Mouse Cytomegalovirus Early M112/113 Proteins Control the Repressive Effect of IE3 on the Major Immediate-Early Promoter

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The mouse cytomegalovirus major immediate-early (IE) transcript is differentially spliced to produce two IE proteins: IE1, which functions partly to maintain its own promoter, the major IE promoter (MIEP), free from repression, and IE3, which functions partly as a repressor of MIEP. Paradoxically, the site where transcription of the viral genome occurs is also the site where the greatest amounts of IE3 accumulate. This raises the question of how the repression capabilities of IE3 are controlled so soon after infection. We detected IE3, an activator of early proteins, contemporaneously with gene products of the early M112/113 locus. Both IE3 and the early M112/113 gene products colocalize and coimmunoprecipitate. Protein interaction most likely occurs between IE3 and the 87-kDa splice form of M112/113, because only the 87-kDa component coimmunoprecipitated with IE3. The complex also includes PML. Transiently expressed M112/113 can form large domains alone, even in the absence of full viral genomes or PML. Coexpression of M112/113 products and IE3 results in segregation of IE3 into newly formed M112/113-based domains. Importantly, coexpression eliminates the IE3-based repressive effect on MIEP, as determined by MIEP-driven reporter assays. The consequence of segregating IE3 into the M112/113-containing prereplication domains appears to make IE3 unavailable for binding and repressing MIEP during the earliest stages of infection. These findings establish a new feedback mechanism between IE and early proteins, a new mechanism of promoter control via segregation of the repressor, and a new function for proteins from the M112/113 locus.

Human cytomegalovirus (HCMV) and mouse cytomegalovirus (MCMV) possess very similar gene structures and manifest similar pathologies but are highly species specific in their replicative processes and do not produce viruses in cross-species infections. This is the case even though CMV is nonspecies specific in infecting both human and mouse cells and in initiating the transcription cascade in these cells (18). The block does not appear to be at the major immediate promoter enhancer (3). Comparison of the two viruses may yield insights into the site where replication progression is inhibited, thus leading to the identification of a potential interference site. This comparison requires a firm understanding of the functions of various proteins in the transcription cascade or replicative process. The major immediate-early (IE) proteins of the two viruses appear to have the same functions. Transcribed from the large and major IE transcription unit (4, 5, 32), a set of differentially spliced proteins synergistically activate the early proteins (7, 9, 16, 21, 30). The IE1 protein of both viruses is not essential but is produced in prodigious amounts that far exceed the levels necessary to interact successfully with the viral promoters. Its absence reduces the success of replication substantially (25, 26, 35), suggesting that IE1 must enhance transcription indirectly from its own promoter, the major IE promoter (MIEP). One function of IE1 appears to be its binding-associated segregation of various repressors. IE1 binds interacting proteins such as Daxx and PML (35), which reportedly function in transcriptional repression (28). IE1 also inhibits histone deacetylases (HDAC), interacting proteins that repress viral genomes through silencing mediated by the deacetylation of histones. IE1 relief of HDAC-mediated deacetylation of viral genomes leads to a higher success rate of infection (35) and leads to the activation of the repressed viral genome of non-permissive cells (23, 27), which in turn can potentially lead to the release from latency. MCMV and HCMV IE1 appear to have the same functional properties despite a relatively low sequence homology.

IE3, the major transactivator of MCMV early proteins, and its HCMV homologue IE2 are spliced isoforms in which the fifth exon of the transcription unit is used, rather than the fourth exon (i.e., IE1). Both are essential for replicative success (4, 37). As with MCMV IE1 and HCMV IE1, no strong sequence similarity exists between the MCMV IE3 and HCMV IE2 homologues, except for an acidic region in the C-terminal portion of each molecule. These proteins interact with several proteins involved in transcription, such as the TATA-binding protein (7) and the transcription-associated factor TFIIID (10) actually functioning like transcription-associated factors (20). MCMV IE3 and HCMV IE2 are essential in the transcriptional activation of early proteins, such as those expressed from the 112/113 locus (22, 25), although these early proteins become recognizable at the same time, about 2 h postinfection (p.i.) (2, 6), suggesting that very little IE3 can activate the early promoter of 112/113. In addition MCMV IE3 and HCMV IE2 are autorepressors in that they can repress MIEP (8, 19, 24, 29, 31) but apparently not at concentrations sufficient to activate early promoters.

IE1 and IE2 have peculiar patterns of temporal and spatial distribution (12). IE1 first segregates to ND10, specific nuclear
domains containing interferon-upregulated proteins such as PML and Sp100, and then later disperses these domains (1, 17, 38). IE2, on the other hand, localizes alongside these domains and surrounds emerging transcripts from transcriptionally active viral genomes (12). The ND10-defined location of IE2 with its transactivators and the SC35 domain into which the viral transcripts discharge represent the immediate transcript environment. An apparent paradox arises related to the distribution of IE2 and the strongly transcribing viral genome. The highest concentrations of IE2, an MIEP repressor, are present at sites of highly active MIEPs, raising the question of what prevents the repressive activity of IE2. For HCMV, early proteins transcribed from UL112/113 bind single- and double-stranded DNA and localize to nuclear inclusions (15) that reside with IE2 situated next to ND10 (2). Expression of UL112/113 products enhances transcription of proteins essential for the replication complexes (15). UL112/113 proteins seem to enhance the IE-mediated activation of the DNA polymerase (UL54) promoter (16). No other effects have been described for the products of the 112/113 locus. This should be expected, however, because the expression kinetics of different 112/113 splice products appear quite different. Surprisingly, individual proteins follow a complicated and quantitatively dynamic accumulation pattern (39). Various methods have identified four gene products each for UL112/113 (34, 43, 50, and 84 kDa) and for M112/113 (33, 36, 38, and 87 kDa) (6, 9, 39). Additional bands may represent secondary modifications, most likely phosphorylation (39), that may modify various additional functions of the 112/113 proteins. In the present study we investigated the possibility that M112/113 gene products act in IE3 segregation and repress IE3-mediated MIEP repression potential.

MATERIALS AND METHODS

Tissue culture and virus. NIH 3T3 (American Type Culture Collection) and 3T3e1 cells (9) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. For infection and transfection, NIH 3T3 (American Type Culture Collection) and 3T3e1 cells (9) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. No other effects have been described for the products of the 112/113 locus. This should be expected, however, because the expression kinetics of different 112/113 splice products appear quite different. Surprisingly, individual proteins follow a complicated and quantitatively dynamic accumulation pattern (39). Various methods have identified four gene products each for UL112/113 (34, 43, 50, and 84 kDa) and for M112/113 (33, 36, 38, and 87 kDa) (6, 9, 39). Additional bands may represent secondary modifications, most likely phosphorylation (39), that may modify various additional functions of the 112/113 proteins. In the present study we investigated the possibility that M112/113 gene products act in IE3 segregation and repress IE3-mediated MIEP repression potential.

Coimmunoprecipitation and immunoblotting. Cells were collected 20 h after transfection, and luciferase activity was determined by using a luciferase assay system (Promega) according to the manufacturer’s instructions. Luciferase activity was measured with a Wallac Victor luminometer (Perkin Elmer Life Science, Turku, Finland). Each assay was performed in triplicate, and luciferase activity values were normalized by the values for the amounts of total protein.

Molecular cloning. The pmMIEP-luc plasmid was made by inserting the MCMV MIEP fragment into a pNFKB-luc plasmid that had been digested with HindIII and MluI to remove the promoter and all the NF-B-luc plasmid that had been digested with MCMV MIEP fragment into a pNF-B antiserum was from Abcam (Cambridge, Mass.). EVMS 55B antibody was obtained from J. Kerry (9), and rabbit anti-GFP green fluorescent protein (GFP) and rabbit anti-ATRX antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Plasmids were transfected into cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Immunocytochemistry and fluorescence in situ hybridization. The procedures for immunostaining of nuclear protein and in situ hybridization of MCMV viral DNA were carried out as described previously (33). The probe used for DNA in situ hybridization was made from a BAC library containing the MCMV DNA genome. pSM3r, or a major IE transcription unit produced by nick translation (33).

RESULTS

Nuclear distribution of IE3 and M112/113 proteins during infection and transfection. Immunohistochemical and in situ hybridization analyses were performed to establish the relative nuclear location of various viral replication components during the IE and early stages of MCMV infection of 3T3 cells and to ascertain whether transfection of certain proteins recapitulate the distribution of these components that occurs during infection. At 2 h p.i., we observed IE transcripts as green signals localized adjacent to anti-PML immunostained ND10 (Fig. 1A, red). These signals represent the location of transcriptionally active viral genomes. Early proteins transcribed from the M112/113 locus appeared alongside a limited number of ND10 as early as 2 h after infection (Fig. 1B). M112/113 also appeared adjacent to ND10 after transfection with plasmids harboring the native promoter (Fig. 1C), thus mimicking the situation found during infection. Since specific antibodies for IE3 are currently unavailable, we cannot directly compare the positions of IE3 and M112/113 after infection. However, IE3, when transiently expressed as a GFP fusion protein, also appeared alongside ND10 (Fig. 1D), which is reminiscent of IE2 localization during infection with HCMV (12).

Next, we examined whether IE3 and M112/113 colocalize by cotransfecting 3T3 cells with plasmids expressing GFP-tagged IE3 and M112/113. IE3 and M112/113 were observed to colocalize soon after transfection (Fig. 1E). Several hours after infection, these M112/113-containing domains (Fig. 1F, red) increased in size, acquired the ND10 matrix protein PML (blue), and eventually replicated viral genomes, as shown by in situ hybridization (green) performed ~24 h p.i. Therefore, the M112/113 domains containing replicated viral DNA can be considered to be replication compartments, and those lacking replicated viral genomes can be considered to be prereplica-
tion domains. Such large compartments were also formed during the transient expression of viral M112/113 proteins alone (Fig. 1G). These large compartments also acquired PML, as indicated by the color-separated images in Fig. 1H and I. Again, domain formation and acquisition of cellular PML were reproduced by transfection, with a concomitant reduction in the number of ND10. When present in small amounts, IE3 not only aggregated next to ND10 but also, when cotransfected with M112/113, colocalized nearly exclusively in M112/113-induced and expanding domains. The densest M112/113 staining was found at the rim of the domain. Large amounts of IE3 appeared to segregate into these domains (Fig. 1J and K). These images appeared slightly different with various strengths in IE3 and M112/113 expression in any given cell, resulting from different proportions of these proteins within these domains. When IE3 expression dominated, more IE3 appeared in the nucleoplasm (data not shown), whereas when M112/113 expression became excessive, M112/113 proteins appeared as aggregates in the cytoplasm (data not shown). As observed with DAPI (4',6'-diamidino-2-phenylindole) staining, the expanding M112/113 appeared to displace cellular DNA (data not shown).

We investigated the time sequence of replication domain formation and found that the prereplication domains were devoid of newly replicated DNA for prolonged times after infection. Even at 24 h p.i., cells in different stages of replication existed (Fig. 1F and L). One cell (Fig. 1L, lower left) possessed various replication compartments with different amounts of replicated DNA. Another cell (Fig. 1L, lower right) had only one sizable hybridization signal in one of the domains, whereas another cell (top) had smaller M112/113 domains with low-level hybridization signals, many of which localized at or outside the area enclosed by M112/113 (magnified in Fig. 1M). The diffraction-sized hybridization signals appear to represent input viral genomes. These observations indicate that prereplication domains exist for extended periods of time. The transient expression of M112/113 proteins mimics events that take place during the prereplication stage; this includes the displacement of cellular DNA (data not shown) and the recruitment of PML and IE3. These observations, as well as those on the HCMV-mediated definition of the immediate transcript environment (12), pose a paradox. Because the greatest amount of the MIEP auto-repressor IE3 (or in HCMV, IE2) was present at the site of the input viral genome at a time of high

FIG. 1. Segregation of IE3 and M112/113 during infection and transient expression. (A) MCMV-infected 3T3 cells at 2 h p.i. In situ hybridization with IE DNA as a probe. IE transcripts localize adjacent to anti-PML-immunostained ND10. (B) The experiment as described in panel A, but cells are stained for ND10 with anti-PML and anti-M112/113 antibodies. M112/113 appears adjacent to ND10. (C) M112/113-transfected 3T3 cells labeled for ND10 and M112/113. M112/113 localizes adjacent to PML at early times after transfection (12 h). (D) IE3-GFP appears adjacent to ND10 at early times after transfection (12 h). (E) IE3-GFP and 112/113 colocalize at early times after transfection (12 h). (F) MCMV-infected 3T3 cells at 24 h p.i. were stained for M112/113 (red) and PML (blue), followed by fluorescence in situ hybridization (green). Replicated viral DNA is located in M112/113- and PML-containing replication compartments. (G to I) 3T3 cells transfected with plasmids expressing M112/113 are shown stained for PML in panel H and for M112/113 in panel I. M112/113 forms large domains with PML present at its center. (J) 3T3 cells transfected with plasmids expressing M112/113 and IE3-GFP. M112/113 and IE1-GFP colocalize and form large domains. (K) The experiment is the same as described for panel J. M112/113 is shown alone. (L) 3T3 cells infected with MCMV at 24 h p.i. and immunostained for M112/113 and by in situ hybridization for total viral DNA. The start of replication is evident in the lower left cell. Very limited replication is apparent in the lower right cell, whereas no signs of replication are apparent in the top cell. (M) Higher magnification of the top cell of panel L.
transcription as shown by in situ hybridization, the question arises, Why was MIEP not suppressed at very early stages of infection?

Interaction of proteins in the replication domain. Suppression of IE3 activity may be mediated through IE3 interactions with other proteins. However, colocalization of proteins such as IE3 and M112/113 within the same domain does not necessarily indicate that these two proteins interact. Thus, to determine whether proteins found within the prereplication domain do indeed interact, we cotransfected 3T3 cells with M112/113 and IE3 driven by their native respective promoters, immunoprecipitated lysates derived from these cells, and then probed the immunoprecipitates via Western blotting (Fig. 2). Immunoprecipitation with rabbit anti-M112/113 antibodies resulted in the coinmunoprecipitation of IE3 and PML (Fig. 2, lane 3). ATRX, a protein present in ND10 and connected to PML through interactions with Daxx (14), did not coprecipitate. Immunoprecipitation of the GFP-tagged IE3 with anti-GFP antibodies resulted in the coinmunoprecipitation of PML and predominantly the 87-kDa splice form of M112/113, its largest molecular mass component (Fig. 2, lane 4). The lower molecular mass components of M112/113 (33, 36, and 38 kDa) appeared only as substantially diminished bands. As expected, nonspecific rabbit serum failed to precipitate any of the proteins tested (Fig. 2, compare lanes 1 and 2). This experiment demonstrates that the three proteins (IE3, PML, and M112/113) found in the prereplication compartment interacted directly or indirectly.

To determine whether PML or the adapter protein Daxx are involved in the formation of the M112/113 domains, we repeated these transfection experiments with PML−/− and Daxx−/− cells. M112/113 formed the expanding domain segregating IE3 in the absence of these two proteins (data not shown). These proteins are therefore not necessary as adapters between IE3 and M112/113.

Effect of M112/113 on the abundance of IE3. UL112/113 has been reported to strongly affect the expression of other viral proteins in cotransfection experiments (15, 16). To determine whether M112/113 affects IE3 expression from MIEP, we used a 3T3-based cell line that constitutively expresses M112/113 that was not upregulated by transiently expressed IE3 (9). To control for the potential effect of endogenously expressed M112/113 on MIEP, that is, on transcription, we transfected IE1 driven by the same promoter as IE3. As shown in Fig. 3 (lanes 4 and 5), the amount of IE1 produced did not differ in the absence or presence of M112/113 products. However, we repeatedly found a substantially larger amount of IE3 in cells expressing M112/113 products than in cells not expressing M112/113 (Fig. 3, compare lanes 4 and 5). This result suggests that not transcription but, instead, either the translation of IE3 or the stability of this protein is enhanced in the presence of M112/113.

Physiological effect of M112/113-dependent increase of IE3. Because IE3 represses its native promoter and M112/113 increases the amount of IE3 in the cell as shown above, increased amounts of IE3 should therefore reduce the MIEP-driven transcription of a reporter protein. To test this hypothesis, we transfected 3T3 cells either with an MIEP-based luciferase reporter plasmid (pmMIEP-luc) alone or with pmMIEP-luc and pBB5.5(e1), a plasmid that expresses M112/113. First, we determined whether M112/113 itself affects the MIEP-driven luciferase reporter. Figure 4A shows that M112/113 failed to significantly affect luciferase activity (bars 1 and 2). As expected, IE3 inhibited MIEP-driven luciferase expression (compare bars 4 and 5). However, when M112/113 and IE3 were cotransfected, the IE3-based repression was eliminated (compare bars 4 and 5), despite the presence of greater amounts of IE3 (Fig. 4C, anti-GFP-stained IE3 fusion protein; lanes correspond to the bars). Expression of IE1 in the presence of the MIEP-luc reporter increased luciferase activity (Fig. 4A, compare bars 4 and 7), as expected from the previously reported deacetylation-associated inhibition of IE1 (35). These results show that, despite increased levels of IE3, the ability of IE3 to
repress MIEP is reduced in the presence of M112/113 gene products.

DISCUSSION

The present study was prompted by a paradoxical observation made initially with HCMV that the autorepressor for MIEP, IE2, a splice product of the major IE transcript, is located in its highest concentration at the site of the highest transcript concentration, i.e., the transcribing viral genome (12). Because the immediate transcript environment develops only where viral genomes transcribe, the presence of viral DNA appears to be the deciding factor dictating where this environment is formed. However, DNA alone fails to segregate to ND10; rather, some sort of DNA-protein complex is necessary for segregation (33, 34). The precise requirements have not yet been determined for CMV, although without pp71, a strong transactivator binding to viral DNA, HCMV forms its immediate transcript environment at sites other than ND10, as determined by the location of IE2 (13). As shown previously, early proteins transcribed from the 112/113 locus concentrate within a few domains very early on during infection with MCMV and HCMV (2, 6). We confirmed this observation in the present study. The immediate transcript environments, partly defined by IE3 aggregates, colocalize with...
M112/113. Thus, the immediate transcript environment develops into prereplication domains for MCMV. Here we show that M112/113 proteins, when transiently expressed, can generate the equivalent of a prereplication domain into which PML is recruited as it is during infection. This suggests that the domain formation is possible in the absence of the full viral genome. Potentially, the transfected viral DNA serves in a similar capacity as the full viral genome in nucleating these domains on a limited number of ND10. The finding that an expanding domain and a chromatinexcluding domain are still produced by PML and Daxx cells transiently expressing M112/113 argues against the possibility that PML and Daxx, both ND10-based proteins, play major roles in domain formation. During infection, proteins expressed from the M112/113 transcription unit can form an expanding prereplication domain in the absence of replication substantially earlier than replication starts.

As the IE protein that transactivates the M112/113 transcription unit, IE2 of HCMV or IE3, the MCMV equivalent, should be present earlier during infection than the 112/113 proteins. However, within the limits of the experiment, in terms of temporal resolution and the ability to accumulate sufficient amounts to be detected microscopically, the two proteins appear contemporaneously (−2 h p.i.) and at the same location (6, 25). This suggests that the transactivation of the 112/113 promoter needs very little IE3, i.e., less than that demonstrable with Western blotting or immunohistochemical techniques. IE3 like IE2 also autoregulates MIEP in that it functions as an autorepressor (25, 29). This is consistent with results of transfection experiments in which a limited amount of IE3 is generated, even in the presence of IE1 with its HDAC-binding and inactivating capacity (Tang et al., submitted). In situ hybridization with a DNA probe derived from the IE region underscores the paradox of our findings: the small amount of IE3 necessary to activate an early promoter is in stark contrast to the high amount of IE3 (i.e., MIEP repressor protein) present at MIEP when transcription from this promoter is highly active.

During infection and transfection, M112/113 proteins and IE3 are present in the same prereplication domain. Replicated viral genomes can be found within this domain much later, at a time after all essential replication proteins have been present for some time. Although not all components of the basic transcription machinery can be found in this domain at this time point, many transcription factors are present such as TATA-binding protein and TFII B (12). Since all the components necessary for viral replication are present in the prereplication domain, the question arises, Why doesn’t the replication of CMV start earlier, as it does in herpes simplex, for instance? The preemptive creation of an expanding domain or privileged space fails to provide an obvious answer to this question as this is quite different from the replication domain of adenovirus, which expands with the expanding edge of newly replicated viral genomes (11).

Surprisingly, despite the high concentrations of IE3 found in the very early appearing prereplication domains, we did not observe a concomitant increase in repression of MIEP. Large amounts of transcripts were present as judged by in situ hybridization. An explanation for the lack of MIEP repression by high concentrations of IE3 during early infection is that IE3 may not be functionally available due to binding to M112/113. These early proteins, then, would repress the interaction of the IE protein with its own promoter. Three models could be constructed to describe this newfound function of M112/113. In the first model, while the IE3 and M112/113 proteins are entering the nuclear space, binding can occur via an essential C-terminal IE3 promoter-binding site, thus rendering IE3 inactive. Such binding also results in the deposition of IE3 at the M112/113 protein-induced prereplication domain. In this case, this deposition would be an irrelevant side effect. In the second model, after nucleation of the viral genome at ND10, M112/113 builds up a dense matrix similar to the PML-based ND10 matrix that can trap incoming IE3. Such a matrix would need sufficient binding sites for IE3 to act as a sink, preventing IE3 from reaching and interacting with MIEP.

These two models are not mutually exclusive. IE3 and the HCMV homologue IE2 have binding sites for a number of cellular factors and probably more than one interface (20). For the M112/113 proteins, at least two interfaces are postulated, one in the common N-terminal region, because all four of the M112/113 translation products seem to aggregate within the prereplication domain, and one in the C-terminal end of the 87-kDa component, which seems to possess the binding site for IE3. From these multiple binding sites, a dense matrix could form around the viral genome. The viral genome may be the nucleation site, because it has many IE3 binding sites, including those for early promoters. However, inconsistent with both models of a simple removal of IE3 from the soluble pool is that such removal should also affect the transactivation of the early protein promoters. By contrast, these promoters appear to be activated synergistically by the presence of 112/113 products (16). The various 112/113 splice products and their secondary modification by phosphorylation may modulate their repressive effect on IE3 and their activating effect on early protein transcription. Alternatively, the N-terminal region necessary for activation of early promoters (22, 29) may remain functional, even though the repressive region in the C-terminal domain remains occupied by the 112/113 proteins.

In the third possible model, many of the infecting viral genomes transcribe upon entry into the nucleus, albeit briefly, but become quickly silenced by HDAC. Thus, many of these genomes can contribute to the production of IE1 and IE3, providing the activation potential for the early proteins. With IE1 segregation at all ND10, and with it, the block of the silencing effect of deacetylation (35), only genomes reaching this site continue to be transcribed. The site containing the viral DNA then attracts 112/113 products that form the prereplication domain and inhibit the repressive properties of IE3. Therefore, the prereplication domain can extend further, generating a privileged space. Instead of silencing, further transcription would be a consequence of the formation of prereplication sites where transcription complexes are preformed and repressive complexes are maintained inactive. All other genomes absent from the immediate vicinity of this privileged space would be silenced either by HDAC or IE3. Such a scenario is consistent with the finding that a large number of competent viruses in the nucleus are silenced when they are not at ND10. The formation of a privileged space by 112/113 resolves a number of difficult-to-explain findings, including the presence of active viral genomes at sites having the highest levels of interferon-activated repressive proteins and having
the highest concentrations of the autorepressive IE proteins HIE2 and MIE3.

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