Role of the cytomegalovirus major immediate early enhancer in acute infection and reactivation from latency

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Received: 1 November 2007 / Published online: 19 December 2007
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Abstract The cytomegalovirus (CMV) major immediate early (MIE) enhancer-containing promoter regulates the expression of the downstream MIE genes, which have critical roles in reactivation from latency and acute infection. The enhancer consists of binding sites for cellular transcription factors that are repeated multiple times. The primate and nonprimate CMV enhancers can substitute for one another. The enhancers are not functionally equivalent, but they do have overlapping activities. The CMV MIE enhancers are located between divergent promoters where the leftward genes are critical and essential for reactivation from latency and acute infection and the rightward gene is nonessential. The rightward transcription unit is controlled by an enhancer for murine CMV. In contrast, human CMV has a set of repressor elements that prevents enhancer effects on the rightward viral promoter. The human CMV enhancer that controls the leftward transcription unit has a distal component that is nonessential at high multiplicity of infection (MOI), but has a significant impact on the MIE gene expression at low MOI. The proximal enhancer influences directly the level of transcription of the MIE genes and contains an essential Sp-1 site. The MIE promoter has a site adjacent to the transcription start site that is essential at the earliest stage of infection. The MIE enhancer-containing promoter responds to signal transduction events and to cellular differentiation. The role of the CMV MIE

enhancer-containing promoter in acute infection and reactivation from latency are reviewed.

Keywords CMV enhancer · MIE promoter · Latency · Reactivation

Introduction

Cytomegaloviruses (CMVs) are members of the betaherpesviruses. These viruses cause disease in the host of origin and replicate slowly relative to the other herpesviruses. In humans, CMV causes disease in multiple organs such as lung, liver, retina, gastrointestinal tract, etc. CMV replicates in differentiated cells of the endoderm, mesoderm and ectoderm which include cell types such as macrophages, dendritic cells, colonic and retinal pigmented epithelial cells, endothelial cells, fibroblasts, smooth muscle cells, neuronal cells, glial cells, hepatocytes and trophoblasts [1–9]. In contrast, the virus fails to replicate in poorly differentiated cells such as progenitor cells of the bone marrow and monocytes of the blood. In these cells the virus is maintained in a latent state [10].

The inactivity of the viral major immediate early (MIE) promoter is characteristic of CMV latency [11, 12]. During latency, the viral genome is associated with enzymes such as histone deacetylase (HDAC) and methyltransferase that is typical of silenced chromatin. The viral major immediate early (MIE) promoter is associated with heterochromatin protein HP1, which selectively binds methylated histone [13].

The viral MIE enhancer-containing promoter can respond to cellular signal transduction events, which activate the MIE promoter and the expression of the downstream genes [14]. Early during productive infection, the viral genome is associated with histone acetylases that

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acetylate the histone and open-up the chromatin structure for transcription [15, 16]. The viral MIE genes are located downstream of the MIE promoter and are necessary to initiate the productive replication cycle. The MIE gene products are rarely expressed during latency and are rate limiting for productive viral replication. The signal transduction events that activate the latent viral genome require further investigation. Our current data suggest that the MIE enhancer-containing promoter switches on or off depending on both cellular signal transduction events and the immune defenses of the host. With murine CMV, occasional MIE gene expression occurs during latency [17–19]. For example, the first gene downstream of the murine CMV MIE promoter, the *ie1* gene, may be expressed without a return to productive infection. Even after the expression of the essential *ie3* gene downstream of the MIE promoter, murine CMV does not necessarily enter a productive phase of infection [19]. In a nonpermissive monocytic cell type in cell culture, THP-1 cells, human CMV does not enter productive infection. Cellular differentiation is required for viral DNA synthesis and virus production [16]. Both the murine CMV studies in the host and the human CMV studies in cell culture indicate that cellular differentiation and the signal transduction events associated with cellular differentiation are necessary for viral reactivation from latency. Therefore, the CMV enhancer-containing promoter is a focal point to start the viral reactivation from latency, but the differentiation state of the cell determines the extent of viral gene expression and the production of infectious virus. Nevertheless, the CMV enhancer-containing promoter has one of the key roles for the genesis of disease by regulating viral latency and reactivation. This chapter will review the role of the CMV enhancer-containing promoter in acute infection and reactivation from latency.

Cytomegalovirus enhancers

Cytomegalovirus infections have been investigated in some detail in seven mammalian species. Figure 1 compares the

MIE enhancers of some of the primate and nonprimate CMVs. The CMV MIE enhancer elements characteristically have an array of *cis*-acting binding sites that are repetitive and bind cellular eukaryotic transcription factors. However, the arrangement and number of sites vary with the different species-specific viruses. The NF- κ B, CREB/ATF, and AP-1 sites are commonly shared transcription factor-binding sites (Fig. 1) [20–30]. The primate CMV enhancers have more CREB/ATF sites and the nonprimate CMVs of mouse and rat have more AP-1 sites. Murine CMV and human CMV have RAR-RXR sites. Murine CMV has numerous NF- κ B sites, but rat CMV does not. Human CMV has some unique far upstream sites like Elk-1, serum response factor, CBP, and gamma-interferon activating sites [26, 31]. These structural variations presumably indicate evolutionary adaptations for more efficient viral replication in various cell types. The different *cis*-acting elements can act independently and cooperatively to attract and enhance RNA polymerase II transcription of the MIE genes [32–34]. It was inferred from transient transfection experiments that the CREB/ATF and NF- κ B sites were the key components of the human CMV enhancer [32]. Surprisingly, mutation of one type of site in the enhancer has no effect on transcription from the MIE promoter in the context of the remaining sites and viral infection of human fibroblast (HF) cells. For example, mutation of all the CREB/ATF sites or all the NF- κ B sites has little to no effect on human CMV replication at high or low multiplicity of infection (MOI) [35, 36]. In addition, murine CMV with the enhancer substituted with the human CMV enhancer containing mutated NF- κ B binding sites replicated like wild type virus in mouse fibroblast cells [36].

Figure 2 demonstrates that there are multiple different binding sites for eukaryotic transcription factors in the human CMV genome and some sites are repeated multiple times. The multiple elements work together to promote transcription from the MIE promoter. It is possible that in the presence of the other multiple transcription factor binding sites of the entire enhancer, deletion of one type of site has little effect on replication in HF cell culture. Either the

Fig. 1 The enhancers of primate and nonprimate cytomegaloviruses. The known consensus binding sites of cellular transcription factors in the major immediate early enhancers are designated

Fig. 2 The human cytomegalovirus major immediate early enhancer and divergent promoters. The viral genes and promoter/transcription start sites are designated by an *arrow*. The enhancer has two components that are proximal and distal. Between the distal enhancer and the

UL127 promoter is a unique region that binds transcriptional repressor proteins. Downstream of the UL127 promoter is a region referred to as the modulator. The transcription factor binding sites are designated

other sites compensate for the loss or virion-associated glycoproteins like gB and gH or tegument proteins like pp71 facilitate a strong activation of the MIE promoter in HF cells [14, 37]. It is known that viral glycoprotein gB stimulates the PI-3'-PK signal transduction pathway which can activate cellular transcription factors such as CREB/ATF and NF- κ B [14, 38]. In addition, human CMV has incorporated into its tegument a cellular casein kinase that can activate the NF- κ B pathway [38]. The viral tegument protein pp71 (UL82) enters the cell and inhibits the transcription repressor hDaxx, which increases transcription from the MIE promoter [37, 39–41]. Even though the CMV enhancers are unique to each species, there are interplays between the diverse array of cellular transcription factors that govern the initiation and magnitude of expression from the MIE promoter. Therefore, the CMV enhancers determine the efficiency of viral replication.

The divergent promoters flanking the CMV enhancers

Betaherpesviruses have their MIE enhancers flanked by divergent transcription units. Bidirectional promoter arrangements are relatively common in the human and mouse genomes and the bidirectional genes they control have a biological relationship [42, 43]. To the leftward direction of the MIE enhancer in the CMV genomes are the MIE genes required for productive replication in permissive cells. To the rightward direction is a gene that is nonessential for replication in cell culture [44, 45]. With murine CMV, the intergenic region is about 1.4 kbp with an enhancer located 680 bp upstream of the *ie1* transcription start site [24]. In series is an enhancer that strongly drives transcription of the murine CMV *ie2* gene in the rightward direction [46]. In the mouse, murine CMV expresses rightward and leftward genes stochastically, i.e. either the right-

ward gene is on and leftward off or vice-versa [45]. The rightward gene appears to be expressed under tissue specific circumstances in the mouse, but the significance of this tissue specific expression is not understood [47]. Human CMV has a similar arrangement, except transcription of one gene of the pair is induced while transcription of the other is inhibited. There is a region between -740 and -550 that represses the rightward transcription of the UL127 gene at all times after infection of permissive HF cells referred to as the unique region (UR) (Fig. 2). The region was initially termed the UR because DNase I protection assays using nuclear extracts prepared from human cells demonstrated multiple interactions between unknown cellular proteins [48]. Deletion of the approximately 200-bp region between the UL127 TATA box and the MIE enhancer, results in rightward gene expression [49–51]. The UR is a complicated region that functions as a boundary domain or an insulator between the UL127 gene and the human CMV MIE enhancer. This region contains sites for the binding of cellular repressor proteins. The DNA binding proteins are transcriptional repressor proteins, some of which have been identified such as CCAAT displacement protein (CDP), special AT-rich sequence binding protein 1 (STAB-1), and pancreatic-duodenal homobox factor-1 (PDX-1) [52, 53]. It has been shown that SATB1 and CDP negatively regulate several viral and cellular genes through interaction with histone deacetylase and histone lysine methyltransferase [54–57]. CDP is a known transcriptional repressor protein that has effects on other viruses such as human papilloma virus and murine leukemia virus [58–61]. The UR of human CMV binds CDP, which represses expression from the human CMV UL127 promoter in transient transfection assays 4- to 6-fold. Down regulation of CDP in the cell by silencing RNA (siRNA) allows for higher expression from the UL127 promoter [52]. The UR also binds STAB1, but STAB1 did not independently repress the UL127 promoter

and the additive effect with CDP was marginal [52]. In transient transfection assays, PDX-1 repressed the MIE promoter [53]. There is a *cis*-acting site just upstream of the UL127 TATA box, referred to as the Fox-like site (Fig. 2) that represses transcription from the UL127 promoter as much as 80 to 100-fold when in the context of the virus in infected HF cells [50]. The repressive function of the UR explains how the human CMV MIE enhancer can selectively activate expression of the MIE genes but not the UL127 gene during productive infection. In the context of the virus, the presence of the UR has no positive or negative effect on the MIE promoter in recombinant virus infected HF cells. The effect of the rightward gene in murine or human CMVs on viral replication or latency is presently an enigma.

There is also a large component that flanks the 3' end of the UL127 ORF termed the modulator (Fig. 2). In transient transfection assays, the modulator augments transcriptional control from the MIE promoter [53, 62]. However, deletion of the modulator in recombinant viruses has no effect on human CMV replication in HF cells and in undifferentiated or differentiated monocytic THP-1 cells [63]. The effect of the modulator may either be redundant or have a role in cell types not tested to date. There is an A-T rich region with a dyad symmetry and multiple binding sites for cellular eukaryotic proteins such as YY1 and silencing binding protein (SBP) in the modulator (Fig. 2). Other binding sites such as ETS2-repressor factor (ERF) and Gfi-1 in the enhancer (Fig. 2) have been proposed to have a repressive effect on transcription from the MIE promoter in transient transfection assays, but this has not been demonstrated in recombinant viruses [64–67]. Removal of these sites by mutation in the viral genome did not relieve quiescence in cell culture model systems like the THP-1 and N-Tera 2 cells [44, 63, 68].

Substitution of CMV enhancers

One approach to study the functions of the CMV enhancers has been to substitute the enhancer from one species with that from a different species. Enhancer substitution experiments demonstrated that the enhancers are not functionally equivalent but they do have overlapping activities. For example, the simian CMV enhancer can fully substitute for the human CMV enhancer, but replacing the human CMV enhancer with the murine CMV MIE enhancer results in diminished MIE gene expression, viral replication and the production of a small plaque phenotype in cell culture [69]. The rat CMV MIE enhancer substituted with the murine CMV enhancer replicated less efficiently in cell culture and reduced levels of virus were detected in the salivary glands of the infected rat [70]. Murine CMV enhancer substituted

with the human CMV enhancer caused the virus to initiate infection less efficiently in the lung, spleen and adrenal glands of the mouse [71]. Lastly, while primate and nonprimate CMV enhancers can substitute for the human CMV enhancer, human herpesvirus 6 enhancer cannot (Isomura and Stinski, unpublished data). In general, the enhancer substitutions resulted in reduced viral replication efficiency, which indicates that the CMV enhancers have species specificity.

The distal and proximal enhancers of human CMV

The human CMV enhancer has a distal component between –550 and approximately –300 and a proximal component between approximately –300 and –39 relative to the transcription start site (+1) of the MIE promoter. Without the distal enhancer, the recombinant virus replicates slowly at a low MOI and has a small plaque phenotype. The effect of deletion of the distal enhancer is detected only after infection at low MOI [72]. UV-inactivated human CMV at a high viral-particle to cell ratio can rescue the recombinant virus with the distal enhancer deletion. The murine CMV distal enhancer cannot substitute for the human CMV distal enhancer [69]. The distal and proximal enhancer components presumably interact because there is at least a 100-fold reduction in virus production when the distal enhancer is deleted or substituted [69, 72].

The distal enhancer is composed of multiple *cis*-acting elements that function in *cis* and in either orientation (Fig. 2). Which *cis*-acting elements are most important for the function of the distal enhancer requires further investigation. There are sites at –510 and –380 that are gamma interferon activation sites (GAS). Recombinant viruses with these sites deleted or with point mutations replicated normally at high MOI and less efficiently at low MOI [31]. It is possible that signal transduction events that activate interferon also activate MIE gene expression. The open reading frames in the region can have stop codons inserted at –345 and –300 without any negative affect on distal enhancer function and MIE gene expression [33].

The proximal enhancer has *cis*-acting elements that directly affect transcription from the MIE promoter. Without the proximal enhancer, the recombinant virus cannot replicate. Successively larger 5'-end truncation of the proximal enhancer results in recombinant viruses that replicate slower and to lower titers. Enhancerless human CMV with just the TATA box containing promoter element does not replicate in HF cell culture [73]. The minimal enhancer element for human CMV replication in HF cell culture is an Sp-1 binding site at either –75 or –55 [73, 74]. Viral replication is greatly diminished with one or two Sp-1 sites, and there is a characteristic minute plaque phenotype [74].

Human CMV infection induces an increase in Sp-1 DNA binding activity and the viral glycoproteins gB and gH in the viral envelope are responsible for this induction [14]. Sp-1 or Sp-3 transcription factors bind the GC boxes at –75 and –55 [74]. Sp-1 is reported to interact with TAF4 [75] and possibly factors binding to the initiator sequence (Inr) at the transcription start site [76]. In addition, Sp-1 is reported to interact with CREB/ATF [77, 78]. The Sp-1/Sp-3 sites are between the upstream CREB/ATF sites and the downstream Inr and may bridge these components at the TATA box (Fig. 2). Sp-1 may also have an effect on the acetylation of histones at the MIE promoter. It is notable that the chimpanzee and simian CMVs also have Sp-1/Sp-3 binding sites in the proximal enhancer near the TATA box (Fig. 1). It is assumed that the cell type, the stage of cellular differentiation, and the activity of the various signal transduction pathways have an impact on both the distal and proximal enhancers.

When using infectious viral DNA in the absence of the distal enhancer and the virion envelope and tegument proteins, the NF-1, AP-1, Sp-1, CREB/ATF, and NF- κ B *cis*-acting sites in the proximal enhancer cumulatively affect transcription from the MIE promoter and recombinant virus replication (Lashmit et al., unpublished data). The NF- κ B sites as well as the AP-1 and NF-1 sites have a moderate effect in the presence of the two Sp-1 sites, but a single CREB/ATF site has a significant effect (Lashmit et al. unpublished data).

The MIE promoter

The human CMV promoter contains a TATA box between –28 and –22, a *cis*-repression sequence (crs) between –13 and +1, and an Inr between +1 and +7 [79]. When the crs is extensively mutated, recombinant virus cannot replicate even if the upstream proximal and distal enhancer elements are present. If the human CMV crs is substituted with the murine CMV crs, the recombinant virus replicates less efficiently (Isomura et al., unpublished data). In contrast, substitution with the simian crs, which has a sequence similar to the human crs, has no effect. When the crs is mutated at only two bases, –10 and –9, there is 100-fold less MIE transcription. In addition, there is a 20-fold reduction in the splicing of the MIE precursor RNA [80]. These data indicate that the crs has an essential role in viral replication and may act as a positive element between the enhancer and the transcription start site in the early stages of MIE transcription. The crs may also act as a critical bridge between the transcription start site and the proximal enhancer for efficient MIE gene expression. An unknown cellular protein of approximately 150 kDa binds to the MIE promoter between –15 and +7 [79]. When the viral MIE protein

designated IE86 accumulates in the infected cell, it competes for binding to the region between –15 and +2 and represses transcription from the MIE promoter. Therefore, the crs element has the following two roles in early viral replication. (a) It plays a critical role in the early stages of transcription from the MIE promoter. (b) It prevents over expression of the MIE genes as the infection progresses.

De-silencing the CMV enhancer containing promoter

During latency, the CMV MIE promoter is associated with relatively high levels of HDACs, hypoacetylated histones and HP1 [13, 15]. An inhibitor of histone deacetylases, trichostatin A, will activate the MIE promoter and viral replication. Reactivation also occurs upon differentiation of CD34+ progenitor cells or monocytes into either dendritic cells or macrophages [15, 81, 82]. Allogeneic stimulation and proinflammatory cytokines are two events that have been linked to blood monocyte differentiation to macrophage or dendritic cells [82, 83]. In differentiated dendritic cells or macrophages, the MIE promoter is associated with relatively low levels of HDACs and higher levels of acetylated histones [13, 15, 81].

Two conditionally permissive cell lines have been used to investigate de-silencing of the viral genome in cell culture, the undifferentiated monocytic THP-1 cell or the neuroembryonic N-Tera-2 cell. These cells can be infected with human CMV and viral DNA can be found in the nucleus, but there is no viral MIE or early gene expression. In the undifferentiated THP-1 cell, the MIE promoter is associated with histone modifications typical of silenced chromatin [84]. In the N-Tera-2 cells, some of the viral genomes can be found in a supercoiled structure which is considered to be representative of herpesvirus latent genomes [68].

The CMVs differ from the alphaherpesvirus, herpes simplex virus, and the gammaherpesviruses, Epstein Barr virus and Kaposi sarcoma virus, by requiring expression of both viral MIE proteins for reactivation from latency in cell culture or in the mouse [16, 18, 19, 85]. The MIE CMV regulatory proteins will induce viral early gene expression. However, human CMV viral DNA synthesis and replication does not occur in the THP-1 cells without cellular differentiation [16]. Sporadic expression of the murine CMV MIE and early genes is not sufficient to signal viral reactivation and replication [19]. Because human CMV and murine CMV do not always replicate in cells expressing MIE or early viral proteins, cellular differentiation appears to be an important step in the viral reactivation process. While it has been assumed that MIE gene expression is the important rate-limiting step for CMV replication, cellular differentiation appears to be the major step for efficient viral reactivation.

We know very little about the events necessary to activate the cell towards differentiation and the latent viral genome towards productive viral replication. Not all events leading to differentiation of the cell also lead to reactivation of the virus. Reactivation appears to be related to cytokine stimulation of the undifferentiated cell. Tumor necrosis factor alpha (TNF- α) activates the murine CMV MIE promoter through the NF- κ B and AP-1 sites in the viral enhancer, but this is not enough to sustain viral reactivation in the mouse [86–88]. TNF- α plus allogeneic stimulation of T cells also induces murine CMV MIE promoter reactivation in the lungs or kidneys of the latently infected mouse [87, 89]. With human CMV, TNF- α and gamma released during the allogeneic stimulation of T cells contribute to activation of human CMV replication in monocytes from the blood [83]. While the MIE distal enhancer of human CMV contains two GAS sites for stimulation by gamma interferon as discussed above, quiescent human CMV in N-Tera-2 cells did not respond to gamma interferon [90]. In contrast, the quiescently viral infected cells did respond to stimulation of the cyclic-AMP pathway, which results in the activation of protein kinase pathways and phosphorylation of CREB/ATF. Inhibition of the protein kinase A pathway, but not the protein kinase C pathway, prevented activation of viral replication in the N-Tera-2 cells. These results suggest that the CREB/ATF sites in the MIE enhancer of the latent viral genome might be one of the most responsive elements for reactivation of the latent virus. However, there are approximately 60 different stimuli for activation of CREB/ATF which include growth factor-, steroid hormone-, peptide-, cyclic nucleotide-, immune cell-, neurologic- and ion channel/intracellular Ca^{++} signaling and effects by viral, bacterial, plant, phospholipid, and lipid components [91]. Saffer and Kalejta have proposed that the cellular protein Daxx mediates repression of the MIE promoter [92]. However, knockdown of hDaxx in nonpermissive undifferentiated cells did not permit human CMV MIE gene expression [93]. Which of the above are critical for reactivation from latency is currently not understood.

Perspectives

The CMV enhancers are responsive to cellular transcription factors. The mechanisms that trigger and sustain CMV reactivation from latency in vivo are largely unknown. A better appreciation of the nonviral induced signal transduction events that activate transcription from the CMV MIE promoter during latency would shed additional light on the life cycle of the virus. The MIE enhancer-containing promoter may be first among multiple check points to control viral reactivation from latency. We also do not

currently understand how the virus and the cell maintain the latent viral genome. Does the viral DNA replicate during cellular DNA replication? If so, where is the viral latent origin of replication? Does the virus use viral or cellular proteins to maintain the latent state? What is the relationship between cellular signal transduction events, cellular differentiation, and reactivation of virus from latency? Answers to these questions and others may lead to better strategies towards CMV disease prevention.

Acknowledgments We thank members of the Stinski lab and Jeffery Meier for critical review of this manuscript. Our work was supported by grant AI-13562 from the National Institutes of Health (M.F. S.) and by Grants-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports, Culture and Technology of Japan (15390153, 17659138 and 16017322 to Tatsuya Tsurumi, and 17590429 to H.I.), Research on Health Sciences focusing on Drug Innovation (SH54412 to H.I.), and Grant-in-aid for Cancer Research (13-01 to H.I.) from the Ministry of Health, Labor and Welfare.

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