not immunologically mediated by mech-
anisms absent in the SCID mouse, namely T and B cells.

Cyclin-deleted virus cannot efficiently reactivate ex vivo. We
wished to study the effect of the absence of the v-cyclin gene on
establishment and reactivation from latency. Based on analogy
to studies of latency with murine cytomegalovirus, we first
needed to establish an equivalent acute infection (30). We
infected mice with 100 times more MHV68^{cy-} than MHV68^{wt}
(a mixture of 5×10^6 PFU of MHV68^{cy-} and 5×10^4 PFU of
MHV68^{wt}). Three days postinfection there was an average of
10^4 PFU/10^6 spleen cells of MHV68^{cy-} and 3.0×10^3 PFU of
MHV68^{wt} virus/10^6 spleen cells. By 5 days, the titers had
dropped significantly but the titers of MHV68^{cy-} remained
higher (Fig. 5A). We were then prepared to analyze the ability
of MHV68^{cy-} to establish and reactivate from a latent infec-
tion.

The operational definition of latency requires the absence of
preformed infectious virus in an assay of high sensitivity along
with the ability to reactivate virus from explanted cells. To
exclude the possibility of persisting infectious virus, we ana-
lyzed spleens at 28 days after infection and tested all samples
containing putative latent virus for infectious virus in an assay
of high sensitivity. Since ruptured cells cannot reactivate latent
virus, disruption of cells allows determination of preexisting
infectious virus. The process of sonication employed reduces
the titer of the introduced infectious virus by 80%. In order to
compensate for this, we sonicated between 30 and 100 times
more spleen cells than were examined for reactivation from
latency. In all cases, we and others detected no infectious virus
in spleens at 28 days after infection (55). CPE observed from
spleen cell explants was therefore thought to be attributable to
viral reactivation.

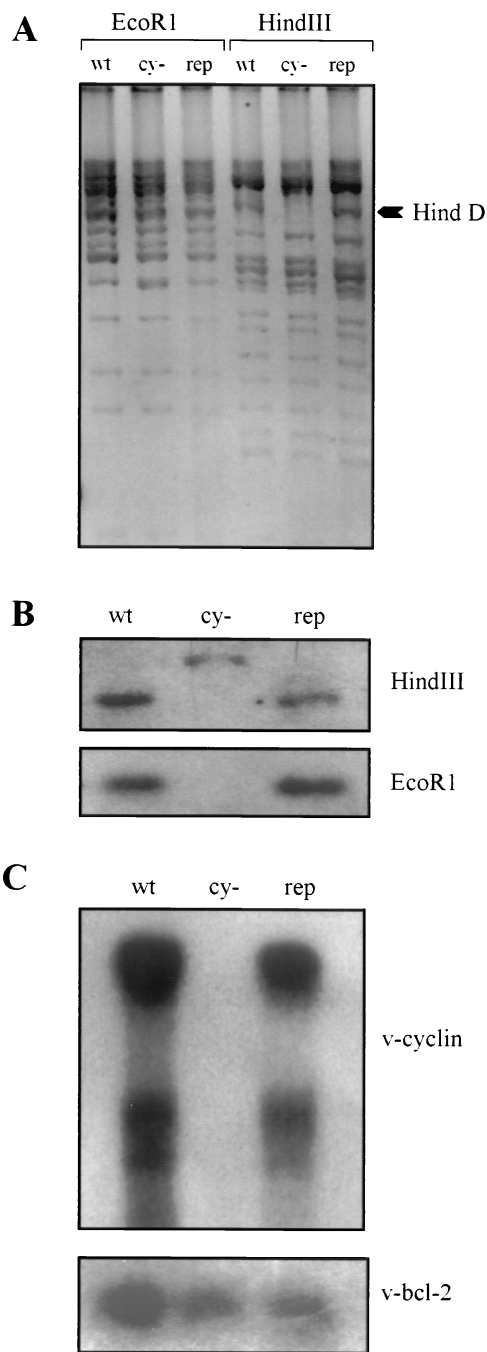In order to assay for latency, spleen cells from mice were

FIG. 2. Analysis of MHV68^{cy-} and MHV68^{rep}. (A) Virion DNA was digested with *Eco*RI and *Hin*dIII, and the restriction pattern of MHV68^{wt} (wt) was compared to those of MHV68^{cy-} (cy−) and MHV68^{rep} (rep). (B) Infected cell DNA was hybridized with Hind D. Cyclin-encoding *Hin*dIII restriction fragment is shown in the upper panel, and the *Eco*RI fragment is shown in the lower panel. (C) Northern analysis of v-cyclin expression is shown in the upper panel and the neighboring gene v-bcl-2 is shown in the lower panel.

explanted over monolayers of REFs. Spleens from mice infected with a mixture of 5×10^6 PFU of MHV68^{cy-} and 5×10^4 PFU of MHV68^{wt} were analyzed. Spleen cells were plated in 10-fold dilutions of 10^3, 10^4, and 10^5 cells per well. Titers of supernatants from wells showing CPE were determined, and plaques were stained for β-Gal expression (Fig. 5B). MHV68^{wt} and MHV68^{rep} reactivate in a cell dose-response manner while

reactivation of MHV68^{cy-} was rarely detected at any dose of spleen cells. In four independent experiments, a total of 250 wells positive for CPE were tested for the presence of MHV68^{cy-}. While 99.2% of the wells containing reactivatable virus were MHV68^{wt}, only 2 wells (0.8%) reactivated MHV68^{cy-}. Mice infected separately with MHV68^{wt} and MHV68^{cy-} yielded similar results (data not shown), indicating that complementation between the viruses was not occurring. Furthermore, complementation would have resulted in more reactivation of MHV68^{cy-}, which was not seen. To determine whether the repair of cyclin would restore the ability of the virus to reactivate from latency, mice were infected with 10^6 PFU of MHV68^{wt} and 10^6 PFU of MHV68^{rep}. Spleen cells were examined 28 days after infection, and reactivation was assessed as described above. MHV68^{rep} reactivated in a dose-response manner at a similar frequency as spleen cells from mice infected with MHV68^{wt} (Fig. 5C).

To address the possibility that reactivating virus may represent MHV68^{cy-} virus that had deleted the β-Gal gene, we amplified v-cyclin with PCR on DNA extracted from 19 pools of reactivating virus from mice infected with 5×10^6 PFU of MHV68^{cy-} and 5×10^4 PFU of MHV68^{wt}. v-cyclin was amplified from all samples tested, but not from cells infected with MHV68^{cy-}, indicating that if deletion of β-Gal occurs during reactivation, it is a rare event (data not shown). The cyclin homologue appears to be required for efficient reactivation from latency.

Cyclin-deleted virus can establish latency. The diminished frequency of reactivation of MHV68^{cy-} could be explained by a deficiency in the establishment of latency or reactivation from latency. To determine if latency was established equivalently by MHV68^{wt} and MHV68^{cy-} under the conditions we used to achieve equivalent acute infections, we employed a real-time PCR method. This method employs flourescently labeled oligonucleotides attached to a quencher that suppresses fluorescence when hybridized to DNA. When the probe is specifically bound to the template it will be released by the exonuclease activity associated with polymerase. Fluorescence can then be quantitatively detected so that the intensity of fluorescence corresponds to the quantity of template, and quantities can be interpolated from a standard curve. To quantitate the viral genomes we utilized a primer and probe set specific to v-cyclin to detect MHV68^{wt}, a set specific to the β-Gal gene to detect MHV68^{cy-}, and a set specific to the double-copy murine RNase P gene, to which viral genomes can be normalized. Standard curves were generated for v-cyclin (Fig. 6A) and β-Gal (Fig. 6B) with known quantities of DNA. The efficiency of amplification of both templates appears to be comparable based on the similar slope of the standard curves. Values for unknowns were then derived from these standard curves. Absolute viral genome copy number was normalized to that of the host genome to generate data shown in Fig. 6C as viral genomes per nanogram of cellular DNA. PCR was performed on DNA extracted from spleen cells from mice 28 days after infection with a mixture containing 100 times as much MHV68^{cy-} as MHV68^{wt} and compared to uninfected spleen cell DNA. At 28 days postinfection, the number of MHV68^{wt} and MHV68^{cy-} genomes was approximately equivalent in two independent experiments. These numbers reflect the acute splenic infection titer data well and predict that with increased MHV68^{cy-} input, MHV68^{cy-} can efficiently establish latency but has a defect in reactivation from latency.

Stimulation of cellular cyclin D2 expression cannot substitute for the absence of MHV68 cyclin in reactivation from latency. The virus may encode the cellular cyclin homologue to regulate the timing of cyclin expression. Alternatively, the
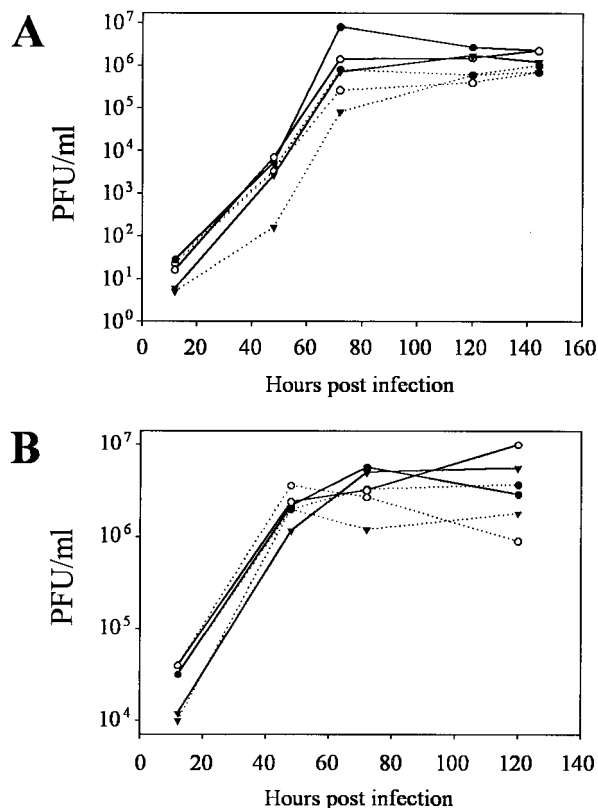
FIG. 3. Single (A) and multistep (B) growth curves comparing growth of MHV68^{wt} (●), MHV68^{cy-} (○), and MHV68^{rep} (▼) in REF cells (filled lines) and BALB/c 3T3 cells (dashed lines). Supernatants were frozen at given times after infection, and titers were determined on monolayers of REF cells. Error generated in determining supernatant titers is not visible.

function or properties of v-cyclin may be qualitatively different than those of cellular cyclin, as has been described for the cyclin encoded by HHV8 (15, 46). To address this question, we asked whether appropriately timed cellular cyclin expression would stimulate MHV68^{cy-} reactivation. Since it has been shown previously that LPS stimulates expression of cyclin D2 in B cells (47) and MHV68 latently infects primarily B cells in the spleen (49; our unpublished observations), we stimulated cellular cyclin D2 expression by culturing latently infected spleen cells in the presence of LPS. A volume of 10^6 spleen cells obtained 28 days postinfection with a mixture of 5 × 10^6 PFU of MHV68^{cy-} and 5 × 10^4 PFU of MHV68^{wt} were cultured in the presence of LPS. Cellular cyclin D2 expression was shown to increase after treatment with LPS (Fig. 7A). After CPE was observed, the titer of the virus from the supernatant was determined, and the plaques were stained with X-Gal. Reactivation of MHV68^{cy-} was virtually absent in the presence or absence of LPS. Reactivation of MHV68^{wt} was not substantially influenced by LPS stimulation of splenocytes. This provides a preliminary indication that the function of v-cyclin cannot be substituted by appropriately timed cellular cyclin D2 expression. It is still possible that v-cyclin can be substituted by cyclin D1 or D3.

DISCUSSION

Most gammaherpesviruses, with the notable exception of EBV (which upregulates cellular cyclin) encode homologues to cyclin D (2, 28, 32, 51; Chang et al., Letter, Nature). This

observation indicates that D-type cyclins may have an important role in the biology of the virus that the host cyclin D cannot provide. There are several possible roles for v-cyclin that can be envisioned. Cyclin may be important in the lytic infection by stimulating progression into S phase, which would increase expression of host proteins required for viral replication. Our data indicate clearly that in tissue culture infection this is not the case. Furthermore, data from our group and others have shown that v-cyclin is expressed with leaky-late kinetics in lytic viral replication (50). If the function of v-cyclin expression is to increase expression of proteins that function in the S phase of cell cycle to optimize viral replication, late expression would seem inappropriate unless the v-cyclin is a virion protein that acts upon entry into the cell. This raises the possibility of roles for v-cyclin in transformation or latency. Many studies have focused on the role of viral cyclins in transformation by the gammaherpesviruses (8, 12, 20, 50). MHV68 v-cyclin expressed in transgenic mice has been shown to function as an oncogene and transform lymphocytes (50). Although interesting, this observation sheds little light on the biologically relevant role of v-cyclin. Another possibility is that v-cyclin plays a role in latency. Our experiments were designed to examine which of these portions of the life cycle of MHV68 require v-cyclin.

We deleted the v-cyclin gene from MHV68 and studied the phenotype of the mutant virus. We confirmed the deletion by PCR, restriction pattern, and Southern and Northern analyses. Expression of the neighboring bcl-2 homologue was not altered in the recombinant virus. The recombinant virus grew efficiently in cell culture but was deficient in replication in vivo.
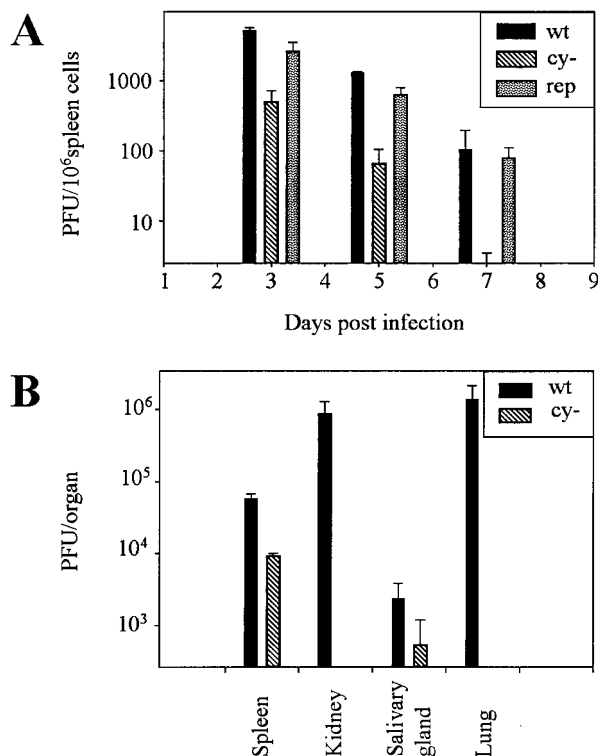

FIG. 4. Analysis of MHV68^{cy-} in acute infection. (A) Titers of sonicated spleen cells from BALB/c mice infected with 10^6 PFU of MHV68^{wt}, MHV68^{cy-}, or MHV68^{rep} were determined at 3, 5, and 7 days postinfection. (B) Titers of sonicated organs from SCID mice infected with 10^4 PFU of MHV68^{cy-} and MHV68^{wt} in a mixture were determined 7 days postinfection.
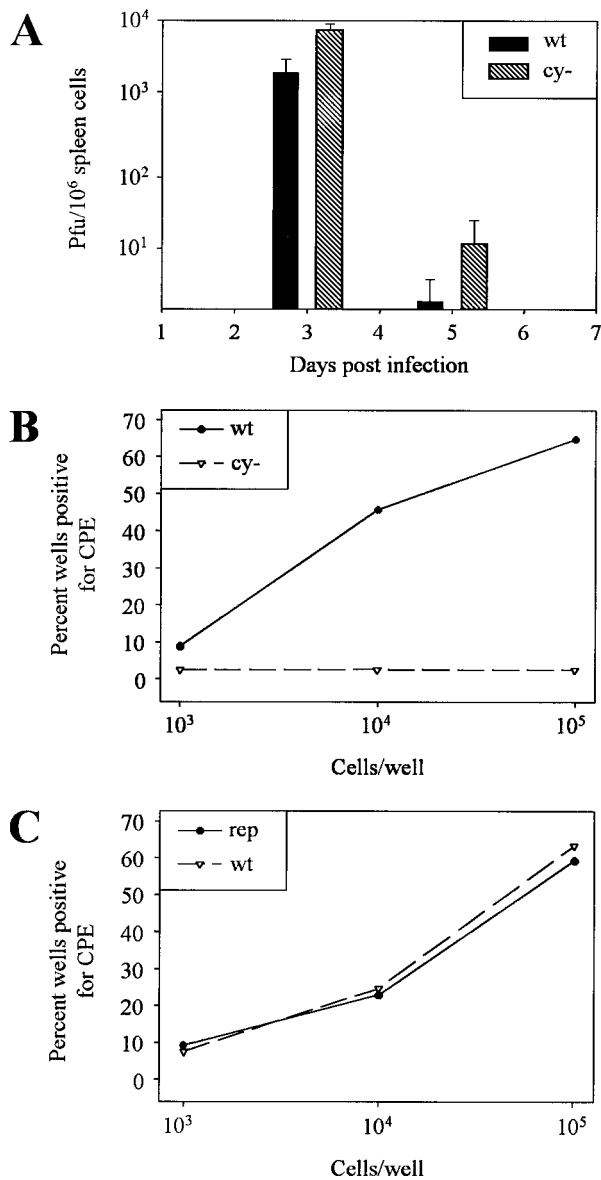
FIG. 5. Analysis of MHV68^{cy-} reactivation from latency. (A) After infection with a mixture of 5×10^6 PFU of MHV68^{cy-} and 5×10^4 PFU of MHV68^{wt}, acute infection titers were similar. (B) Splenocytes were isolated from spleens of BALB/c mice 28 days after infection. Cells were serially diluted as 10^3, 10^4, and 10^5 cells/well in 96-well plates over monolayers of REFs. After 3 weeks, titers of supernatants from CPE-positive wells were then determined, and supernatants were stained with X-Gal to differentiate between MHV68^{wt} and MHV68^{cy-}. Plates were then scored for percent wells positive for MHV68^{wt} and MHV68^{cy-}. (C) To compare MHV68^{rep} to MHV68^{wt}, mice were infected with 10^6 PFU of MHV68^{rep} and MHV68^{wt} per ml separately, and spleen cells were explanted and scored for percent wells positive for CPE.

We infected mice with a mixture with MHV68^{cy-} and MHV68^{wt} to provide a control for each animal. This deficiency appeared to be inherent to the virus and not immunologically mediated at least by T or B lymphocytes since MHV68^{cy-} replicated deficiently in the immunologically deficient SCID mouse.

Work with murine cytomegalovirus indicated that the number of latently infected genomes correlates with the titer of the virus in acute infection (30). In order to create equivalent acute infections, we adjusted the input virus to 100 times more

MHV68^{cy-} than MHV68^{wt}. We were thus able to establish an acute infection with MHV68^{cy-} that was comparable to wild-type infection. In order to investigate the role of v-cyclin in reactivation, it was critical to establish that equivalent latent genomes were present. We used quantitative real-time PCR to establish that there were equivalent numbers of latent MHV68^{cy-} as MHV68^{wt} genomes present in latently infected spleen cells. Under these conditions, we observed a dramatic deficiency in the ability of MHV68^{cy-} to reactivate from latency. This phenotype was corrected by repair of the cyclin-deleted virus.

What would be the advantage to the virus to encode cyclin, when the cells it infects contain a functional cyclin? Viral cyclin
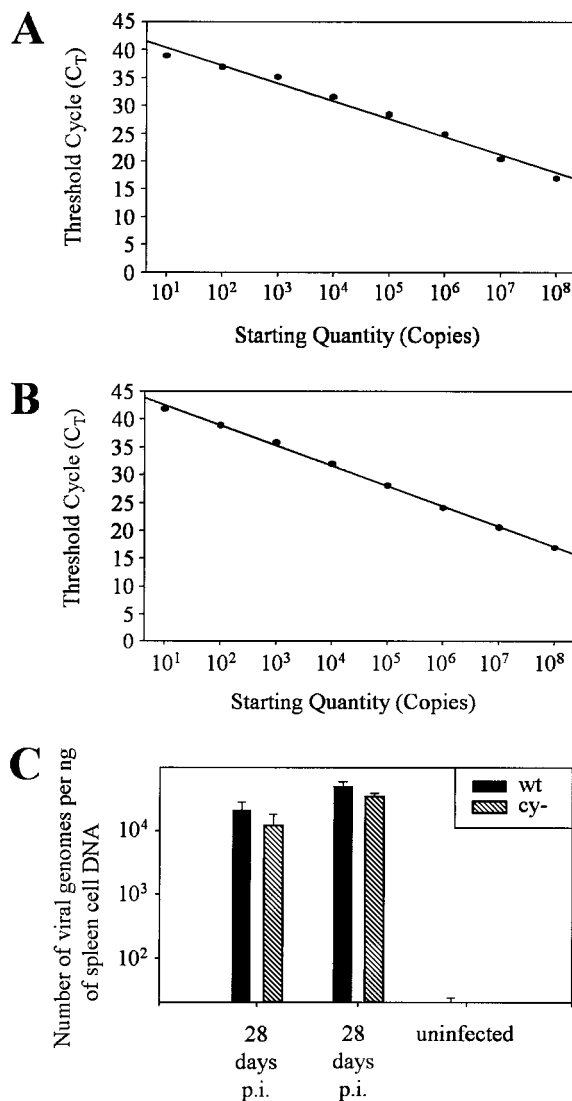

FIG. 6. Equivalent numbers of MHV68^{wt} and MHV68^{cy-} genomes are present in latently infected spleen cells. Real-time PCR was used to generate a standard curve from C_T values of serially diluted plasmids for detection of MHV68^{wt} (v-cyclin [A]) and MHV68^{cy-} (β-Gal [B]). DNA was extracted from spleen cells of mice 28 days postinfection with 5×10^6 PFU of MHV68^{cy-} and 5×10^4 PFU of MHV68^{wt} and from uninfected mice. Copy number was determined from standard curves and normalized to the quantity of genomic DNA determined by amplification of RNase P and then represented as the number of genomes per nanogram of spleen cell DNA. Data shown were generated from two independent experiments.
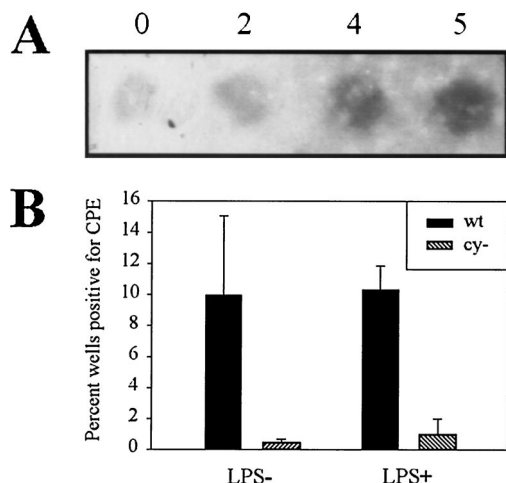
FIG. 7. Effect of LPS stimulation on MHV68^{cy-} reactivation. Spleen cells from mice infected with a mixture of 5×10^6 PFU of MHV68^{cy-} and of 5×10^4 PFU MHV68^{wt} were cultured in the presence or absence of LPS. RNA from spleen cells was extracted at the designated numbers of days after explantation. (A) An RNA dot blot from these samples was probed with murine cyclin D2. (B) The effect of LPS on reactivation was determined by scoring percent of wells positive for CPE attributable to MHV68^{wt} or MHV68^{cy-}.

may be qualitatively different from cellular cyclin. HHV8 and HVS v-cyclins preferentially bind CDK 6 and have extended substrate specificity which includes phosphorylation of a histone protein (15, 22). More recent investigations have demonstrated that HHV8- and HVS-encoded cyclins are resistant to inhibition by the CDK inhibitors, p16, p21, and p27 and that HHV8 cyclin can downregulate p27 (25, 46). Furthermore, HHV8 cyclin activates cyclin A expression in a manner that is distinct from cellular cyclin D or E (12). The usefulness of these alterations for the virus is not clear.

It is possible that MHV68-encoded cyclin may have evolved similar qualities distinct from cellular cyclin. Other possibilities might include an altered pattern of degradation through ubiquitination, or it may interact with proteins which modify its function in unexpected ways. Alternatively, the viral cyclin could be encoded by the virus so that the virus may control the timing of expression. This might be particularly true for reactivation if this event were to occur in quiescent cells unprepared for DNA replication. Our observation that the efficiency of MHV68^{cy-} reactivation is only slightly increased with LPS stimulation doesn't support this hypothesis, at least with respect to cyclin D2. To address this question more precisely, experiments are underway to place the open reading frame of the cellular D-type cyclins under the regulatory elements of v-cyclin in the viral genome.

The timing of cyclin expression is consistent with a role in reactivation. Results from us and others indicate that MHV68 v-cyclin is not expressed during latency and is expressed in lytic infection (37, 52). This contrasts with the expression pattern in HHV8, in which cyclin is expressed in a cell line derived from a latently infected primary effusion lymphoma (11). v-cyclin may have a different expression pattern in tumor cells than in cells in which the virus is normally latent. Genes like cyclin that have described roles in transformation may or may not have been selected in evolution for their transformative properties. If not, they have been selected for a different function in the life cycle of the virus and lead to transformation as a secondary effect or as a result of dysregulation of viral genes.

The role of cell cycle regulatory elements in herpes viral reactivation is not without precedent. Cell cycle proteins may

regulate herpesvirus latency and reactivation through distinct mechanisms. The alphaherpesvirus bovine herpesvirus expresses a single RNA during latency, the latency-related gene product. This RNA has been shown to bind to cyclin A and prevent cell cycle progression (33). In HSV, the immediate-early protein Vmw110 is required for efficient reactivation from latency and has also been shown to inhibit progression of transfected cells though mitosis and at the G_1/S phase border (16, 24, 57). Apparently paradoxically, it has also been shown to stabilize a D-type cyclin (21). The EBV-encoded gene product Zta, which contributes to the switch from latent to lytic gene expression, inhibits cell cycle progression prior to S phase (6, 10, 23). The apparent discrepancy in the stimulation and arrest of cell cycle could be explained in many cases by the possibility that genes expressed late in G_1 could be important for viral replication and reactivation. In any case, cell cycle positioning is an important element in regulating latency and reactivation from latency.

If latency is to have any value as a survival strategy the viral genome must persist intact so that at some later time a productive acute infection can the initiated. Gene products in EBV and HHV8 that trigger reactivation have been identified by utilizing latently infected cell lines (10, 42). While latently infected cell lines are useful for identification of proteins that trigger reactivation, they allow limited conclusions to be made about the role of these proteins in vivo. The observation that cyclin is required for reactivation in MHV68 will allow further investigation into the mechanism of reactivation of the gammaherpesviruses in vivo.

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