



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Regulation of the transcription and replication cycle of human cytomegalovirus is insensitive to genetic elimination of the cognate NF-kappaB binding sites in the enhancer

Citation for published version:

Gustems, M, Borst, E, Benedict, CA, Pérez, C, Messerle, M, Ghazal, P & Angulo, A 2006, 'Regulation of the transcription and replication cycle of human cytomegalovirus is insensitive to genetic elimination of the cognate NF-kappaB binding sites in the enhancer', *Journal of Virology*, vol. 80, no. 19, pp. 9899-904.
<https://doi.org/10.1128/JVI.00640-06>

Digital Object Identifier (DOI):

[10.1128/JVI.00640-06](https://doi.org/10.1128/JVI.00640-06)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Journal of Virology

Publisher Rights Statement:

Copyright © 2013 by the American Society for Microbiology.

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Regulation of the Transcription and Replication Cycle of Human Cytomegalovirus Is Insensitive to Genetic Elimination of the Cognate NF- κ B Binding Sites in the Enhancer

Montse Gustems, Eva Borst, Chris A. Benedict, Carmen Pérez, Martin Messerle, Peter Ghazal and Ana Angulo
J. Virol. 2006, 80(19):9899. DOI: 10.1128/JVI.00640-06.

Updated information and services can be found at:
<http://jvi.asm.org/content/80/19/9899>

These include:

REFERENCES

This article cites 28 articles, 18 of which can be accessed free at: <http://jvi.asm.org/content/80/19/9899#ref-list-1>

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), [more»](#)

Information about commercial reprint orders: <http://journals.asm.org/site/misc/reprints.xhtml>
To subscribe to to another ASM Journal go to: <http://journals.asm.org/site/subscriptions/>

Regulation of the Transcription and Replication Cycle of Human Cytomegalovirus Is Insensitive to Genetic Elimination of the Cognate NF- κ B Binding Sites in the Enhancer

Montse Gustems,¹ Eva Borst,^{2,3} Chris A. Benedict,⁴ Carmen Pérez,¹
Martin Messerle,² Peter Ghazal,⁵ and Ana Angulo^{1*}

Institut d'Investigacions Biomèdiques August Pi i Sunyer, 08036 Barcelona, Spain¹; Department of Virology, Hannover Medical School, 30625 Hannover, Germany²; Medical Faculty, University of Halle, 06120 Halle, Germany³; Division of Molecular Immunology, La Jolla Institute for Allergy and Immunology, San Diego, California 92121⁴; and Scottish Centre for Genomic Technology and Informatics, University of Edinburgh, Edinburgh EH16 4SB, United Kingdom⁵

Received 30 March 2006/Accepted 10 July 2006

The role of NF- κ B in regulating human cytomegalovirus (HCMV) replication and gene transcription remains controversial. Multiple, functional NF- κ B response elements exist in the major immediate-early promoter (MIEP) enhancer of HCMV, suggesting a possible requirement for this transcription factor in lytic viral replication. Here we demonstrate by generating and analyzing HCMVs with alterations in the MIEP-enhancer that, although this region is essential for HCMV growth, none of the four NF- κ B response elements contained within the enhancer are required for MIE gene expression or HCMV replication in multiple cell types. These data reveal the robustness of the regulatory network controlling the MIEP enhancer.

The major immediate-early promoter (MIEP) of human cytomegalovirus (HCMV) is responsive to a multitude of transcription factors and plays a pivotal role in initiating the viral transcription/replication cycle (7, 16; reviewed in references 22 and 23). Regulation of the MIEP has been postulated to be critical in determining HCMV permissiveness and the transition between latent and lytic infection. Thus, deciphering the molecular mechanisms of the MIEP regulation may reveal key control points contributing to HCMV pathogenesis.

The MIEP enhancer includes four cognate NF- κ B recognition sites, and NF- κ B activates MIEP transcription in transient-transfection assays (20, 25–27). HCMV infection results in rapid induction of cellular NF- κ B (19, 27, 30), and several groups have reported a potential contribution of NF- κ B to the replication strategy of HCMV through regulation of the MIEP (8, 13). In contrast, we and others have reported a neutral or even a negative role of NF- κ B activation on HCMV transcription/replication cycle in different cell types (3, 4, 11, 14, 15). However, the basis for these experimental discrepancies is currently unclear. Importantly, a direct test of the requirement for the MIEP NF- κ B binding sites in HCMV transcription/replication has still not been performed. Here we report on formally assessing the direct requirement of the cognate binding sites for NF- κ B in contributing to major immediate-early (MIE) transcription and viral growth.

As a first step toward understanding NF- κ B regulation of the HCMV MIEP, we deleted enhancer sequences from –52 to –667 (including all NF- κ B response elements), in HCMV AD169. A parental HCMV bacterial artificial chromosome (BAC) (5, 6) containing the E-GFP open reading frame

(ORF) under control of the murine cytomegalovirus (MCMV) MIEP (Fig. 1A, line 1) was used to construct two enhancerless HCMV recombinant mutants. In HCMVdE, MIEP sequences from –52 to –667 were removed (Fig. 1A, line 2), and in HCMVdE::Kan, enhancer sequences were replaced with a 1-kbp stuffer region to maintain the genomic spatial integrity of the *ie1/ie2* and *UL127* promoters (Fig. 1A, line 3). Once the integrity of constructed HCMV genomes was confirmed by restriction analysis (data not shown), they were transfected in MRC-5 fibroblasts. Three days posttransfection, ~100 single cells expressing green fluorescent protein (GFP) could be detected in all cultures (see Fig. 1B, panels D, G, and J). Cells transfected with the parental HCMV BAC yielded viral plaques (Fig. 1B, panel E) that progressed to complete cytopathicity (panel F), whereas cultures transfected with the enhancerless HCMV BACs did not result in viral spread (panels H, I, K, and L). These data indicate that deletion of the entire MIEP enhancer region of HCMV genome is lethal.

To verify that the generated HCMV mutants were defective due to deletion of the enhancer, the ability of an IE1/IE2 expression plasmid (pSVH) (29) to rescue replication was tested. Cotransfection of enhancerless BACs with pSVH resulted in spread of GFP-expressing virus to adjacent cells (Fig. 1B, panels M to O and P to R), ultimately leading to a complete cytopathic effect. In addition, a revertant HCMVdE virus was generated by the ET BAC mutagenesis method (5, 24) and shown to replicate with identical kinetics to the parental HCMV in MRC-5 cells (Fig. 1C). Thus, these results indicate that deleting the entire MIEP enhancer in HCMV AD169 abolishes lytic viral replication in cultured fibroblasts and are consistent with previous results resecting MIEP enhancer sequences in the Towne strain of HCMV (18, 21). Here, through the rescue experiments, we have eliminated the possibility that the replication defects seen in the enhancerless HCMV recombinants were due at least in part to alterations in other regions

* Corresponding author. Mailing address: Institut d'Investigacions Biomèdiques August Pi i Sunyer, C/ Villarroel 170, Barcelona 08036, Spain. Phone: 34 647 450269. Fax: 34 93 4021907. E-mail: aangulo@ub.edu.

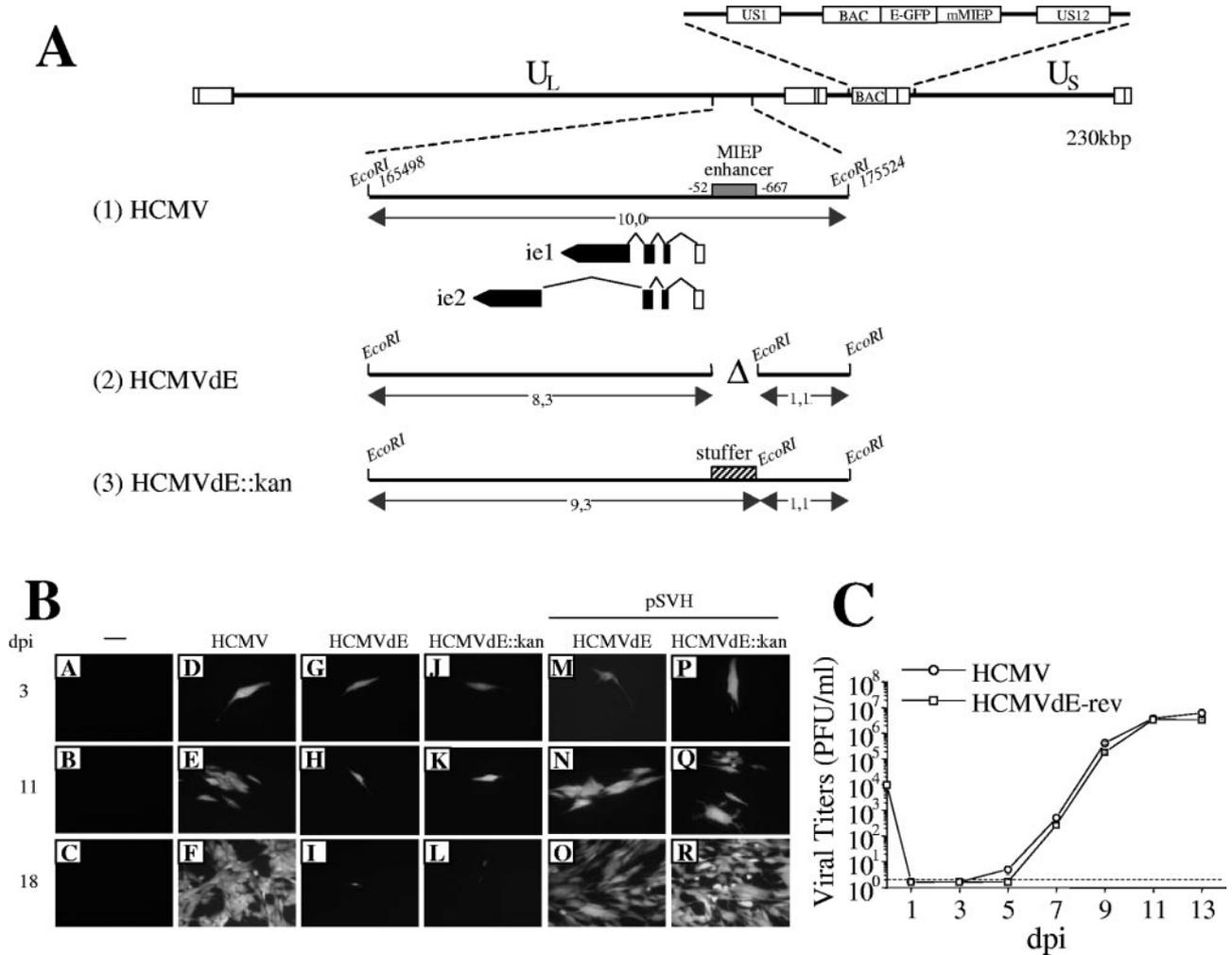


FIG. 1. Effect of a deletion of the entire MIEP enhancer on HCMV. (A) Schematic representation of the enhancerless HCMV BAC genomes constructed. The top line represents the map of the parental HCMV BAC genome with the US1-US12 region expanded above, indicating the BAC sequences and the E-GFP gene under control of the HCMV MIEP (mMIEP) that replace the US2-US11 region. Below, the EcoRI J fragment (nucleotide sequences from 165498 to 175524 of the HCMV genome [28]) encompassing the HCMV MIE region is enlarged (line 1), with the structures of the *ie1* and *ie2* transcripts indicated. The first noncoding exon of the *ie1/ie2* transcription unit is depicted as an open rectangle, and coding exons are shown solid. The shaded box marks the HCMV MIEP enhancer (extending from nucleotides -52 to -667 relative to the *ie1/ie2* HCMV transcription start site). The two enhancerless HCMV BAC genomes, HCMVdE and HCMVdE::Kan, were derived from the parental HCMV BAC by the ET BAC mutagenesis method (5, 6, 24). The deletion of the entire HCMV enhancer (nucleotide sequences from 174713 to 175328 of the HCMV genome) in HCMVdE (line 2) is indicated by a “Δ” symbol. In HCMVdE::Kan (line 3), the 1-kbp fragment from the kanamycin resistance gene that replaces the HCMV MIEP enhancer is represented by a cross-hatched box. A new EcoRI restriction site was introduced at the location of the enhancer deletion (in HCMVdE) or the stuffer insertion (in HCMVdE::Kan) to facilitate the characterization of the mutant genomes by an EcoRI restriction digestion (data not shown). Sizes of the natural and new EcoRI J DNA fragments for each recombinant BAC are indicated. The illustration is not drawn to scale. (B) Transfection of HCMV enhancerless genomes in cultured fibroblasts. MRC-5 cells were cotransfected by the calcium phosphate precipitation technique with 2 μg of either HCMV (D to F), HCMVdE (G to I and M to O), or HCMVdE::Kan (J to L and P to R) BAC DNAs together with 1 μg of a vector expressing the tegument protein pp71 (2), and when indicated with 1 μg of the HCMV IE1 and IE2 expression vector pSVH (29). GFP expression was detected by fluorescence microscopy at the indicated days after infection (dpi). Magnification, ×20. (C) Growth kinetics of HCMVdE-rev. MRC-5 cells were infected at a multiplicity of infection (MOI) of 0.025 with HCMV or HCMVdE-rev, a revertant of HCMVdE in which sequences from -52 to -667 of the MIEP were reintroduced in the HCMVdE genome by the ET BAC mutagenesis method. At the indicated days postinfection supernatants from the infected cultures were harvested, and titers were determined by standard plaque assays on MRC-5 cells. Each datum point represents the average and standard deviation from three separate cultures. The dashed line represents the limit of detection.

of their genome. These observations are also in line with the absolute requirement of the enhancer during the acute HCMV infection (17).

To directly analyze the role of NF-κB in regulating the MIEP, we specifically disrupted the four enhancer NF-κB binding sites. The point mutations introduced in the MIEP (3)

are illustrated in Fig. 2A. It should be noted that these four sites are the only NF-κB binding sites present in the MIE enhancer and that, in the context of the whole genome, NF-κB recognition sites have only been described to date in another location, the US3 immediately-early (IE) enhancer (9). Transfection of human U937 cells with reporter plasmids containing

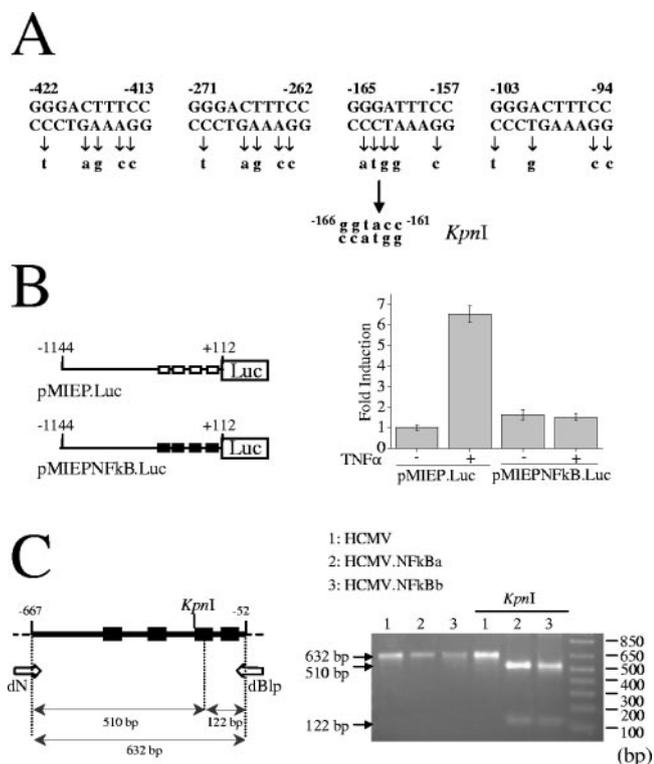


FIG. 2. Construction of recombinant HCMVs with point mutations in the NF-κB binding sites of the enhancer region. (A) The sequence and location of the four NF-κB binding sites within the HCMV MIEP are shown, and the point mutations introduced in each specific element are indicated below the wild-type sequence. Mutation of the NF-κB recognition site at position -157 to -165 generates a KpnI restriction site at position -161 to -166, which is shown. Coordinates refer to the HCMV *ie1/ie2* transcription start site. (B) The structures of the luciferase (Luc) reporter constructs pMIEP.Luc and pMIEPNFkB.Luc containing MIEP sequences from -1144 to +112 (relative to the HCMV *ie1/ie2* transcription start site) without (□) and with (■) the NF-κB binding sites disrupted, respectively, are shown. 5×10^5 U937 cells were electroporated with 2 μg of pMIEP.Luc or pMIEPNFkB.Luc, along with 0.6 μg of the internal control plasmid pRL-TK, and cultured for 46 h before the luciferase activity was assayed. Six hours before harvesting, cultures were treated with TNF-α (10 ng/ml; +) or vehicle (phosphate-buffered saline; -). The results are presented as the fold induction, taking “1” as the activity presented by the pMIEP.Luc in the absence of TNF-α. The values shown represent the average ± the standard deviation (bars) of four determinations. (C) Schematic diagram of the HCMV MIEP enhancer region, with the location of the four NF-κB binding sites marked by solid boxes. The KpnI restriction site at position -161 to -166 introduced when the NF-κB binding site located between positions -157 and -165 is mutated is indicated, as well as the sizes of the expected KpnI fragments derived from the PCR-amplified enhancer fragment with primers dN and dBlp (3) (flanking the enhancer and indicated with white arrows). Coordinates refer to the HCMV *ie1/ie2* transcription start site. To confirm the correct mutagenesis of the NF-κB binding sites, enhancer sequences were amplified from stocks of HCMV (lanes 1 and 4), HCMV.NFkBα (lanes 2 and 5), and HCMV.NFkBβ (lanes 3 and 6) by PCR. Marked by arrows are the amplified products before (lanes 1 to 3) or after (lanes 4 to 6) digestion with KpnI resolved by gel electrophoresis. Size markers are shown at the right margin.

either the wild-type or the NF-κB mutant MIEP revealed that tumor necrosis factor alpha (TNF-α)-induced MIEP activity was abolished in the mutant, whereas basal transcription was not affected (Fig. 2B). These results are consistent with previ-

ous studies indicating a role for NF-κB in regulating MIEP activity in transfection-based assays. To then test whether the four cognate NF-κB response elements in the MIEP are required for HCMV replication, two independent HCMV recombinants mutated in the NF-κB binding sites were generated (HCMV.NFkBα and HCMV.NFkBβ). HCMV.NFkBα was constructed by using the ET mutagenesis method and subsequent transfection of the mutant BAC in MRC-5 cells, whereas HCMV.NFkBβ was generated by cotransfecting MRC-5 cells with HCMVdE, pSVH, and pUChEnh.NFkB, a plasmid that carries HCMV sequences from nucleotide 171443 to 176844 (10) in which the four NF-κB elements of the enhancer were mutated. Infectious recombinant viruses were recovered from the transfections, used to infect new cell monolayers, and plaque purified three times. Viral stocks of both HCMV.NFkBα and HCMV.NFkBβ were prepared, their genomic integrity was verified by restriction digestion (data not shown), and the successful disruption of the enhancer NF-κB binding sites was confirmed by PCR analysis (Fig. 2C) and the nucleotide sequence of the MIEP region (data not shown).

We next assessed the effect of abrogating the enhancer NF-κB recognition sites on expression emanating from the MIEP in the context of the infection of human embryonic lung (HEL) fibroblasts with either wild-type or mutant viruses. As shown by real-time PCR, IE1 mRNA levels were comparable or even slightly higher (48 h postinfection [hpi]) in cells infected with HCMV.NFkBα and HCMV.NFkBβ than in parental HCMV-infected cells (Fig. 3A). In addition, HEL cells were infected throughout a 72-h period with the three viruses and subjected to Western blots by using a monoclonal antibody specific for the IE1 protein. We could not detect significant differences in the expression of the IE1 protein between HCMV and the HCMV.NFkB mutants at any of the time points analyzed (Fig. 3B). Furthermore, treatment of cells with the NF-κB inducer TNF-α (Fig. 3B) did not result in differential expression of IE1 in cells infected with wild-type or mutant viruses. Consequently, we conclude that the NF-κB binding sites in the MIEP do not significantly influence *ie1* gene transcription or expression in lytically infected fibroblasts.

To examine the requirement for the enhancer NF-κB binding elements on HCMV growth, kinetic studies with HCMV, HCMV.NFkBα, or HCMV.NFkBβ were performed on HEL fibroblasts. No difference in viral production at any time point was observed between the mutants and the parental virus (Fig. 4A). Treatment of cultures with TNF-α drastically inhibited HCMV growth, likely due to the induction of beta interferon (4; data not shown). In order to examine the replication capacity of HCMV.NFkB mutants in other cell types, we tested lung fibroblasts (MRC-5), U373 MG cells derived from glioblastoma, retinal pigment epithelium (RPE) cells, and differentiated embryonal carcinoma cells NTERA2 (NT2/D1). Importantly, parental and mutant viruses replicated in a comparable manner (Fig. 4B to E), strongly suggesting a neutral role of NF-κB for HCMV acute transcription/replication cycle in different cell types in culture.

The molecular details on the activation of the MIEP during HCMV infection are still poorly understood. In the present study, we demonstrate by generating and characterizing HCMV recombinants with alterations in the MIEP enhancer that (i) the enhancer region is necessary for HCMV growth

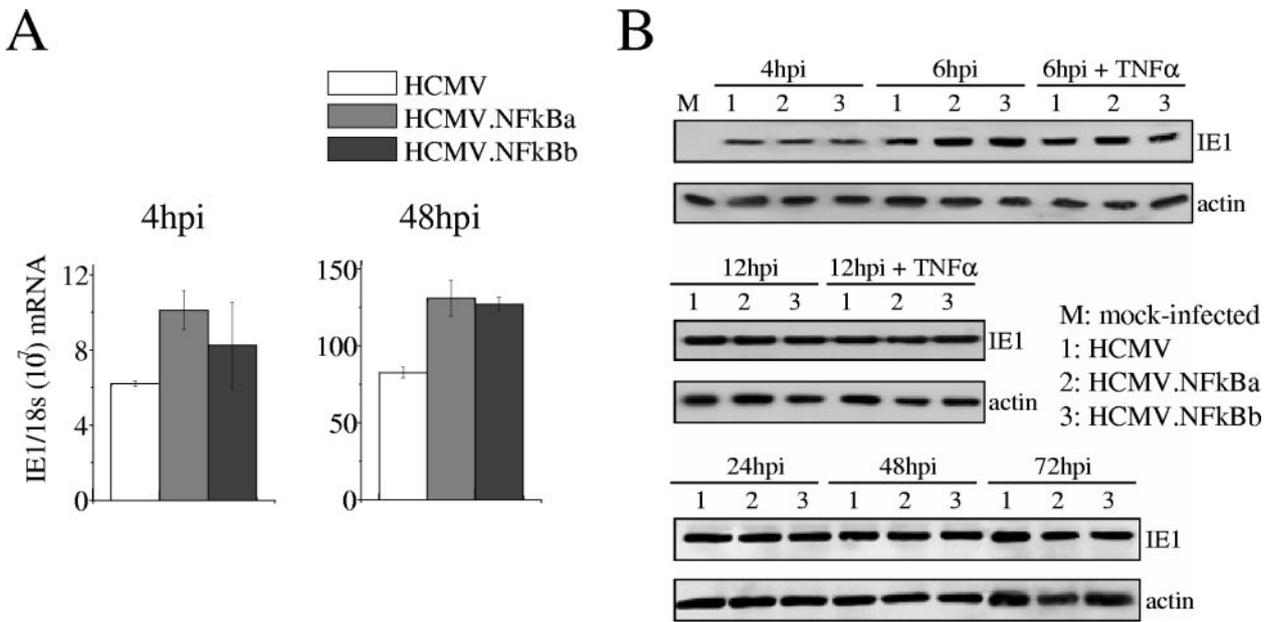


FIG. 3. Analysis of *ie1* gene expression in HCMV.NFkB-infected cells. (A) Real-time PCR analysis of *ie1* RNA expression in cells infected with HCMV.NFkB. HEL fibroblasts were infected at an MOI of 0.01 with HCMV, HCMV.NFkB, or HCMV.NFkBb and harvested at the time points after infection indicated for isolation of RNA and subsequent analysis by real-time PCR using primers within exon 4 of the HCMV *ie1* gene as previously described (3). The results are presented as the relative amount of *ie1* mRNA normalized to 18S rRNA, and error bars represent the standard errors of the means. (B) Expression kinetics of the IE1 protein by HCMV.NFkB mutants. HEL fibroblasts were mock infected or infected at an MOI of 0.6 (for the 4-, 6-, and 12-h time points) or 0.1 (for the 24, 48, and 72 h time points). Where indicated, cells were treated with 10 ng of TNF- α /ml 2 h before infection, during, and immediately after the adsorption period. At the indicated time (in hours) postinfection (hpi), samples were lysed, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 7% gels, transferred to nitrocellulose as previously described (1), and probed with an HCMV IE1 specific monoclonal antibody (MAB810; Chemicon, Temecula, CA). As an internal control, actin immunodetection was performed with a monoclonal antibody (A2066; Sigma, St. Louis, MO).

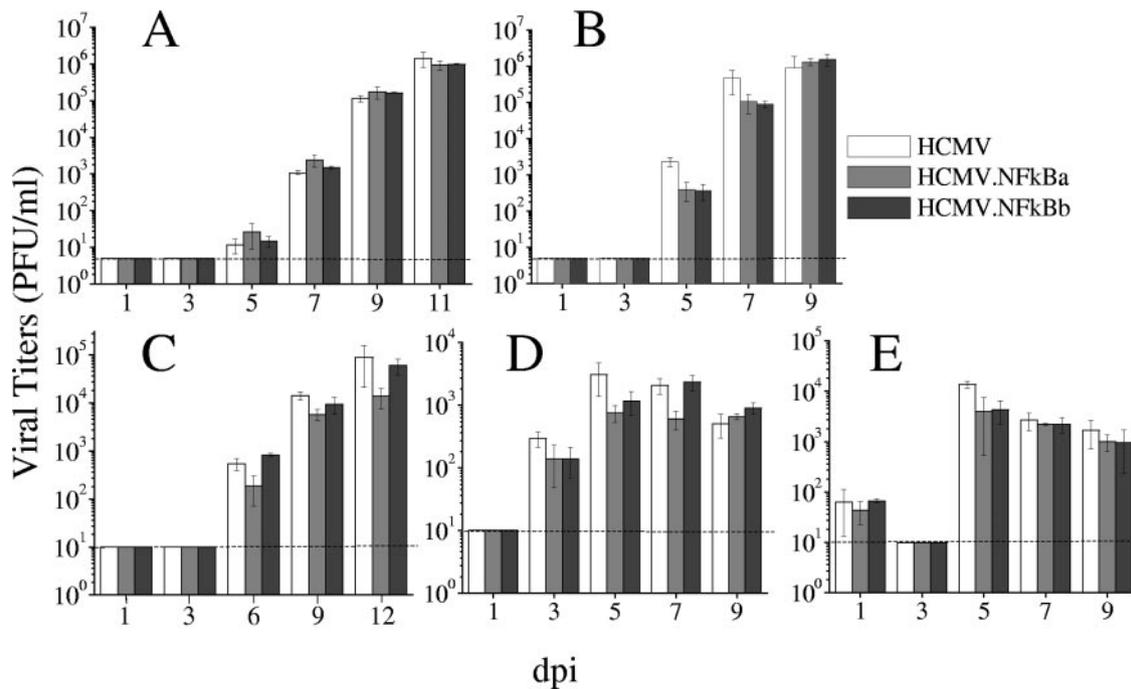


FIG. 4. Growth kinetics of HCMV.NFkB mutants. HEL (A), MRC-5 (B), RPE (C), U373 (D), and NT2/D1 cells (E; differentiated for 5 days with 10^{-5} M retinoic acid) were infected at an MOI of 0.025 (HEL and MRC-5) or 1 (RPE, U373, and NT2/D1, resulting in ca. 10 to 25% of GFP-positive cells in the culture at 48 hpi) with HCMV, HCMV.NFkB, or HCMV.NFkBb. At the indicated time in days postinfection, the amount of extracellular (HEL, MRC-5, and RPE) or cell associated (U373 and NT2/D1) infectious virus present in the cultures was determined by plaque titration assays on MRC-5 cells. Each datum point represents the average and standard deviation from three separate cultures. Dashed lines represent the limits of detection.

and (ii) the MIEP NF- κ B response elements do not contribute to lytic replication of HCMV in multiple cell types.

The involvement of NF- κ B activity on MIE expression and HCMV replication has been a controversial issue (3, 4, 8, 11–15). The discrepancy pivots around whether NF- κ B directly or indirectly influences HCMV transcription and/or replication. It is generally assumed in the field that the activation of NF- κ B results in the direct stimulation of the MIEP and, as a consequence, in increased, i.e., gene expression and viral replication. Our observations are in agreement with the fact that NF- κ B contributes to HCMV MIEP-enhancer activation in transient-transfection assays, as has been documented in a number of reports. However, we clearly show here that the cognate NF- κ B binding sites within the enhancer do not play a major independent role in the transcription/replication strategies of HCMV in a variety of cell types. We demonstrate in a direct manner that, in the context of the infection, the enhancer is insensitive to mutations in the NF- κ B binding sites, underlining a high level of robustness of the associated regulatory network controlling this region. While these results are in line with previous observations (3, 11, 14, 15), they are in contrast with others (8, 12, 13). In these apparently contradictory studies, a positive involvement of NF- κ B in MIEP transcription and HCMV replication has been observed by blocking NF- κ B activity using a variety of pharmacologic agents exhibiting a range of specificity and selectivity. These studies provide an indirect test and, moreover, the selectivity of the agents (e.g., aspirin or MG-132) used to inhibit NF- κ B signaling pathways should be taken in consideration. It must be also noted that although to date only NF- κ B sites have been found in the MIEP and in the US3 IE enhancer (9), the presence of additional NF- κ B responsive genes in the HCMV genome could account in part for some of the discrepancies found in different studies.

Our data do not exclude the possibility that NF- κ B may regulate HCMV MIE gene expression or growth in cell types not examined here or are required for in vivo replication and/or reactivation from latency. The fact that the viruses used in the present study derive from the HCMV laboratory strain AD169 prevented their analysis in other cell types more relevant for HCMV infection, including macrophages, endothelial, or dendritic cells. Mouse CMV recombinants containing the HCMV MIEP (with or without mutations in the NF- κ B response elements) replicate and establish latency in the mouse (1, 3; A. Angulo unpublished results), permitting us to explore these aspects in the context of an in vivo infection.

We thank Anett Rassmann for technical assistance.

This study was supported by grants from the Ministerio de Ciencia y Tecnología (MCYT; SAF2002-00270 and SAF2005-05633 to A.A.), the Wellcome Trust (to P.G.), and the American Heart Association (0330064N to C.A.B.) and by the Deutsche Forschungsgemeinschaft (SFB 587, project A13 to M.M.). A.A. is a fellow from the Ramón y Cajal program (MCYT).

REFERENCES

1. Angulo, A., M. Messerle, U. H. Koszinowski, and P. Ghazal. 1998. Enhancer requirement for murine cytomegalovirus growth and genetic complementation by the human cytomegalovirus enhancer. *J. Virol.* **72**:8502–8509.
2. Baldick, C. J., Jr., A. Marchini, C. E. Patterson, and T. Shenk. 1997. Human cytomegalovirus tegument protein pp71 (ppUL82) enhances the infectivity of viral DNA and accelerates the infectious cycle. *J. Virol.* **71**:4400–4408.
3. Benedict, C. A., A. Angulo, G. Patterson, S. Ha, H. Huang, M. Messerle, C. F. Ware, and P. Ghazal. 2004. Neutrality of the NF- κ B-dependent pathway for human and murine cytomegalovirus transcription and replication in vitro. *J. Virol.* **78**:741–750.
4. Benedict, C. A., T. A. Banks, L. Senderowicz, M. Ko, W. J. Britt, A. Angulo, P. Ghazal, and C. F. Ware. 2001. Lymphotoxins and cytomegalovirus cooperatively induce interferon-beta, establishing host-virus detente. *Immunity* **15**:617–626.
5. Borst, E. M., and M. Messerle. 2005. Analysis of human cytomegalovirus oriLyt sequence requirements in the context of the viral genome. *J. Virol.* **79**:3615–3626.
6. Borst, E., G. Hahn, U. H. Koszinowski, and M. Messerle. 1999. Cloning of the human cytomegalovirus (HCMV) genome as an infectious bacterial artificial chromosome in *Escherichia coli*: a new approach for construction of HCMV mutants. *J. Virol.* **73**:8320–8329.
7. Boshart, M., F. Weber, G. Jahn, K. Dorsch-Hasler, B. Fleckenstein, and W. Schaffner. 1985. A very strong enhancer is located upstream of an immediate-early gene of human cytomegalovirus. *Cell* **41**:521–530.
8. Caposio, P., M. Dreano, G. Garotta, G. Gribaudo, and S. Landolfo. 2004. Human cytomegalovirus stimulates cellular IKK2 activity and requires the enzyme for productive replication. *J. Virol.* **78**:3190–3195.
9. Chan, Y.-J., W.-P. Tseng, and G. S. Hayward. 1996. Two distinct upstream regulatory domains containing multicopy cellular transcription factor binding sites provide basal repression and inducible enhancer characteristics to the immediate-early IES (US3) promoter from human cytomegalovirus. *J. Virol.* **70**:5312–5328.
10. Chee, M., S. A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchison, T. Kouzarides, J. A. Martignetti, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barel. 1990. Analysis of the protein coding content of the sequence of human cytomegalovirus strain AD169. *Curr. Top. Microb. Immunol.* **154**:125–169.
11. Cinatl, J., S. Margraf, J.-U. Vogel, M. Scholz, J. Cinatl, and H. W. Doerr. 2001. Human cytomegalovirus circumvents NF- κ B dependence in retinal pigment epithelial cells. *J. Immunol.* **167**:1900–1908.
12. DeMeritt, I. B., J. P. Podduturi, A. M. Tilley, M. T. Nogalski, and A. D. Yurochko. 2006. Prolonged activation of NF- κ B by human cytomegalovirus promotes efficient viral replication and late gene expression. *Virology* **346**:15–31.
13. DeMeritt, I. B., L. E. Milford, and A. D. Yurochko. 2004. Activation of the NF- κ B pathway in human cytomegalovirus-infected cells is necessary for efficient transactivation of the major immediate-early promoter. *J. Virol.* **78**:4498–4507.
14. Eickhoff, J. E., and M. Cotten. 2005. NF- κ B activation can mediate inhibition of human cytomegalovirus replication. *J. Gen. Virol.* **86**:285–295.
15. Eickhoff, J., M. Hanke, M. Stein-Gerlach, T. P. Kiang, K. Herzberger, P. Habenberger, S. Muller, B. Klebl, M. Marschall, T. Stamminger, and M. Cotten. 2004. RICK activates a NF- κ B-dependent anti-human cytomegalovirus response. *J. Biol. Chem.* **279**:9642–9652.
16. Ghazal, P., and J. A. Nelson. 1993. Transcription factors and viral regulatory proteins as potential mediators of human cytomegalovirus pathogenesis, p. 360–383. *In* Y. Becker, G. Darai, and E.-S. Huang (ed.), *Molecular aspects of human cytomegalovirus diseases*. Springer-Verlag Publishers, Heidelberg, Germany.
17. Ghazal, P., M. Messerle, K. Osborn, and A. Angulo. 2003. An essential role of the enhancer for murine cytomegalovirus in vivo growth and pathogenesis. *J. Virol.* **77**:3217–3228.
18. Isomura, H., T. Tsurumi, and M. F. Stinski. 2004. Role of the proximal enhancer of the major immediate-early promoter in human cytomegalovirus replication. *J. Virol.* **78**:12788–12799.
19. Kowalik, T. F., B. Wing, J. S. Haskill, J. C. Azizkhan, A. S. Baldwin, and E. S. Huang. 1993. Multiple mechanisms are implicated in the regulation of NF- κ B activity during human cytomegalovirus infection. *Proc. Natl. Acad. Sci. USA* **90**:1107–1111.
20. Lee, Y., W.-J. Sohn, D.-S. Kim, and H.-J. Kwon. 2004. NF- κ B- and c-Jun-dependent regulation of human cytomegalovirus immediate-early gene enhancer/promoter in response to lipopolysaccharide and bacterial CpG-oligodeoxynucleotides in macrophage cell line RAW 264.7. *Eur. J. Biochem.* **271**:1094–1105.
21. Meier, J. L., and J. A. Pruessner. 2000. The human cytomegalovirus major immediate-early distal enhancer region is required for efficient viral replication and immediate-early gene expression. *J. Virol.* **74**:1602–1613.
22. Meier, J. L., and M. F. Stinski. 1996. Regulation of cytomegalovirus immediate-early genes. *Intervirology* **39**:331–342.
23. Mocarski, E. S., and C. T. Courcelle. 2001. Cytomegaloviruses and their replication, p. 2629–2673. *In* D. M. Knipe and P. M. Howley (ed.), *Fields virology*, 4th ed. Lippincott-Raven Publishers, Philadelphia, Pa.
24. Muyrers, J. P., Y. Zhang, V. Benes, G. Testa, W. Ansoorge, and A. F. Stewart. 2000. Point mutation of bacterial artificial chromosomes by ET recombination. *EMBO Rep.* **3**:239–243.
25. Prosch, S., K. Staak, J. Stein, C. Liebenenthal, T. Stamminger, H.-D. Volk, and D. H. Kruger. 1995. Stimulation of HCMV cytomegalovirus IE enhancer/

- promoter in HL-60 cells by TNF α is mediated via induction of NF- κ B. *Virology* **208**:197–206.
26. **Prosch, S., R. Wuttke, D. H. Kruger, and H.-D. Volk.** 2002. NF- κ B-A potential therapeutic target for inhibition of human cytomegalovirus (re)activation? *Biol. Chem.* **383**:1601–1609.
 27. **Sambucetti, L. C., J. M. Cherrington, G. W. G. Wilkinson, and E. S. Mocarski.** 1989. NF- κ B activation of the human cytomegalovirus enhancer is mediated by a viral transactivator and by T-cell stimulation. *EMBO J.* **8**:4251–4258.
 28. **Spector, D. H., L. Hock, and J. C. Tamashiro.** 1982. Cleavage maps for human cytomegalovirus DNA strain AD169 for restriction endonucleases EcoRI, BglII, and HindIII. *J. Virol.* **42**:558–582.
 29. **Stenberg, R. M., J. Fortney, S. W. Barlow, B. P. Magrane, J. A. Nelson, and P. Ghazal.** 1990. Promoter-specific trans activation and repression by human cytomegalovirus immediate-early proteins involves common and unique protein domains. *J. Virol.* **64**:1556–1565.
 30. **Yurochko, A. D., E. S. Hwang, L. Rasmussen, S. Keay, L. Pereira, and E. S. Huang.** 1997. The human cytomegalovirus UL55 (gB) and UL75 (gH) glycoprotein ligands initiate the rapid activation of Sp1 and NF- κ B during infection. *J. Virol.* **71**:5051–5059.