Initiation of Cytomegalovirus Infection at ND10

G. G. Maul

Contents

Abstract As a large double-stranded DNA virus, CMV replicates in the nucleus, a highly structured environment. Diffusional and solid phases exist as interdependent sets of interactions between many components that determine either replicative success of an infecting virus or the defensive success of the host cell. In their extremes, cell death may be part of the lytic release of viral particles, or, in defense terms, the ultimate sacrifice preventing virus release. Between these extremes exists an evolutionarily derived standoff between virus and cell. Exogenous shifts in homeostasis can disturb this balance, diminishing the cell's defensive powers and reactivating the silenced viral genome. Many of the solid-phase aspects of this process can be seen in situ and analyzed. This review evaluates structural information derived from CMV-infected cells in situ at very early times of infection and the conceptional advances derived from them, mostly centering on the major immediate early gene products, specifically IE1. A scientific basis for considering the major immediate early proteins as potential targets in suppressing CMV disease is discussed.

G.G. Maul
The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19014, USA
maul@wistar.org

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Structural Observations in the First Hours After CMV Infection and Their Limits in Interpretative Value

The last physical hurdle for the infecting viral genome appears to be the nuclear pore complex. How viral DNA enters the host nucleus is not entirely clear, nor is it clear how it is protected from degradation by endonucleases once released from the capsid. One must assume that the viral DNA is first neutralized before or as it enters the host nucleus. This could be mediated either by the host's histones – specifically during S-phase when free histones are abundant – forming an easily silenced chromatin package or by positively charged polyamines neutralizing the virus DNA's negative charge, as has been reported for HSV1 (Gibson and Roizman 1971). Spermidine may still be associated with the viral genome when it leaves the capsid and enters the host nucleus. Because the nucleus is a highly structured environment, viral genomes are excluded from certain domains, such as the tightly packed nucleolus. Any observed nonrandom distribution of viral genomes in the nucleus may therefore be due to exclusion.

We do not know the physical dimensions of the large viral genome of CMV in the nucleus. A tightly coiled, chromatinized genome may be slightly larger than an encapsidated genome. Nor do we know whether the viral genome can move through the nucleus, either by passive diffusion or by active transport. This question has been difficult to address, because we cannot see the virus in real time and must therefore construct a likely sequence of events from observations of fixed material. The large size of CMV genomes aids microscopic identification and localization by in situ hybridization, which allows individual viral genomes to be visualized (Ishov et al. 1997). The size of these signals is close to that obtained by imaging tegument proteins like pp71 (Fig. 1a and b), i.e., the diffraction point size, which is limited by the wavelength of light. Signals can only become weaker when originating from smaller sources. Comparison of encapsidated viruses and in situ hybridization signals representing CMV genomes suggests that the infected host nucleus contains genomes that are highly condensed (Ishov et al. 1997). One caveat is that the completely extended genome (78 μm) would not be visible using this technique, because signals from any point along the approximately 260-Mb genomes would be too weak to register with our current techniques. Therefore, the detectable size, as estimated from what is visible, is equal to or smaller than the wavelength of light (~300 nm).

Fig. 1 (continued) early transcripts (*green*), for IE2 (*blue*) and PML (*red*). IE2 is located like a collar around the emerging transcripts. **d** 3T3 cell infected with MCMV (24 h p.i.). The cell is labeled with antibodies to the 112/113 gene product (*red*) and for viral genomes by in situ hybridization (*green*). Viral genomes are seen on the outside of the 112/113 labeled prereplication domains. **e** HFF 3 h p.i. by HCMV. Cell is triple-labeled for viral immediate early transcripts (*green*), for the splicing compartment delineating SC35 (*blue*) and the PML defining ND10 (*red*). The nucleus is outlined in *blue*. **f** MCMV infected 3T3 cells (24 h p.i.). Cells were probed for viral DNA by in situ hybridization (*green*) and for the 112/113 gene product by antibodies (*red*). The *hollow red spheres* inside the nucleus represent the replication compartments. The nucleus is outlined in *blue*

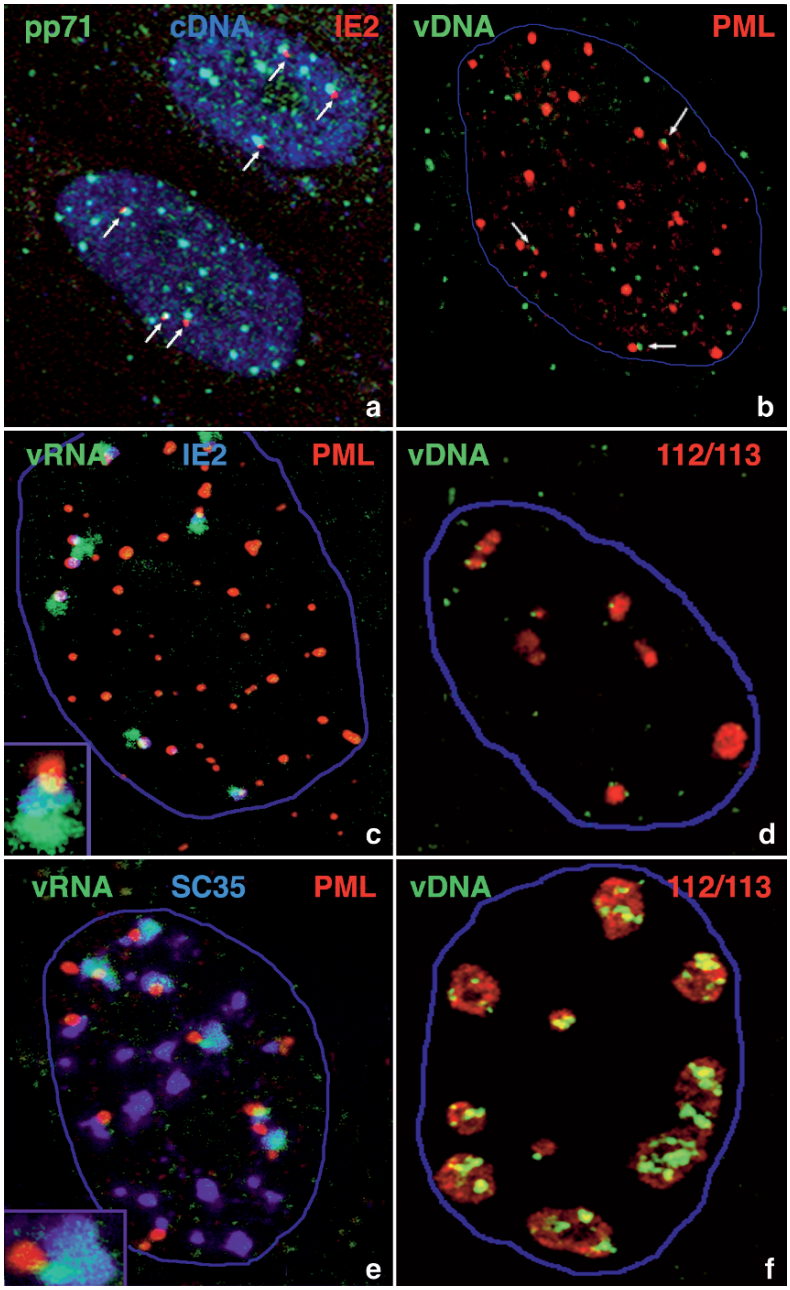

Fig. 1 **a** HCMV infected HFF (3 h p.i.). *Small green dots* represent pp71 antibodies bound to viral particles. *Larger green dots* in the (*blue*) nucleus represent pp71 antibodies bound to Daxx, which is associated with PML at ND10. The three *red domains* located beside the pp71 stained ND10 denote the localization of IE2 aggregates. **b** Same as **a**, but stained with anti-PML antibodies in *red* indicating ND10 and by in situ hybridization with HCMV DNA labeling viral genomes (*green*). *Arrows* point to viral genomes juxtaposed to ND10. The *blue line* was introduced to show the approximate boundary of the nucleus. **c** Same as **a**, but triple-labeled for the major immediate

The opposite technical problem is the much larger appearance of a structure with dimensions below the wavelength of light due to the dispersion of light from a high-intensity source. In this instance, a structure can appear much larger than it really is. One such type of structure is ND10 (nuclear domain 10), most often identified by immunofluorescence using antibody to the promyelocytic leukemia protein (PML; Fig. 1b, c, e). ND10 figure prominently in the early stages after DNA virus infections and appear to function like nuclear depots (Ishov and Maul 1996; Maul 1998). Most ND10 appear to be substantially larger than 300 nm when examined by fluorescent microscopy but smaller than 300 nm when examined by electron microscopy (Maul et al. 1995). This disparity is important to consider when interpreting images that show physical association between viral genomes and ND10. In human fibroblasts examined at 3 h postinfection (p.i.) (Fig. 1b), HCMV genomes appear throughout the nucleus as diffraction-spot-sized signals. Only a few of these signals localize beside ND10; none localize in these domains. Localization of genomes next to ND10 could be due to random events, particularly when one excludes the large volume of the nucleolus and the apparent location of various DNA viruses in only the interchromosomal space, that is, not within the chromosomal territories but the space occupied where ND10 are positioned (Bell et al. 2001).

Shortly after infection, IE transcripts appear to emanate only from a few ND10 (Fig. 1c and e) (Ishov et al. 1997). The conclusion drawn from these images was that most of the viral genomes that had reached the nucleus were incompetent to transcribe and only those that reached ND10 found a congenial space where transcription was possible. However, most of the major ND10-associated proteins, such as PML and Sp100, are upregulated by interferon (Maul 1998). More ominous, all of these proteins, including the PML-interacting Daxx, are transcriptional repressors involved in the formation of heterochromatin (Seeler et al. 1998; Xu et al. 2001; Ishov et al. 2004). ND10, therefore, appear more like sites for nuclear defense. Also, CMV and other DNA viruses possess genes whose products can eliminate or disperse these nuclear domains, as first identified for herpes simplex virus (Maul et al. 1993). For CMV, the dispersing protein is IE1, which first accumulates at ND10 for some time before ND10 are dispersed (Ishov et al. 1997; Ahn et al. 1998). Surprisingly, the highest concentration of the major immediate early viral transactivator and major immediate early promoter (MIEP) repressor, IE2, is at the site of the highest concentration of viral IE transcripts (Fig. 1c). These transcripts seem to pass from the IE2-covered site into the splicing-factor-containing domain (Fig. 1e). The number of transcripts is much larger than expected, suggesting either a very high transcription rate from a single transcription unit or, as we suspect presently, an inhibition of splicing and thus accumulation of unspliced transcripts. The general arrangement of ND10, the region of accumulated transcripts, and the IE2-containing site were described as the immediate transcript environment which resolves approximately 6 h p.i (Ishov et al. 1997). At first glance, these observations indicate that the virus dispersed a site inhibitory to its replicative success. The immediate early transcript environment also includes the UL112/113 gene products (Fig. 1d). The UL112/113 gene product is

part of the expanding prereplication domain and viral genomes are present at the outside of this domain. Prereplication domains apparently become the replication compartments many hours later when they appear hollowed out (Fig. 1f). More than a decade after the first observation that viruses transcribe predominantly at ND10, it remains unclear what advantage the virus gains by dispersing ND10-associated proteins. Is it possible that the questions asked are not relevant and that transcription at ND10, and ultimately replication where ND10 had been present, is simply a consequence of various layers of interactions, none of them simply representing either advantages for the virus or defense mechanisms of the host. Clearly though, those domains focus our mind on the activities that take place in these microenvironments.

Because of the limited resolving power of light microscopy, we still do not know the precise location of transcribing viral genomes relative to ND10 beyond 300 nm resolution, a huge molecular gap. This is important, because it could mean the virus has become part of ND10, where its interaction with a solid interface would have the same mechanistic relevance as recently argued for genes with matrix elements (Kumar et al. 2007). The viral genome may be localizing at the ND10 interface within the interchromosomal space, i.e., randomly localizing in the limited interchromosomal space where ND10 also resides. However, the observations that viral transcription occurs at ND10, that IE1 disperses ND10-associated proteins, and that interferon induces ND10-associated proteins suggest that the association of viral transcription and ND10 is causal rather than casual. To understand this relationship better, we may have to reconstruct the evolutionary balance achieved by a multitude of interactions, each modifying others.

Are ND10 Really the Start Sites of CMV Transcription?

The static images obtained from fixed material 3 h p.i., certainly suggest as much. However, the lateral infection sequence employed for HSV1 (infection from a neighboring cell in the same culture flask plane) indicates that the ND10-associated proteins leave their initial segregated state and move to the viral genomes (Everett and Murray 2005). According to this scenario, new aggregates of ND10-associated proteins form on the virus genome and even prevent the virus genome from moving into the center of the nucleus. Our early observations for HSV1 and HCMV then come from the infection and nuclear pore penetration on the large upper surface of the nuclei, thus requiring a short migration downward to preexisting ND10 (or a short migration of ND10-associated proteins to the virus). We have shown that foreign DNA/foreign protein complexes of bacterial or viral origin attract ND10-associated proteins whether introduced into cells by infection or transfection (Tang et al. 2000, 2003). Reiterated HPV11 origins of replication plus the origin binding protein E2 or integrated bacterial reiterated operon sequences plus GFP-labeled LacI repressor protein attracted ND10-associated proteins (Tang et al. 2001). Foreign DNA alone did not. One interesting finding was that foreign DNA/foreign

protein complexes accumulate a limited amount of ND10-associated proteins, i.e., smaller incorporated reiterations had smaller dot size. The dot size did not increase with interferon upregulation of PML or Sp100, indicating these sites were not ND10 nucleating sites. Rather they are new sites of DNA or chromatin that bound PML or Sp100 and entered the visual range because of the reiteration of binding sites. Since most HCMV viral genomes have no visually recognizable PML attached even after entering the nucleus (see Fig. 1b), except for the few particles at ND10 that transcribe, this virus should be re-evaluated for association with ND10. The key experiment would be to determine whether the transcribing viruses are at ND10 and whether such apparent ND10-associated aggregates had moved or were newly formed. Because transcribing genomes cannot presently be labeled in a time-resolved manner, we may use immediate early transcript environment formation as a surrogate, by tracking the accumulation of GFP-tagged IE2 in cells containing Cherry-tagged ND10.

Structural and Functional Aspects of IE1

IE1 is the viral protein that disperses ND10 (Korioth et al. 1996; Ahn and Hayward 1997; Ishov et al. 1997; Wilkinson et al. 1998). In contrast to the immediate early protein ICP0 of HSV-1, it does not do so by the proteosome-dependent destruction of PML and Daxx. It was suggested that IE1 disperses ND10 by binding to constituents and their removal from ND10 over time, because IE1 and PML interact (Ahn et al. 1998). Neither the mechanism of interaction nor the means of dispersal are known. One preliminary finding on MCMV IE1 casts doubt on the idea that stochastic removal of PML by IE1 is the sole mechanism. Removing a 7-amino acid sequence from one of the helices in the N-terminal region of MCMV IE1 eliminates the dispersive function but not the binding function (Q. Tang and G. Maul, unpublished data). We may have to search for a mechanism that includes a function other than simple binding of PML, for dispersion of ND10. We also may have to compare IE1 of HCMV and MCMV; such a comparison could help illuminate the different domains necessary for certain functions.

IE1 has several functional properties that have been used to probe its structure through mutational analysis. It augments viral and host gene transcription, disperses ND10 and binds repressor proteins. These functions are driven either by the indirect augmentation of transcription, possibly by alleviating repression of IE1 binding to p107 (Poma et al. 1996) or HDAC (Tang and Maul 2003; Nevels et al. 2004b), or by the direct augmentation of transcription through IE1 binding of transcription factors (Lukac et al. 1997). Determining how these proteins bind to IE1, and to which interface, is important for developing effective interference strategies. Because IE1 plays an important role during low-particle infections (assumed to be the normal infection mode), a strategy aimed at IE1 inactivation might be successful in blocking the HDAC-binding capacity of IE1 and allowing the host cell to silence competent viral genomes.

Deletion analysis has revealed that the HDAC-binding site in MCMV IE1 is between amino acid residues 100 and 310. A peptide comprised of these amino acids retains HDAC-binding capability and the potential to augment transcription from the MIEP. A deletion in a similar helical region of HCMV IE1 also eliminates the augmenting effect (Stenberg et al. 1990) and abolishes IE1-mediated dispersal of ND10 (Ishov et al. 1997; Lee et al. 2004). More detailed deletion analysis revealed that removing amino acid sequences surrounding the HDAC binding domain eliminates the ability of IE1 to disperse ND10 (Q. Tang and G. Maul, unpublished data). Structurally, this suggests that IE1 possesses a bipartite ND10-binding domain that differs from the HDAC-binding domain. A bipartite p107-binding domain has also been reported for HCMV IE1 (Poma et al. 1996).

HCMV IE1 and MCMV IE1 share only 12% amino acid homology, mostly in the highly acidic C-terminal region. Moreover, HCMV IE1 is 20% shorter than MCMV IE1. Therefore these two proteins appear to have very little in common. However, they share the same genetic structure, as well as the ability to disperse ND10 and augment viral transcription. We need additional comparative and functional analyses, particularly if the mouse system is to be used as an experimental small animal model. Although the primary structures of HCMV IE1 and MCMV IE1 differ significantly, their secondary structures are surprisingly similar. One unresolved aspect of the apparent structural similarity between HCMV IE1 and MCMV IE1 is the position of the small ubiquitin modifier (SUMO). In HCMV IE1, SUMO is at aa 450 (Xu et al. 2001), and in MCMV IE1 we find the SUMO consensus sequence at aa 223. These rather large covalent modifications could have a strong differential influence on the 3D structure of IE1, and thus could influence the functional properties of the two different IE1s. Since SUMO modifies very small amounts of protein at any given time (Johnson 2004), these SUMO subsets may have additional functions. In HCMV IE1, deletion of the SUMO modification site reduces the levels of IE2 transcript and their translation products (Nevels et al. 2004a) and PML desumoylation (Lee et al. 2004). In MCMV IE1, the putative SUMO modification site is within the HDAC binding site (G. Maul, unpublished observations); its precise functions, however, remain unknown.

Though IE1 is not essential for productive infection, it appears to be very important for replicative success. Isolating the respective functions of this molecule and assigning them to its different interfaces may provide a rational basis for the search for small interfering molecules. Such molecules may induce an IE1 minus phenotype, substantially lowering productive infection.

IE1 Counteracts the Host Cell's Silencing Mechanisms

The major immediate early transcript is differentially spliced to produce a number of proteins. The two major and best-investigated proteins, IE1 and IE2, have in common exons 2 and 3 but differ in the larger exon 4 (IE1) and exon 5 (HCMV IE2 and its MCMV homolog, IE3). These proteins act synergistically to activate early

viral protein expression, but antagonistically to autoregulate the MIEP (Cherrington and Mocarski 1989; Pizzorno and Hayward 1990; Stenberg et al. 1990; Cherrington et al. 1991; Liu et al. 1991). Both activate or augment viral and host gene transcription (Hagemeier et al. 1992). Most transactivators become part of the basal transcription machinery when they bind to DNA in the promoter region. Apparently, IE1 and IE2 are no exceptions. IE2 binds to specific sequences on early promoters (Cherrington et al. 1991; Meier and Stinski 1997). Some, but not all, transcription factors (TBP, TFIIB) accumulate in the domain adjacent to ND10 together with IE2, where there is just one viral genome (Ishov et al. 1997). IE2 is therefore the likely accumulator of pretranscription complexes, although it might create this enriched microenvironment in some association with the UL112/113 gene products.

IE1's mechanisms of action are not understood as well as those of IE2. Apparently, IE1 is not essential to produce viral progeny, but is necessary for the more natural mode of infection involving low levels of particles. Fibroblasts infected with an IE1-deletion mutant of HCMV require a much larger number of mutant viral particles to achieve the same degree of replicative success as that of wild type viruses, indicating the necessity of multiple genomes of the IE1 mutant. Mocarski and colleagues also found that viral transactivators, such as tegument proteins can compensate for IE1 at high multiplicities of infection (moi) (Mocarski et al. 1996). At low moi, infected cells produce no replication compartments, despite the nearly equal amount of IE2 synthesis (Greaves and Mocarski 1998). This does not support the idea that IE1 is a necessary component of the transcription machinery as IE2 is. The temporal localization of IE1 in specific nuclear compartments, along with the potential interactions of IE1 with nuclear proteins in these compartments, points to additional functions of IE1 that are in line with the often noted augmentation of transcription observed in transfection experiments.

Intuitively, one might suppose that the nuclear site with the highest concentration of a protein is where it functions. IE1 should therefore function in all ND10, and IE2 should function beside a few ND10. However, because not all ND10 have transcribing viral genomes, it follows that IE1 would not act on viral genomes. IE1 does not act on host genes as no host genes have been found in ND10. IE1 must therefore have functions other than transactivation with the basal transcription machinery.

Identifying proteins that interact with IE1 is one way to determine its other functions. IE1 colocalization with ND10 proteins has been used to identify IE1 interaction partners. The interaction between IE1 and PML in HCMV (Ahn et al. 1998) has also been confirmed in MCMV (Tang and Maul 2003). In immunoprecipitation analyses, both of the ND10-associated proteins, PML and Daxx, co-immunoprecipitate with MCMV IE1, suggesting that all three proteins form a complex. At present, no functional assay exists for examining the interaction between IE1 and Daxx. However, MCMV replicates more successfully in $Daxx^{-/-}$ cells (Tang and Maul 2006). However, analysis of the influence of the ND10-associated proteins on overall replicative success has just begun in cells where these proteins and another ND10-associated protein, such as Sp100, have been eliminated or strongly downregulated by siRNA.

Other functions of IE1 are observed. IE1 apparently functions as an antagonist to type1 interferon signal transduction (Paulus et al. 2006). Where this antagonist interferes is of interest since it may be at several levels of interaction. It might be the downstream effect of dispersing PML and thus releasing and possibly changing the Sp100 isotype composition. Circumstantial evidence comes from work that shows that elimination of PML and thus release of all ND10-associated proteins strongly reduces the detrimental Sp100 isotypes (Everett et al. 2006; Negorev et al. 2006).

Effect of ND10-Associated Proteins on CMV

PML

PML is the matrix protein of ND10. Without PML, specifically the SUMO modified form, the aggregation of various ND10-associated proteins does not take place (Ishov et al. 1999). Numerous proteins have been shown to accumulate at ND10, mostly when overexpressed, and many reviews suggest a plethora of supposed functions of these protein accumulations (Negorev and Maul 2001). There seems to be no nuclear function that has not been fingered as dependent, influenced or modulated by these structures, except perhaps splicing. This often indiscriminate assignment of function, based on mostly spurious evidence of colocalization after overexpression, has muddied the field considerably. However, a clearing and clear effect on the replicative cycle of HCMV has recently been provided by Stamminger's group, who showed that the depletion of PML through shRNA substantially increases replicative success. This was all the more convincing because it could be reversed by the reconstitution of a single PML isotype (Tavalai et al. 2006). The finding that depletion of PML can enhance the number of cells replicating HCMV and plaque formation by a factor of 4 shows that IE1 cannot completely overcome the repressive effect, unlike ICP0 of HSV-1 (Everett et al. 2006). However, the much higher (20 times) enhancement of replicative success of the IE1 deletion mutant also shows that IE1 has a suppressive effect on the PML-based inhibition of immediate early transcriptional events and replicative success.

PML may repress the initiation of immediate early transcription, or its progression, or both. PML may repress transcription by interfering directly with Daxx-mediated NFkB binding on the MIEP, or by indirect means such as retaining repressive factors (Daxx, ATRX, Sp100). IE1 in turn may enhance transcriptional activity by lowering free PML, as IE1's concentration generally exceeds that of PML. Observations on HCMV and MCMV show that PML, Daxx and Sp100 initially accumulate in HCMV IE2 or MCMV IE3 domains and the UL112/113 outlined prereplication domains. From there, they disperse at later stages when IE1 is present. In the absence of IE1 in either virus, these ND10-associated proteins remain in the prereplication domains, and later in the replication domains (Tavalai

et al. 2006). These observations may merely represent minor shifts in the availability of free repressive proteins, but could also signify inactivation by segregation. The repressors in these domains may also affect the reduction of replicative success. We do not even know whether PML and IE1 interact directly, except that evidence from Daxx$^{-/-}$ cells seems to exclude Daxx as an adapter (Tang and Maul 2003). The mechanism, or at least the molecular domains of PML essential for PML-induced inhibition, can now be investigated by using the PML-depleted cells and manipulating the individual PML isotype, specifically PML IV. This includes the manipulation of its ND10-forming capability. Such investigations may determine whether the HCMV replication sequence is dependent on the ability of PML to multimerize and separately to form complexes by attracting Daxx, and through Daxx, ATRX (Ishov et al. 2004).

Daxx

Daxx interacts at its C-terminal end with sumoylated PML (Ishov et al. 2004), and the SUMO-interacting motif (SIM) at the very end of the molecule is the necessary domain for interaction with may other repressors. Single amino acid changes in the I I V L sequence of Daxx abrogate ND10 association and functional repression of the glucocorticoid receptors (Lin et al. 2006). Daxx therefore likely functions as an adaptor protein or corepressor. The repressive effects of Daxx on HCMV have been documented by siRNA suppression of hDaxx (Cantrell and Bresnahan 2005, 2006; Saffert and Kalejta 2006) and for MCMV, by inference, using mouse Daxx$^{-/-}$ cells (Tang and Maul 2006). The viral counter-defenses seem to center on the tegument protein pp71, which was identified as the first Daxx-interacting protein. Pp71 and Daxx interact in the N-terminal half of the Daxx molecule (Ishov et al. 1999, 2002; Hofmann et al. 2002; Marshall et al. 2002) away from the PML-interacting C-terminal end (Ishov et al. 1999). pp71 is enriched in all ND10 after infection (Ishov et al. 2002). We assume the viral genome deposition at ND10 to come about by interaction of the viral DNA binding to pp71, which in turn is bound to Daxx and thus deposited at highly increased frequency to the high concentration of PML at ND10 (Ishov et al. 1999, 2002; Hofmann et al. 2002; Marshall et al. 2002). If Daxx is a repressor and binds pp71 to produce an inactive transactivator complex, the viral genome should be suppressed at ND10, a nuclear defense. Here IE1 may counter this defense by its binding of Daxx. The balance between these separate interactions should help reveal the choreography of sequential, temporal and spatial interactions that set the stage for the progress or suppression of the lytic cycle.

Our experiments also show that MCMV IE1 appears to interact with PML or Daxx independently. Because Daxx interacts with the histone deacetylases (HDAC) (Li et al. 2000), we tested the possibility that IE1 binds indirectly to HDAC. Identifying an IE1-HDAC interaction, however, may have been fortuitous, since HDAC does not normally localize to ND10, but does so in the presence of IE1. HDAC may be recruited to ND10 by IE1 during the early stages of infection, when

IE1 accumulates at ND10. The IE1-dependent segregation of HDAC to ND10 may not be significant, because there is little segregated, relative to the amount present in the nucleus. On the other hand, the large amounts of IE1 expressed, especially after ND10 dispersal, may be more significant, because IE1 might flood the nucleus sufficiently to reduce free HDAC, relieving the HDAC-associated suppression of chromatinized viral genomes. This is consistent with results from HDAC activity assays, which show that IE1 binding to HDAC inhibits HDAC deacetylation (Tang and Maul 2003). IE1, therefore, may not exert its primary effects at sites where it is most concentrated; rather, it appears that IE1 functions throughout the nucleus as an HDAC scavenger (at least HDAC 1 and 2 or their complexes), and possibly, as a scavenger for other host proteins.

ATRX

ATRX (alpha thalassemia-mental retardation, X linked) protein has not yet been associated with CMV biology. However, ATRX was the first cellular protein found to interact with Daxx at an N-terminal region and localize to ND10 by the adapter function of Daxx (Ishov et al. 2004). ATRX is a member of the SWI/SNF family of helicases or ATPases with chromatin remodeling activity, and it associates with HP1 (Picketts et al. 1996, 1998; Gibbons et al. 1997) and with the SET domain of chromatin modifying proteins (Cardoso et al. 1998). ATRX and Daxx are only removed from ND10 for a short time during the S/G2 interphase, suggesting reestablishment of the epigenetic properties of newly replicated heterochromatin (Ishov et al. 2004). Conditional genetic ablation of ATRX in mice has similar effects on developing brain structures, as has congenital HCMV infection (Berube et al. 2005). It is tempting to speculate that Daxx/ATRX is removed by IE1 during stochastic reactivation events of latent HCMV in the developing embryo. If so, HCMV silencing and development of latency during congenital infection may not be a totally benign cellular defense. We are now investigating the possibility that permissive cells can silence competent virus, and anticipate that ATRX is involved in the suppression of CMV genomes.

Sp100

Sp100 is a constitutive ND10-associated protein that has been shown to affect HSV1 immediate early protein expression by affecting the promoter of these proteins (Taylor et al. 2000; Wilcox et al. 2005; Isaac et al. 2006; Negorev et al. 2006). Sp100A, the dominant isotype found at ND10, produces a mild activation. All other isoforms that have apparent DNA- or chromatin-binding domains (Sp100B-SAND domain; Sp100C-PhD and Bromo domain; Sp100HMG-HMG domain) are repressive, with the Sp100B having the strongest effect. Preliminary

experiments, testing the effect of Sp100 isotypes on the MIEP driven-luciferase reporter assay, show that these Sp100 isotypes likely affect CMV replicative properties, but the isotypes that show inhibition are quite different from those affecting HSV1. The properties of Sp100, such as insolubility, extremely low abundance, and cell cycle modifying characteristics do not allow many common approaches to analyze potential interactions directly. However, the differential effect of its isotypes on HSV1 (Negorev et al. 2006), and their differential interferon upregulation and accumulation into the immediate transcript environment and prereplication sites of HCMV (Tavalai et al. 2006), make these proteins worthy of further investigation.

HDAC

When entering the host nucleus, the viral genome may become chromatinized to reduce its size. This would facilitate diffusion through the nucleus and aid repression by the host's deacetylating agents. Such a silencing mechanism would be an effective host defense. Indeed, evidence for silenced viral genomes has been found by precipitating deacetylated chromatinized MIEP with antibodies against deacetylated histones (Meier 2001; Tang and Maul 2003; Reeves et al. 2006) shortly after infection of permissive cells. Consistent with such a host defense mechanism, as well as with the viral counteracting mechanism (IE1-mediated inhibition of the deacetylation of chromatinized viral DNA), is the finding that the deacetylation inhibitor trichostatin A (TSA) rescues an MCMV IE1 deletion mutant (Tang and Maul 2003) and also the HCMV deletion mutant (Nevels et al. 2004b). Somewhat unexpectedly, TSA substantially enhances the viral productivity of permissive cells infected with wild type virus and significantly increases the number of cells exhibiting signs of productive infection. The latter observation suggests that even permissive cells, in the absence of an immune system or the cytokine-based innate immune response, can suppress viral replication after infection by a competent virus and can limit the initial production of IE1 and IE2 (or IE3, in the case of MCMV) (Tang and Maul 2003). The suppression of many individual viral genomes may take place in the same nucleus where some other genomes are actively transcribing (those at ND10).

Perspectives

The ability of the host cell to completely suppress the initiation of the viral replication cycle without complete inhibition of IE transcription may rely on several factors: (1) the number of viral genomes entering the nucleus; (2) the increased amount of tegument-associated transcription factors internalized as a result of fusing dense bodies, i.e., virus particle lacking a capsid and genome but filled with

tegument proteins; (3) the cell cycle stage of the cell; and (4) the amounts of silencing factors, such as HDAC, and repressors, such as Daxx, Sp100 and PML. Those factors are likely to affect the immediate early transcription and specifically the production of the major immediate early proteins. Success at the margin appears to depend on the amount of IE1 produced to overcome such repressive properties of the cell in a cell culture environment. Thus, IE1 plays a critical role at the start of infection and might prove to be a target for antiviral drug development. In order to assess the potential of IE1 as a drug development candidate, we need to determine its influence on viral success in an organismal context.

Additionally, we do not know the mechanism of ND10 dispersion and the effect of the release of ND10-associated proteins. The mechanism may be due to desumofication and as such IE1 may be a desumoylation enzyme. Such a function needs to be directly searched for since it may affect more than ND10-associated proteins presently recognized. The release of ND10-associated proteins by IE1 may mimic interferon upregulation of these proteins (PML and Sp100), i.e., sudden availability of additional and in general detrimental proteins, we know now to be specifically segregated and thus inactivated. Interferon downstream effects of Sp100 as the intermediary protein are preliminarily associated with specific inflammation-inducing proteins. Their occasional induction by abortively reactivating latent virus may account for the atherosclerotic association of HCMV despite never finding replicating virus in plaques.

Low priority has been given to splicing of the major immediate early transcription unit. Exon skipping is involved, and how this is regulated will have a major impact on reactivation. We expect to find cellular defense mechanisms that work at the level of splicing inhibitors. A priori they must exist, since many cells may reactivate to produce only IE1 but not IE2 or the MCMV IE3. The splice enhancers and silencers in the nucleotide sequence will be difficult to identify, but it may be essential to do so, so that when mutant viruses are produced with deletions in exon 4 to 5 for in vivo verification of IE1 or IE2 effects, we do not inadvertently assay a splice phenomenon.

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