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The Major Immediate-Early Proteins IE1 and IE2 of Human Cytomegalovirus Colocalize with and Disrupt PML-Associated Nuclear Bodies at Very Early Times in Infected Permissive Cells

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The major immediate-early (MIE) gene products of human cytomegalovirus (HCMV) are nuclear phosphoproteins that are thought to play key roles in initiating lytic cycle gene regulation pathways. We have examined the intranuclear localization pattern of both the IE1 and IE2 proteins in virus-infected and DNA-transfected cells. When HCMV-infected human diploid fibroblast (HF) cells were stained with specific monoclonal antibodies, IE1 localized as a mixture of nuclear diffuse and punctate patterns at very early times (2 h) but changed to an exclusively nuclear diffuse pattern at later times. In contrast, IE2 was distributed predominantly in nuclear punctate structures continuously from 2 to at least 12 h after infection. These punctate structures resembled the preexisting PML-associated nuclear bodies (ND10 or PML oncogenic domains [PODs]) that are disrupted and dispersed by the IE110 protein as a very early event in herpes simplex virus (HSV) infection. However, HCMV differed from HSV by leading instead to a change in both the PML and SP100 protein distribution from punctate bodies to uniform diffuse patterns, a process that was complete in 50% of the cells at 2 h and in 90% of the cells by 4 h after infection. Confocal double-label indirect immunofluorescence assay analysis confirmed that both IE1 and IE2 colocalized transiently with PML in punctate bodies at very early times after infection. In transient expression assays, introduction of IE1-encoding plasmid DNA alone into Vero or HF cells produced the typical total redistribution of PML into a uniform nuclear diffuse pattern together with the IE1 protein, whereas introduction of IE2-encoding plasmid DNA alone resulted in stable colocalization of the IE2 protein with PML in the PODs. A truncated mutant form of IE1 gave large nuclear aggregates and failed to redistribute PML, and similarly a deleted mutant form of IE2 failed to colocalize with the punctate PML bodies, confirming the specificity of these effects. Furthermore, both Vero and U373 cell lines constitutively expressing IE1 also showed total PML relocalization together with the IE1 protein into a nuclear diffuse pattern, although a very small percentage of the cells which failed to express IE1 reverted to a punctate PML pattern. Finally, the PML redistribution activity of IE1 and the direct association of IE2 with PML punctate bodies were both confirmed by infection with E1A-negative recombinant adenovirus vectors expressing either IE1 or IE2 alone. These results confirm that transient colocalization with and disruption of PML-associated nuclear bodies by IE1 and continuous targeting to PML-associated nuclear bodies by IE2 are intrinsic properties of these two MIE regulatory proteins, which we suggest may represent critical initial events for efficient lytic cycle infection by HCMV.

The nucleus in eukaryotic cells contains several discrete domains in which different cellular processes such as DNA replication, transcription, pre-mRNA processing, and ribosome assembly progress. Among them, the punctate nuclear bodies known as ND10 (nuclear dot domains) or PODs (PML oncogenic domains) contain several cellular proteins including SP100 and the PML proto-oncogene product (1, 54). PML was originally identified as part of a fusion with retinoic acid receptor alpha (RARα) that resulted from the t(15:17) translocation in acute promyelocyte leukemia (APL) (11, 16, 26, 27, 45). In APL cell lines, the localization of PML changes from the punctate nuclear bodies to a nuclear and cytoplasmic micropunctate pattern. Interestingly, treatment of APL tumor cells with retinoic acid restores the normal punctate PML staining pattern (12, 17, 29), which correlates with regaining normal cell growth controls and the ability to differentiate.

In herpes simplex virus (HSV)-infected cells, PML-associated nuclear bodies are lost early in the replication cycle (39). From the analysis of HSV type 1 immediate-early (IE) gene mutants, the IE110 (ICP0) protein is known to be necessary for the loss of PML punctate bodies (13, 39, 40). IE110 is a nuclear relatively nonspecific transcriptional activator protein (44), which plays a role in triggering efficient initiation of the lytic cycle (21, 35, 50, 60), especially during reactivation from the latent state. Both IE110 and PML are RING class zinc finger proteins (14, 26), and IE110 also localizes in nuclear punctate structures (3, 42, 60). At early times after either virus infection or DNA transfection, IE110 initially colocalizes with PML in the PODs and then leads to displacement and loss of the PML signal, although the transient colocalization becomes stabilized if IE110 RING finger mutants are used (15). Furthermore, expression of IE110 alone is sufficient to reorganize PML punctate bodies after infection with a recombinant adenovirus (Ad) carrying the IE110 gene (Ad5-IE110) (13, 39, 40). In both

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Epstein-Barr virus (EBV)-infected B cells and in EBV-transformed lymphoblast cell lines, the EBNA5 protein also apparently accumulates in the PML-associated nuclear bodies (55). Ad infection causes a redistribution of PML from spherical punctate nuclear bodies to fibrous structures (4, 49), and the product encoded by E4-ORF3 is responsible for this change (4). In addition, the E1A oncoprotein concentrates in the PML punctate bodies (4).

Transcription of the major immediate-early (MIE) gene of human cytomegalovirus (HCMV) can occur even in the absence of de novo protein synthesis and produces two principal nuclear regulatory phosphoproteins, referred to as IE1 (UL123) and IE2 (UL122), that are thought to play key roles in initiating and maintaining HCMV gene regulation pathways in both lytic and latent infections. The abundant 1.9-kb IE1 mRNA species encodes the acidic 72-kDa phosphoprotein (53), which associates with metaphase chromosomes (32) and has been suggested to transactivate the MIE promoter through upstream NF-κB sites (7) but otherwise has little activity on its own as a transactivator. On the other hand, IE2 (86 kDa) is a powerful nonspecific transactivator of many viral and cellular promoters (10, 22, 47, 48) and down-regulates its own promoter through binding to the *cis*-repression signal (CRS) target DNA motif near the cap site in transient cotransfection assays (6, 8, 33, 36, 46). Only the C-terminal region of the IE2 proteins of all other betaherpesviruses shows relatively high levels of amino acid conservation (5, 8), and the HCMV IE2 protein has been reported to bind to TBP, Rb, p53, CREB, and EGR-1 in vitro (19, 20, 34, 52, 58). Interestingly, HCMV infection also appears to lead to cell cycle arrest with associated increased levels of cyclins, phosphorylated Rb, and p53 (24), and HCMV IE1 and IE2 together are able to fully complement E1A-negative mutants of Ad (51, 56). In addition, IE1 and IE2 are known to be able to inhibit some apoptosis pathways (59).

HCMV infection has also recently been shown to disrupt PML punctate bodies (28), suggesting that interactions with nuclear structures containing PML may be a necessary event at early stages of infection for many DNA viruses. The fact that the IE110 protein of HSV, EBNA-5 of EBV, and E1A and E4-ORF6 of adenovirus all colocalize within PML-associated nuclear punctate bodies (3, 4, 21, 35, 55, 60) led us to investigate whether any of the MIE gene products of HCMV might also associate with PML punctate bodies. In the present study, we show that the HCMV IE2 protein continuously associates with the same nuclear punctate structures that contain PML and SP100 in both virus-infected and DNA-transfected cells, whereas IE1 transiently targets to and colocalizes with PML in the punctate bodies followed by relocalization of both IE1 and PML into a uniform nuclear diffuse pattern.

MATERIALS AND METHODS

Cell cultures and cell lines. Permissive human diploid fibroblast (HF) cells and Vero cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The astrocytoma cell line U373-A1b (2, 58) and the Vero cell line VN68cl7.8 (32) both stably express IE1 of HCMV. They were prepared as G-418-resistant subclones from cell cultures cotransfected with pSV_2NEO and the HCMV(Towne) plasmid pIE1 (for U373-A1b) or pRL103 (for VN68cl7.8). Plasmid pRL103 contained the cloned 20.8-kb HCMV(Towne) *Hin*dIIIC DNA fragment (31), and pIE1 contained a 4.0-kb *Cla*I DNA subfragment from pRL103. Master cultures of the G-418-resistant sublines were usually maintained in G-418 at 100 μg/ml.

Viruses. The HCMV(Towne) virus stock used was prepared as described by LaFemina et al. (32). An HCMV(AD169) IE1 cDNA under the control of the HCMV promoter/enhancer region from position −299 to +69 was expressed from an Ad type 5 vector with E1A deleted (Ad5-IE1), referred to previously as RAd31 (57). The IE2-expressing Ad5 vector (Ad5-IE2) was constructed by cloning an intact cDNA cassette encoding exons 2, 3, and 5 of HCMV(Towne) IE2 derived from a modified version of plasmid pCJC186 (8). The IE2 cDNA from pCJC186 was first placed into the unique *Bam*HI site of the expression plasmid pMV10 containing the HCMV IE promoter/enhancer region from −299 to +60 together with a polyadenylation signal (+2757 to +3025). The IE2 expression cassette was then moved into the Ad transfer vector pMV60 and cotransfected together with the Ad-dl309 genome vector plasmid pJM17 (57). Generation of recombinant Ad5 containing an extragenic HCMV enhancer-driven β-galactosidase (β-Gal) expression cassette has been described elsewhere (2). Ad5-IE1, Ad5-IE2, and Ad5-β-Gal were routinely grown in human 293 complementing cells expressing E1A and E1B. For infection, the cells were seeded into four-well slide chambers (0.6×10^5/well), and the subconfluent cells were infected with the HCMV or recombinant Ad vectors at a multiplicity of infection (MOI) of <1.0 PFU per cell. Virus was absorbed for 1.5 h, and then medium was replaced with fresh medium at time zero.

Expression plasmids and transient DNA transfection. Genomic versions of the HCMV(Towne) IE1 and IE2 coding regions were all derived from pRL103, which contains the 20.8-kb *Hin*dIIIC fragment containing the entire leftward-oriented MIE gene transcription unit (31). The parent plasmid pRL45 contains a 6.6-kb *Eco*RI/*Sal*I subfragment expressing the wild-type forms of both IE1(1–491) and IE2(1–579) under the control of their natural transcriptional and splicing signals, whereas pMP17 and pMP18 express either IE1 or IE2 only from within the same genetic background (48). Plasmid pRL74 expressing a truncated mutant form of IE1(1–347) only (triple terminator at codon 347) and plasmid pMP88 expressing both intact IE1 and an internally deleted mutant form of IE2 (Δ136–289) were also described previously (32, 47, 48).

For DNA transfection experiments, Vero or HF cells were seeded into two-well slide chambers (0.8×10^5/well) and DNA (3 μg/well) was introduced for transient expression assays in subconfluent cells by using the *N*,*N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid-buffered saline version of the calcium phosphate procedure described previously (46).

Antibodies. Mouse monoclonal antibodies (MAbs) 6E1 and 12E2 against HCMV IE1 (exon 4) and IE2 (exon 5), respectively, were obtained from Vancouver Biotech (Vancouver, British Columbia, Canada), and MAb CH810, which detects epitopes present in both IE1 and IE2 (exons 2 and 3), was purchased from Chemicon (Temecula, Calif.). Rabbit antipeptide polyclonal antibody (PAb) (P3) directed against amino acids (aa) 367 to 382 of HCMV IE2 was described previously (47). Rabbit antipeptide PAb (E) against aa 459 to 473 of the HCMV IE1 protein and antipeptide PAb against aa 484 to 498 of the PML oncoprotein were prepared according to the methods described previously (47) and were provided by I. Waheed and D. Ciufo, respectively. Rabbit PAb against SP100 was kindly provided by Carin Szostecki (Max Plank Institüt für Biochimie, Martinsried, Germany).

Indirect immunofluorescence assay (IFA). Both virus-infected and DNA-transfected cells were fixed with either methanol or paraformaldehyde. For the methanol procedure, the cells were washed in Tris-saline and then fixed with absolute methanol at −20°C for 10 min and permeabilized in ice-cold Tris-saline for 5 min. For the paraformaldehyde procedure, the cells were washed in phosphate-buffered saline (PBS), fixed with 1% paraformaldehyde solution in PBS at 20°C for 5 min, and then permeabilized in ice-cold 0.2% Triton X-100 solution in PBS for 20 min. The cells were incubated with mouse MAbs at 1:200-fold dilutions or rabbit PAbs at a 1:100-fold dilution for PAb P3, at a 1:400-fold dilution for PAb E, at a 1:1,000-fold dilution for the PML antibody, and at a 1:500-fold dilution for the SP100 antibody in Tris-saline at 37°C for 1 h, followed by fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin G (IgG) or by rhodamine-coupled goat anti-rabbit IgG antibody at a 1:100-fold dilution at 37°C for 45 min. For double labeling, both MAbs and PAbs were incubated together. Slides were screened and photographed with a 40× oil immersion objective on a Leitz Dialux 20EB epifluorescence microscope with Kodak T-Max P3200 film and appropriate FITC or rhodamine filters. For confocal microscopy, Texas red was used instead of rhodamine and samples were visualized by using a confocal microscope (Bio-Rad; MRC600).

RESULTS

HCMV infection displaces PML and SP100 from nuclear bodies at early times. To study the effects of HCMV infection on POD protein distribution, we initially investigated the localization patterns of two cellular POD proteins (PML and SP100) and the MIE proteins (IE1 and IE2) after HCMV infection in HF cells. Six hours after infection of HF cells with HCMV(Towne) at a low MOI (<1.0), IFA experiments were performed to detect the MIE proteins and cellular POD proteins by double-staining approaches (Fig. 1). Detection with mouse MAb CH810 (FITC signal), which recognizes an epitope present in the exon 2/3 region common to both IE1 and IE2 of HCMV, showed mixed nuclear diffuse and punctate staining patterns by the methanol fixation procedure (Fig. 1a, c, and e), with about 10% of all infected cells showing a nuclear punctate pattern. In addition, the normal nuclear punctate patterns of both PML and SP100 detected with rabbit PAbs

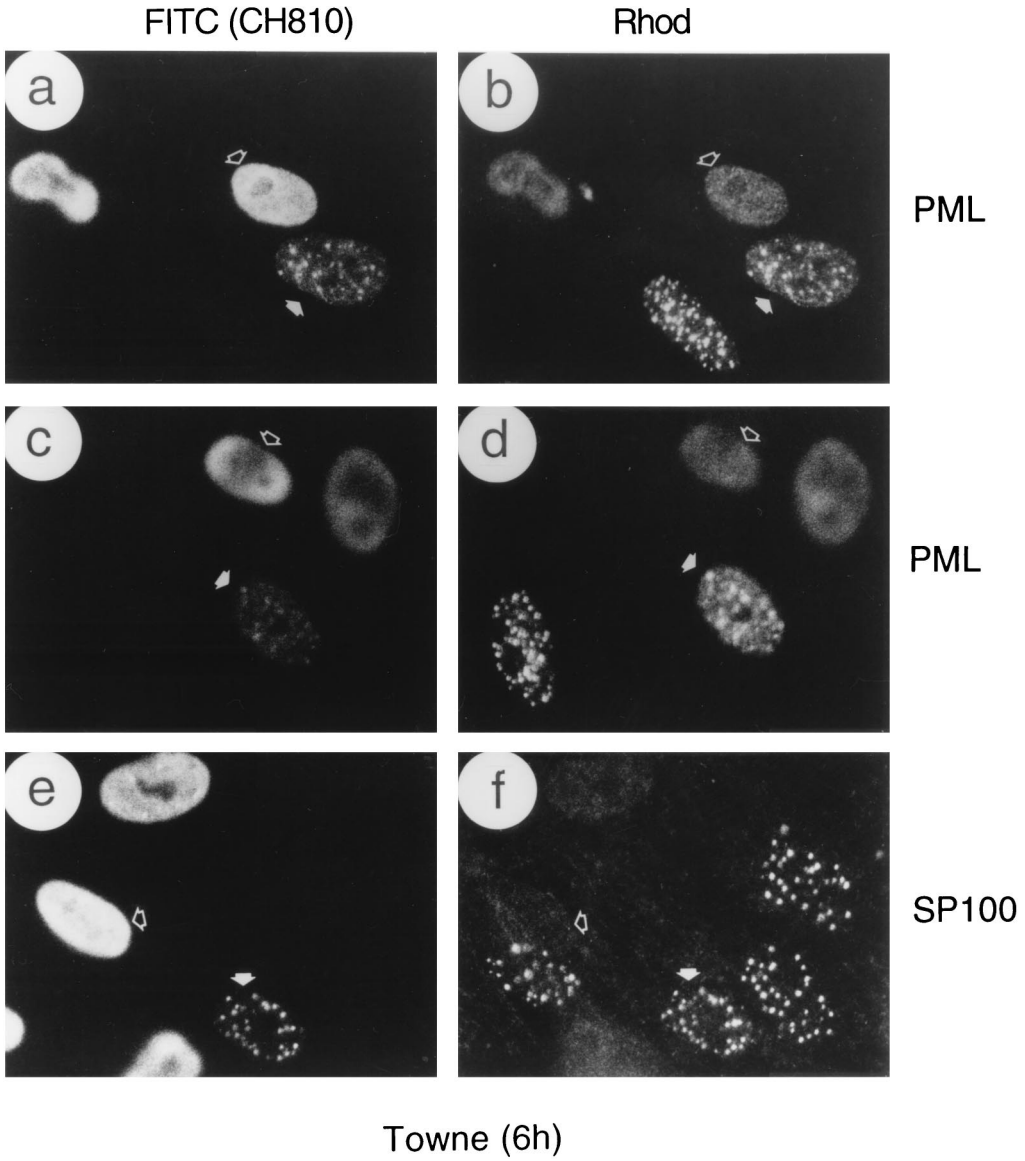


FIG. 1. Disruption of PODs (ND10) containing PML and SP100 after HCMV infection. HF cells were infected with HCMV(Towne) at a low MOI (<1.0 PFU/cell). At 6 h after infection, the cells were fixed in methanol and double-label IFA was carried out with mouse MAb CH810 recognizing both IE1 and IE2 (exons 2 and 3) and rabbit PAb against either PML (b and d) or SP100 (f). (a, c, and e) Detection of IE1 or IE2 with MAb CH810 and FITC-labeled anti-mouse IgG. (b and d) Detection of PML in the same fields with rabbit anti-PML PAb and rhodamine-coupled anti-rabbit IgG. (f) Detection of SP100 in the same fields with rabbit anti-SP100 PAb and rhodamine-coupled anti-rabbit IgG. Infected cells showing a change from the normal punctate pattern of PML or SP100 into a nuclear diffuse pattern are indicated by open arrows, and infected cells showing a punctate colocalization pattern between viral protein(s) and PML or SP100 are indicated by closed arrows. Note the lack of antibody cross-reaction in the uninfected cells (all panels), which display a normal punctate pattern of PML or SP100 but without expressing viral protein(s).

(rhodamine signals) were changed to uniform nuclear diffuse patterns in the majority of infected cells that showed the nuclear diffuse pattern of MIE proteins, although the intensity of the remaining signal for SP100 was much weaker than that for PML (Fig. 1, open arrows). In contrast, both PML and SP100 remained punctate in those infected cells that showed the punctate MIE protein staining pattern (Fig. 1a and b; c and d; e and f, closed arrows). However, we could not tell from this experiment whether the 10% of MIE-expressing cells showing the punctate staining pattern with CH810 MAb contained just IE1 or IE2 alone or expressed both viral proteins. Overall, these results confirmed that up to 90% of all MIE-expressing cells produced displacement of the PML and SP100 signals from the POD structures by 6 h after infection.

Some IE1 colocalizes with PML punctate structures in infected cells, whereas most IE1 is distributed in a nuclear diffuse pattern. To separately investigate the roles of each of the two MIE regulatory proteins, we investigated the localization pattern of IE1 and IE2 in infected HF cells with two mouse MAbs that specifically recognize either IE1 (exon 4) or IE2 (exon 5) only. In double-label IFA experiments with mouse MAb 6E1 directed against IE1 and rabbit antipeptide PAb against PML at 6 h after infection, we found that IE1 was distributed exclusively in a nuclear diffuse pattern when fixed

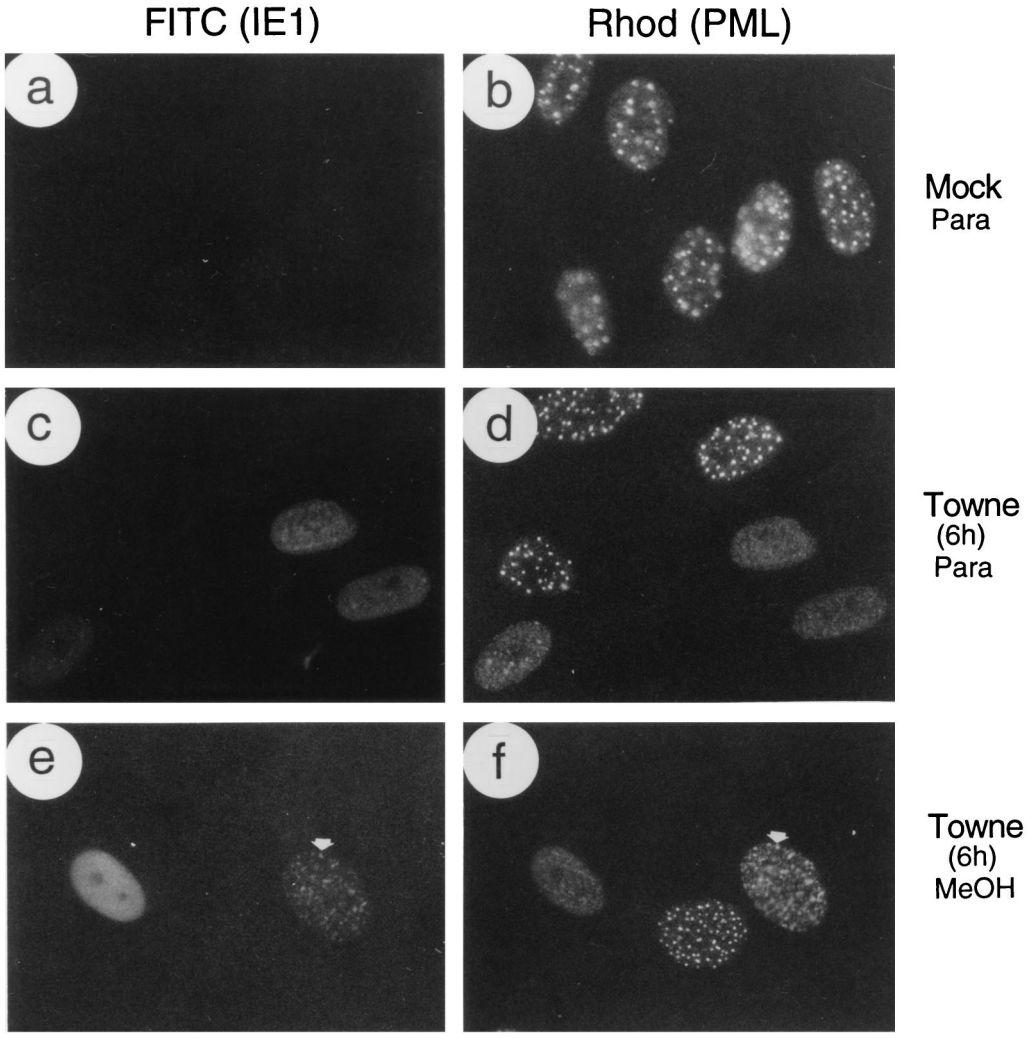


FIG. 2. Mixed localization patterns of IE1 after HCMV infection in permissive cells. HF cells were infected (c to f) or mock infected (a and b) with HCMV(Towne) at an MOI of <1.0. At 6 h after infection, the cells were fixed with paraformaldehyde (a to d) or with methanol (e and f) followed by double-label IFA with mouse MAb 6E1 recognizing IE1 only and rabbit PAb against PML. (a, c, and e) Detection of IE1 with MAb 6E1 and FITC-labeled anti-mouse IgG. (b, d, and f) Detection of PML in the same fields with rabbit anti-PML PAb and rhodamine-coupled anti-rabbit IgG. Most infected cells (about 90%) show uniform nuclear diffuse IE1 patterns, but examples of cells showing colocalization between IE1 and PML in punctate patterns after methanol fixation (10%) are indicated by closed arrows in panels e and f. Note the lack of antibody cross-reaction in the uninfected cells displaying a normal punctate pattern of PML (b) but no IE1 (a).

by the paraformaldehyde–Triton X-100 procedure (Fig. 2c) but was distributed as a mixture of nuclear diffuse and nuclear punctate patterns when fixed by the methanol procedure (Fig. 2e). Double-label IFA of uninfected cells in the same field with 6E1 (Fig. 2a) or with PAb against PML (Fig. 2b) showed that there was no cross-reaction between the two antibodies. With the methanol procedure, about 90% of the IE1-positive cells showed a nuclear diffuse pattern, whereas about 10% displayed a punctate distribution pattern of IE1. The proportion of cells with a punctate IE1 distribution detected with MAb 6E1 was the same as that of cells with punctate staining detected as described above by MAb CH810. This observation suggested that those 10% of MIE-positive cells showing punctate staining patterns with MAb CH810 must all be expressing at least IE1. Interestingly, in these 10% of the cells, IE1 always colocalized with PML, although the punctate pattern often appeared changed into a more network-like distribution (Fig. 2e and f, closed arrows). In contrast, PML was always redistributed into a nuclear diffuse pattern in the 90% of MIE-positive cells displaying a nuclear diffuse pattern of IE1 at 6 h after infection (Fig. 2c and d; e and f). This result suggests that the localization pattern of IE1 may determine the redistribution of PML.

IE2 targets predominantly to punctate structures in infected cells. We also carried out double-label IFA experiments on HF cells at 6 h after HCMV(Towne) infection with mouse MAb 12E2 against IE2 and rabbit PAb against PML (Fig. 3). IE2 was also distributed in a predominantly nuclear punctate pattern with both the paraformaldehyde (Fig. 3c) and methanol (Fig. 3e) fixation procedures in almost all IFA-positive cells. The punctate distribution of IE2 was also observed at 6 h after infection of U373 astrocytoma cells with HCMV(Towne) (data not shown). As expected, double labeling showed that PML was displaced from the punctate bodies in virtually all of the IE2-expressing infected cells at this time point (Fig. 3c and d; e and f).

Unlike the double-labeling result between IE1 and PML (Fig. 2), very few infected cells were observed to show both punctate IE2 and a punctate PML distribution with methanol

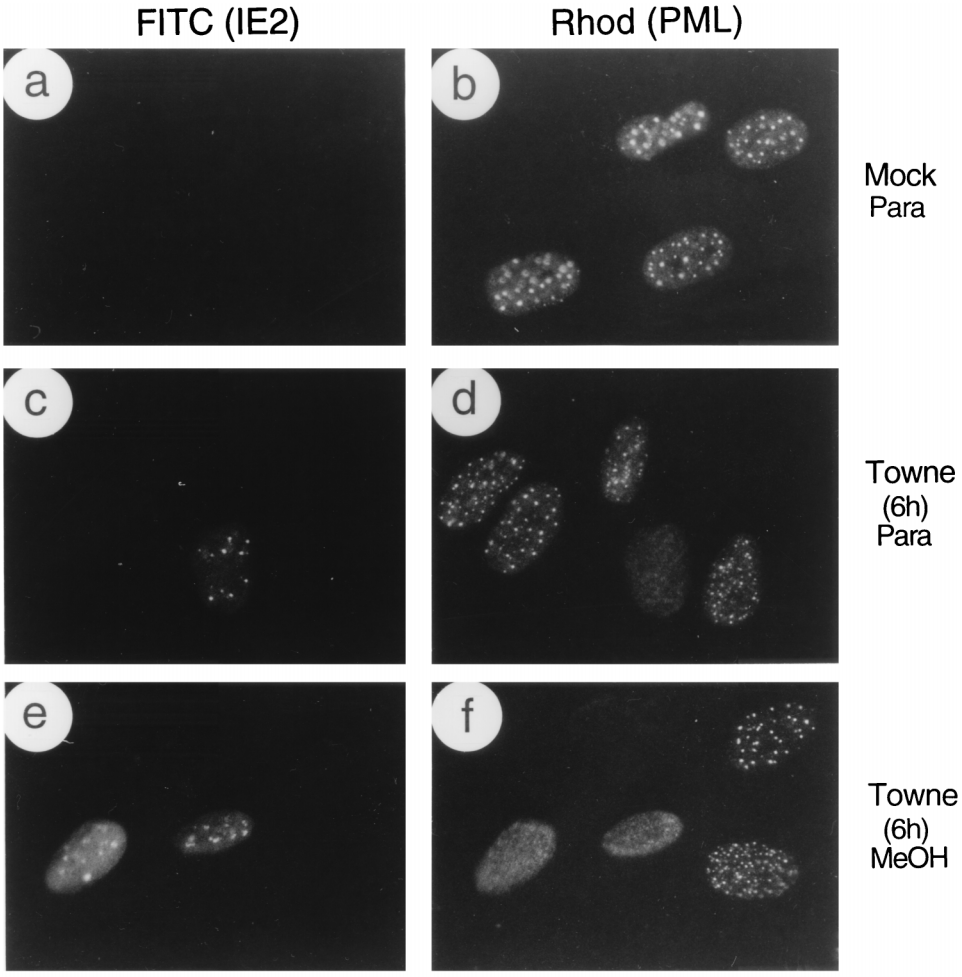


FIG. 3. Nuclear punctate distribution of IE2 after HCMV infection in permissive cells. HF cells were infected (c to f) or mock infected (a and b) with HCMV(Towne) at an MOI of <1.0. At 6 h after infection, the cells were fixed with paraformaldehyde (a to d) or with methanol (e and f) and subjected to double-label IFA with mouse MAb 12E2 recognizing IE2 only and rabbit PAb against PML. (a, c, and e) Detection of IE2 with MAb 12E2 and FITC-labeled anti-mouse IgG. (b, d, and f) Detection of PML in the same fields with rabbit anti-PML PAb and rhodamine-coupled anti-rabbit IgG. Note the uniform diffuse pattern of PML in infected cells showing a nuclear punctate pattern of IE2 (c and d; e and f) and the lack of antibody cross-reaction in the uninfected cells displaying a normal punctate pattern of PML (b) but no IE2 (a).

fixation at 6 h. This observation suggests that the 10% of MIE-positive cells showing a punctate colocalization pattern between IE1 and PML after methanol fixation (Fig. 2) may not be expressing IE2. To examine this question more directly, double-label IFA between IE1 and IE2 at 6 h after infection was performed with a rabbit antipeptide PAb (E) prepared against IE1 together with mouse MAb 12E2 against IE2 (Fig. 4). As expected, all cells displaying a normal nuclear diffuse pattern of IE1 (Fig. 4a) showed a punctate pattern of IE2 (Fig. 4b). However, no clear IE2 punctate pattern was detected in most of the cells that showed a network-like punctate pattern of IE1 (Fig. 4c and d, arrows). Therefore, this result confirmed that some infected cells expressed only IE1, but not IE2, and that in these cells IE1 was distributed in a network-like punctate pattern that colocalized with PML. In those few cells that did give punctate staining patterns for both IE1 and IE2, the two appeared to show either partial or complete colocalization (Fig. 4e and f).

Both IE1 and IE2 present in punctate bodies colocalize with PML as determined by confocal microscopy. To investigate the distribution of IE1 relative to PML punctate bodies at very early times after infection, confocal microscopy and double-label IFA were performed. When the HF cells were fixed with methanol at just 2 h after infection, the proportion of IE1-positive cells displaying a punctate pattern of IE1 protein increased to more than 50%. Furthermore, when assayed by double-label IFA between IE1 and PML (Fig. 5a), the merge image (yellow) revealed that the punctate pattern of IE1 (green) clearly overlapped completely with that of PML (red).

To examine whether the IE2 punctate bodies were also related to PML punctate bodies, double-label confocal IFA between IE2 and PML was performed at 2 h after infection. Three different types of IE2 distribution were observed. In a small proportion of infected cells, few or none of the IE2 punctate bodies (green) colocalized with PML punctate bodies (red) (Fig. 6a). In contrast, some other cells showed colocalization between most of the IE2 punctate bodies (green) and the PML bodies (red) (Fig. 6b). However, in the majority of cells with IE2 punctate patterns the PML distribution had already become nuclear diffuse. Therefore, IE2 is distributed into nuclear punctate structures that at least transiently colo-

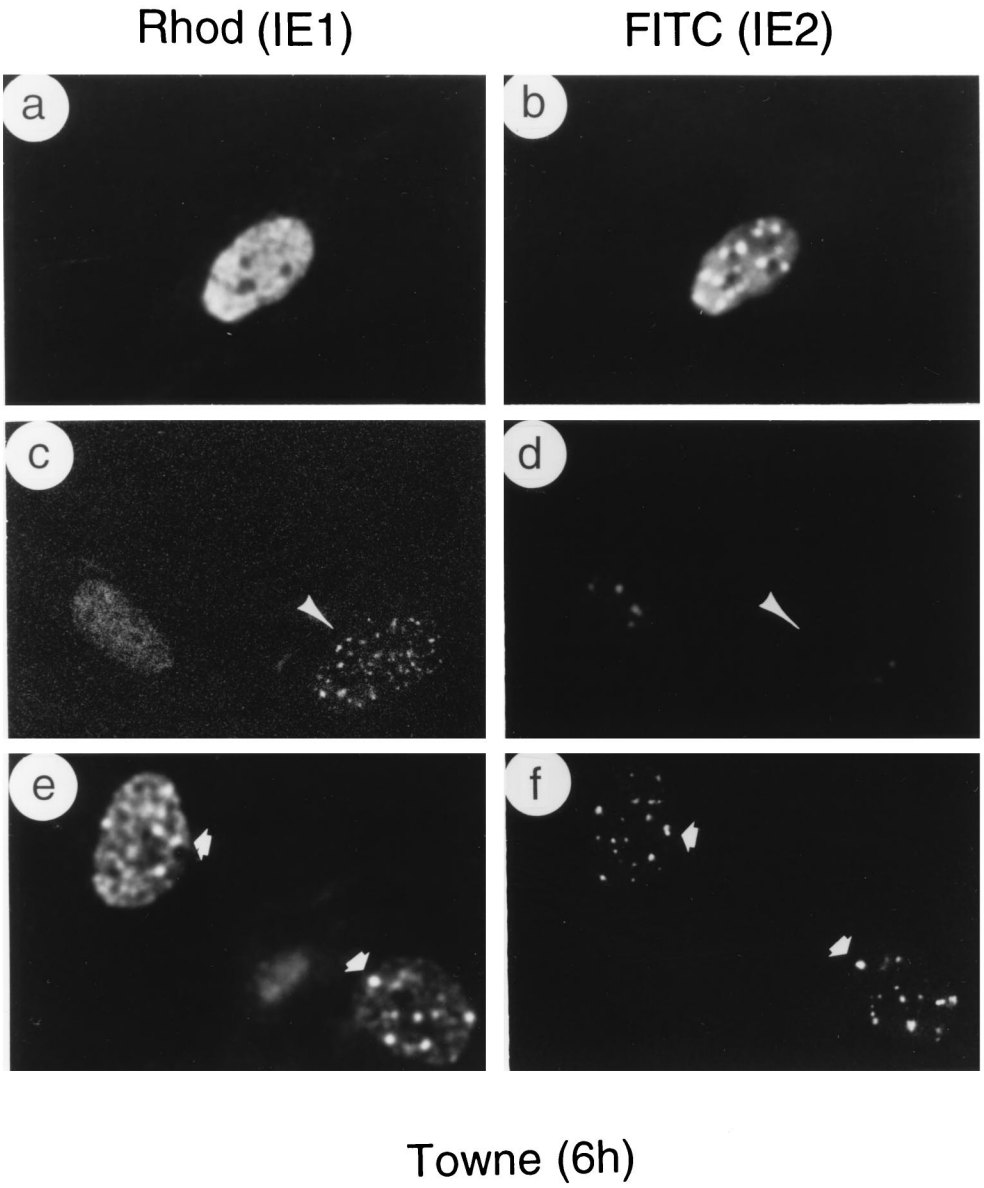


FIG. 4. Detection of infected cells expressing IE1 but not IE2 and occasional cells with colocalized punctate IE1 and IE2. HF cells were infected with HCMV(Towne) at an MOI of <1.0. At 6 h after infection, the cells were fixed with methanol followed by double-label IFA with rabbit PAb against IE1 and mouse MAb against IE2. Three double-label images representing different distribution patterns of IE1 and IE2 are shown. (a, c, and e) Detection of IE1 with rabbit antipeptide PAb (E) and rhodamine-coupled anti-rabbit IgG. (b, d, and f) Detection of IE2 in the same fields with MAb 12E2 and FITC-labeled anti-mouse IgG. Long arrowheads show an IE1-positive, IE2-negative cell, and short arrows denote cells with partial colocalized IE1 and IE2.

calize with PML in HCMV-infected cells and apparently stay there after PML is redistributed.

Time course of punctate and displacement interactions with PML. The possibility that the relative localization patterns of IE1, IE2, and PML might change significantly at different stages early after HCMV infection was investigated by examining cells from the same culture at various time points (2, 3, 4, 6, and 12 h). HF cells were infected with HCMV(Towne) at a low MOI (0.6 PFU/cell), and double-label IFA experiments between either IE1 or IE2 and PML were performed with MAb 6E1 or 12E2 and PAb against PML. The results are summarized in Table 1. Double-label detection of IE1 and PML revealed that the IE1 protein was distributed as a mixture of punctate (54%) and nuclear diffuse (46%) patterns at 2 h but then gave a predominantly nuclear diffuse pattern (94%) at 6 h. However, the punctate form of IE1 colocalized with PML at all time points. The proportion of cells showing this colocalization as a punctate pattern gradually decreased from 33% at 3 h to 12% at 4 h and 6% at 6 h and then remained at 5% at 12 h. The punctate patterns of both IE1 and PML at 6 and 12 h were more network-like as shown in Fig. 2e and f (closed arrows). No cell showing a punctate pattern of IE1 and a nuclear diffuse pattern of PML was detected. Taken together, these results demonstrate that IE1 first targets to PML punctate bodies at very early times and then both IE1 and PML change to a nuclear diffuse pattern in almost all cells. Furthermore, IE1 was always found to colocalize with PML after infection irrespective of whether both gave a punctate or a nuclear diffuse pattern.

On the other hand, IE2 was always distributed in a punctate

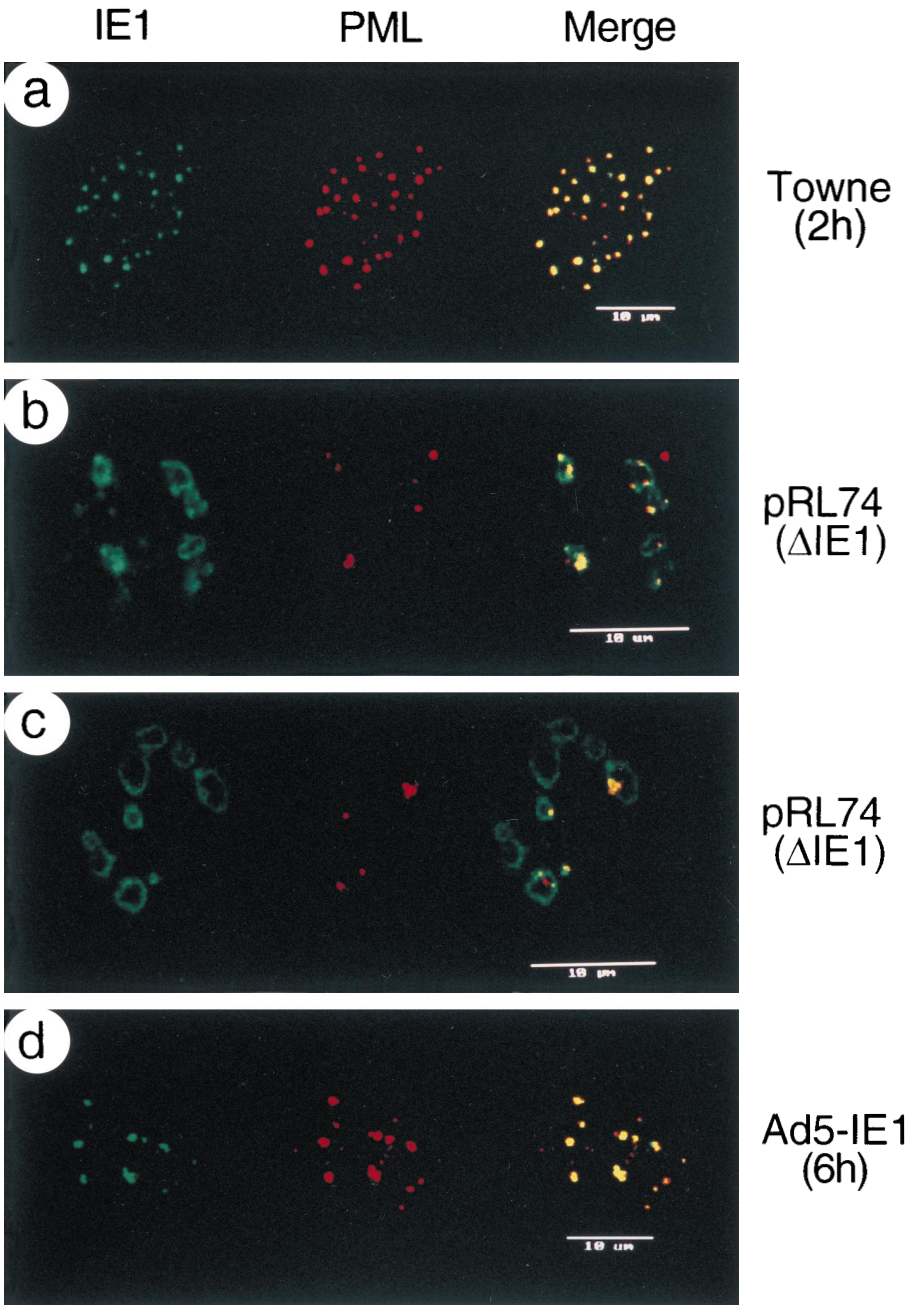


FIG. 5. Demonstration of the colocalization of IE1 with PODs after virus infection as visualized by confocal microscopy. The fixed cells were subjected to double-label IFA with mouse MAb 6E1 recognizing IE1 and rabbit PAb against PML. (Left-hand frames [green fluorescence]) Detection of IE1 with MAb 6E1 and FITC-labeled anti-mouse IgG. (Center frames [red fluorescence]) Detection of PML in the same fields with rabbit anti-PML PAb and Texas red-labeled anti-rabbit IgG. (Right-hand frames [yellow merge pattern]) Confocal images from each fluorochrome were recorded and superimposed. (a) HF cells were infected with HCMV(Towne) at an MOI of <1.0 and fixed with methanol at 2 h. (b and c) Two different images of Vero cells transfected with plasmid pRL74 expressing IE1(Δ347–491) containing a deletion of the acidic domain at the C terminus. The cells were fixed with paraformaldehyde at 48 h after transfection. (d) HF cells were infected with an E1A-negative recombinant Ad vector expressing wild-type HCMV IE1 (Ad5-IE1) and fixed at 6 h with methanol.

pattern from 2 h until at least 6 h after infection, although a mixture of punctate and nuclear diffuse patterns was detected at 12 h (Table 1). Double-label IFA comparison of IE2 and PML showed that the proportion of cells displaying punctate patterns for both IE2 and PML was 21% at 2 h and then gradually decreased to 2% at both 6 and 12 h. The total number of cells in the culture expressing IE1 at 2 h was 42%, which was higher than the 32% of cells expressing IE2 as assayed by IFA. In addition, at 6 h the proportion of IE1-positive cells (65%) also remained a little higher than that of IE2-positive cells (61%), consistent with our earlier observation that some infected cells still expressed IE1 alone even at that stage.

IE1 expressed alone is sufficient for PML redistribution in both permanent cell lines and transiently transfected cells. The double-label IFA experiment comparing IE1 and PML in

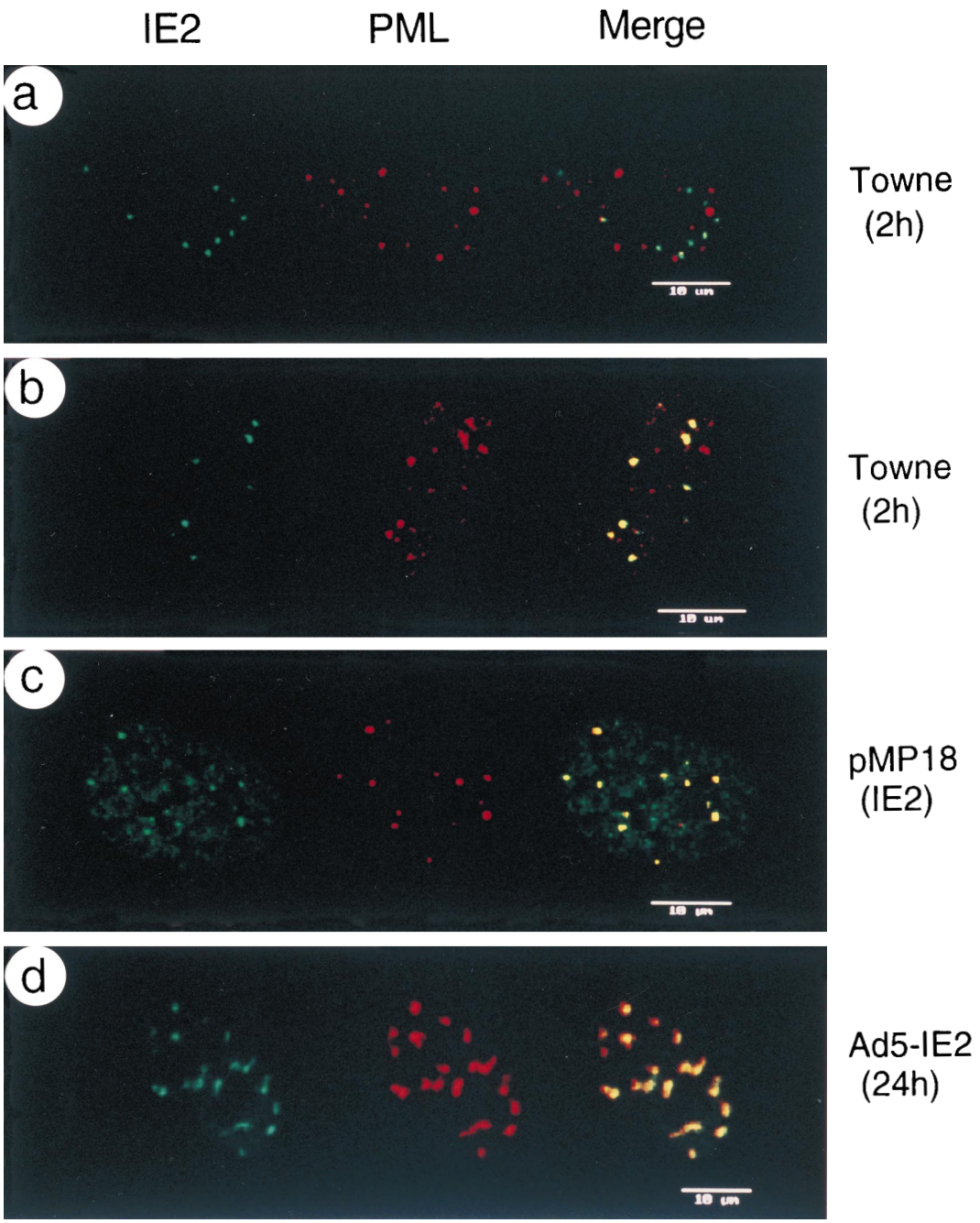


FIG. 6. Confocal microscopy demonstrating colocalization of IE2 with PML in both virus-infected and DNA-transfected cells. The fixed cells were subjected to double-label IFA with mouse MAb 12E2 recognizing IE2 only and rabbit PAb against PML. (Left-hand frames [green fluorescence]) Detection of IE2 with MAb 12E2 and FITC-labeled anti-mouse IgG. (Center frames [red fluorescence]) Detection of PML in the same fields with rabbit anti-PML PAb and Texas red-labeled anti-rabbit IgG. (Right-hand frames [merge fluorescence]) Confocal images from each fluorochrome were recorded and superimposed. Overlapping of green and red staining produces a yellowish color. (a and b) HF cells were infected with HCMV(Towne) at a low MOI (<1.0 PFU/cell). The cells were fixed with methanol at 2 h after infection. (c) Vero cells were transfected with plasmid pMP18 which expresses HCMV IE2 only. The cells were fixed with paraformaldehyde at 48 h after transfection. (d) HF cells were infected with an E1A-negative recombinant Ad vector expressing wild-type HCMV IE2 (Ad5-IE2) and fixed at 24 h with methanol.

Fig. 2 suggested that the nuclear diffuse distribution of IE1 correlates with and might be responsible for PML disruption. Therefore, to determine whether IE1 alone is sufficient for the redistribution of PML, we investigated the effect of IE1 on PML distribution in two different stable cell lines that constitutively express HCMV IE1. In both the permissive U373-A1b astrocytoma cell line (58) and the nonpermissive VN68cl7.8 Vero cell line (32), the punctate distribution of PML was changed into a nuclear diffuse pattern in all cells expressing IE1 (Fig. 7a and b for U373-A1b; c and d for VN68cl7.8). Interestingly, in the few cells in each culture that had lost or failed to express IE1 the normal punctate PML distribution was fully restored (Fig. 7, arrows).

The displacement of PML from punctate bodies by IE1

TABLE 1. Summary of IFA localization patterns for IE1 or IE2 and PML in HF cells at early times after infection with HCMV(Towne)

Intranuclear protein distribution patterns by double-label IFA	% Positive cells (no. scored/no. tested) at time (h) after infection (MOI, 0.6)				
	2	3	4	6	12
Subsets of protein localization patterns[a]					
IE1/PML					
P/P	54 (55/101)	33 (96/293)	12 (22/182)	6 (6/105)	5 (8/172)
P/ND	0	0	0	0	0
ND/P	0	0	0	0	0
ND/ND	46 (46/101)	67 (197/293)	88 (160/182)	94 (99/105)	95 (164/172)
IE2/PML					
P/P	21 (23/111)	13 (35/260)	9 (9/103)	2 (3/156)	2
P/ND	79 (88/111)	87 (225/260)	91 (94/103)	98 (153/156)	98 (150/153)
ND/ND or P	0	0	0	0	NS[b]
Proportion of cells expressing viral protein[c]					
IE1	42 (98/233)	51 (134/265)	65 (142/217)	65 (164/254)	66 (158/238)
IE2	32 (86/266)	46 (101/220)	59 (160/271)	61 (178/290)	62 (135/218)

[a] P, nuclear punctate; ND, nuclear diffuse.
[b] NS, IE2 is distributed as a mixed punctate with nuclear diffuse pattern at 12 h but was scored only as punctate.
[c] Percentage of cells expressing IE1 or IE2 among total cells in the culture.

alone was also examined by double-label IFA after transient expression from plasmid DNA introduced into Vero cells. Transfection with plasmid pMP17, which expresses wild-type HCMV IE1 only in a nuclear diffuse pattern, led to either a change of the PML signal from the punctate pattern into a nuclear diffuse pattern in most cells (Fig. 8A, panels a and b, open arrows) or a total loss of the PML IFA signal in some cells (Fig. 8A, panels c and d, open arrows). The apparent disappearance rather than redistribution of PML correlated with the highest levels of IE1 expression as judged by the strength of the IFA signal. Note that in these experiments IE1 expressed at 48 h after transfection was always nuclear diffuse and never punctate.

Therefore, the results with cells expressing wild-type IE1 (from both the permanent cell lines and the transient assays) demonstrate that IE1 alone is both necessary and sufficient for PML disruption. Furthermore, the observation that PML redistribution was ablated in the 2 to 3% of cells in the stably transfected culture that failed to express IE1 strongly reinforces this correlation.

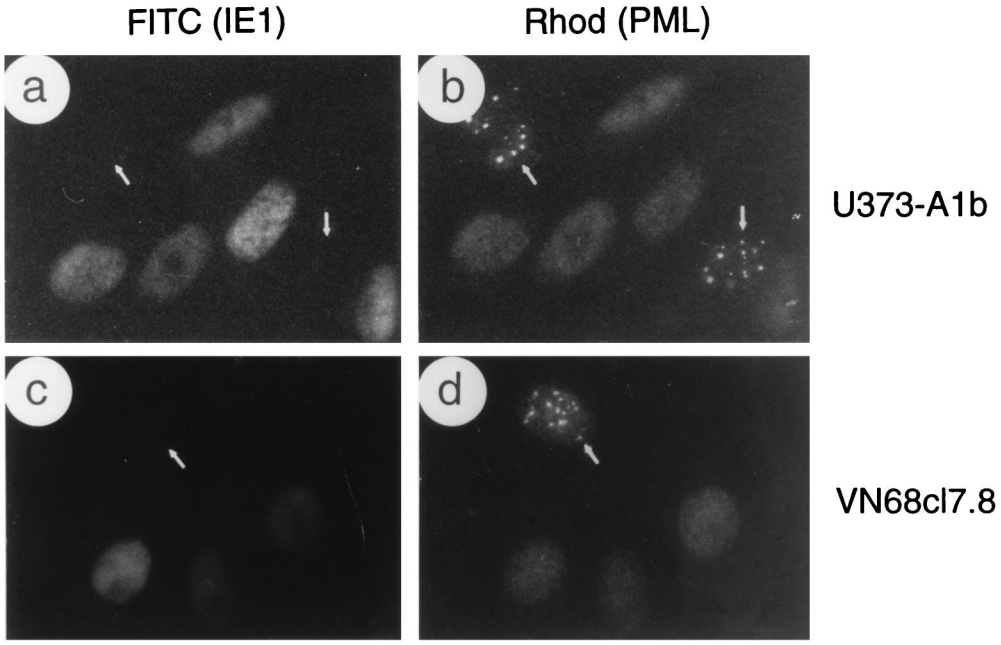
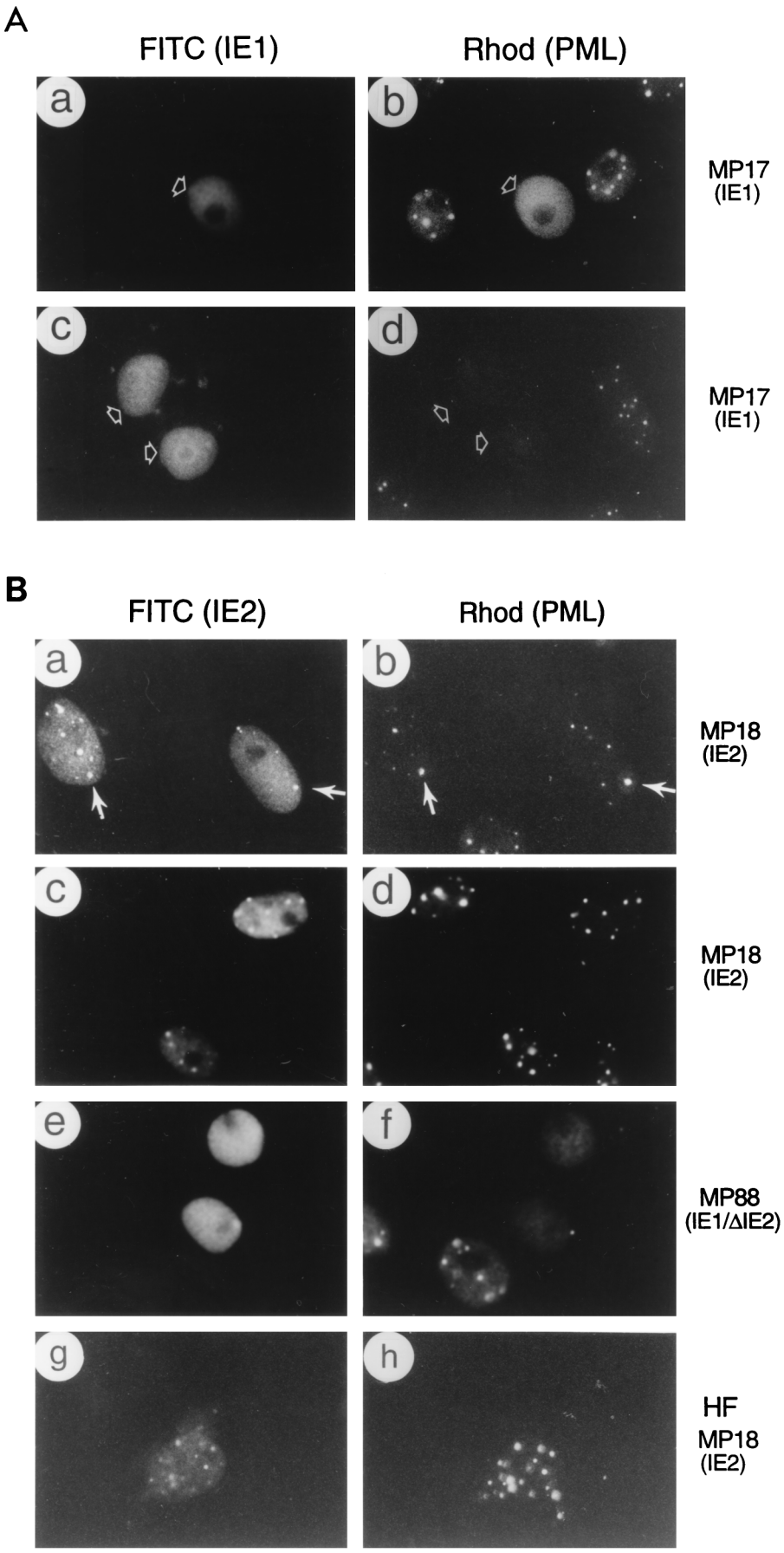


FIG. 7. Disruption of PML punctate bodies in permanent cell lines that constitutively express IE1. The cells were seeded into two-well slide chambers and fixed with paraformaldehyde while still subconfluent followed by IFA with mouse MAb 6E1 recognizing IE1 and rabbit PAb against PML. Arrows indicate occasional cells that have lost IE1 expression and restored the normal punctate PML pattern. (a and b) Astrocytoma cell line U373-A1b (2, 58); (c and d) Vero cell line VN68cl7.8 (32) expressing IE1 of HCMV. Panels show double-label detection in the same fields of IE1 with MAb 6E1 and FITC-labeled anti-mouse IgG and of PML with rabbit anti-PML PAb and rhodamine-coupled anti-rabbit IgG.


A
FITC (IE1)
Rhod (PML)
a
b
c
d
MP17
(IE1)
MP17
(IE1)
B
FITC (IE2)
Rhod (PML)
a
b
c
d
e
f
g
h
MP18
(IE2)
MP18
(IE2)
MP88
(IE1/ΔIE2)
HF
MP18
(IE2)

Consistent with previous results (32), a mutant IE1 protein containing a truncation in the C-terminal region at codon 347 (pRL74) gave large spherical granular structures within the nucleus rather than the typical nuclear diffuse distribution pattern (Fig. 5b and c, green fluorescence). To characterize the PML distribution pattern associated with these abnormal structures, the cells transfected with the mutant IE1 protein lacking the C-terminal region were examined by double-label IFA and confocal microscopy. Two examples of Vero cells transfected with pRL74 are shown in Fig. 5b and c. The mutant IE1 protein (green) was distributed in large granular structures, whereas PML (red) remained in small punctate bodies, although the merge image of the same field showed that the PML punctate bodies were included within the IE1 granular structures. Therefore, the mutant IE1 protein lacking the Glu-rich C-terminal region from codon 347 to 491 was distributed in an aberrant globular rather than a uniform diffuse pattern and failed to displace PML from the punctate bodies.

IE2 expressed alone shows apparently stable colocalization with PML in punctate bodies. When Vero cells were transfected with plasmid pMP18 expressing wild-type IE2 alone, the IE2 protein was distributed as a mixed nuclear pattern combining a background diffuse distribution with a typical POD-like punctate pattern (Fig. 8B, panels a and c), rather than giving the fully punctate distribution found during HCMV infection of HF cells (Fig. 3c and e). Double-label IFA comparison of wild-type IE2 and PML showed that most (but not all) IE2 punctate bodies were closely associated with PML punctate bodies (Fig. 8B, panels a and b, arrows; panels c and d) and that they directly colocalized in the yellow merge patterns by confocal microscopy (Fig. 6c). This apparently stable colocalization property of IE2 with PML punctate bodies in the absence of IE1 was also obtained in permissive HF cells transfected with wild-type IE2 (Fig. 8B, panels g and h). When the cells were transfected with a plasmid encoding wild-type forms of both IE1 and IE2 (pRL45), the IE2 pattern remained mixed punctate and diffuse but PML was redistributed into a diffuse pattern (not shown). In contrast, double-label IFA of Vero cells transfected with plasmid pMP88, which expresses both wild-type IE1 and mutant IE2(Δ136–289), showed that this form of the IE2 protein gave an exclusively nuclear diffuse pattern when detected with the IE2-specific MAb (Fig. 8B, panel e), although the PML punctate bodies were still disrupted by IE1 (Fig. 8B, panel f).

Infection with recombinant Ad confirms that IE1 alone displaces PML but that IE2 alone colocalizes with PML. The PML-disrupting activity of IE1 and the colocalization property of IE2 for PML punctate bodies were further investigated by infection of permissive HF cells with E1A-negative recombinant Ad5 vectors expressing β-Gal (Ad5-β-Gal), HCMV IE1 alone (Ad5-IE1), or HCMV IE2 alone (Ad5-IE2). No viral IE1- and IE2-related signals were detected in uninfected HF cells or with Ad5-β-Gal-infected cultures (Fig. 9a and c), which both gave a normal punctate distribution of PML in almost all cells (Fig. 9b and d). However, infection with Ad5-IE1 gave a normal nuclear diffuse pattern of IE1 in virtually all cells at 24 h (Fig. 9e), which correlated with the typical redistribution of PML from nuclear punctate bodies into a nuclear diffuse pattern (Fig. 9f). Interestingly, at 6 h 50% of the Ad5-IE1-expressing cells showed punctate IE1 patterns that colocalized with PML punctate bodies as detected by confocal analysis (Fig. 5d). Therefore, expression of IE1 alone by Ad infection was sufficient to both transiently target to and disrupt the PML bodies in HF cells, resulting in diffuse nuclear colocalization of both proteins at later time points just as in HCMV-infected cells, although the process was much slower with the Ad vector.

In similar experiments, HF cells infected with Ad5-IE2 expressing IE2 alone gave very few IE2-positive cells detectable at 6 h but produced abundant punctate patterns containing both IE2 (Fig. 9g) and PML in nearly all cells at 24 h (Fig. 9h). Therefore, again expression of IE2 from the cDNA gene in the Ad vector was very much slower than that from the genomic context in HCMV-infected cells. When confocal microscopy was employed to examine HF cells after infection with Ad5-IE2, the IE2 punctate structures (green) were found to be completely colocalized with those of PML (red) as shown in the merge image (yellow) (Fig. 6d). These observations confirmed that the IE2 protein expressed alone, in the absence of IE1 and all other HCMV proteins, is capable of efficiently colocalizing with PML punctate bodies without affecting PML distribution in HF cells infected with Ad.

DISCUSSION

The results of our IFA experiments with MAbs that are specific for HCMV IE1 or IE2 show unambiguously that within the first few hours after HCMV infection at a low MOI both proteins target to and interact with the preexisting PML-containing nuclear bodies called PODs or ND10. By 2 h after infection of permissive HF or U373 cells, up to 50% of the cells contained newly synthesized IE1 protein that was distributed in a punctate intranuclear pattern that precisely colocalized with punctate bodies containing PML and SP100 as detected by double-label confocal microscopy. However, this interaction was only transient, with IE1 and both PML and SP100 simultaneously becoming relocalized into a uniform diffuse nuclear pattern within most cells by 4 to 6 h after infection. The pattern of nuclear diffuse IE1 and PML then persisted throughout the late stages of infection. In contrast, a portion of the newly synthesized IE2 protein also targeted to and colocalized with PODs within 4 h after infection, but it remained stably associated with punctate structures for up to 12 h even though PML and SP100 had been displaced by IE1. Similar consistent phenotypes were displayed by each of the intact wild-type IE1 or IE2 proteins when expressed alone from isolated genomic or cDNA genes introduced either into permissive HF or U373 cells or into nonpermissive Vero cells by DNA transfection procedures, or by infection with E1A-negative Ad vectors. Mutant forms of each protein either gave aberrant globular structures without disrupting PML (IE1) or failed to colocalize to the PODs (IE2) in transient assays. Therefore, the ability of both proteins to target to PODs and, in the case of IE1, the additional property of displacement of PML and SP100 are intrinsic characteristics of each protein that are not signifi-

FIG. 8. PML colocalization patterns of IE1 and IE2 after transient expression in DNA-transfected cells. The cells were fixed with paraformaldehyde followed by double-label IFA at 48 h after transfection. (A) (IE1) Two double-label images in the same fields of Vero cells transfected with expression plasmid pMP17 encoding wild-type IE1 only. (a and c) Detection of IE1 with mouse MAb 6E1 and FITC-labeled anti-mouse IgG; (b and d) detection of PML with anti-PML PAb and rhodamine-coupled anti-rabbit IgG. Open arrowheads denote IE1-expressing cells with diffuse PML signals. (B) (IE2) (a to d) Two double-label images of Vero cells transfected with pMP18 expressing wild-type IE2 only. (e and f) Double-label images of Vero cells transfected with pMP88 expressing both wild-type IE1 and the in-frame deletion mutant IE2(Δ136–289). (g and h) Double-label images of HF cells transfected with pMP18 expressing wild-type IE2 only. (a, c, e, and g) Detection of IE2 with mouse MAb 12E2 and FITC-labeled anti-mouse IgG. (b, d, f, and h) Detection of PML in the same fields with anti-PML PAb and anti-rabbit IgG. Long arrows indicate PODs containing colocalized IE2 and PML proteins.

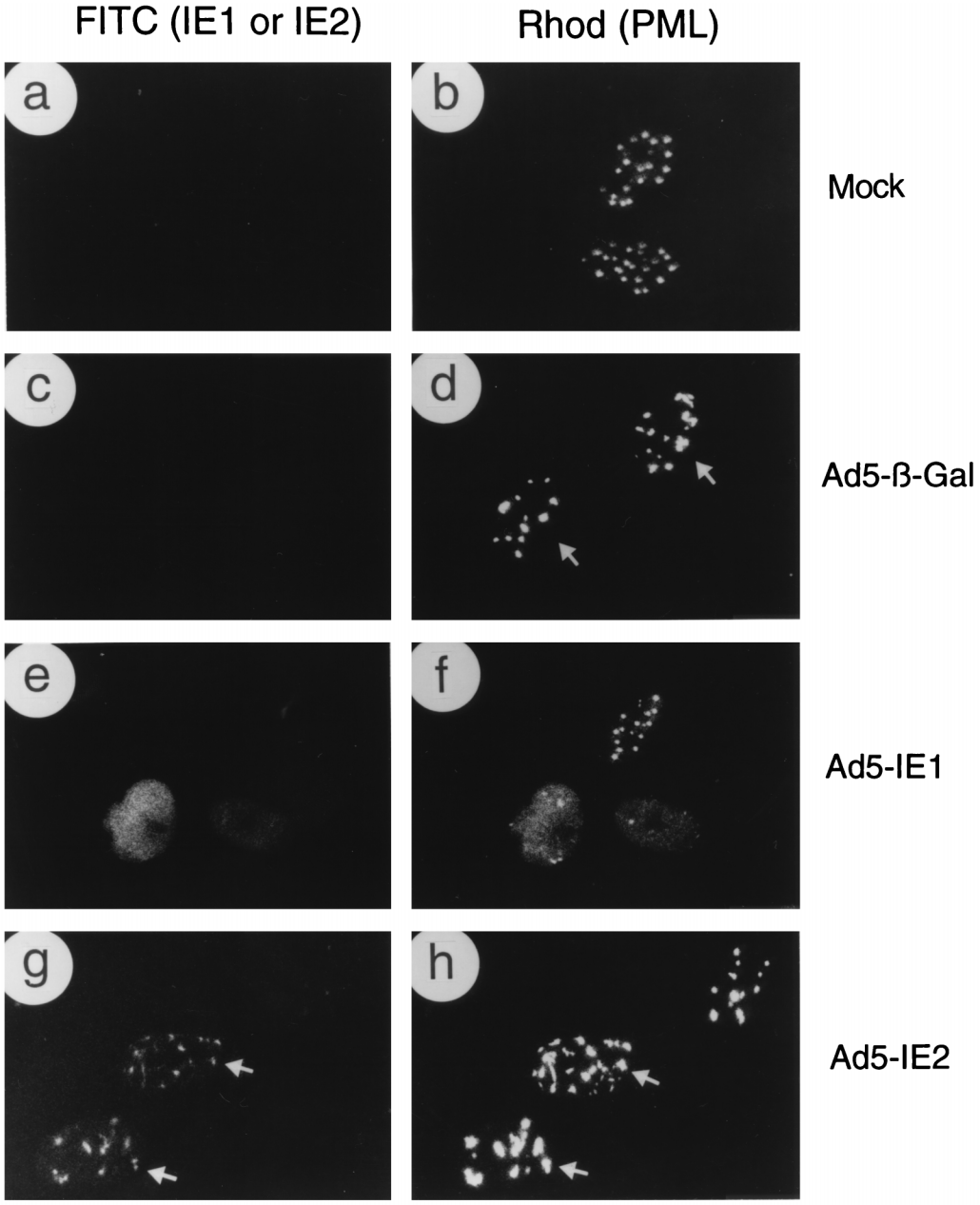


FIG. 9. Infection of HF cells with recombinant Ad vectors expressing isolated IE1 or IE2 cDNAs reveals disruption of PML punctate bodies by IE1 and colocalization of IE2 with nuclear bodies containing PML. The cells were fixed with methanol at 24 h after infection followed by double-label IFA with mouse MAb recognizing either IE1 or IE2 and rabbit PAb against PML. (a and b) Mock-infected HF cells. (c to h) Infection of HF cells with E1A-negative recombinant Ad vectors expressing either β-Gal, IE1, or IE2 at a low MOI (<1.0); (c and d) Ad5-β-Gal; (e and f) Ad5-IE1; (g and h) Ad5-IE2. (a and c) Negative controls for detection of IE1 and IE2 with a mixture of MAbs 6E1 and 12E2 and FITC-labeled anti-mouse IgG; (e) detection of IE1 with MAb 6E1 and FITC-labeled anti-mouse IgG; (g) detection of IE2 with MAb 12E2 and FITC-labeled anti-mouse IgG; (b, d, f, and h) detection of PML in the same fields with rabbit anti-PML PAb and rhodamine-coupled anti-rabbit IgG.

cantly dependent upon any other modifying viral factors or potential virion components. Our findings confirm and extend those of Kelly et al. (28), who first described the displacement of PML from PODs by HCMV infection but did not ascribe these properties to any particular virus-encoded gene products.

In uninfected cells, the distribution of PML in PODs is highly cell cycle dependent, with the typical punctate pattern representing primarily the G_1 phase, whereas the PODs are smaller and more numerous in S-phase cells, fewer in number in G_2, and virtually absent in parts of M phase (30). The size and number of PODs are increased in response to alpha interferon and gamma interferon and by estrogens and heat shock treatment, and they show complex alterations in patterns in proliferating tissues and at different stages of tumor development and metastasis (18, 29, 30, 38). In APL tumor cells, in which translocated PML has become fused to the RARα gene, the colocalization of the hybrid protein shows a direct and reversible correlation with the transformed phenotype. In the tumor cells, PML/RAR is diffuse and dispersed or microspeckled, whereas in the presence of retinoic acid a conformational

change in the PML/RAR protein returns it to the PODs, and both normal growth control and the ability to differentiate are restored (29). Overexpressed PML leads to reduced growth rates in cell lines (30) and also appears to act as a tumor suppressor in assays for focus formation by the *Neu* oncogene (43). The PML protein contains a RING finger class zinc binding motif (43) similar to that found in the HSV IE110 protein, which is believed to represent a protein-protein interaction domain (14). POD-associated PML and HSV IE110 are both tightly bound to the nuclear matrix, and in electron microscopy studies PML appears to decorate the outside of smaller spherical bodies presumably consisting of matrix proteins.

Evidence that perhaps all DNA viruses have proteins that interact with PODs in various ways at very early stages of infection is accumulating. Ad E1A and E4-ORF3 (4) as well as simian virus 40, large-T antigen, and EBNA5 of EBV (55) do so in addition to HSV IE110. In the case of HCMV, the interaction is subtly different than that for HSV, in which IE110 initially transiently colocalizes with PML in the PODs, followed by PML and SP100 becoming dispersed and largely undetectable by IFA, while IE110 persists in smaller and more numerous punctate bodies. RING finger mutants of IE110 still target to the PODs but fail to disperse PML and SP100 (9, 15). In contrast, HCMV IE1 relocalizes PML into the nucleoplasm rather than dispersing it and stably colocalizes with it in a uniform nuclear diffuse pattern, whereas IE2 targets to the POD sites irrespective of whether PML is present and is continuously retained there throughout infection without affecting PML in any obvious way. However, there appears to be an inverse relationship between the level of IE1 expressed and the amount of PML that remains detectable.

The pattern of interaction between IE2 and the PODs appears to involve only partial colocalization in both transfected cells, where much of the IE2 IFA signal was also present in a uniform diffuse pattern, and in infected cells, where not all PML bodies contained IE2 and not all IE2 punctate structures contained PML. In contrast, IE2 expressed from the E1A-negative Ad5 vector displayed a virtually total colocalization with PODs. These differences may be accounted for by the multiple forms of IE2 expressed from the viral genome and from genomic plasmids giving different interaction patterns. Because the Ad5 version of IE2 represents a cDNA form, both the absence of splicing and that of positive virion factors may also account for the surprisingly slow time course of both IE1 and IE2 expression from the Ad5 vectors compared to that with HCMV infection.

Constitutive expression of IE2, like that of all four of the HSV IE nuclear proteins (25), appears to be toxic to cells, but expression of IE1 is compatible with cell survival (32). Furthermore, as in APL cells, the loss of PML from the PODs in stable Vero and U373 cell lines was compatible with both progression of the cell cycle and cell survival. Although we did not detect any punctate IE1 in either the stable IE1-positive cell lines or in transient expression assays, this presumably reflects the very transient nature of the colocalization interaction between newly synthesized IE1 and the PODs as seen at 2 h after HCMV infection or at 6 h after Ad5-IE1 infection. In comparison to this, the short-term transfected cells have presumably completed the displacement of PML when fixed at 48 h. It will also be interesting to determine whether the small percentage of revertant cells in the stable IE1-expressing Vero and U373 cell lines represents an essential but cell-cycle-associated feature in which IE1 expression is lost and PML returns to the PODs in a transient fashion or whether these cells have simply accidentally lost the resident IE1 gene.

Obvious questions arise as to what are the consequences of POD interactions for IE1 and IE2 function and HCMV infection. The highly abundant acidic IE1 protein appears to have little intrinsic transcriptional activity on its own, although it clearly boosts the overall level of IE2 transcriptional activity in situations in which IE2 is only weakly expressed. IE1 has been suggested to have positive autoregulatory effects via NF-κB sites on the MIE promoter/enhancer in some cell types (7), although in other assays it displays generalized negative effects on highly expressed reporter genes (46). Very recently, Mocarski et al. (41) reported that HCMV with IE1 deleted has greatly reduced growth capacity at a low MOI but displays few defects at a high MOI in permissive cell culture. Preliminary mapping of the domains of IE1 that contribute to the POD interaction properties has revealed that the highly acidic C terminus of IE1 is necessary for displacement and localization of both IE1 and PML into a uniform diffuse pattern. Deletion of this region produced large globular or ring-shaped IE1 protein aggregates that appeared to encompass PML-positive POD-like bodies. On the other hand, IE2 is a specific DNA-binding transcriptional transactivator and repressor protein (47, 48) that is expected to be absolutely essential for progression of the lytic cycle under all conditions. However, like HSV IE110, it displays a broad promoter target specificity for transactivation in transient cotransfection assays. A mutant form of IE2 with a deletion within the conserved C-terminal dimerization and DNA binding domain that fails to either transactivate or autoregulate also failed to colocalize with the PODs.

Current ideas about the role of the nuclear matrix-associated PODs include the following general possibilities. (i) They merely represent storage sites or intermediate stages in the nuclear trafficking of a subclass of nuclear proteins. (ii) They are storage sites for a subclass of transcription factors or transcription control factors (such as SP100 perhaps) that when released by removal of PML lead to overall increases in transcriptional activity in the cell. (iii) They represent the normal sites of action of growth suppressor or cell-cycle-regulatory proteins including PML that are inactive when released from the PODs. (iv) They represent a cellular antiviral (or anti-DNA) defense mechanism, which sequesters input viral genomes (or DNA fragments) at the PODs in an inactive form, unless the process is disrupted (23, 37). In the case of HSV, Maul et al. (23, 37) have also suggested that, by sequestering input viral genomes adjacent to the PODs, the latter may become the initial loci at which viral transcription and DNA replication occur. Obviously, much more study will be required to determine which, if any, of these types of mechanisms might apply in the case of HCMV. However, our results clearly strengthen the suspicion that disruption and reorganization of the PODs may represent a helpful or even necessary event at very early stages in the invasion of the host cell by DNA viruses.

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