Originally published as:

Schumacher, U., Handke, W., Jurak, I., Brune, W.
Mutations in the M112/M113-coding region facilitate murine cytomegalovirus replication in human cells
(2010) Journal of Virology, 84 (16), pp. 7994-8006.

DOI: 10.1128/JVI.02624-09

Mutations in the M112/M113-Coding Region Facilitate Murine Cytomegalovirus Replication in Human Cells

Uwe Schumacher, Wiebke Handke, Igor Jurak and Wolfram Brune*

Division of Viral Infections, Robert Koch Institute, Nordufer 20, 13353 Berlin, Germany

*Corresponding author. Present address: Heinrich Pette Institute for Experimental Virology and Immunology at the University of Hamburg, Martinstr. 52, 20251 Hamburg, Germany.
Phone: 49 40 48051-351. Fax: 4940 48051-103. E-mail: wolfram.brune@hpi.uni-hamburg.de.

Cytomegaloviruses, representatives of the Betaherpesvirinae, cause opportunistic infections in immunocompromised hosts. They infect various cells and tissues in their natural host but are highly species specific. For instance, human cytomegalovirus (HCMV) does not replicate in mouse cells, and human cells are not permissive for murine cytomegalovirus (MCMV) infection. However, the underlying molecular mechanisms are so far poorly understood. In the present study we isolated and characterized a spontaneously occurring MCMV mutant that has gained the capacity to replicate rapidly and to high titers in human cells. Compared to the parental wild-type (wt) virus, this mutant formed larger nuclear replication compartments and replicated viral DNA more efficiently. It also disrupted promyelocytic leukemia (PML) protein nuclear domains with greater efficiency but caused less apoptosis than did wt MCMV. Sequence analysis of the mutant virus genome revealed mutations in the M112/M113-coding region. This region is homologous to the HCMV UL112-113 region and encodes the viral early 1 (E1) proteins, which are known to play an important role in viral DNA replication. By introducing the M112/M113 mutations into wt MCMV, we demonstrated that they are sufficient to facilitate MCMV replication in human cells and are, at least in part, responsible for the efficient replication capability of the spontaneously adapted virus. However, additional mutations probably contribute as well. These results reveal a previously unrecognized role of the viral E1 proteins in regulating viral replication in different cells and provide new insights into the mechanisms of the species specificity of cytomegaloviruses.

Cytomegaloviruses (CMVs) are prototypes of the subfamily of the Herpesviridae. Representatives of this subfamily have been identified in various animal species, and these viruses cause similar symptoms in their respective hosts (36). HCMV is an opportunistic pathogen that causes generally mild infections in people with a fully functional immune system. However, this virus is also responsible for serious medical problems, particularly in newborns and immunocompromised patients (39).

Since their first isolation in cell culture, CMVs have been recognized as highly species specific (57). They replicate only in cells of their own or a closely related species. For instance, simian CMV can replicate in human fibroblasts (32), and HCMV can replicate in chimpanzee skin fibroblasts (41). Similarly, murine cytomegalovirus (MCMV) productively infects rat cells (7, 46), but a rat cytomegalovirus did not replicate in murine fibroblasts (7). However, cells of other more distantly related species are usually nonpermissive to infection. Several studies have shown that CMVs can enter cells of other species and express a subset of viral genes (19, 20, 29, 32). This finding has led to the conclusion that the restriction to CMV replication in nonpermissive cells is associated with a postpenetration block to viral gene expression and DNA replication but not due to a failure to enter the cell (36).

Recently, we picked up on this topic and tried to gain new insights into the molecular mechanisms underlying the species specificity of CMVs. We showed that CMVs of mice and rats induce apoptosis when they infect human fibroblasts or retinal epithelial cells (26). The induction of apoptosis prevented a sustained replication of these viruses in human cells and reduced progeny production to insignificant levels. When apoptosis was inhibited by the overexpression of Bcl-2 or a functionally similar protein, MCMV was able to replicate to substantial titers in human cells. These results indicated that the induction of apoptosis is an important limitation to cytomegalovirus cross-species infections (26). However, the fact that MCMV replication in human cells in the presence of apoptosis inhibition was somewhat delayed and less efficient than that in murine cells indicated that other limiting factors likely

exist. Another study suggested that MCMV can replicate to low levels in human cells with the help of HCMV immediate- early 1 (IE1) and HCMV tegument proteins (50).

In the present study, we describe the isolation and characterizationof a mutant MCMV that has spontaneously acquired the ability to replicate rapidly and to high titers in human retinal pigment epithelial (RPE-1) cells. We show that this virus induces less apoptosis and replicates its DNA faster than the parental wild-type (wt) MCMV. Moreover, the mutant virus disrupts intranuclear sites of intrinsic antiviral defense more efficiently than the wt virus. Sequence analysis of the human cell-adapted MCMV strain revealed several alterations, including mutations in the M112/M113- coding region. By targeted mutagenesis we showed that mutations in M112/M113 are sufficient to facilitate MCMV replication in human cells. However, additional mutations most likely contribute to the remarkably efficient replication of the adapted strain.

Materials and methods

Cells and viruses.
hTERT RPE-1 (ATCC CRL-4000) cells are telomeraseimmortalized retinal pigment epithelial cells (6). MRC-5 cells (ATCC CCL-171) are primary human embryonic lung fibroblasts. 10.1 cells are spontaneously immortalized murine embryonic fibroblasts (22). Primary human foreskin fibroblasts (HFFs) were a gift from Jens von Einem (University of Ulm, Ulm, Germany), and primary human umbilical vein endothelial cells (HUVECs) were provided by Regine Heller (University of Jena, Jena Germany). HUVECs were cultured in complete endothelial cell growth medium (Promocell). All other cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37°C and 5% CO_2 in a humidifiedatmosphere. wt and mutant MCMVs were propagated in 10.1 fibroblasts as described previously (26). MCMV/h was propagated in human RPE-1 cells. Bacterial artificial chromosome (BAC)-derived HCMV strains AD169 and TB40/E (23, 45) were propagated on MRC-5 fibroblasts. MCMV titers were determined on mouse 10.1 cells and HCMV titers were determined on MRC-5 cells by using the median tissue culture infective dose ($TCID_{50}$) method (33).

Plasmids and transfection.
The M112/M113-coding sequence plus 50-nucleotide (nt) extensions on either side (for homologous recombination) were PCR amplified from wt MCMV and MCMV/h by using primers M112_Not_fwd (GTT-CCT-GCG-GCC-GCG-GTA-GAT-TAC-GTG-CCC-ACT-TTTC) and M113_Pst_Mfe_rev (CAA-CTG-CAG-TCA-GTT-AGA-GTT-TAC-AGA-GCATCA-TTT-CTT-TAT-CCA-TCT-TTCAATTGA-GAT-CAA-TTA-AGA-TCATCG-AACACA). The amplification products were cleaved with NotI and PstI (underlined), cloned into pBluescript KS(+), and verified by sequencing. An Flp recombination target (FRT)-flanked kanamycin resistance gene (excised with EcoRI from pSLFRTkn [5]) was cloned into the MfeI site (underlined) located behind the M112/M113-coding region, yielding plasmids pBS-M112-kn and pBSM112*mut*-kn. For protein expression by transient transfection, the M112/M113- coding sequence (without the kan cassette) was excised with NotI and PstI from the pBluescript backbone and inserted into pFlagCMV5a (Sigma). Plasmid pp89UC, encoding MCMV IE1, and pcDNA-IE1, encoding HCMV IE1, were kindly provided by Martin Messerle (Hannover Medical School, Hannover, Germany) and Michael Nevels (University of Regensburg, Regensburg, Germany), respectively. The green fluorescent protein (GFP) expression plasmid pEGFP-C1 was obtained from Clontech. Plasmid transfections were done by using Polyfect transfection reagent (Qiagen).

Generation of gene knockdown cells.
Plasmids carrying the short hairpin RNA (shRNA) expressing retroviral vectors pHM2237/empty, pHM2238/shC, pHM2240/shDaxx1, and pHM2243/shPML2 (52) were kindly provided by Thomas Stamminger and Nina Tavalai (University of Erlangen, Erlangen, Germany). Retroviruses were produced by transfecting the vector plasmids into the Phoenix Ampho packaging cell line by calcium phosphate transfection, as described previously (48), and were used for the transduction of RPE-1 cells. Nonclonal cell populations stably expressing shRNA were obtained by selection with 5 μg/ml puromycin for 7 days.

Antibodies and immunodetection.
Monoclonal antibodies against the MCMV IE1 (CROMA101) and early 1 (E1) (CROMA103) proteins were provided by Stipan Jonjic (University of Rijeka, Rijeka, Croatia), monoclonal antibodies against M44 (3B9.22A) and MCMV glycoprotein B (gB) (2E8.21A) were provided by Lambert Loh (University of Saskatchewan, Saskatoon, Saskatchewan, Canada), and monoclonal antibodies against HCMV IE1 (1B12) were provided by Thomas Shenk (Princeton University, Princeton, NJ). A polyclonal rabbit antiserum against MCMV IE3 was provided by Eva Borst (Hannover Medical School, Hannover, Germany). Antibodies recognizing the promyelocytic leukemia (PML) protein (H-238; Santa Cruz Biotechnology), hDaxx (E94; Epitomics), and β-actin (AC-74; Sigma) were purchased from the indicated suppliers.

For immunofluorescence analyses cells were seeded onto coverslips on the day before infection/transfection. After 24 h, cells were washed twice with phosphatebuffered saline (PBS), fixed for 30 min at 4°C with 4% paraformaldehyde, neutralized with 50 mM ammonium chloride, permeabilized with 0.3% Triton X-100, and blocked with 0.2% cold-water fish gelatin (Sigma). Proteins of interest were detected by indirect immunofluorescence using secondary antibodies coupled to Alexa Fluor 568 or Alexa Fluor 488 (Invitrogen). Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI). Confocal laser scanning microscopy was performed by using a Zeiss LSM510 Meta microscope. To analyze PML disruption or the formation of replication compartments, at least three different experiments were done, and a minimum of 150 infected cells from each experiment were evaluated.

For Western blot analyses, cells were infected at a multiplicity of infection (MOI) of 5 $TCID_{50}$/cell, harvested at the indicated time points, and lysed with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1% Na-deoxycholate 1% Triton X-100, and 0.1% SDS. Proteins samples were separated by SDS-PAGE and transferred onto positively charged nitrocellulose membranes. Proteins of interest were detected by using protein-specific primary antibodies, horseradish peroxidase-coupled secondary antibodies (Dako Cytomation), and enhanced chemiluminescence (ECL) reagents (Amersham).

BAC mutagenesis.
All recombinant viruses were constructed on the basis of MCMV-GFP (9) by using BAC technology (11). To construct the ΔM112/M113 mutant, a zeocin resistance gene was PCR amplified by using primers M112_zeo_fwd (5'-ACG-TGC-CCA-CTT-TTC-TCG-TCG-CGA-CCG-GTGAAA-AGA-CCT-TCG-TTC-GGA-CCT-gtt-gac-aat-taa-tca-tcg-gcat-3) and M113_zeo_rev (5'-AGT-CAG-TTA-GAG-TTT-ACA-GAG-CAT-CAT-TTCTTT-ATC-CATCTTT-CAT-GAG-At-cag-tcc tgc-tcc-tcg-gcca-3) to introduce 50-nt homology arms (shown in uppercase type) upstream and downstream of the M112/M113-coding region. This linear PCR fragment was used for homologous recombination in Escherichia coli strain DY380 containing the MCMVGFP BAC (10). Mutant BACs were analyzed by restriction digestion and agarose gel electrophoresis. In a second step, linear fragments containing the wt or mutant M112/M113-coding sequence and the kanamycin cassette were excised with NotI and PstI from plasmids pBS-M112-kn and pBS-M112mut-kn, respectively, and used for homologous recombination. Clones were selected with kanamycin, and recombinant BACs were analyzed by restriction digestion. The kanamycin cassette was removed by using Flp recombinase as previously described (10). An independent M112/M113 mutant virus was constructed by using the *gal*K system (56). Briefly, an Δ M112/M113 mutant was constructed as described above by using *gal*K instead of zeo for positive selection. In a second step, the mutant M112/M113 sequence was reinserted by using *gal*K for negative selection. Mutant and control viruses were reconstituted by transfecting purified BAC DNA into mouse fibroblasts using Polyfect transfection reagent (Qiagen).

Genome sequencing.
MCMV/h was derived from a single plaque of infected RPE-1 cells and was passaged continuously on RPE-1 cells. For the preparation of MCMV/h virion DNA, viral particles in the supernatant of infected RPE-1 cultures were pelleted by centrifugation for 3 h at 25,000 x g. Pellets were resuspended in a solution containing 10 mM Tris-HCl (pH 7.8), 5 mM EDTA, and 0.5% SDS; digested overnight with 50 µg/ml proteinase K at 56°C; and finally subjected to phenol-chloroform extraction and DNA precipitation. Purified DNA was analyzed by restriction digestion. High-quality virion DNA was sent to Macrogen for shotgun sequencing. The resulting contigs were aligned, and the remaining gaps were filled by primer walking. In this way, almost the entire MCMV/h genome was sequenced save for a few kilobases at the genome termini.

Growth kinetics.
For growth kinetics, cells were seeded into six-well dishes and infected with the viruses of interest. At 4 h postinfection (hpi), the medium was removed, cells were washed twice with PBS, and fresh medium was added. At the indicated time points, titers in the supernatant were determined by using the $TCID_{50}$ method. All growth kinetic experiments were done in triplicate.

Southern blot analysis and real-time PCR.
Cells were infected at an MOI of 5 $TCID_{50}$/cell for 4 h. At the indicated time points, adherent and detached cells were collected, and complete genomic DNA was extracted by proteinase K digestion and phenol-chloroform extraction. For slot blot analysis, 1 g of DNA of each sample was denatured for 10 min at 95°C in 6 SSC (0.9MNaCl, 90 mM Na-citrate [pH 7.0]), cooled rapidly on ice, transferred onto a nylon membrane by vacuum suction using a slot blot apparatus (Roth), and fixed by using a UV cross-linker (Stratagene). For Southern blot analysis, equal amounts of DNA were digested with EcoRI, separated by gel electrophoresis, and blotted onto a nylon membrane. Hybridization with a digoxigenin (DIG)-labeled MCMV M45 probe and detection by ECL were done by using a DIG-High Prime DNA labeling and detection kit (Roche).

For real-time PCR analysis cells were infected at an MOI of 0.5 $TCID_{50}$/cell, and DNA was extracted at the indicated time points by using the DNeasy blood and tissue kit (Qiagen). A SYBR green PCR master mix (ABI Applied Biosystems) was used for the quantitative detection of the cellular myc gene and the viral IE1 gene. The appropriate primers were described previously (8, 55). For the determination of viral and cellular genome copies, defined dilutions of plasmids containing the cloned PCR products were used. Genome copies were determined in triplicates from three separate infection assays.

Cell viability and cell death assays.
Cell viability was measured by using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium (MTS) assay (CellTiter 96 AQueous; Promega). Mean values and standard deviations of data from at least six parallel experiments are shown. Significance levels were calculated by using a Student's t test. To analyze DNA fragmentation as a sign of apoptosis, cells were grown on coverslips and infected at an MOI of 5 $TCID_{50}$/cell. Nuclear DNA fragmentation was detected by using a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling (TUNEL) assay (TMR red in situ cell death detection kit; Roche) and evaluated by using a Zeiss Axiovert 200 M epifluorescence microscope.

Results

Isolation of a mutant MCMV spontaneously adapted to human cells.
In a previous study we showed that MCMV does not replicate to significant levels in human cells and that infected human cells undergo apoptosis. The inability of MCMV to replicate and spread in human cells could be overcome by overexpressing a Bcl-2-like antiapoptotic protein (26). During one of our experiments, we fortuitously observed the spontaneous outgrowth of a rapidly spreading virus in a cell culture dish of human retinal pigment epithelial (RPE-1) cells that had been infected 2 weeks earlier with MCMV-GFP, a GFP-expressing MCMV variant. We named this spontaneously arisen mutant MCMV/h to indicate its adaptation to human cells. MCMV/h expressed GFP, caused a typical cytopathic effect (CPE), formed plaques, and could be serially passaged on RPE-1 cells. Moreover, it grew much faster and to higher titers than MCMV-bclXL (26), a mutant MCMV that overexpresses Bcl-x_L, in RPE-1 cells, human embryonic lung fibroblasts (MRC-5), human foreskin fibroblasts (HFFs), and human umbilical vein endothelial cells (HUVECs) (Fig. 1A to F). In MRC-5 fibroblasts, MCMV/h did not replicate as efficiently as in RPE-1 cells upon low-MOI infection (Fig. 1D). By microscopic inspection we observed that MCMV/h was capable of spreading from the initially infected fibroblasts to neighboring cells, forming foci and even small plaques of infected (GFPpositive) cells. After a few days, these foci stopped expanding and gradually regressed (data not shown). The same replication phenotype was observed for HFFs, and the virus replicated to almost identical titers in these cells (data not shown).

The reason for this limited replication in human fibroblasts after low-MOI infection is so far unknown. In murine fibroblasts, however, the virus replicated to titers similar to those of

parental MCMV-GFP even after low-MOI infection (Fig. 1G). MCMV/h replicated only transiently in HUVECs after high- MOI infection (Fig. 1F) and to titers at or below the detection limit after low-MOI infection (data not shown). Virion DNA was extracted from an MCMV/h stock grown on RPE-1 cells and subjected to EcoRI and NheI restriction. The restriction patterns of MCMV/h DNA were very similar to the patterns of MCMV-GFP. However, a few differences were evident (Fig. 1H), suggesting that genetic alterations had occurred. The transfection of purified MCMV/h DNA into RPE-1 cells resulted in a reconstitution of infectious MCMV/h. This largely ruled out the possibility that the replication of MCMV/h in human cells was caused by the presence of an undetected helper RNA virus, as RNA viruses would not withstand the DNA purification procedure. To exclude a possible contamination with HCMV, BAC DNA of HCMV laboratory strain AD169 was included. The digestion pattern of MCMV/h is completely different from that of AD169. Hence, the adapted virus is not an HCMV.

Phenotypic characterization of MCMV/h.

We have previously described that MCMV induces apoptosis in infected human cells (26). Compared to wt MCMV, MCMV/h induced less apoptosis, as seen morphologically by microscopic inspection as well as by analyzing nuclear DNA fragmentation with a TUNEL assay (Fig. 2A and B). MCMV/h decreased the viability of infected RPE-1 cells, as measured by an MTS assay, but the loss of viability was less pronounced than that after infection with the parental virus, MCMV-GFP (Fig. 2C). The viability of murine fibroblasts remained largely unaltered between 24 and 72 hpi, no matter whether the cells had been infected with wt or mutant MCMVs (Fig. 2D). Next, we analyzed viral gene expression in human and murine cells. The expressions of the immediate-early 1 (IE1), early 1 (E1), M44, and glycoprotein B (gB) proteins as representatives
of the immediate-early, early, early-late, and late kinetic classes, respectively, were measured by Western blotting at different times postinfection. In murine fibroblasts, viral protein expression levels in cells infected with MCMV/h were very similar to those in cells infected with the parental virus with the exception of gB, whose expression level rose slightly earlier in MCMV/h-infected cells (Fig. 3A). In RPE-1 cells, the MCMV/h protein expression pattern was quite similar to the one observed for 10.1 fibroblasts. However, M44 expression was delayed in RPE-1 cells, and gB expression was lower at 24 hpi. In contrast, viral protein expression vanished in wt MCMV-infected cells between 48 and 72 hpi, because infected cells had died (Fig. 3A), consistent with previous observations (26). Viral DNA replication was observed only transiently in wt MCMV-infected RPE-1 cells but occurred efficiently in MCMV/h-infected cells, as determined by slot blot analysis and real-time PCR (Fig. 3B and C).

Sequence analysis of MCMV/h reveals genetic alterations.

We wondered whether a genetic alteration or an epigenetic modification of the MCMV genome was responsible for the ability of MCMV/h to replicate efficiently in human cells. As MCMV/h retained its ability to replicate in RPE-1 cells even after repeated passage in murine fibroblasts (data not shown), we considered it unlikely that an epigenetic modification, which might have occurred in human cells, was responsible. Moreover, the changes in the restriction pattern of MCMV/h compared to the expression pattern of the parental virus (Fig. 1H) suggested that the phenotype could have been caused by one or more mutations in the viral genome. As the sequencing of a few candidate genes (such as genes with known antiapoptotic functions) did not reveal any differences from wt MCMV, we decided to have the whole MCMV/h genome sequenced. The MCMV/h sequence was then compared to the previously reported MCMV Smith sequence (42). The results of this comparison are listed in Table 1. The most striking alteration was a 10.3-kb deletion (nt 9561 to 19848, open reading frames [ORFs] m10 to m18) combined with a 12.5-kb inverted duplication of ORFs m58 to m73 (nt 91689 to 104152). In addition, several point mutations and small deletions were detected. Some of these were also found in the parental MCMV BAC, suggesting that they are probably not responsible for the phenotype of MCMV/h. As MCMV/h originated from a single plaque of infected RPE-1 cells and was passaged repeatedly on the same cell line, we assumed that MCMV/h represents a single mutant clone. However, we did not subject this virus to numerous rounds of plaque purification, which would be necessary to formally exclude the possibility that MCMV/h consists of more than one mutant clone.

To analyze which of the detected mutations contributed to the extended-host-range phenotype of MCMV/h, we decided to engineer selected mutations into the wt MCMV-GFP genome. The deletion of the region of m10 to m18 did not facilitate MCMV replication in RPE-1 cells (data not shown), indicating that this region does not contain a gene that actively restricts MCMV's host range. The reconstruction of the duplication spanning ORFs m58 to m73 is technically very difficult and was therefore deferred.

Mutations in M112/M113 facilitate MCMV replication in RPE-1 cells.
Next, we focused on the mutations in M112/M113. This region encodes the E1 proteins, which exist in at least four differentially spliced isoforms (13, 16) (Fig. 4A). It is highly homologous in its structure to the UL112-113 region of human cytomegalovirus (16, 47, 60). Although the mechanisms of action of the E1 proteins have not been fully elucidated, a number of studies have shown that they play an important role in the formation of nuclear replication compartments (4, 38, 40), in viral DNA replication (37, 61), and in the regulation of gene expression (24, 28, 49). Hence, we thought that the mutations in M112/M113 might play a role in the ability of MCMV/h to replicate in human cells.

MCMV/h contains a 9-nucleotide deletion in M112/M113 that should result in a 3-amino-acid deletion affecting all four E1 isoforms (Table 1 and Fig. 4A). In addition, two point mutations were present, which were predicted to cause amino acid exchanges only in the large 87-kDa isoform. A third point mutation at position 163813 is likely silent. To test whether these mutations influence MCMV replication in RPE-1 cells, we first deleted the entire E1-coding region from the MCMVGFP BAC as shown in Fig. 4B. We then reinserted either the wt or the mutant M112/M113 sequence. The resulting BACs were named RevM112 and M112mut, respectively. The recombinant MCMV BACs were verified by restriction digestion (Fig. 4C) and transfected into fibroblasts to obtain recombinant viruses. The M112mut and RevM112 viruses were easily reconstituted, but numerous attempts to reconstitute the M112/M113 deletion mutant were unsuccessful. This indicated either that the E1 proteins are essential for MCMV replication or that viral replication is so severely compromised in their absence that a deletion mutant can be regenerated only in complementing cells. RevM112 and M112mut replication in murine fibroblasts was indistinguishable from that of the parental virus (data not shown). RevM112 also behaved like the parental wt virus in that it did not replicate in RPE-1 cells. In contrast, M112mut replicated to high titers in RPE-1 cells (Fig. 5A).

However, it did not replicate as rapidly as MCMV/h, suggesting that additional mutations present in MCMV/h must contribute to its highly efficient replication in human cells. To exclude the possibility that the FRT site, which remained behind the M112/M113-coding region (Fig. 4B), had a negative influence on the replication of M112mut, we constructed an independent M112/M113 mutant MCMV using the *gal*K system (56). The resulting virus replicated with the same kinetics in RPE-1 cells as M112mut (data not shown), indicating that the FRT site did not have a detrimental effect. We also tested to which extent the constructed viruses induced cell death in RPE-1 cells. Again, RevM112 behaved like the parental wt virus, and M112mut displayed an intermediate phenotype; it induced apoptosis less efficiently than wt MCMV but more efficiently than MCMV/h (Fig. 5B). Next, we asked whether the mutations in M112/M113 had an impact on the abundance of individual E1 protein isoforms in infected RPE-1 cells. As shown in Fig. 6A, the abundances of the 36- and 38-kDa E1 proteins were similar for all viruses up to 48 hpi. The 33-kDa isoform was not detected by the CROMA103 antibody, presumably because CROMA103 recognizes an epitope encoded by exon 2. The expression of the 87-kDa E1 protein was maintained up to 96 hpi in cells infected
with MCMV/h or the M112mut virus but was reduced at 48 hpi and almost absent at later times in cells infected with wt MCMV or the revertant, RevM112. As the large E1 isoform was previously reported to control the repressive effect of IE3 on the major immediate-early (MIE) promoter (49), we tested whether the loss of the 87-kDa E1 protein correlated with a educed level of expression of the MIE proteins IE1 and IE3. No obvious differences in the abundances of IE1 and IE3 were detected up to 24 hpi (Fig. 6A), but at later times (particularly at 72 and 96 hpi), the IE1 and IE3 levels were reduced. However, it is difficult to determine to which extent this effect was caused by the 87-kDa E1 protein, because the onset of cell death, which starts at around 48 hpi and is more pronounced in wt- and RevM112-infected cells, impairs viral protein and DNA levels in general.

The E1 proteins form intranuclear structures that also contain IE3 and viral DNA polymerase and accumulate viral DNA (4, 49). Therefore, they are considered to represent replication compartments. They start out as small punctate foci that coalesce to a large lobulated compartment at late times after infection (13, 40). To test whether wt and mutant MCMVs differ in their abilities to form replication compartments, we analyzed the distributions of the E1, IE3, and M44 proteins in infected RPE-1 cells by immunofluorescence. In all cases, IE3 colocalized in nuclear replication compartments with the DNA polymerase processivity factor M44 and the E1 proteins, but there were clear differences in the sizes of the replication ompartments (Fig. 6B and C). A statistical evaluation revealed that the majority of cells infected with MCMV/h contained large lobulated replication compartments, whereas only
small punctate structures were found in the vast majority (> 80%) of cells infected with wt MCMV or the revertant, RevM112 (Fig. 6D). As in previous experiments, the constructed M112mut mutant showed an intermediate phenotype, with large lobulated replication compartments in approximately

40% of infected RPE-1 cells and small punctate replication foci in approximately 60% of infected RPE-1 cells.

HCMV and MCMV DNA replication compartments initiate from the periphery of promyelocytic leukemia protein-associated nuclear bodies (PML bodies, also known as ND10) (4, 49). The PML bodies contain proteins such as PML, Daxx, and Sp100, which can function as transcriptional repressors (18, 34, 53). At early times after CMV infection, PML bodies are dispersed, and the PML protein is found diffusely distributed throughout the nucleus (3, 21, 31, 44, 58). ND10 disruption has been correlated with efficient viral gene transcription and DNA replication (2). Hence, we wondered whether MCMV was capable of disrupting PML domains in human RPE-1 cells. As shown in Fig. 7A and B, ND10 disruption was observed for only a minority of wt MCMV-infected cells but for the majority of MCMV/h-infected cells. The M112mut mutant also had an increased ability to disrupt PML bodies but was less efficient than MCMV/h (Fig. 7B). This raised the question of whether the E1 proteins encoded by the M112/M113 locus were responsible for the ND10 disruption or whether these proteins promote ND10 disruption more indirectly. To resolve this question, we tested if E1 proteins expressed from a plasmid vector were able to disrupt PML bodies. Neither the E1 proteins expressed from a wt M112/M113 gene sequence nor those expressed from the MCMV/h-derived mutant M112/M113 gene were able to disperse PML nuclear domains. In contrast, the transfection of an MCMV IE1 expression plasmid resulted in the efficient disruption of PML domains, similar to the effect of HCMV IE1 (Fig. 7C). These results suggested that the viral E1 proteins did not disrupt ND10 by themselves but rather promoted ND10 disruption by an indirect mechanism.

Recent studies have corroborated the repressive effect of PML and hDaxx on HCMV replication by showing that the elimination of these proteins by small interfering RNA (siRNA)-mediated gene knockdown enhances viral replication (51, 52, 59). Based on these observations we reasoned that a knockdown of PML or hDaxx might also facilitate MCMV replication in human RPE-1 cells. Therefore, we used previously described shRNA-expressing retroviruses (52) to generate stable PML and hDaxx knockdown cells (Fig. 7D) and analyzed MCMV replication in these cells. As shown in Fig. 7E, PML knockdown did not facilitate the replication of wt MCMV in RPE-1 cells but enhanced the replication of the M112mut mutant. In contrast, the knockdown of hDaxx decreased the replication of M112mut in RPE-1 cells (Fig. 7E). The reason for the decreased replication in Daxx knockdown cells is unclear. However, it seems likely that a previously reported increased sensitivity of Daxx knockdown cells to proapoptotic stimuli is responsible (14, 35).

Taken together, this study shows that MCMV can spontaneously gain the ability to replicate in human cells by acquiring a few small mutations in the M112/M113-coding region. These mutations decrease the proapoptotic effect of MCMV infection on human cells and increase the formation of replication compartments and the efficiency of viral DNA replication. The mutations also enhance the ability of MCMV to disrupt PML nuclear domains. However, PML-mediated repression of viral transcription and/or replication does not seem to be the primary barrier to cross-species infection but rather limits its efficiency. The introduction of the M112/M113 mutations into wt MCMV did not fully reproduce the remarkably rapid replication of MCMV/h in human RPE-1 cells, suggesting that additional mutations must contribute to its phenotype.

Discussion

In this study we describe and characterize an MCMV mutant that has spontaneously obtained the capacity to replicate in human cells. At first, the identification of this mutant virus was surprising to us. However, a literature search revealed that a spontaneous adaptation of MCMV to human cells has been seen before. In 1969, Raynaud and colleagues described the adaptation of a field mouse (Apodemus sylvaticus) cytomegalovirus to monkey kidney cells and human diploid cells by first passaging it through embryonic hamster cells and a baby hamster kidney (BHK21) cell line (43). In monkey kidney cells, their virus replicated to a titer of 107 $TCID_{50}$/ml, a titer similar to the titer that our MCMV/h isolate reached in human RPE-1 cells. A later study by Kim et al. compared the Raynaud strain (prior to adaptation) to the MCMV Smith strain and found that the two strains were indistinguishable in terms of replication kinetics, CPE morphology, virus particle density, and antibody cross-reactivity, suggesting that the two viruses were identical (30). Kim and colleagues concluded that the virus must have undergone a remarkable change during tissue culture passage or that the virus that replicated in human cells was a contaminant and not the original mouse virus. As the studies

by Raynaud et al. and Kim et al. were done before the advent of molecular biology, it is impossible to tell whether the two MCMV strains were genetically identical or only similar in their phenotypes. Be that as it may, the present study clearly shows that even the MCMV Smith strain can spontaneously adapt to human cells and reveals a genetic basis for this extended- host-range phenotype.

In a previous study we showed that MCMV induces apoptosis in human cells and that an inhibition of apoptosis allows the virus to replicate in human cells (26). Surprisingly, the human cell-adapted MCMV/h still induced apoptosis in a large proportion of infected RPE-1 cells, although it was less than that induced by the parental wt virus (Fig. 2C). A sequence analysis of MCMV/h also did not reveal any mutations in known antiapoptotic genes of MCMV or in MCMV homologs of HCMV cell death suppressors. Instead, we demonstrated that mutations in the M112/M113-coding sequence facilitate MCMV replication in human cells and are at least in part responsible for the remarkably efficient growth of MCMV/h in RPE-1 cells. The mutations in MCMV/h enabled the virus to express its proteins and replicate its DNA faster or more efficiently than the parental wt virus (Fig. 3 and 6A). On the other hand, the mutant virus also induced less apoptosis than wt MCMV (Fig. 5B), and this most probably contributed to the enhanced protein expression and DNA replication. However, MCMV/h still has a more pronounced apoptosis-inducing effect on human cells than on murine cells. MCMV/h titers drop quite rapidly after high-MOI infection of human cells, particularly if sensitive cells such as HUVECs are used (Fig. 1F). Hence, MCMV/h is not yet perfectly adapted to human cells, and apoptosis of infected cells must still be considered an important limitation for MCMV cross-species infections.

The MCMV M112/M113 and the HCMV UL112-113 regions are highly similar in their locations within the viral genome and share an almost identical splicing pattern (16). Differentially spliced mRNAs encode at least four protein products of 33, 36, 38, and 87 kDa in the case of MCMV (16) and 34, 43, 50, and 84 kDa in the case of HCMV (60). Secondary modifications such as phosphorylation are responsible for differences between the predicted and the apparent molecular weights of some of the protein variants (13, 60). Although the E1 proteins were identified many years ago, their mechanisms of action remain poorly understood. E1 proteins become detectable shortly after the major immediate-early proteins (4,
13), suggesting that very little IE protein is necessary to activate the 112/113 promoter. Together with the MIE proteins, the E1 proteins can enhance the expression of other viral genes, particularly those involved in DNA replication (24, 28). In addition, the E1 proteins may also have a more direct role in viral DNA replication, as they can bind single- and doublestranded DNA and accumulate at nuclear replication sites (25). Moreover, UL112-113 proteins are necessary for the transient complementation of HCMV oriLyt-dependent DNA replication (37), and antisense RNA to UL112-113 transcripts inhibits viral DNA replication (61).

It seems likely that the different E1 protein isoforms have nonidentical functions, but how the different isoforms function and how they interact with each other and with other proteins have only begun to be understood. Of the three relevant mutations in MCMV/h, one mutation (the 3-amino-acid deletion) affects all E1 protein isoforms, whereas the two point mutations lead to amino acid exchanges only in the large 87-kDa isoform (Fig. 4A). However, we cannot exclude that the point mutations have an impact on splicing efficiency and shift the balance between the various E1 isoforms. The higher abundance of the 87-kDa E1 protein at late times postinfection (Fig. 6A) might indeed reflect altered splicing but might also be based on differences in protein stability between the wt and the mutant 87-kDa proteins. Interestingly, it was recently shown that the 87-kDa protein interacts with IE3 and modulates the repressive effect of IE3 on the MIE promoter (49). However, marked differences in IE1 or IE3 levels between wt and mutant viruses were detected only from 48 hpi onward (Fig. 6A), the same time when massive cell death starts, particularly in cells infected with wt MCMV. Hence, it is difficult to determine the contributions of the 87-kDa E1 protein and cell death to the reduced IE1 and IE3 levels, respectively. In fact, previous work has shown that extended cell survival due to the expression of an antiapoptotic protein correlated with higher levels of viral protein expression at 72 and 96 hpi (26). PML nuclear domains (ND10) consist predominantly of proteins functioning as transcriptional repressors (18, 34, 53). Viral genomes are frequently detected in close association with PML domains, suggesting that the PML domains exert a cellintrinsic antiviral defense. This notion is supported by the fact that some DNA viruses are capable of dispersing PML nuclear bodies (18, 34, 53). Moreover, the inactivation of PML, Daxx, or Sp100 by gene knockdown significantly enhances viral replication in permissive cells (52, 53, 59). However, can the disruption of PML structures also facilitate virus replication in cells that are usually nonpermissive for a particular virus? We previously constructed MCMV-hIE1 (an MCMV mutant expressing HCMV IE1), and this virus did not replicate to significant titers in human RPE-1 cells or MRC-5 fibroblasts (26), even though it disrupted ND10 in more than 60% of infected RPE-1 cells (data not shown). Moreover, a knockdown of PML or Daxx in

the same cells did not facilitate the replication of wt MCMV (Fig. 7E), indicating that ND10 disruption alone is not sufficient for MCMV to replicate and spread efficiently. However, PML knockdown did enhance the replication of the M112mut virus. Interestingly, using the same MCMV-hIE1 mutant, Tang and Maul previously showed a low level of replication in human fibroblasts (50). Those authors concluded that HCMV IE1 can help MCMV to cross the species barrier. The reason for this minor discrepancy between the two studies is unclear, and further investigation is needed to consolidate these findings. However, we believe that it is fair to draw the conclusion that PML disruption has little (or no) effect on MCMV replication in human cells, unless the virus carries additional mutations facilitating its replication in these cells. Mutations in M112/M113 were sufficient to afford MCMV replication in human RPE-1 cells. However, the M112mut virus clearly did not replicate and spread as rapidly as MCMV/h (Fig. 5A). Hence, additional mutations in MCMV/h must contribute to its remarkably efficient replication. Many of the mutations listed in Table 1 are in largely uncharacterized ORFs or noncoding regions of the MCMV genome. Therefore, it is difficult to predict which of them contributes to the phenotype. The identification and characterization of additional contributing mutations should provide new insights into the functions of as-yet-uncharacterized genes or regulatory regions and into the mechanisms of the species specificity of cytomegaloviruses in general. Once we have a better understanding of how MCMV can overcome the obstacles to its replication in human cells, we might be able to construct HCMV mutants that can replicate in murine cells or even in the mouse, thereby providing the long-sought-after small-animal model for the study of HCMV infection in vivo.

Acknowledgments

We thank E. Borst, R. Heller, S. Jonjic, L. Loh, M. Messerle, M. Nevels, T. Shenk, T. Stamminger, and J. von Einem for providing cells, plasmids, and antibodies; K. Berger for technical assistance; and M. Budt, M. Nevels, and S. Voigt for critically reading the manuscript.
This work was supported by grant BR 1730/3-1 from the Deutsche Forschungsgemeinschaft and an intramural research grant from the Robert Koch Institute.

References

1. Ahasan, M. M., and C. Sweet. 2007. Murine cytomegalovirus open reading frame m29.1 augments virus replication both in vitro and in vivo. J. Gen. Virol. 88:2941–2951.
2. Ahn, J. H., and G. S. Hayward. 2000. Disruption of PML-associated nuclear bodies by IE1 correlates with efficient early stages of viral gene expression and DNA replication in human cytomegalovirus infection. Virology 274:39–55.
3. Ahn, J. H., and G. S. Hayward. 1997. The major immediate-early proteins IE1 and IE2 of human cytomegalovirus colocalize with and disrupt PMLassociated nuclear bodies at very early times in infected permissive cells. J. Virol. 71:4599–4613.
4. Ahn, J. H., W. J. Jang, and G. S. Hayward. 1999. The human cytomegalovirus IE2 and UL112-113 proteins accumulate in viral DNA replication compartments that initiate from the periphery of promyelocytic leukemia proteinassociated nuclear bodies (PODs or ND10). J. Virol. 73:10458–10471.
5. Atalay, R., A. Zimmermann, M. Wagner, E. Borst, C. Benz, M. Messerle, and H. Hengel. 2002. Identification and expression of human cytomegalovirus transcription units coding for two distinct Fcgamma receptor homologs. J. Virol. 76:8596–8608.
6. Bodnar, A. G., M. Ouellette, M. Frolkis, S. E. Holt, C. P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteiner, and W. E. Wright. 1998. Extension of life-span by introduction of telomerase into normal human cells. Science 279:349–352.
7. Bruggeman, C. A., H. Meijer, P. H. Dormans, W. M. Debie, G. E. Grauls, and C. P. van Boven. 1982. Isolation of a cytomegalovirus-like agent from wild rats. Arch. Virol. 73:231–241.
8. Brune, W., M. Hasan, M. Krych, I. Bubic, S. Jonjic, and U. H. Koszinowski. 2001. Secreted virus-encoded proteins reflect murine cytomegalovirus productivity in organs. J. Infect. Dis. 184:1320–1324.
9. Brune, W., C. Ménard, J. Heesemann, and U. H. Koszinowski. 2001. A ribonucleotide reductase homolog of cytomegalovirus and endothelial cell tropism. Science 291:303–305.
10. Brune, W., M. Nevels, and T. Shenk. 2003. Murine cytomegalovirus m41 open reading frame encodes a Golgi-localized antiapoptotic protein. J. Virol. 77:11633–11643.

11. Brune, W., M. Wagner, and M. Messerle. 2006. Manipulating cytomegalovirus genomes by BAC mutagenesis: strategies and applications, p. 61–89. In M. J. Reddehase (ed.), Cytomegaloviruses: molecular biology and immunology. Caister Academic Press, Norfolk, United Kingdom.
12. Buck, A. H., J. Santoyo-Lopez, K. A. Robertson, D. S. Kumar, M. Reczko, and P. Ghazal. 2007. Discrete clusters of virus-encoded microRNAs are associated with complementary strands of the genome and the 7.2-kilobase stable intron in murine cytomegalovirus. J. Virol. 81:13761–13770.
13. Bühler, B., G. M. Keil, F. Weiland, and U. H. Koszinowski. 1990. Characterization of the murine cytomegalovirus early transcription unit e1 that is induced by immediate-early proteins. J. Virol. 64:1907–1919.
14. Chen, L. Y., and J. D. Chen. 2003. Daxx silencing sensitizes cells to multiple apoptotic pathways. Mol. Cell. Biol. 23:7108–7121.
15. Child, S. J., L. K. Hanson, C. E. Brown, D. M. Janzen, and A. P. Geballe. 2006. Double-stranded RNA binding by a heterodimeric complex of murine cytomegalovirus m142 and m143 proteins. J. Virol. 80:10173–10180.
16. Ciocco-Schmitt, G. M., Z. Karabekian, E. W. Godfrey, R. M. Stenberg, A. E. Campbell, and J. A. Kerry. 2002. Identification and characterization of novel murine cytomegalovirus M112-113 (e1) gene products. Virology 294:199–208.
17. Dölken, L., J. Perot, V. Cognat, A. Alioua, M. John, J. Soutschek, Z. Ruzsics, U. Koszinowski, O. Voinnet, and S. Pfeffer. 2007. Mouse cytomegalovirus microRNAs dominate the cellular small RNA profile during lytic infection and show features of posttranscriptional regulation. J. Virol. 81:13771–13782.
18. Everett, R. D. 2006. Interactions between DNA viruses, ND10 and the DNA damage response. Cell. Microbiol. 8:365–374.
19. Fioretti, A., T. Furukawa, D. Santoli, and S. A. Plotkin. 1973. Nonproductive infection of guinea pig cells with human cytomegalovirus. J. Virol. 11:998–1003.
20. Garcia-Ramirez, J. J., F. Ruchti, H. Huang, K. Simmen, A. Angulo, and P. Ghazal. 2001. Dominance of virus over host factors in cross-species activation of human cytomegalovirus early gene expression. J. Virol. 75:26–35.
21. Ghazal, P., A. E. Visser, M. Gustems, R. Garcia, E. M. Borst, K. Sullivan, M. Messerle, and A. Angulo. 2005. Elimination of ie1 significantly attenuates murine cytomegalovirus virulence but does not alter replicative capacity in cell culture. J. Virol. 79:7182–7194.
22. Harvey, D. M., and A. J. Levine. 1991. p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. Genes Dev. 5:2375–2385.
23. Hobom, U., W. Brune, M. Messerle, G. Hahn, and U. H. Koszinowski. 2000. Fast screening procedures for random transposon libraries of cloned herpesvirus genomes: mutational analysis of human cytomegalovirus envelope glycoprotein genes. J. Virol. 74:7720–7729.
24. Iskenderian, A. C., L. Huang, A. Reilly, R. M. Stenberg, and D. G. Anders. 1996. Four of eleven loci required for transient complementation of human cytomegalovirus DNA replication cooperate to activate expression of replication genes. J. Virol. 70:383–392.
25. Iwayama, S., T. Yamamoto, T. Furuya, R. Kobayashi, K. Ikuta, and K. Hirai. 1994. Intracellular localization and DNA-binding activity of a class of viral early phosphoproteins in human fibroblasts infected with human cytomegalovirus (Towne strain). J. Gen. Virol. 75(Pt. 12):3309–3318.
26. Jurak, I., and W. Brune. 2006. Induction of apoptosis limits cytomegalovirus cross-species infection. EMBO J. 25:2634–2642.
27. Kattenhorn, L. M., R. Mills, M. Wagner, A. Lomsadze, V. Makeev, M. Borodovsky, H. L. Ploegh, and B. M. Kessler. 2004. Identification of proteins associated with murine cytomegalovirus virions. J. Virol. 78:11187–11197.
28. Kerry, J. A., M. A. Priddy, T. Y. Jervey, C. P. Kohler, T. L. Staley, C. D. Vanson, T. R. Jones, A. C. Iskenderian, D. G. Anders, and R. M. Stenberg. 1996. Multiple regulatory events influence human cytomegalovirus DNA polymerase (UL54) expression during viral infection. J. Virol. 70:373–382.
29. Kim, K. S., and R. I. Carp. 1972. Abortive infection of human diploid cells by murine cytomegalovirus. Infect. Immun. 6:793–797.
30. Kim, K. S., V. Sapienza, and R. I. Carp. 1974. Comparative studies of the Smith and Raynaud strains of murine cytomegalovirus. Infect. Immun. 10: 672–674.
31. Korioth, F., G. G. Maul, B. Plachter, T. Stamminger, and J. Frey. 1996. The nuclear domain 10 (ND10) is disrupted by the human cytomegalovirus gene product IE1. Exp. Cell Res. 229:155–158.
32. Lafemina, R. L., and G. S. Hayward. 1988. Differences in cell-type-specific blocks to immediate early gene expression and DNA replication of human, simian and murine cytomegalovirus. J. Gen. Virol. 69:355–374.
33. Mahy, B. W. J., and H. O. Kangro. 1996. Virology methods manual. Academic Press, San Diego, CA.

34. Maul, G. G. 2008. Initiation of cytomegalovirus infection at ND10. Curr. Top. Microbiol. Immunol. 325:117–132.
35. Michaelson, J. S., and P. Leder. 2003. RNAi reveals anti-apoptotic and transcriptionally repressive activities of DAXX. J. Cell Sci. 116:345–352.
36. Mocarski, E. S., and C. T. Courcelle. 2001. Cytomegaloviruses and their replication, p. 2629–2673. In D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
37. Pari, G. S., and D. G. Anders. 1993. Eleven loci encoding trans-acting factors are required for transient complementation of human cytomegalovirus ori- Lyt-dependent DNA replication. J. Virol. 67:6979–6988.
38. Park, M. Y., Y. E. Kim, M. R. Seo, J. R. Lee, C. H. Lee, and J. H. Ahn. 2006. Interactions among four proteins encoded by the human cytomegalovirus UL112-113 region regulate their intranuclear targeting and the recruitment of UL44 to prereplication foci. J. Virol. 80:2718–2727.
39. Pass, R. F. 2001. Cytomegalovirus, p. 2675–2705. In D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
40. Penfold, M. E., and E. S. Mocarski. 1997. Formation of cytomegalovirus DNA replication compartments defined by localization of viral proteins and DNA synthesis. Virology 239:46–61.
41. Perot, K., C. M. Walker, and R. R. Spaete. 1992. Primary chimpanzee skin fibroblast cells are fully permissive for human cytomegalovirus replication. J. Gen. Virol. 73:3281–3284.
42. Rawlinson, W. D., H. E. Farrell, and B. G. Barrell. 1996. Analysis of the complete DNA sequence of murine cytomegalovirus. J. Virol. 70:8833–8849.
43. Raynaud, J., P. Atanasiu, C. Barreau, and M. Jahkola. 1969. Adaptation d'un virus cytomègalique provenant do Mulot (Apodemus sylvaticus) sur differantes cellules hètèrologiques, y compris les cellules humaines. C. R. Hebd. Seances Acad. Sci. Hebd. Seances Acad. Sci. D 269:104–106.
44. Sandford, G. R., U. Schumacher, J. Ettinger, W. Brune, G. S. Hayward, W. H. Burns, and S. Voigt. 2009. Deletion of the rat cytomegalovirus immediate early 1 gene results in a virus capable of establishing latency but with lower levels of acute virus replication and latency that compromise reactivation efficiency. J. Gen. Virol. 91:616–621.
45. Sinzger, C., G. Hahn, M. Digel, R. Katona, K. L. Sampaio, M. Messerle, H. Hengel, U. Koszinowski, W. Brune, and B. Adler. 2008. Cloning and sequencing of a highly productive, endotheliotropic virus strain derived from human cytomegalovirus TB40/E. J. Gen. Virol. 89:359–368.
46. Smith, C. B., L. S. Wei, and M. Griffiths. 1986. Mouse cytomegalovirus is infectious for rats and alters lymphocyte subsets and spleen cell proliferation. Arch. Virol. 90:313–323.
47. Staprans, S. I., and D. H. Spector. 1986. 2.2-kilobase class of early transcripts encoded by cell-related sequences in human cytomegalovirus strain AD169. J. Virol. 57:591–602.
48. Swift, S., J. Lorens, P. Achacoso, and G. P. Nolan. 2001. Rapid production of retroviruses for efficient gene delivery to mammalian cells using 293T cell-based systems. Curr. Protoc. Immunol. 10:10.17C.
49. Tang, Q., L. Li, and G. G. Maul. 2005. Mouse cytomegalovirus early M112/ 113 proteins control the repressive effect of IE3 on the major immediateearly promoter. J. Virol. 79:257–263.
50. Tang, Q., and G. G. Maul. 2006. Mouse cytomegalovirus crosses the species barrier with help from a few human cytomegalovirus proteins. J. Virol. 80:7510–7521.
51. Tavalai, N., P. Papior, S. Rechter, M. Leis, and T. Stamminger. 2006. Evidence for a role of the cellular ND10 protein PML in mediating intrinsic immunity against human cytomegalovirus infections. J. Virol. 80:8006–8018.
52. Tavalai, N., P. Papior, S. Rechter, and T. Stamminger. 2008. Nuclear domain 10 components promyelocytic leukemia protein and hDaxx independently contribute to an intrinsic antiviral defense against human cytomegalovirus infection. J. Virol. 82:126–137.
53. Tavalai, N., and T. Stamminger. 2008. New insights into the role of the subnuclear structure ND10 for viral infection. Biochim. Biophys. Acta 1783: 2207–2221.
54. Valchanova, R. S., M. Picard-Maureau, M. Budt, and W. Brune. 2006. Murine cytomegalovirus m142 and m143 are both required to block protein kinase R-mediated shutdown of protein synthesis. J. Virol. 80:10181–10190.
55. Voigt, S., A. Mesci, J. Ettinger, J. H. Fine, P. Chen, W. Chou, and J. R. Carlyle. 2007. Cytomegalovirus evasion of innate immunity by subversion of the NKR-P1B:Clr-b missing-self axis. Immunity 26:617–627.
56. Warming, S., N. Costantino, D. L. Court, N. A. Jenkins, and N. G. Copeland. 2005. Simple and highly efficient BAC recombineering using *gal*K selection. Nucleic Acids Res. 33:e36.
57. Weller, T. H. 1970. Cytomegaloviruses: the difficult years. J. Infect. Dis. 122:532–539.

58. Wilkinson, G. W., C. Kelly, J. H. Sinclair, and C. Rickards. 1998. Disruption of PML-associated nuclear bodies mediated by the human cytomegalovirus major immediate early gene product. J. Gen. Virol. 79:1233–1245.
59. Woodhall, D. L., I. J. Groves, M. B. Reeves, G. Wilkinson, and J. H. Sinclair. 2006. Human Daxx-mediated repression of human cytomegalovirus gene expression correlates with a repressive chromatin structure around the major immediate early promoter. J. Biol. Chem. 281:37652–37660.
60. Wright, D. A., and D. H. Spector. 1989. Posttranscriptional regulation of a class of human cytomegalovirus phosphoproteins encoded by an early transcription unit. J. Virol. 63:3117–3127.
61. Yamamoto, T., S. Suzuki, K. Radsak, and K. Hirai. 1998. The UL112/113 gene products of human cytomegalovirus which colocalize with viral DNA in infected cell nuclei are related to efficient viral DNA replication. Virus Res. 56:107–114.

Tables and Figures

Figure 1. Replication of MCMV/h in human and murine cells. (A to F) Human RPE-1 cells, HFFs, MRC-5 cells, and HUVECs were infected at an MOI of 0.2 or 5 $TCID_{50}$/cell with MCMV/h, MCMV-bclXL, or the parental wt virus. HCMV strains AD169 and TB40/E are shown for comparison. (G) Murine 10.1 fibroblasts were infected with the same viruses described above (A) at an MOI of 0.1 $TCID_{50}$/cell. Virus replication was determined by titration. DL, detection limit. (H) Virion DNA was extracted from MCMV/h and the parental wt MCMV, digested with restriction enzymes, and separated by gel electrophoresis. MCMV and HCMV (strain AD169) BAC DNAs are shown for comparison. Differences in the restriction fragment patterns of BAC and virion DNAs are expected, as virion DNA is linear and BAC DNA is circular, and BAC DNA contains the BAC replicon in addition to the viral genome. The terminal fragments of the virion DNA and the corresponding joined fragment of the BAC DNA are indicated by arrowheads.

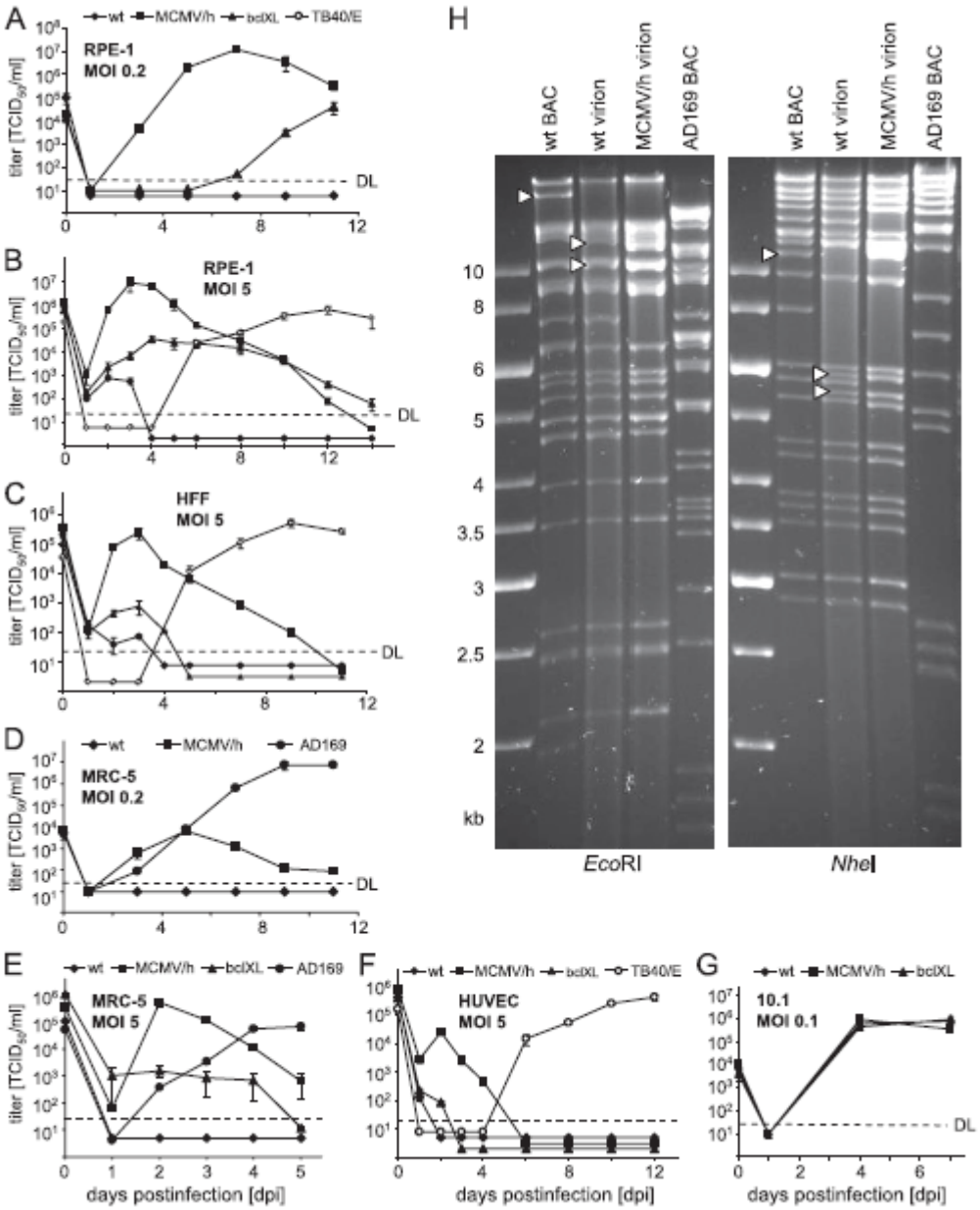

Figure 2. MCMV/h induces less cell death than the parental wt MCMV. (A) RPE-1 cells were infected at an MOI of 5 $TCID_{50}$/cell. Fluorescent images were taken 24 and 48 hpi. Infected cells show green fluorescence, as both viruses express GFP. (B) RPE-1 cells were infected as described above, and nuclear DNA fragmentation as a sign of apoptosis was determined at 48 hpi by using a TUNEL assay. Fragmented DNA is indicated by red fluorescence. Nuclei were stained with DAPI. (C and D) Human RPE-1 cells (C) and murine 10.1 fibroblasts (D) were infected at an MOI of 5 $TCID_{50}$/cell with MCMV/h, the parental wt virus, or MCMV-bclXL. Cell viability was measured by using an MTS assay and is shown relative to the viability at 0 hpi. The differences in viability between the wt and MCMV/h at 48 and 72 hpi were significant (P 0.01 by a Student's t test).

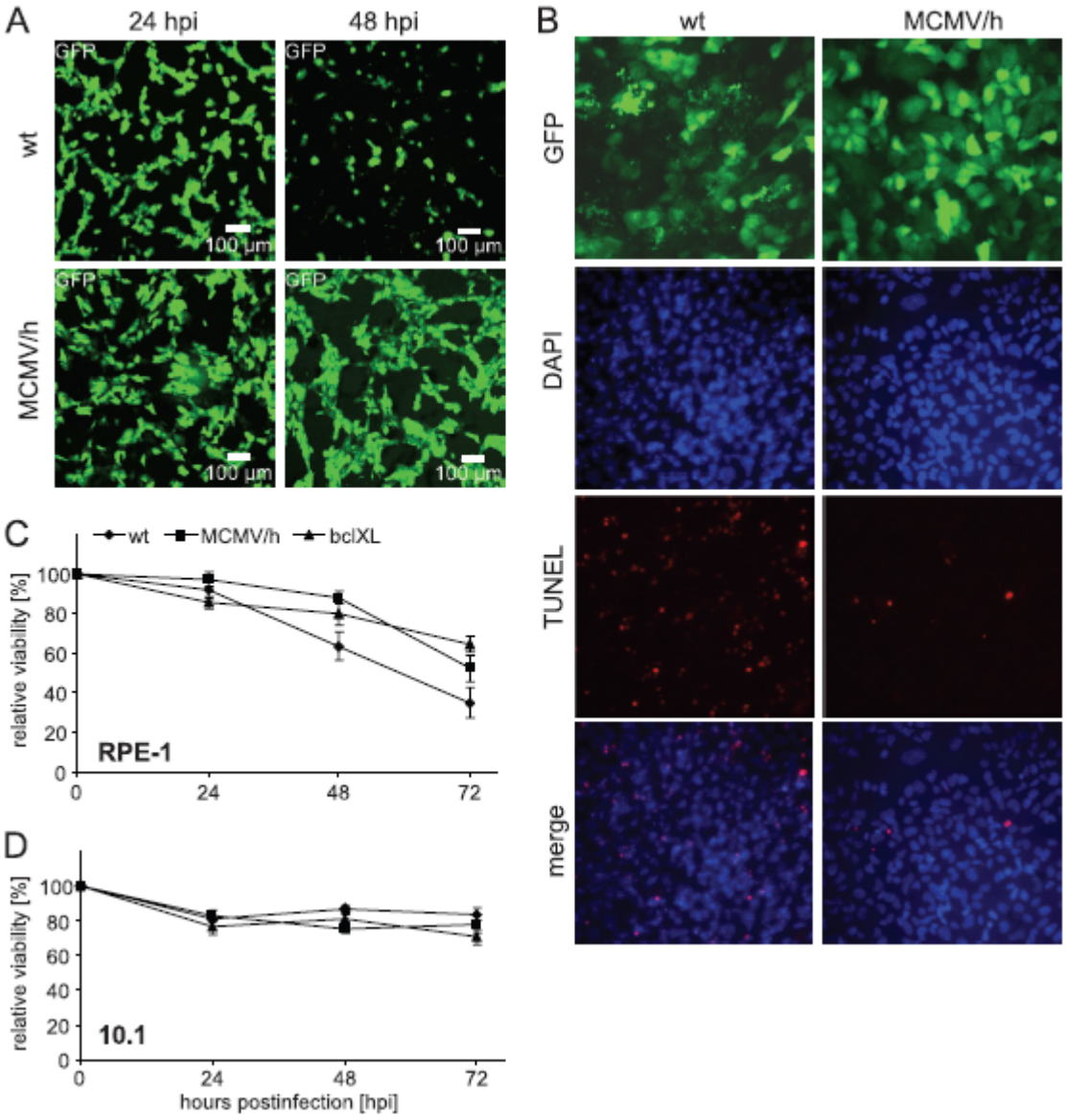

Figure 3. Viral gene expression and DNA replication. (A) Human RPE-1 and murine 10.1 cells were infected with wt MCMV or MCMV/h. The levels of expression of the viral IE1, E1, M44, and gB proteins were determined by Western blotting. (B and C) Viral DNA replication was determined by slot blot hybridization (B) or by real-time PCR (C). Viral genome amplification is shown relative to the value determined at 8 hpi (representing input viral genomes before replication).

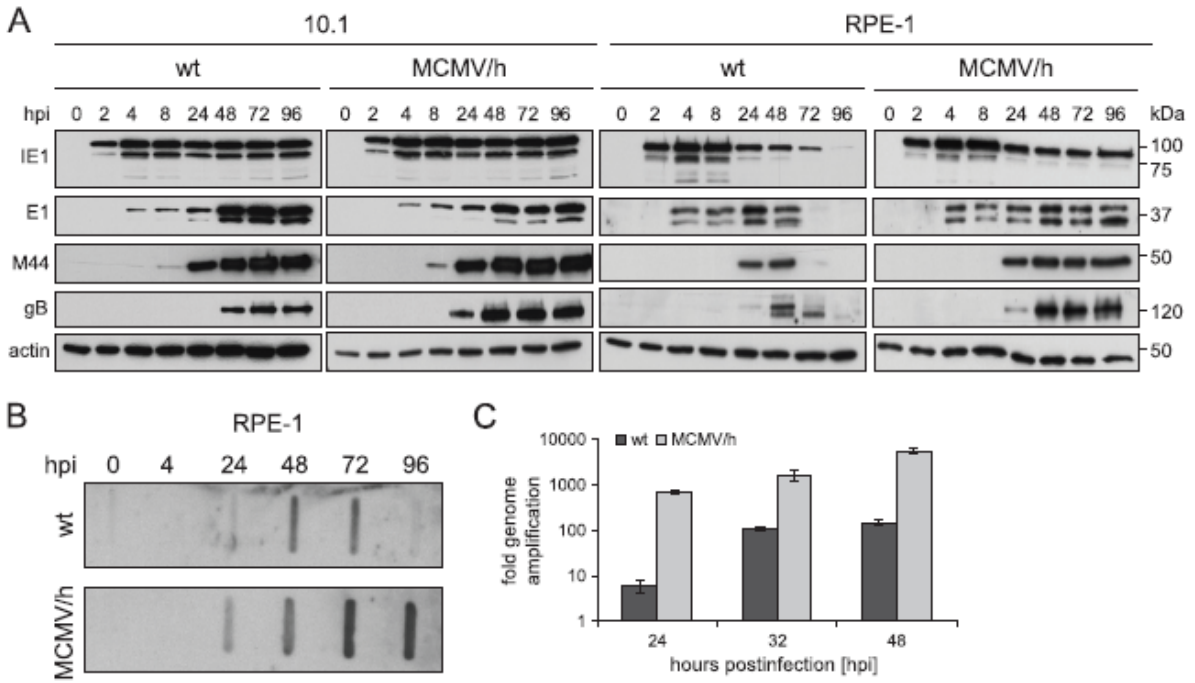

Figure 4. Construction of mutant MCMV genomes. (A) Structure of the M112/M113-coding region according to data described previously by Ciocco-Schmitt et al. (16). Mutations detected in MCMV/h are indicated by arrows, and the predicted changes in the amino acid sequence are shown below. (B) An MCMV M112/M113 deletion mutant was constructed by the insertion of a zeocin resistance gene (zeo) replacing the M112/M113-coding region. The wt or mutant M112/M113 sequence was then reinserted by using a kan gene flanked by FRT sites as a selectable marker. The kan gene was subsequently removed by Flp recombination. NheI restriction sites are indicated with N, and the expected NheI fragment sizes are shown. (C) wt and mutant BACs were digested with NheI and separated by gel electrophoresis.

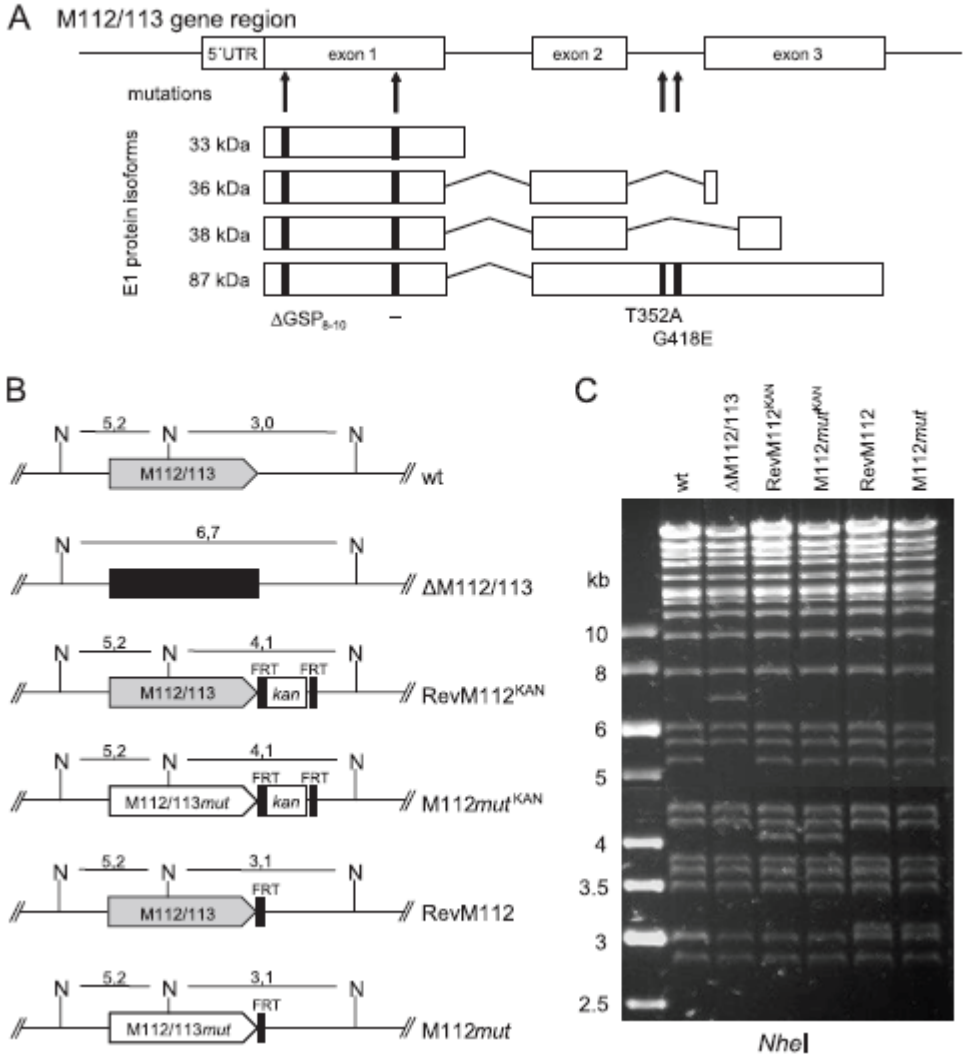

Figure 5. Viral replication and cell death induction in RPE-1 cells. (A) RPE-1 cells were infected at an MOI of 0.2 $TCID_{50}$/cell with wt and mutant MCMVs as indicated. Virus replication was determined by titration. DL, detection limit. (B) RPE-1 cells were infected at an MOI of 5 $TCID_{50}$/cell. Cell viability was measured by using an MTS assay and is shown relative to the viability at 0 hpi. The differences in viability between the wt and M112mut at 48 and 72 hpi were significant (P 0.01 by a Student's t test), but there was no significant difference between the wt and RevM112.

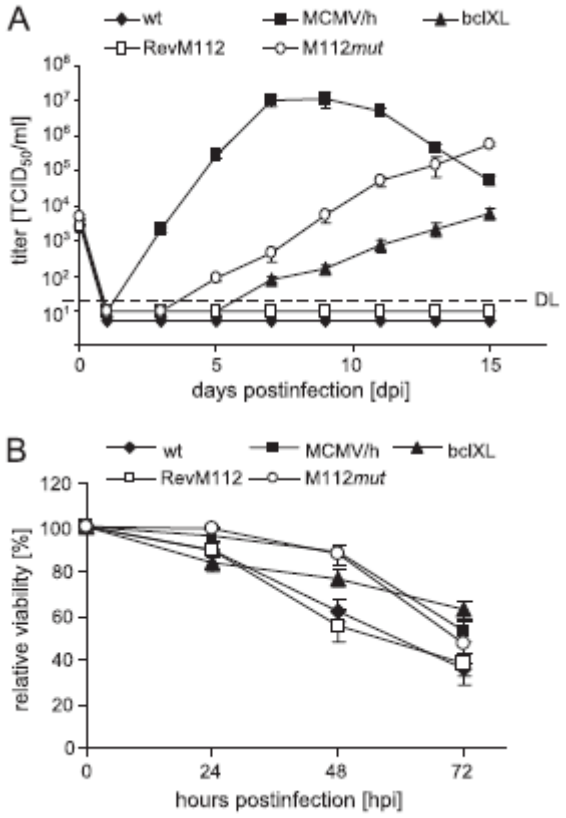

Figure 6. M112/M113 protein expression and formation of replication compartments. (A) Cells were infected with wt and mutant MCMVs. The levels of expression of the IE1, IE3, E1, M44, and gB proteins were determined by Western blotting. The 36-, 38-, and 87-kDa E1 proteins were detected on the same blot, but a longer exposure is shown for the large 87-kDa isoform, as its level of expression is much lower than the levels of expression of the 36- and 38-kDa isoforms. Viral DNA (vDNA) levels were determined by Southern blotting. (B and C) Viral replication compartments were stained at 24 hpi with antibodies against IE3, M44, and E1. (D) The relative abundances of the two different forms of replication compartments at 24 hpi were determined by evaluating more than 150 nuclei per sample in four independent experiments. The differences between wt and mutant MCMVs (MCMV/h and M112mut, respectively) were significant (P 0.01 by a Student's t test).

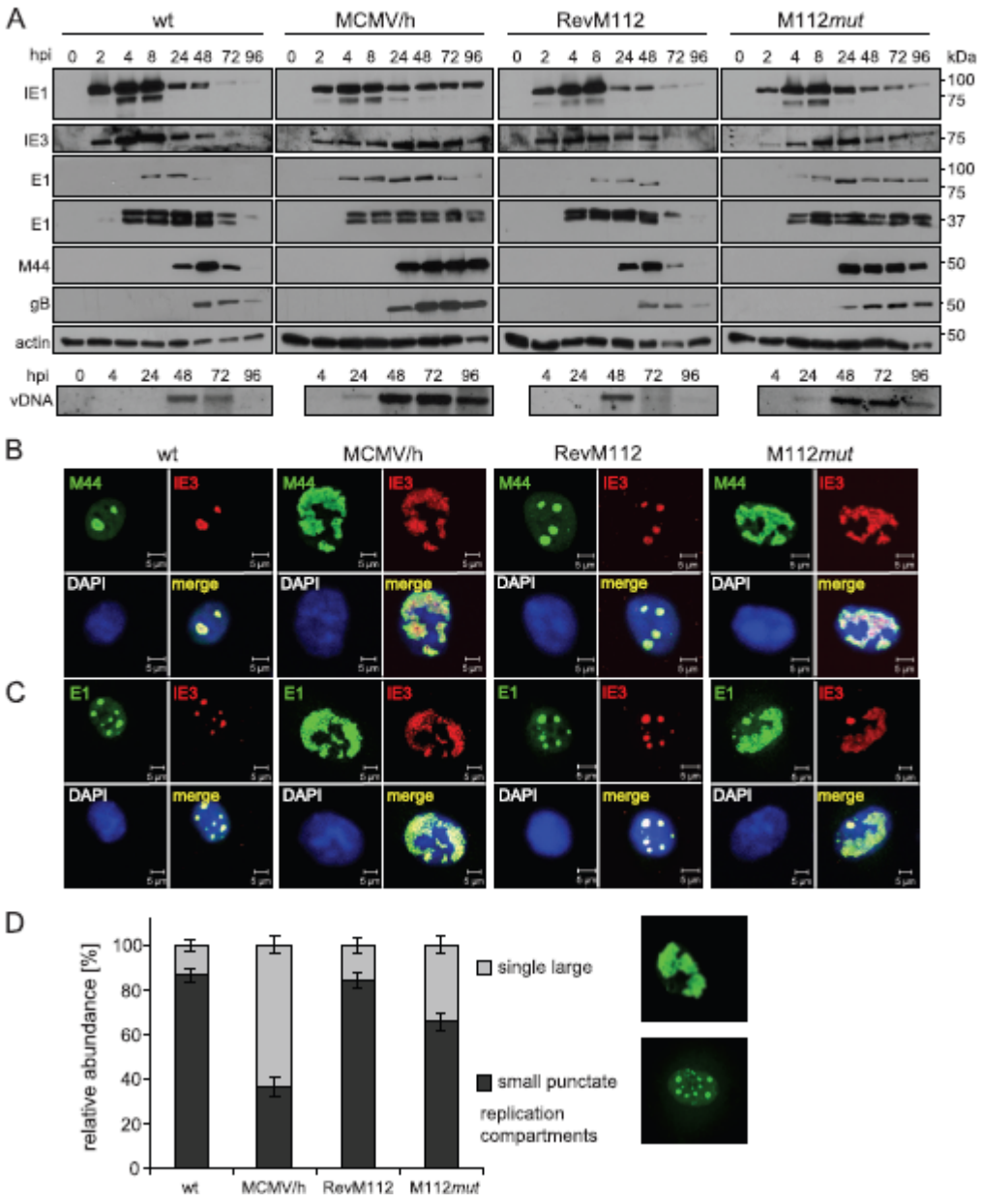

Figure 7. ND10 disruption by MCMV in human cells. (A) RPE-1 cells were infected with wt MCMV, MCMV/h, or HCMV strain AD169. Infected cell nuclei were stained at 24 hpi with antibodies against the viral E1 (for MCMV) or IE1 (for HCMV) protein. ND10 was stained with a PML-specific antibody. (B) The relative abundance of punctate versus dispersed ND10 was determined by evaluating more than 150 nuclei per sample in three independent experiments. Differences between wt and mutant MCMVs were significant (P 0.01 by a Student's t test). (C) RPE-1 cells were transfected with plasmids encoding enhanced GFP (EGFP), HCMV or MCMV IE1, and wt or mutant M112/M113 (E1) proteins. Transfected cells were stained with antibodies specific for the transiently expressed proteins, and ND10 was stained with an anti-PML antibody. (D) RPE-1 cells were transduced with retroviral vectors expressing PML- or Daxx-specific shRNAs, a control shRNA, or an empty vector as described previously (52). Successful gene knockdown was verified by Western blotting. An unspecific background band recognized by the anti-PML antibody is marked with an asterisk. (E) The shRNA-expressing cells were infected at an MOI of 0.2 $TCID_{50}$/cell, and the replication kinetics of wt MCMV (solid symbols) and the M112mut virus (open symbols) were determined by titration.

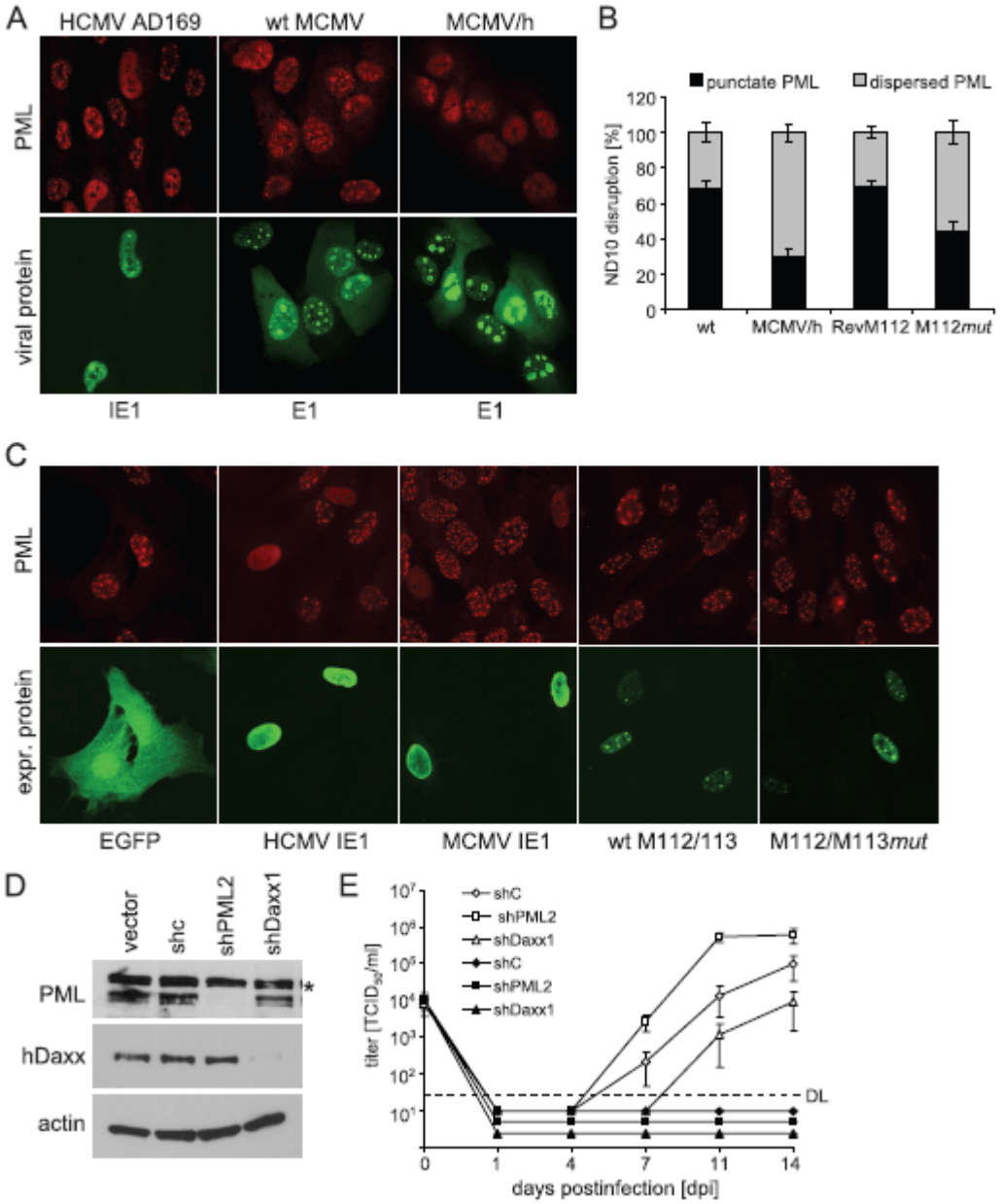

Table 1. Sequence alterations detected in MCMV/h compared to the published sequence of the MCMV Smith strain[f]

ORF(s)	Position(s)	Sequence alteration	Predicted consequence	Presence in parental virus
m10–m18	9561–19848	Δ10288 nt	Δm10–m18	No
	104152–91689	Inverse duplication	Duplication of m58–M71	No
m20	20958	ΔG	ORF extended	Yes[a]
M26–M27	31973	+G	Intergenic region	Yes
m29	36197	+G	Frameshift, ORF truncated	Yes[b]
m29.1	36197	+G	Frameshift, ORF extended	Yes[b]
m30	37263	+C	Frameshift, ORF extended	Yes[b]
M31	38803	+G	Frameshift, ORF extended	Yes[a]
M32	40000	C→T	Silent mutation	Yes
M45	61917	+C	Fusion of ORFs M45 and m45.1	Yes[c]
m58	92088	ΔA	Frameshift	Yes
	92178	C→G	?	Yes
	92179	+CG	Frameshift	Yes
	92206	CG→GC	?	Yes
	92226	G→A	?	Yes
	92230	G→A	?	Yes
	92358	ΔG	Frameshift	Yes
M71–M72	102942	ΔA	? (intergenic region)	Yes
M78–M79	112624	+T	? (intergenic region)	Yes
M86	123941	A→C	N1104H	Yes
	123951	T→G	L1107R	Yes
M93	135803	A→C	Silent mutation	Yes
M102	146853	G→A	D420N	Yes
m108	162352–162389	Δ37 nt	microRNA[d] deleted (positions 162364–162385)	No
	162374	A→G	Silent mutation	Yes[d]
	162380	A→G	Silent mutation	Yes[d]
M112	163118–163126	Δ9 nt	$\Delta GSP_{8\text{-}10}$	No
	163813	C→T	Silent mutation	No
	164243	A→G	T352A	No
	164442	G→A	G418E	No
M116	168256	G→A	G206V	No
	168370	G→A	A256V	No
	168376	G→A	S258F	No
	168526	C→A	T296I	No
m119.1	172403	C→A	G197W	No
M122 Ex5	179136	G→A	S227F	Yes
m129	187786	ΔT	Frameshift, ORF truncated	Yes
m143	201402	+G	Frameshift, ORF extended	Yes[e]

[a] See reference 27.
[b] See reference 1.
[c] See reference 9.
[d] See references 12 and 17.
[e] See references 15 and 54.
[f] Reported under GenBank accession number NC_004065 (42).