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Enhancerless Cytomegalovirus Is Capable of Establishing a Low-Level Maintenance Infection in Severely Immunodeficient Host Tissues but Fails in Exponential Growth▽

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Major immediate-early transcriptional enhancers are genetic control elements that act, through docking with host transcription factors, as a decisive regulatory unit for efficient initiation of the productive virus cycle. Animal models are required for studying the function of enhancers paradigmatically in host organs. Here, we have sought to quantitatively assess the establishment, maintenance, and level of *in vivo* growth of enhancerless mutants of murine cytomegalovirus in comparison with those of an enhancer-bearing counterpart in models of the immunocompromised or immunologically immature host. Evidence is presented showing that enhancerless viruses are capable of forming restricted foci of infection but fail to grow exponentially.

Transcriptional enhancers consist of modules of transcription factor binding sites and mediate gene desilencing and promoter activation in an orientation-independent manner and even from remote sites (4). Whereas the "rheostatic" model, also known as "rate" or "progressive-response" model, proposes that enhancers function by increasing the rate of transcription from a cognate gene, the "binary" model, also known as "on-or-off" or "probability" model, proposes that they function by raising the probability for a cognate gene being in a desilenced state (12). In either case, the result of enhancer action is an increased amount of transcripts. Major immediate-early (MIE) enhancers of herpesviruses enhance the transcription of MIE genes that encode transactivator proteins critically involved in the expression of viral early-phase (E) genes as well as host cell genes. Enhancers are therefore regarded as key regulators for initiating the productive cycle in acute infection as well as in the reactivation from latency. The current knowledge of MIE enhancers of cytomegaloviruses (CMVs), members of the beta subfamily of the herpesviruses, and of the role of the cognate MIE proteins in the viral replication cycle have been comprehensively reviewed recently (3, 6, 26, 27, 28, 36, 42, 43). In the specific case of murine CMV (mCMV), the MIE locus consists of a bidirectional gene pair with a promoter-enhancer-enhancer-promoter element (8, 11, 24, 34, 40) flanked to the left and to the right by transcription unit *ie1-ie3* and gene *ie2* that are transcribed in opposite directions and encode the transactivator proteins IE1 and IE3 as well as IE2, respectively (7, 21, 22, 30, 31). IE3/M122, corresponding to IE2/UL122 of human CMV (hCMV), has been identified as the essential transactivator for E gene expression (30) and thus for viral replication (1). The two enhancer elements can act independently if engineered in isolation in viruses mCMV-ΔEnh1 or mCMV-ΔEnh2, and act synergistically after infection with wild-type (WT) virus (24).

Whereas much is known from infected cell cultures, studies of MIE enhancer function *in vivo* are restricted to animal models. Accordingly, our knowledge of the *in vivo* role for MIE enhancers is still incomplete. Notably, orthologous enhancers of different CMV species can replace each other more or less efficiently but with directional differences. Specifically, "enhancer swap" mutants of mCMV in which the MIE enhancer was replaced with the hCMV MIE enhancer (2) replicated in host organs with no obvious difference compared to WT virus in organ and cell type tropism (15), local spread within tissues (15), and establishment of latency as well as capability of reactivation and recurrence (16). In contrast, the reciprocal enhancer swap replacing the hCMV MIE enhancer with the mCMV MIE enhancer in a chimeric hCMV resulted in reduced MIE gene transcription in human fibroblast cell culture at low multiplicities of infection (19). Likewise, the mCMV MIE enhancer did not completely replace the rat CMV enhancer in cell culture and *in vivo* (37). The fact that the presence of an MIE enhancer is a crucial determinant of viral replicative fitness in host organs was concluded from the complete absence of detectable infectious particles and pathogenesis of an "enhancerless" mCMV in otherwise highly susceptible immunodeficient CB17.SCID mice (13), suggesting an absolute requirement for the MIE enhancer for virus growth *in vivo*.

As already discussed in the original work by Ghazal and colleagues (13), CB17.SCID mice, although deficient in adap-

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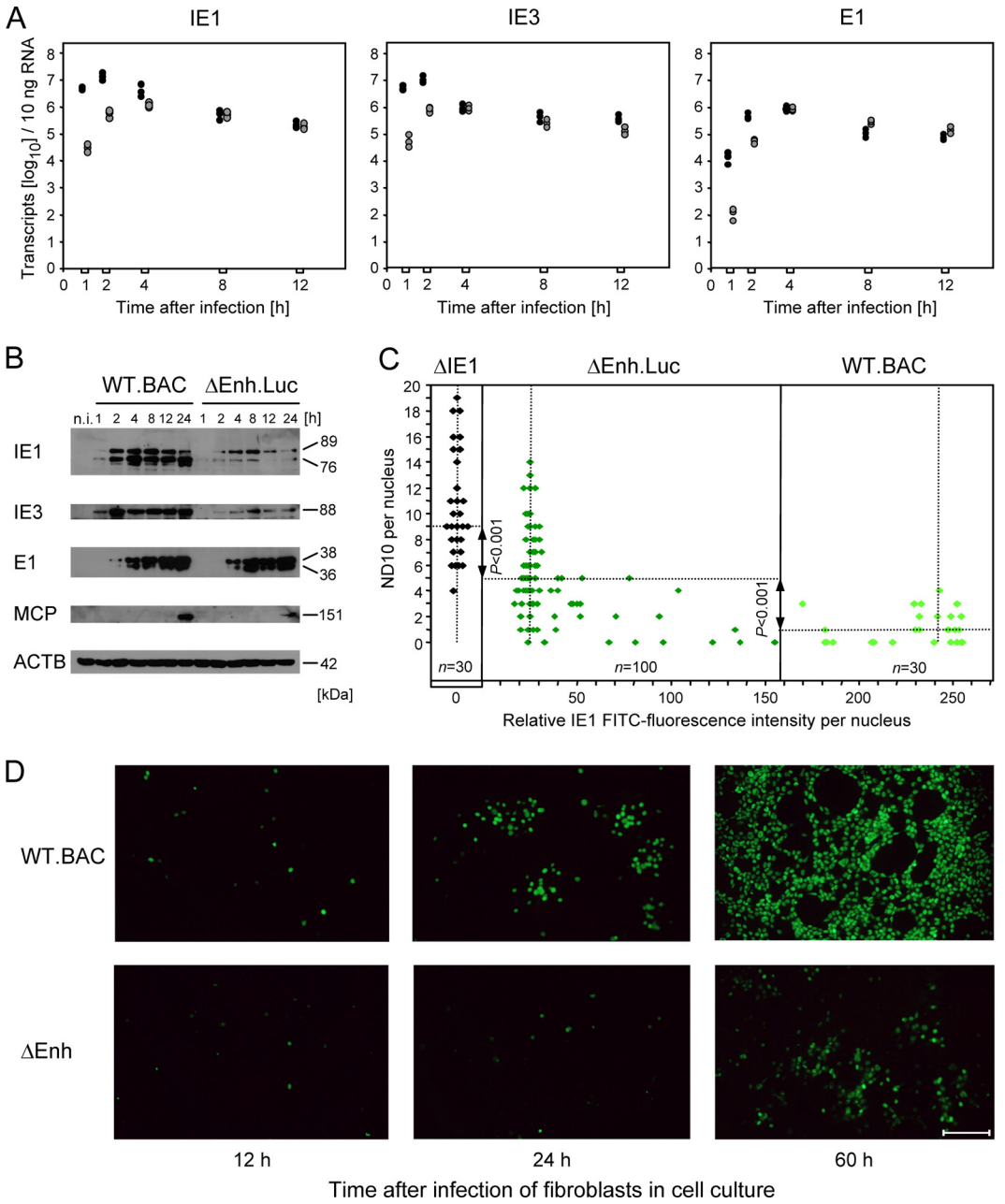

FIG. 1. MIE enhancer function in cultured fibroblasts. (A) Enhancement of viral transcription. BALB/c MEFs were infected under conditions of centrifugal enhancement of infectivity with 0.2 PFU per cell of mCMV-WT.BAC (black circles) or mCMV-ΔEnh.Luc (gray circles), resulting in an effective MOI of 4 (25, 32). Total RNA was isolated at the indicated times after the end of the 30-min centrifugation period. Absolute quantitation of IE1, IE3, and E1 transcripts was performed by reverse transcriptase quantitative PCR (RT-qPCR) as described in detail previously (39), and data were normalized to 10 ng of total RNA corresponding to ~1,000 cells. Symbols represent data from individual, triplicate six-well cultures. (B) Time course of viral protein expression. Western blot analysis was performed after infection of MEFs at an MOI of 4 (see above) essentially as described previously (18). n.i. (not infected), lysates from uninfected MEFs. Proteins IE1/m123 (23) and E1/M112-M113 (5, 10) were detected with monoclonal antibodies (MAbs) CROMA 101 and CROMA 103, respectively (kindly provided by S. Jonjic, Rijeka, Croatia). IE3/M122 (30) was detected with a polyclonal rabbit antiserum (kindly provided by M. Messerle, Hannover, Germany), and MCP/M86 with affinity-purified polyclonal rabbit antibodies, custom-made as described previously (46). ACTB, β-actin detected with affinity-purified polyclonal rabbit antibody. (C) Expression and function of IE1 in individual cells. CLSM analysis was performed essentially as described and documented previously (39, 46) for correlating ND10 numbers and levels of IE1 expression in the nuclei of the indicated numbers (*n*) of individual MEFs at 4 h after infection at an MOI of 4 with mCMV-ΔIE1 (ΔIE1, black diamonds), mCMV-ΔEnh.Luc (ΔEnh.Luc, dark-green diamonds), and mCMV-WT.BAC (WT.BAC, bright-green diamonds). Horizontal and vertical dotted lines mark the median values of ND10 numbers (ordinate) and relative IE1 fluorescein (FITC) fluorescence intensities (abscissa), respectively. The indicated *P* values for differences in ND10 numbers were calculated with the nonparametric and distribution-free Kolmogorov-Smirnov test. Data sets differ significantly if the *P* value is <0.05. (D) Imaging of multistep virus growth in fibroblast cell cultures documenting focal spread of infection. BALB/c MEFs grown on glass coverslips in 24-well plates were infected noncentrifugally at an MOI of 0.05 with mCMV-WT.BAC (top panels) and mCMV-ΔEnh (bottom panels). FITC fluorescence images were taken after IE1 (CROMA 101) staining at the indicated times after infection. The bar marker represents 200 μm.

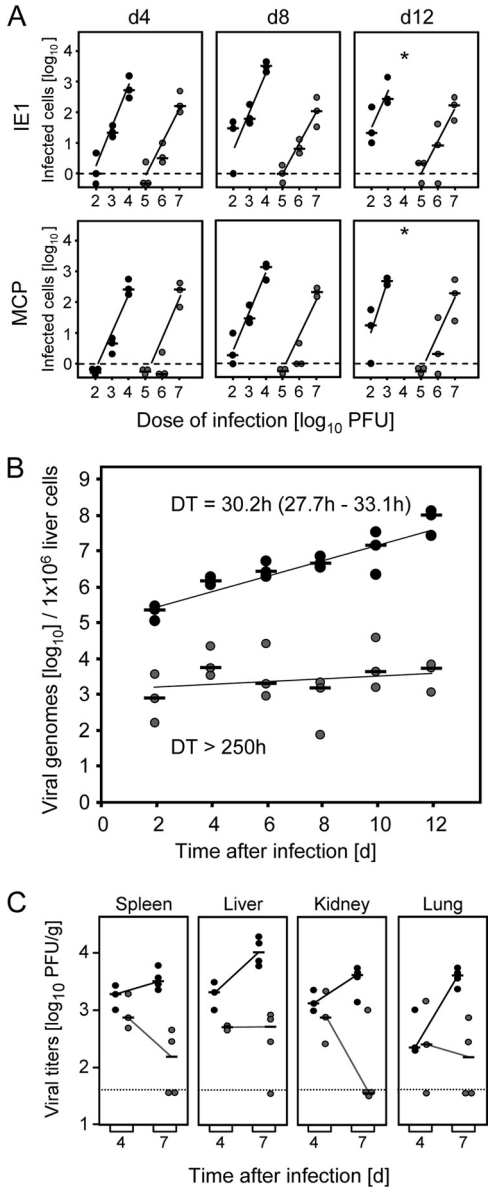

FIG. 2. Virus multiplication in host organs. Throughout, symbols indicate three to four individual mice tested for each day and group, with the median values marked by a short horizontal bar. (A and B) Virus multiplication in the liver of the severely immunocompromised host. Female BALB/c mice (8 weeks old) underwent hematoablative treatment by total-body gamma irradiation with a single dose of 7 Gy, 2 h prior to intravenous infection with mCMV-WT.BAC (black circles) or mCMV-ΔEnh.Luc (gray circles). (A) Virus dose dependence and kinetics of infection. Liver tissue infection was assessed by quantitating infected liver cells, mostly hepatocytes, in representative 10-mm^2 areas of tissue sections by IHC specific for the intranuclear proteins IE1/m123 (for the method, see references 15 and 32) and MCP/M86 (for the method, see reference 46), representing the IE and L phases of the viral replicative cycle. To compensate for reduced IE1 expression after infection with mCMV-ΔEnh.Luc, the period of color reaction was extended from the usual 2 min (sufficient for mCMV-WT.BAC) to 10 min. *, all mice succumbed to infection with 10^4 PFU of mCMV-WT.BAC. (B) Multiplication of viral genomes in the liver. Viral genome numbers in the liver were quantitated at the indicated days after infection with 10^3 PFU of mCMV-WT.BAC (black circles) or 10^6 PFU of mCMV-ΔEnh.Luc (gray circles) by qPCR specific for gene *M55*/*gB* as described previously (38), using linearized plasmid pDrive_gB_PTHrP_Tdy as the standard (41). Log-linear growth curves were determined by regression analysis, and doubling times (DT) as well as their 95% confidence intervals (shown in parentheses) were calculated as explained in greater detail elsewhere (46). (C) Virus multiplication in the immunologically immature neonatal host. Three-day-old BALB/c pups were infected intraperitoneally with 5×10^2 PFU of mCMV-WT.BAC (black circles) or 2×10^6 PFU of mCMV-ΔEnh (gray circles). At days 4 and 7 after infection, virus titers in homogenates of the indicated organs were determined by PFU assay with centrifugal enhancement of infectivity, performed on monolayers of MIE-complementing NIH 3T3-Bam cells (16). Dotted lines indicate the detection limit of the assay. Note that infection of pups with 5×10^4 PFU of mCMV-ΔEnh did not yield detectable virus in any of the organs tested (data not depicted).

tive immunity, are not completely devoid of innate immunity, which might have led to an early resolution of a low-level infection by the enhancerless virus. However, it still remains to be determined whether the enhancerless mCMV can, in principle, establish and disseminate a low-level maintenance infection. We therefore have here revisited the enhancer requirement in an even more sensitive model for detecting mCMV replication, namely, the infection of genetically susceptible BALB/c mice after immunoablative total-body gamma irradiation, a relevant and well-established preclinical model originally designed for studying CMV infection in the context of hematopoietic stem cell transplantation (17). Evidence is provided to conclude that enhancerless mCMV can replicate in various host organs and spread locally to form infectious foci for maintaining a low-level infection in the absence of exponential growth.

(A part of this work is included in the M.D. thesis of R. Pintea at the University Medical Center of the Johannes Gutenberg University Mainz.)

Enhancerless virus mCMVdE::Luc, here named mCMV-ΔEnh.Luc, contains a luciferase gene sequence as a stuffer in place of the mCMV MIE enhancer (13) and is based on bacterial artificial chromosome (BAC) plasmid pSM3fr that corresponds to reconstituted WT-like virus MW97.01 (45), here named mCMV-WT.BAC. To overcome IE transactivator deficiency and to eliminate attenuating BAC sequences, mCMV-ΔEnh.Luc was propagated, and its infectivity was quantitated on complementing NIH 3T3-Bam cells (13).

Upon infection of noncomplementing BALB/c-derived mouse embryo fibroblasts (MEFs) at a multiplicity of infection (MOI) of 4 with mCMV-WT.BAC and mCMV-ΔEnh.Luc, steady-state levels of IE1, IE3, and E1 transcripts were found to be reduced for the mutant by ~2 $\log_{10}$ grades early after infection (Fig. 1A), which was associated with reduced levels of MIE proteins IE1 and IE3 (Fig. 1B). Interestingly, in hCMV, GC boxes located in the proximal enhancer mediate MIE transcription and viral replication in human fibroblasts through binding of transcription factors SP-1 and/or SP-3 (20). In this context, it is important to note that a putative SP-1 site located at positions −93 to −102 relative to the *ie1-ie3* MIE transcription start site (+1) of mCMV (24) is deleted in mCMV-ΔEnh.Luc (see Fig. 4A). In fact, a search for motifs by using the MEME/MAST algorithm combined with the JASPAR database (24) did not predict any transcription factor binding sites between positions −1 and −47 that are retained in

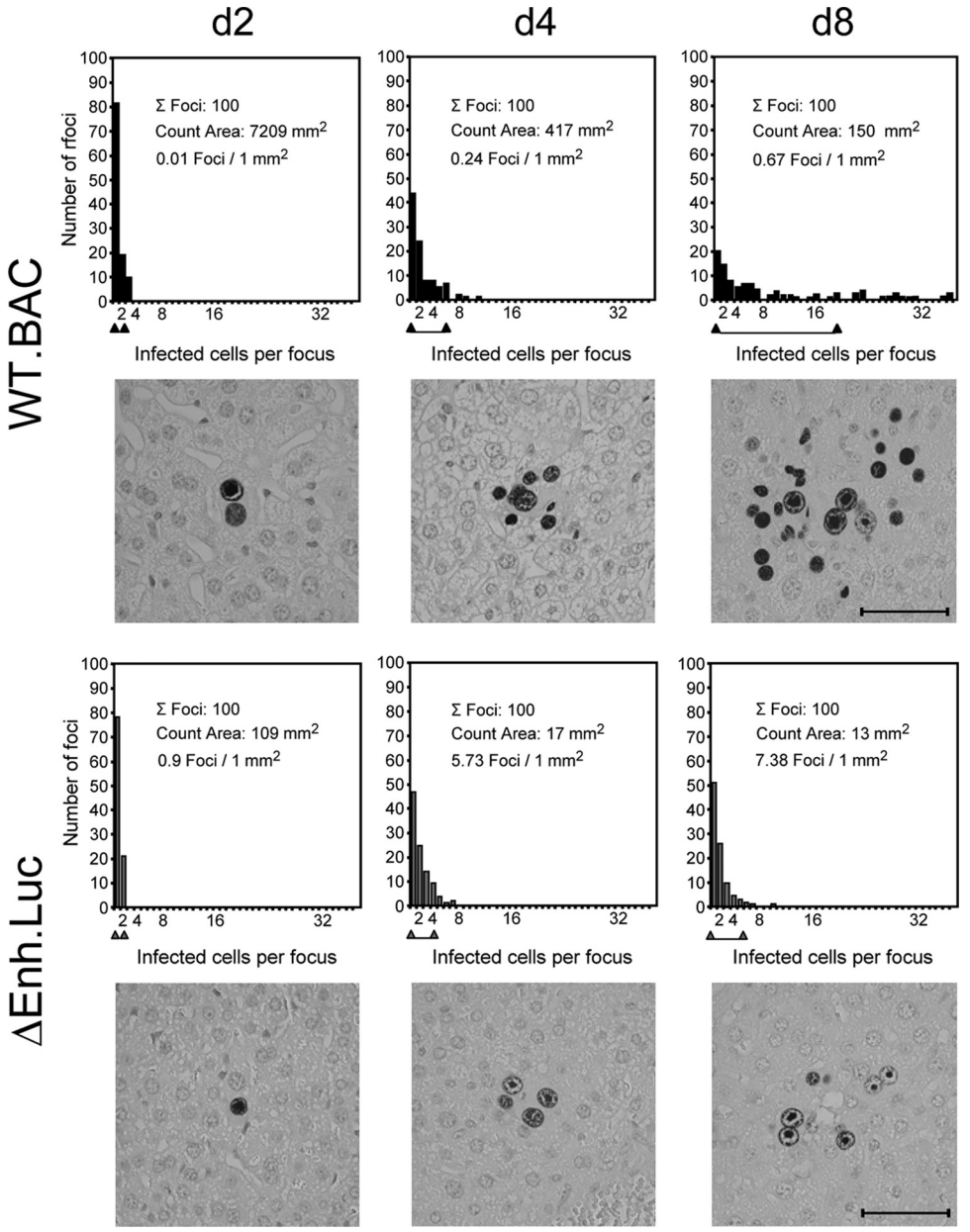

FIG. 3. Focal spread of infection in liver tissue. Immunocompromised mice (see legend of Fig. 2) were infected intravenously with 10^3 PFU of mCMV-WT.BAC (black bars) or 10^7 PFU of mCMV-ΔEnh.Luc (gray bars). IHC specific for IE1/m123 (MAb CROMA 101) was performed in liver tissue sections at the indicated days after infection by using the peroxidase-diaminobenzidine-nickel method for black staining (15, 32), followed by light hematoxylin counterstaining. Data were collected and pooled from three mice per day and group. Bar diagrams show the size distributions of 100 infectious foci counted. Caliper rules underneath the abscissas as well as representative IHC images highlight the increase in median focus size over time. Bar markers represent 50 μm.

mCMV-ΔEnh.Luc. While absence of the enhancer had little impact on the expression of the E-phase protein E1, there appears to be some reduction in the late (L)-phase major capsid protein MCP/M86 (Fig. 1B), consistent with reduced viral DNA replication and release of infectivity (24).

Expression and functional activity of protein IE1 was studied on a single-cell level by confocal laser scanning microscopy (CLSM) correlating nuclear IE1 fluorescence intensity and IE1's capacity to dissociate repressive nuclear domains 10 (ND10), as described and illustrated with images in previous reports (14, 39, 44, 46) (Fig. 1C). Compared with mutant virus mCMV-ΔIE1 (14), the low level of IE1 expressed by mCMV-ΔEnh.Luc was able to dissociate ND10, although with a broad overlap in the cell distributions. As expected, the high level of IE1 expressed by mCMV-WT.BAC dissociated most ND10 in all cells. Of importance is the observation that individual cells infected with mCMV-ΔEnh.Luc expressed higher levels of IE1 that were associated with efficient dissociation of ND10 and were most likely due to a stochastically elevated basal activity of the core promoter. In striking accordance with these data, a

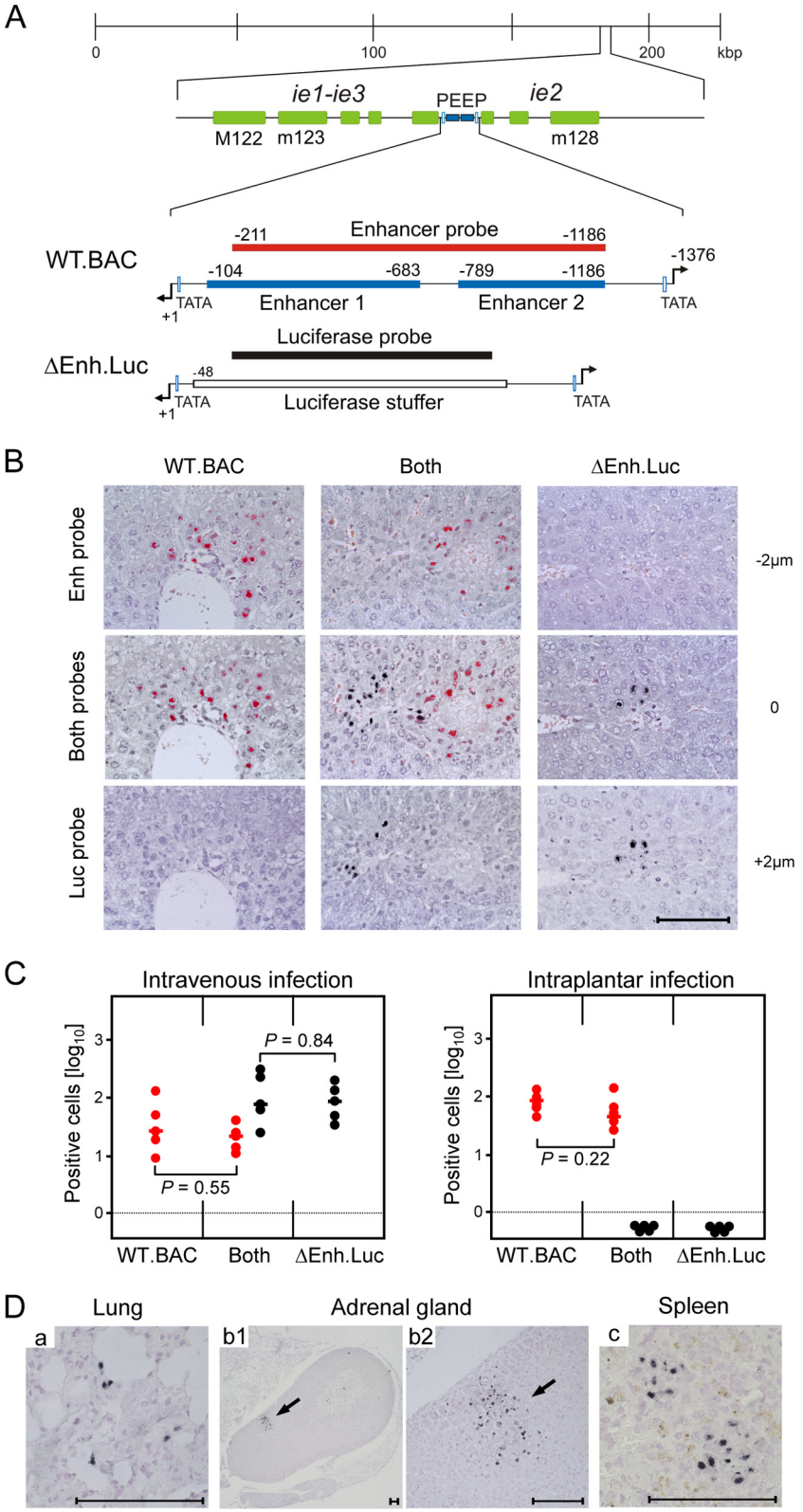

FIG. 4. *In situ* verification of virus authenticity. (A) Maps of the mCMV genome (not drawn to scale), illustrating the organization of the mCMV MIE locus with the regulatory promoter-enhancer-enhancer-promoter (PEEP) region and the exons of the flanking IE genes as well as the positions of the ISH DNA probes. Map positions refer to the 5′ transcription start site (+1) of the *ie1-ie3* transcription unit, equivalent to nucleotide 182,895 in the mCMV Smith strain genome (33) (GenBank accession no. NC_004065). Arrows indicate the DNA strand and the divergent 5′-to-3′ directions of transcription for the bidirectional MIE gene pair. (B) Virus identification by two-color ISH in the coinfection model. Immunocompromised mice were infected with 10^3 PFU of mCMV-WT.BAC alone, with 10^7 PFU of mCMV-ΔEnh.Luc alone, or with a

multistep growth analysis of the related mCMV-ΔEnh (mCMVdE with no Luc stuffer; see reference 13) in MEF cultures revealed a low number of individual cells staining with an intermediate brightness for IE1 and capable of forming fluorescent foci, albeit with delayed spread compared to WT virus (Fig. 1D). Thus, individual cells infected with enhancerless virus can show an almost WT phenotype with regard to IE1 expression and function, at least in the first round of infection, but appear to have a deficiency in replicative capacity and spread. In this context, it is worth recalling that mCMV-ΔEnh.Luc virions were found to be poorly released into the cell culture supernatant (24), suggesting that focus formation may depend on cell-to-cell spread. Accordingly, we wondered if infected host tissue cells with such a phenotype might be founders of a progressive *in vivo* infection.

Since the virus dose used for infection determines the probability for successful hits, immunocompromised recipients (see above) were infected intravenously with graded virus doses, and infected cells in the liver, mostly hepatocytes (35), were detected by immunohistochemistry (IHC) specific for the intranuclear viral proteins IE1 and MCP (Fig. 2A). At a glance, mCMV-ΔEnh.Luc proved to be capable of infecting liver cells in a log-log dose-effect relationship, but with a shift to higher doses by 3 to 4 $\log_{10}$ grades. The graphs also show, however, that the number of cells infected with mCMV-ΔEnh.Luc did not increase over time. This was further corroborated by measuring the doubling times of viral genomes in liver tissue. Whereas the log-linear growth curves indicate exponential growth of mCMV-WT.BAC with a doubling time of ~30 h, mCMV-ΔEnh.Luc barely multiplied, with no doubling in the observation period (Fig. 2B). Importantly, these findings were not restricted to the particular model of a severely immunocompromised host after hematoablative treatment, since essentially the same behavior of enhancerless and WT viruses was revealed in various organs of 3-day-old BALB/c pups representing an immature immune system in the completely independent and likewise medically relevant model of neonatal infection (Fig. 2C).

Stationary numbers of infected cells could reflect a nonproductive first-hit infection of cells or indicate a balance between focal spread starting from some individual cells, as is suggested by Fig. 1C and D, and the extinction of many others. Back to the model of the iatrogenically immunocompromised host, these alternatives were distinguished by measuring the size of infectious foci in the time course by IE1-specific IHC in the liver (Fig. 3). Clearly, at an early time, only single cells were infected, whereas foci consisting of increasing numbers of infected cells were formed with time for both viruses, although with a faster spread of mCMV-WT.BAC. Thus, mCMV-ΔEnh.Luc can establish further rounds of infection in host tissue even though the net number of infected cells increases only slightly over time.

The fateful question remained if the observed foci really resulted from infection with mCMV-ΔEnh.Luc or if possibly revertant virus was formed accidentally during virus propagation in the MIE-complementing NIH 3T3-Bam cells. Although we could not detect the MIE enhancer sequence by quantitative PCR in virus stocks of mCMV-ΔEnh.Luc (data not shown), minute contaminants in the high doses used for infection might have escaped detection. The identity of focus-forming viruses in liver tissue sections was therefore tested most rigorously by *in situ* hybridization (ISH) specific for the mCMV MIE enhancer or the luciferase gene stuffer sequence, resulting in red and black staining, respectively (Fig. 4). After separate intravenous infections with either mCMV-WT.BAC or mCMV-ΔEnh.Luc, infectious foci present in serial 2-μm sections hybridized exclusively with the cognate ISH probe. This unequivocally demonstrated that mCMV-ΔEnh.Luc replicated in the absence of the MIE enhancer. Accordingly, upon intravenous coinfection with both viruses, distinct red and black foci representing mCMV-WT.BAC and mCMV-ΔEnh.Luc, respectively, were detected in the same liver tissue section by using a mixture of both ISH probes (Fig. 4B). We did not find cases of *trans*-complementation through cellular coinfection, which would have become visible by red-and-black speckled nuclear inclusion bodies. Independent replication of both viruses is also indicated by quantitation of infected cells after coinfection in comparison to single infections. As shown in Fig. 4C (left panel), mCMV-ΔEnh.Luc did not profit in any way from the presence of mCMV-WT.BAC. Interestingly, after intraplantar coinfection with equal doses of both viruses (Fig. 4C, right panel), mCMV-WT.BAC did not help mCMV-ΔEnh.Luc to disseminate to the liver, which also indicated a lack of *trans*-complementation. This is in striking contrast to findings made previously for the intraplantar coinfection with mCMV-WT.BAC and the dissemination-deficient mCMV-ΔM36 (9), a virus in which deletion of the antiapoptotic protein M36 prevents replication in macrophages (29). In this example, cellular coinfection was frequent, and *trans*-complementation helped the mutant to travel to the liver (9). Explain-

mixture of both viruses in the 10^3:10^7 PFU ratio. ISH images of serial 2-μm liver tissue sections were taken on day 10 after infection. Sections were hybridized in a chessboard scheme with the mCMV enhancer probe (Enh probe, red staining) alone identifying cells infected with mCMV-WT.BAC, with the luciferase gene stuffer probe (Luc probe, black staining) alone identifying cells infected with mCMV-ΔEnh.Luc, or with a mixture of both probes identifying both viruses simultaneously. Staining methods were described in detail previously (15, 32). Light counterstaining was performed with hematoxylin. The bar marker represents 100 μm. (C) Independent growth of the enhancerless mutant. A possible *trans*-complementation of mCMV-ΔEnh.Luc by mCMV-WT.BAC was tested by comparing single infection quantitatively with coinfection. Corresponding to the experiment shown in panel B, ISH-positive cells were counted for representative 10-mm^2 areas of liver tissue sections. Red and black circles represent data from five mice per group, with the median values marked by a short horizontal bar. The dotted lines indicate the detection limit of the assay, that is, 1 cell in 10 mm^2. Statistical comparisons of interest were made with the distribution-free Wilcoxon-Mann-Whitney (rank sum) test. *P* values (two-tailed) of >0.05 indicate that there is no significant difference. The left panel shows intravenous single infection or coinfection with 10^3 PFU of mCMV-WT.BAC (red) and 10^7 PFU of mCMV-ΔEnh.Luc (black). The right panel shows intraplantar single infection or coinfection with 10^5 PFU of either virus. (D) Organ tropism of mCMV-ΔEnh.Luc. Corresponding to the experiment shown in panel B (lower right ISH image), infected cells were identified with the Luc probe (black) in lung (a), adrenal gland (b1; whole organ overview section resolved to greater detail in b2; arrows point to a particularly extended viral focus), and spleen (c). Bar markers represent 100 μm throughout.

ing this difference is an interesting issue and challenge for future research. Finally, Fig. 4D verifies the authenticity of enhancerless virus replicating in other organs of relevance in CMV disease.

Conclusion. We have here revisited MIE enhancer function and refine the previous view of its absolute requirement for mCMV replication *in vivo*. Enhancerless viruses can in fact grow independently and establish infectious foci in various organs relevant to CMV disease. Presence of the enhancer, however, increases the probability of initiating infection and is required for exponential growth. The enhancer is thus a major determinant of viral replicative fitness and pathogenicity.

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