toward 0° to 180°; at this site there was a distinctly bimodal distribution toward SE and W, and 32 tracks toward 210° to 350° were excluded), and two sites at Baffin Island (50 tracks towards 90° to 270°; 4 tracks toward 270° to 90° excluded).

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# A Ribonucleotide Reductase Homolog of Cytomegalovirus and Endothelial Cell Tropism

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**Human cytomegalovirus infects vascular tissues and has been associated with atherogenesis and coronary restenosis. Although established laboratory strains of human cytomegalovirus have lost the ability to grow on vascular endothelial cells, laboratory strains of murine cytomegalovirus retain this ability. With the use of a forward-genetic procedure involving random transposon mutagenesis and rapid phenotypic screening, we identified a murine cytomegalovirus gene governing endothelial cell tropism. This gene, *M45*, shares sequence homology to ribonucleotide reductase genes. Endothelial cells infected with M45-mutant viruses rapidly undergo apoptosis, suggesting that a viral strategy to evade destruction by cellular apoptosis is indispensable for viral growth in endothelial cells.**

Human cytomegalovirus (HCMV) establishes a persistent lifelong infection. Infection of the immunocompetent individual is usually subclinical, but the virus can cause severe and life-threatening disease in transplant patients and people with acquired immunodeficiency syndrome (AIDS). HCMV infects a wide variety of cells and tissues (*1*). Several studies have implicated HCMV in the genesis of atherosclerosis, and particularly in rapidly progressing coronary artery disease and endothelialitis in cardiac transplant patients, and in the development of coronary restenosis after angioplasty (*2*). Migration of vascular smooth muscle cells stimulated by a CMV-encoded chemokine receptor has been proposed to promote vascular stenosis (*3*). In addition, increased endothelial and smooth muscle cell proliferation that is not counterbalanced by increased apoptosis may also result in thickening of the intima and media of arteries (*4*). This suggests a mechanism by which a viral inhibitor of apoptosis contributes to vascular disease. Moreover, HCMV-infected endothelial cells circulate in the blood of patients with CMV disease and contribute to viral dissemination (*5*).

Studies on the interaction of HCMV with vascular tissues could clarify the contribution of HCMV to vascular disease. Unfortunately, the HCMV laboratory strains do not replicate in endothelial cell cultures. Clinical isolates, by contrast, can be propagated in endothelial cells, but this property is lost after virus propagation in fibroblasts (*6*, *7*). Although there is evidence for a genetic basis of cell tropism (*6*), the gene(s) responsible are difficult to find, owing to the large genome of CMV, the lack of candidate genes, and the difficulty generating mutants of clinical HCMV isolates.

The HCMVs and murine CMVs (MCMVs) share a similar pathobiology and have collinear genomes. In the mouse, endothelial cells are known to play a role in viral dissemination, latency, and vascular disease (*8*). The genomes of the MCMV and HCMV laboratory strains were recently cloned as infectious bacterial artificial chromosomes (BACs) in *Escherichia coli* (*9*, *10*), where they can be mutated rapidly particularly with the use of random transposon (Tn) mutagenesis (*11*). MCMV derived from the BAC clone retains the capacity to propagate on cultured endothelial cells. In this work, we focused on the genetic basis of the tropism of MCMV for vascular endothelial cells.

In the absence of specific candidate genes, we constructed a library of virus mutants randomly mutated at a single position by combining a refined Tn mutagenesis procedure with a phenotypic screening approach. MCMV Tn mutants were generated by a single-step procedure using a Tn derivative, Tn*Max16*, bearing the enhanced green fluorescent protein (GFP) gene (*12*). To convert mutant genomes into a library of mutant viruses rapidly for phenotypic analysis, we directly transferred BAC DNA from *E. coli* to mammalian cells. The transfer of small multicopy plasmids can be done using naturally invasive bacteria or *E. coli* expressing the invasin gene of *Yersinia pseudotuberculosis* and the listeriolysin O gene of *Listeria monocytogenes* from the plasmid pGB2Ωinv-hly (*13*). We adapted this approach for the transfer of the 240-kb MCMV BAC into fibroblasts (*14*, *15*). A library of 576 *E. coli* clones, each carrying the MCMV BAC, Tn*Max16*, and pGB2Ωinv-hly, was deposited in six 96-well microtiter plates. Two microliters of each bacterial culture was used to inoculate NIH-3T3 fibroblasts grown in 96-well tissue culture plates. Viable mutant viruses were easily detected because only MCMV with a Tn forms green fluorescent plaques, whereas wild-type MCMV or nonviable MCMV mutants do not. In this way, we retrieved 199 viable mutants.

Fibroblast and endothelial cells were infected in parallel with individual virus mutants to screen for loss of ability to grow on endothelial cells (*16*). Viral growth was assessed visually by observation of green fluorescent plaques and by titration. We identified six mutants that did not grow and spread in endothelial cells but did grow well in fibroblasts (Fig. 1). Using BAC DNA extracted from the corresponding *E. coli* clones we determined the Tn insertion sites by direct sequencing from within the Tn (*11*). Remarkably, the insertions in all six mutants mapped to two adjacent open reading frames

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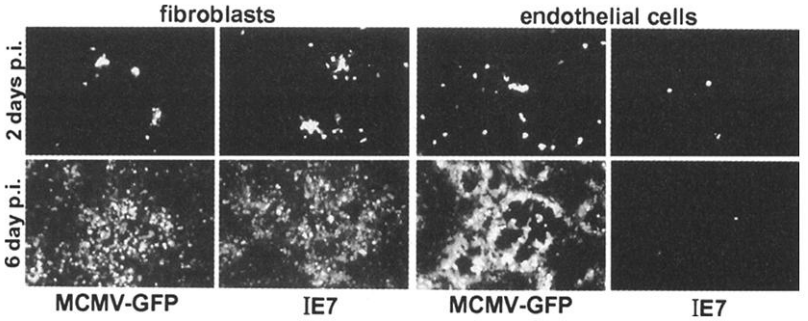


**Fig. 1.** Screening for nonendotheliotropic mutants. Fibroblast and endothelial cell cultures were infected with mutant viruses and with MCMV-GFP, a virus containing the GFP gene at an innocuous position (*12*), as control. Inspection of the cultures with a fluorescent microscope at days 2 and 6 post infection (p.i.) revealed that mutant IE7 did not grow on endothelial cells but did grow, apparently normally, in fibroblasts. Five additional mutants with the same phenotype were found.

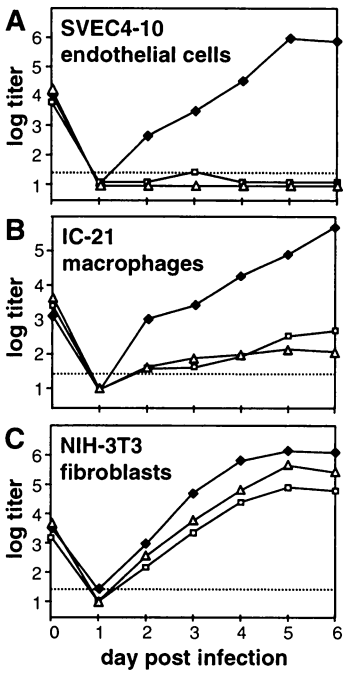


**Fig. 2.** Different cell types were infected with M45 insertion mutants, IE7 (□) and IIIG2 (△), and with MCMV-GFP (◆) as control. The mutants did not grow on endothelial cells (**A**) and grew poorly on macrophages (**B**), but grew almost normally on fibroblasts (**C**), bone marrow stromal cells, and hepatocytes (*15*). Each symbol represents the mean of three samples from parallel experiments. Dotted line, detection limit.

(ORFs), M45 and m45.1, according to the published MCMV sequence (*17*), suggesting that these ORFs encode a common, possibly spliced transcript. To detect splicing, we amplified the area of overlap of these two ORFs by reverse transcriptase–polymerase chain reaction (RT-PCR) (*18*). Instead of splicing, we found an additional cytosine residue at nucleotide position 61918 in comparison to the published sequence. This was confirmed by repeated sequencing of this region in the MCMV genome and in the cloned MCMV Hind III B fragment. Correction of the published sequence results in a single M45 ORF, which explains the common phenotype. To further confirm the correlation between M45 disruption and failure to grow on endothelial cells, we retrieved an additional six mutants with Tn insertions close to the beginning and at the end of M45 from pre-existing libraries of MCMV mutants (*18*). Four mutants with Tn insertions within M45 showed the same growth deficit in endothelial cells. We were unable to recover infectious virus from the two mutant MCMV genomes carrying insertions outside of M45. This is probably caused by interference with the expression of the adjacent genes, *M44* and *M46* (*17*), whose HCMV homologs are essential for virus replication (*19*). To exclude polar effects on neighboring genes, we replaced the Tn insertion in one mutant with a frame-shift mutation (*18*). This mutant showed the same phenotype, whereas a revertant virus grew like wild-type virus. This shows that the phenotype results from an effect on *M45* and not on adjacent genes.

To assess the specificity of the growth deficit, two of the mutants were used to determine their growth kinetics on various cell lines. Data for multistep growth curves were generated from fibroblasts, bone marrow stromal cells, hepatocytes, macrophages, and two different endothelial cell lines (*16*). MCMV-GFP, a recombinant virus that carries the GFP gene at an innocuous position (*12*), was used as control. As shown in Fig. 2, the mutants did not grow on endothelial cells but did grow with no or minimal deficit in all other cells analyzed, except in macrophages where the mutants grew to very low titers. This is strikingly similar to HCMV, where a loss of growth in endothelial cells is associated with a replication deficit in macrophages (*1*, *6*, *7*).

Next, we investigated the reason for the inability to grow on endothelial cells. The M45 insertion mutants clearly infected these cells, because viral genes were expressed as indicated by GFP (Fig. 1). The infection, however, failed to spread to adjacent cells. To define a potential block in the cascade of viral gene expression, infected endothelial cells were analyzed for the expression of an early and a late gene product. Immunofluorescence showed that both glycoprotein 40 (gp40) (*20*) and glycoprotein B were expressed (*15*). However, when endothelial cells were infected at a high multiplicity of infection (MOI), most cells infected with mutant virus were dead at 30 hours after infection (post infection), whereas cells infected with MCMV-GFP showed cytopathic effect but were still alive (Fig. 3, A and B). To differentiate between apoptosis and necrosis, infected endothelial cells were analyzed at an earlier time for hallmarks of apoptosis (*18*). At 22 hours post infection, a large number of cells infected with mutant virus displayed phosphatidyl serine on the outer leaflet of the cell membrane (Fig. 3, C and D). In addition, we detected DNA fragmentation by nick end-labeling (TUNEL) and morphological changes characteristic of apoptosis (Fig. 3, E and F). These phenomena were not observed with ultraviolet (UV)–inactivated virus. This suggests that the *M45* gene encodes or activates an inhibitor of apoptosis that is indispensable for virus growth and spread in endothelial cells.

In this study, we analyzed the genetic basis of endothelial cell tropism by establishing a forward-genetic procedure involving random mutagenesis and rapid phenotypic library screening. This approach differs from functional analysis of isolated viral genes, because it identifies a biological role for a viral protein by subtracting its activity from the viral context. A variant of Tn mutagenesis, signature-tagged mutagenesis, has been developed for bacteria and yeast (*21*, *22*) for simultaneous screening of numerous mutants in a single, complex pool. Unfortunately, this elegant approach cannot be applied to viral mutagenesis because pooling of viral mutants in cell culture would inevitably lead to trans-complementation and recombination in cells infected with more than one mutant, thus obscuring defective phenotypes. Our approach avoids pooling but still allows high-throughput screening. Furthermore, the principle of this forward-genetic approach should also be applicable to many other large DNA viruses that have already been cloned as BACs (*23*).

Random, unbiased methods of mutagenesis are especially useful in the absence of candidate genes that can be analyzed by reverse genetics. Identification of the M45 ORF in MCMV, a homolog of HCMV UL45, is surprising because these genes are homologous to the large (R1) subunit of cellular ribonucleotide reductase genes (*17*). A recent analysis of the homologous gene of a related β-herpesvirus, human herpesvirus 7, revealed that the gene does not encode a functional ribonucleotide reductase subunit, and the authors concluded that this gene has a different, as-yet-unknown function not only in human herpesvirus 7 but also in other β-herpesviruses (*24*). Our results suggest

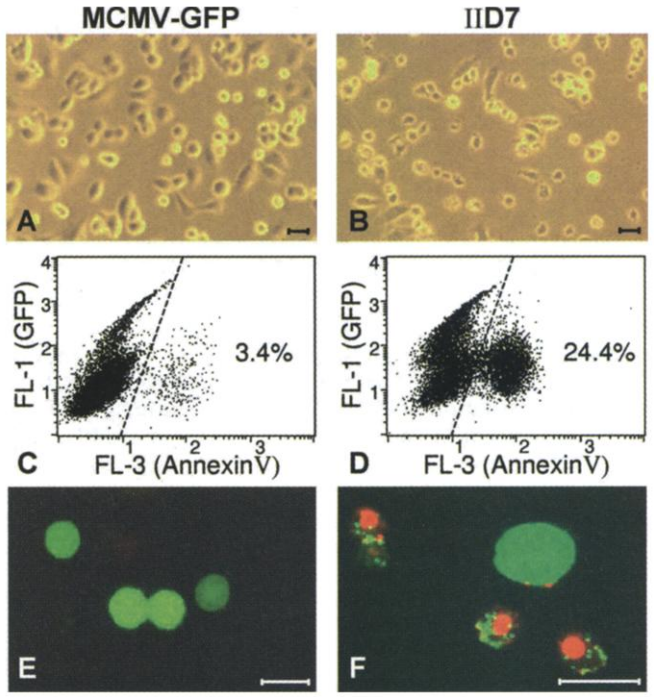


**Fig. 3.** Endothelial cells infected with M45 mutants rapidly undergo apoptosis. At 30 hours post infection, cells infected with MCMV-GFP at an MOI of 10 show a typical cytopathic effect (cell swelling and rounding), whereas cells infected with an M45 mutant (IID7) at the same MOI appear mostly dead (**A** and **B**). At 22 hours post infection, a large proportion of cells infected with IID7 were apoptotic as detected by AnnexinV staining (**C** and **D**). Control staining with propidium iodide was positive in 2 and 5.5% of MCMV-GFP and IID7-infected cells, respectively (not shown). DNA fragmentation was detected by TUNEL assay in numerous IID7-infected cells [(F), red nuclei] but only occasionally in MCMV-GFP–infected (**E**) or uninfected cells. Formation of apoptotic bodies was also observed (***F***) (green vesicles). Bars, 20 μm.

that the M45 ORF encodes or activates an inhibitor of apoptosis and that its physiological expression is essential for virus replication in endothelial cells. However, it is conceivable that the observed phenotype is not strictly confined to endothelial cells and macrophages, but generally applies to cells that are more prone to undergo apoptosis.

Apoptosis of infected cells is an efficient cellular defense mechanism against infectious agents, which is triggered either by immune effector cells or as a direct result of viral infection. Many viruses actively evade apoptotic destruction. Three HCMV gene products have previously been shown to inhibit apoptosis at the level of expression of the individual genes (*25*, *26*). The M45 gene product identified here shares no homology to any known inhibitor of apoptosis. It acts in a cell type–specific manner and in context of the viral infection, which is not yet proven for the other three CMV genes with anti-apoptotic function. Remarkably, unpublished observations indicate that inactivation of the R1 homolog in HCMV also promotes apoptosis (*27*) and that the R1 homolog of herpes simplex virus type 2 has anti-apoptotic function (*28*). This suggests that the gene function represents an escape mechanism used by herpesviruses in general, and it will be exciting to determine to what extent strain-specific differences in tropism are reflected by differences in the sequence or in the expression profile of the respective proteins.

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12. The Tn donor plasmid, pTsTM8 (*11*), was partially digested with Bgl II, blunted with Klenow polymerase, and re-ligated to eliminate one of the two Bgl II sites within Tn*Max8*. The resulting plasmid, pTsTM8B, was used to generate pTsTM16 by inserting the enhanced GFP gene from pEGFP-C1 (Clontech, Palo Alto, CA) into the Bgl II site. MCMV-GFP, which was generated inserting the GFP gene into the *ie2* region of the full-length MCMV-BAC, was provided by M. Messerle (Max von Pettenkofer Institute, Munich).
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14. Tn mutagenesis was performed essentially as described (*11*). The Tn donor plasmid, pTsTM16, and the MCMV-BAC were maintained in *E. coli* at the permissive temperature for replication of pTsTM16 (30°C), where transposition occurs at low frequency. A 100-μl aliquot of this liquid culture was used to inoculate 500 ml of LB medium preheated to the nonpermissive temperature of 43°C, selecting with chloramphenicol and kanamycin for bacteria containing the MCMV genome and Tn*Max16*. This culture containing a library of mutants was grown to an optical density at 600 nm ($OD_{600}$) of 0.6, and electrocompetent cells were prepared. The invasion plasmid, pGB2Ωinv-hly, was introduced by electroporation and selection with spectinomycin as described (*13*). This plasmid was introduced only at this stage to prevent it from serving as substrate for Tn insertion. Bacterial clones were transferred to 96-well microtiter plates. To determine the quality of the library, BAC DNA was extracted from 16 random clones, and 15 of these were found to contain an MCMV-BAC with a single Tn insertion. Two microliters of bacterial culture was used to inoculate fibroblast cultures grown in 96-well dishes without antibiotics. This corresponded to a multiplicity of infection of ~300 bacteria per fibroblast. Two hours later, the medium was replaced with addition of ampicillin and gentamicin (100 μg/ml each). Mutant viruses were amplified on fibroblasts in 12-well dishes. Supernatants of completely infected cells served as crude viral stocks (*15*).
15. Web figures 1 through 4 are available at *Science* Online at www.sciencemag.org/cgi/content/full/291/5502/303/DC1.
16. NIH-3T3, SVEC4-10, M2-10B4, and IC-21 cells were obtained from the American Type Culture Collection. MHEC-5T (*29*), and mHTC-K2 cells were provided by J. Plendl (University of Munich, Germany) and G. Jennings (HepaVec, Berlin, Germany). For screening, 200 μl of crude viral stock (*14*) were used to infect 35-mm dishes of NIH-3T3 and SVEC4-10 cells in parallel. End-point titers were obtained 6 days post infection. For growth curves, titered stocks were used to infect cells at a MOI of 0.1, and samples of the supernatants were harvested each day. All titrations were done on NIH-3T3 cells using the $TCID_{50}$ (median tissue culture infectious dose) method.
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18. Total RNA extracted from infected cells using an RNAeasy kit (Qiagen, Hilden, Germany) was reverse transcribed with avian myeloblast virus (AMV) reverse transcriptase (Roche, Indianapolis, IN) and was used for PCR amplification with primers 5′-AACTCGAGACGGTTGGCGCTGGA-3′ and 5′-TGTACGCGGCGACGATGC-3′, which were also used for sequencing of viral DNA. Mutant Tc38 was taken from a previous collection of Tn mutants (*11*). Mutants 3H9, 4F2, 15G6, 17F10, and 21B7 were obtained by PCR-screening of a library of uncharacterized MCMV::Tn*Max16* mutants (C. Ménard, unpublished data) using primers 5′-ATAACAGTATTTCGGGCTACG-3′ and 5′-CGGCTTTTATTTCAAGACGAG-3′, respectively. See *Science* Online (*15*) for a map of Tn mutants. To obtain the revertant virus, a 1-kb fragment (nt 62020 – 63038) that overlaps the Tn insertion site was cloned into pGS284. Homologous recombination in *E. coli* was performed as described (*30*). To introduce a frame-shift mutation, the Bam HI site in the same fragment was filled in with Klenow polymerase and re-ligated. To detect apoptosis, endothelial cells were infected at an MOI of 10 and were harvested 22 hours post infection, stained in parallel either with AnnexinV-Alexa568 (Roche) or propidium iodide, and analyzed by flow cytometry. DNA fragmentation was detected using a TUNEL assay (Roche) on paraformaldehyde-fixed cells.
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