Synergism between the components of the bipartite major immediate-early transcriptional enhancer of murine cytomegalovirus does not accelerate virus replication in cell culture and host tissues


Institute for Virology, University Medical Center of the Johannes Gutenberg-University, Mainz, Germany

Major immediate-early (MIE) transcriptional enhancers of cytomegaloviruses are key regulators that are regarded as determinants of virus replicative fitness and pathogenicity. The MIE locus of murine cytomegalovirus (mCMV) shows bidirectional gene-pair architecture, with a bipartite enhancer flanked by divergent core promoters. Here, we have constructed recombinant viruses mCMV-DEnh1 and mCMV-DEnh2 to study the impact of either enhancer component on bidirectional MIE gene transcription and on virus replication in cell culture and various host tissues that are relevant to CMV disease. The data revealed that the two unipartite enhancers can operate independently, but synergize in enhancing MIE gene expression early after infection. Kick-start transcription facilitated by the bipartite enhancer configuration, however, did not ultimately result in accelerated virus replication. We conclude that virus replication, once triggered, proceeds with a fixed speed and we propose that synergism between the components of the bipartite enhancer may rather increase the probability for transcription initiation.

Transcriptional enhancers are defined as cis-acting genetic elements that are composed of modules of transcription factor-binding sites to increase transcription in an orientation-independent fashion and even from a distance (Blackwood & Kadonaga, 1998; Fiering et al., 2000). The major immediate-early (MIE) locus of murine cytomegalovirus (mCMV) (Busche et al., 2008; Dorsch-Häsler et al., 1985; Keil et al., 1987a; Maul & Negorev, 2008), like that of its human cytomegalovirus (hCMV) analogue (Boshart et al., 1985; Meier & Stinski, 2006; Stinski & Isomura, 2008), is a key regulatory region decisive for the initiation of the productive virus cycle in acute infection and in reactivation from latency. In both hCMV and mCMV, MIE locus activity is associated with gene desilencing by chromatin remodelling (Bain et al., 2006; Liu et al., 2008; Murphy et al., 2002).

As discussed previously (Chatellard et al., 2007; Reddehase et al., 2008; Simon et al., 2007), the mCMV MIE locus shows a gene arrangement that is reminiscent of a bidirectional gene pair, a gene architecture frequently used in mammalian genomes for coordinated parallel or alternate expression of genes involved in vital functions such as DNA repair (Adachi & Lieber, 2002; Trinklein et al., 2004). By definition, a bidirectional gene pair consists of two divergently transcribed neighbouring genes arranged head to head on opposite strands of the DNA and regulated by a shared bidirectional promoter. Alternatively, bidirectionality can be achieved by placing two core promoters divergently on both upstream and downstream sides of a duplicated enhancer, a construct used in gene technology for enhanced and coordinated expression of transgenes (Li et al., 2004).

Notably, such a promoter–enhancer–enhancer–promoter (PEEP) configuration has been naturally adopted by mCMV for regulating the expression of MIE genes. Specifically, a bipartite enhancer is flanked to the left and to the right by core promoters driving the divergent expression of transcription unit ie1–ie3 and gene ie2, respectively, giving rise to differentially spliced transcripts and the corresponding proteins IE1, IE3 and IE2. Transcript IE1 specifies the 89/76 kDa IE1 protein (Keil et al., 1985, 1987b), which is involved in the early disruption of nuclear domains 10 (Ghazal et al., 2005; Maul, 2008) and acts as a pleiotropic transactivator of
cellular genes (Gribaudo et al., 2000; Lembo et al., 2000) and as a co-transactivator of virus early (E)-phase genes (Messerle et al., 1992). Whilst IE1 is dispensable for virus reactivation from latency (Busche et al., 2009) as well as for the transactivation of cellular genes involved in nucleotide metabolism (Wilhelmi et al., 2008), apparently due to redundant virus regulation of these crucial functions, IE1 contributes to virus fitness in vivo (Ghazal et al., 2005). Transcript IE3 specifies the 88–90 kDa IE3 protein, which is the essential transactivator of virus E-phase genes (Angulo et al., 2000; Messerle et al., 1992). Finally, transcript IE2 specifies the 43 kDa IE2 protein (Messerle et al., 1991), which can act as a pleiotropic transactivator, but no essential function has been assigned to it as yet (Cardin et al., 1995; Manning & Mocarski, 1988).

The bipartite nature of mCMV’s MIE enhancer has been recognized only recently by Chatellard et al. (2007), who have used reporter-gene transfection assays to map two independent enhancers within the MIE locus. So far, however, it remained unknown whether the two enhancers would function independently in the infection of cells in cell culture, as well as in the more natural context of host tissues in vivo. Notably, the bipartite mCMV enhancer can be replaced with the hCMV enhancer without modulation of cell tropism or loss of reactivation potential (Angulo et al., 1998; Grzimek et al., 1999; Gustems et al., 2008).

![Fig. 1. Maps of the bipartite MIE enhancer and enhancer deletion recombinants.](image-url)

(Top) Map of the mCMV genome with the MIE locus shown expanded (not drawn to scale) to reveal the exon–intron structure of the MIE genes. Green boxes represent exons of the IE structural genes; blue boxes indicate the promoter–enhancer–enhancer–promoter (PEEP) configuration of the bipartite regulatory region. Open reading frames are designated according to the nomenclature proposed by Rawlinson et al. (1996). The ie1–ie3 transcription unit is located on the complementary strand. (Centre) MIE PEEP region resolved to greater detail. The indicated nucleotide positions refer to the transcription start site (+1) of the ie1–ie3 transcription unit, which corresponds to nt 182895 in GenBank accession no. NC_004065. Arrows indicate the divergent 5′/3′ directions of transcription. Coloured symbols represent predicted transcription factor-binding sites. Corresponding sequence motifs were searched by using the MEME/MAST algorithm (Bailey & Gribskov, 1998) trained with available MIE enhancer sequences from various CMV species, and were identified by comparison with the JASPAR database (Sandelin et al., 2004). (Bottom) Maps of unipartite enhancer component deletion mutants mCMV-ΔEnh1 and mCMV-ΔEnh2, which were generated from BAC plasmid pSM3fr (Wagner et al., 1999) by single-step recombinase engineering in Escherichia coli SW105 (Warming et al., 2005), using kanamycin resistance as a positive selection marker subsequently eliminated by FLPe recombination (Lee et al., 2001). FRT, FLPe-recombination target. In the ΔEnh1 mutant, nt −104 to −683 (referring to WT.BAC) were deleted, comprising the core enhancer described previously (Chatellard et al., 2007; Dorsch–Hässler et al., 1985). In the ΔEnh2 mutant, nt −693 to −1193 (referring to WT.BAC) were deleted, encompassing Enh2 mapped by Chatellard et al. (2007). For fidelity of the clonings, see Supplementary Fig. S1.
Here, we pursued to answer four questions. (i) Are the two components of the bipartite MIE enhancer functional independently in the context of virus infection? (ii) Do they differ in their potency to enhance MIE gene expression, downstream E-phase gene expression and virus replication? (iii) Do they synergize in enhancing MIE transcription and downstream events? (iv) Is there any tissue-type selectivity in their functions? To address these basic questions, we used bacterial artificial chromosome (BAC) mutagenesis and BAC plasmid pSM3fr (Wagner et al., 1999) to construct recombinant viruses mCMV-ΔEnh1 and mCMV-ΔEnh2 (Fig. 1). Fidelity of the clonings was verified by sequencing (see Supplementary Fig. S1, available in JGV Online).

We first sought to test the dependence of MIE gene transcription on the presence of enhancers as a function of m.o.i. in fibroblast cell cultures (Fig. 2a) in order to establish conditions under which an enhancer is required at all. The data from quantitative RT-PCR for the three MIE genes (triplex RT-qPCR) indicate that basal promoter activity in an enhancerless control virus, mCMV-C3杏E::Luc (Angulo et al., 1998), here named mCMV-ΔEnh, is sufficient to mediate a basal level of MIE gene transcription that increases with increasing m.o.i. Presence of the bipartite, complete enhancer Enh1–Enh2 in virus MW97.01 (Wagner et al., 1999), here named mCMV-WT.BAC, enhanced MIE gene transcription by more than 100-fold, with the highest difference seen for the IE3 transcript. The unipartite enhancer components proved to be functional and equally potent in enhancing MIE gene transcription in both orientations, that is with no preference of Enh1 and Enh2 for IE1–IE3 and IE2, respectively. Notably, however, except at high m.o.i., transcription with mCMV-WT.BAC was approximately 5–10-fold higher than with the unipartite enhancers. This is more than additive, thus revealing synergism.

![Fig. 2.](Image)

Enhancer potencies in mediating the enhancement of MIE gene transcription. (a) Dependence of transcription on m.o.i. Triplex RT-qPCR was performed for the simultaneous quantification of the spliced IE1 and IE3 (Simon et al., 2006), as well as IE2 (Simon et al., 2007), transcripts in total RNA derived from BALB/c mouse embryo fibroblasts (MEFs) 90 min after centrifugal infection (Kurz et al., 1997) with the indicated viruses (■, WT.BAC; □, ΔEnh; ○, ΔEnh1; ●, ΔEnh2) at the indicated m.o.i., increasing in log₅ steps. Graphs for MIE transcripts are arranged in genomic map orientation relative to the MIE PEEP, which is symbolized by boxes with arrows indicating divergent transcription. (b) Time-course of MIE transcript amounts present in MEFs at the indicated times after centrifugal infection with the indicated viruses at an m.o.i. of 0.4. Time zero is defined as the end of the 30 min period of centrifugal infection. E1 transcripts were quantified by specific RT-qPCR (Simon et al., 2006). Throughout, symbols represent the median values for three independent six-well cell cultures, with error bars showing the range. The shaded areas highlight the time period during which synergism between the two enhancer components becomes evident. The arrowhead marks the 45 min time point of highest enhancer synergy. (c) Western blot analysis of IE1 protein (Wilhelmi et al., 2008) after 2, 4 and 6 h of protein synthesis from mRNA present 45 min after infection with an m.o.i. of 0.4. ActD, Irreversible blockade of transcription with actinomycin D (5 μg ml⁻¹).
Next, we measured the time-course of transcription after infection with an intermediate m.o.i. of 0.4, at which the difference between mCMV-WT.BAC and mCMV-ΔEnh is high in both relative and absolute terms (Fig. 2b). The data show peak amounts of the three MIE transcripts at 120 min, followed by a decline for IE2 and IE3 due to their shorter molecular half-lives compared with the extremely stable IE1 transcript (Simon et al., 2006, 2007). Again, enhancerless virus showed significantly reduced MIE gene transcription. Although steady-state transcript levels were comparable for all enhancer-carrying viruses at later times, a clear kick-start advantage provided by the bipartite enhancer became obvious at very early times. Specifically, at 45 min, expression of the essential transactivator transcript IE3 with mCMV-ΔEnh1 and mCMV-ΔEnh2 exceeded that of the enhancerless virus only by a factor of approximately 3, whereas it was already about 70-fold increased with mCMV-WT.BAC. As the IE3 protein is the essential transactivator of downstream E-phase genes, we expected to find an impact on e1 (M112–M113) (Bühler et al., 1990; Ciocco-Schmitt et al., 2002) transcription. To our surprise, however, the differences between the four viruses were levelled rather than expanded, although the enhancerless virus still showed a disadvantage in terms of a
Fig. 3. (a) Enhancer requirement for virus growth in fibroblast cell cultures. Multistep virus growth curves were determined for BALB/c MEFs infected centrifugally at m.o.i.s of 0.4 and 0.004 with the indicated viruses (■, WT.BAC; □, ΔEnh; ○, ΔEnh1; ●, ΔEnh2; ◊, ΔEnh+ Bam25). (Upper panels) Virus genomes quantified by gB/M55-specific qPCR using plasmid pDrive_gB_PTHp_Tdy (Simon et al., 2005) as standard. (Lower panels) Virus infectivity, measured as p.f.u. released into the cell supernatant. Symbols show the individual results for three six-well cultures per time point. Dotted lines help to compare plateau levels of DNA replication and virus productivity. As growth attenuation of mCMV-ΔEnh affects virus production in MEF test and assay cultures, supernatants were retested on the MIE protein-complementing cell line NIH 3T3-Bam25 (Ghazal et al., 2003) (∇). (b) Enhancer requirement for virus growth in host organs. (Left panels) Virus replicative fitness in liver tissue. Virus growth curves were determined for the liver of immunocompromised (6.5 Gy single-dose γ irradiation) BALB/c mice after intraplantar infection with 1 × 10^7 p.f.u. of the viruses indicated. Log-linear regression lines, doubling times (DTs) and 95 % confidence intervals of DTs (given in parentheses) were calculated as described previously (Simon et al., 2006; Wilhelmi et al., 2008). Virus fitness was assessed by the DTs of (i) virus genome numbers, as determined by gB/M55-specific qPCR; (ii) infectivity, measured as p.f.u. present in liver-tissue homogenates; and (iii) numbers of infected hepatocytes, as determined by immunohistological staining of intranuclear IE1 protein pp89/76 (Grzimek et al., 1999; Podlech et al., 2002). In the case of infection with enhancerless virus, NIH 3T3-Bam25 cells were used for the detection of infectivity. (Right panels) Lack of organ-specific differences in enhancer requirement. Virus replicative fitness in spleen, lungs and salivary glands was assessed for the indicated viruses by the DTs of virus genome numbers. Similar results were obtained on the basis of virus infectivity (data not shown). Throughout, symbols represent data for three individually tested mice per time point.

delayed onset of e1 transcription and lower amounts of transcripts (Fig. 2b, outer right panel).

Notably, when transcription was blocked at 45 min, enhancer synergism on the transcriptional level also translated into an increased amount of IE1 protein compared with both unipartite enhancers, and IE1 protein levels were below the detection limit of the Western blot after infection with the enhancerless virus (Fig. 2c). IE3 protein was detectable only after 6 h of protein synthesis and only after infection with mCMV-WT.BAC (data not shown).

How do these early differences in transcription and translation influence virus DNA replication and production of infectious progeny virions? This was studied first by virus growth curves in fibroblast cultures for intermediate and low m.o.i.s of 0.4 and 0.004, respectively (Fig. 3a). Whilst the enhancerless virus was replication-attenuated, in particular at low m.o.i., the three enhancer-carrying viruses replicated to comparable genome levels and were indistinguishable with regard to the release of infectious virus. Notably, even at an intermediate m.o.i. with mCMV-ΔEnh, virus release stagnated at input levels for the entire time-course.

Finally, virus growth was studied in organs of immunocompromised mice after intraplantar infection with the four viruses (Fig. 3b). In accordance with the previously noted, almost absolute growth attenuation of mCMV-ΔEnh (Ghazal et al., 2003), we detected only minute amounts of virus genomes and only at the latest time point monitored, namely day 10, and infectivity and numbers of infected cells were below the limit of detection. Thus, for in vivo growth, there is a strict enhancer requirement, which is probably related to a very low m.o.i. in vivo. Despite the differences in enhancer strengths, the three enhancer-carrying viruses multiplied in the liver with doubling times that were identical within 95 % confidence intervals and regardless of whether virus multiplication was measured on the basis of genome numbers, infectivity or numbers of infected tissue cells (Fig. 3b, left panel). In the liver, hepatocytes account for most of the virus productivity (Sacher et al., 2008), whereas diverse stromal, endothelial and epithelial cell types, as well as macrophages and dendritic cells, replicate CMVs in other organs (Podlech et al., 1998, 2002; Sinzger et al., 2008). Specifically, in salivary glands, acinar epithelial cells are responsible for the tropism of persistent infection (Campbell et al., 2008; Jonić et al., 1989). As cell type-specific differences were reported for the hCMV enhancer (Baskar et al., 1996), we wondered whether differences exist in the requirement for Enh1, Enh2 or the complete enhancer, in particular because the pattern of putative transcription factor-binding sites differs between Enh1 and Enh2 (Fig. 1). Data shown in Fig. 3(b) (right panel), however, revealed almost-identical growth of the three enhancer-carrying viruses in spleen, lungs and salivary glands. Thus, at least for the organs and under the infection conditions studied, we found no notable tissue selectivity of enhancer function.

In conclusion, whilst an enhancement of MIE transcription is important to proceed efficiently with virus replication, as shown by the attenuation of the enhancerless virus, the two components of the bipartite MIE enhancer proved to be independently functional and of comparably poten in surmounting a threshold of MIE transcription required for initiating virus replication. Interestingly, although the bipartite enhancer accelerates MIE gene expression through synergistic action of its two components, this kick-start advantage does not result in faster virus replication in cell culture or in host tissues.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, collaborative research grant 490, individual project E2. We thank Peter Ghazal, Martin Messerle and Ana Angulo for the chance to work with the enhancerless virus mutant.
References


