General blockade of human cytomegalovirus immediate-early mRNA expression in the S/G2 phase by a nuclear, Daxx- and PML-independent mechanism

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The onset of human cytomegalovirus (HCMV) lytic replication is strictly controlled by the host cell division cycle. Although viral entry of S/G2-phase cells is unperturbed expression of major immediate-early (MIE) genes IE1 and IE2 is tightly blocked in these cells. Besides the finding that cyclin-dependent kinase (CDK) activity is required for IE1/IE2 repression little is known about the nature of this cell cycle-dependent block. Here, we show that the block occurs after nuclear entry of viral DNA and prevents the accumulation of IE1/IE2 mRNAs, suggesting an inhibition of transcription. Remarkably, the presence of cis-regulatory regions of the MIE locus is neither sufficient nor necessary for IE1/IE2 repression in the S/G2 phase. Furthermore, the block of viral mRNA expression also affects other immediate-early transcribed regions, i.e. the US3 and UL36–38 gene loci. This suggests a mechanism of repression that acts in a general and not a gene-specific fashion. Such a nuclear, genome-wide repression of HCMV is typically mediated by the intrinsic immune defence at nuclear domain 10 (ND10) structures. However, we found that neither Daxx nor PML, the main players of ND10-based immunity, are required for the block to viral gene expression in the S/G2 phase. In addition, the viral tegument protein pp71 (pUL82), a major antagonist of the intrinsic immunity at pre-immediate-early times of infection, proved to be functional in S-phase cells. This suggests the existence of a yet undiscovered, CDK-dependent mechanism exerting higher-level control over immediate-early mRNA expression in HCMV-infected cells.

INTRODUCTION

Human cytomegalovirus (HCMV, human herpesvirus 5) is a ubiquitous pathogen that is highly adapted to its host and after primary infection enters a latent, asymptomatic state in immunocompetent individuals. Under conditions of immunosuppression, immunodeficiency or immunological naivety, however, lytic replication of HCMV is favoured and can cause fatal disease.

Viral immediate-early (IE) gene expression marks the onset and is a prerequisite for all subsequent steps of the lytic replication cycle. The initiation of IE gene transcription, that by definition is independent of de novo viral gene expression, is facilitated by potent stimulatory elements in the IE promoter regions and by the action of pre-made viral proteins that enter the host cell as part of the infectious virion. The major IE promoter-enhancer (MIEPE) is by far the best-understood cis-acting control region of HCMV and drives transcription of the essential and most abundant IE gene products, IE1 and IE2. Through NF-κB-, AP-1- and GAS-binding sites in the MIEPE, pro-inflammatory signalling induced by the process of virus entry is effectively exploited for kick-starting IE1/IE2 expression (Caposio et al., 2007; Isaacson et al., 2008; Isern et al., 2011; Netterwald et al., 2005). At the same time the viral tegument protein pUL83 (pp65) blocks induction of cellular interferon- and NF-κB-target genes (Abate et al., 2004; Browne & Shenk, 2003) and recruits the interferon-inducible factor IFI16 for further stimulation of MIEPE activity (Cristea et al., 2010). Another tegument factor, pUL82 (pp71), is critical for neutralizing Daxx and ATRX, cellular repressors that in...
concert with PML and other components of the nuclear domains 10 (ND10) are able to silence viral transcription (Tavalin & Stamminger, 2011). In the absence of pp71, a general block of IE gene transcription can occur (Bresnahan & Shenk, 2000) that is assumed to result from Daxx-mediated assembly of repressive chromatin (Woodhall et al., 2006) and to contribute to the establishment of viral latency (Saffert & Kalejta, 2007; Saffert et al., 2010). Thus, both promotor-specific and more broadly acting mechanisms help to overcome the intrinsic cellular immune defence in the pre-IE phase of infection.

Whether lytic replication of HCMV can be successfully initiated also depends on the host cell cycle state at the time of infection. While G0/G1 cells in general support the onset of lytic gene expression, cells cycling through later phases of the cell cycle block the accumulation of major IE (MIE) proteins IE1 and IE2 (Fortunato et al., 2002; Salvant et al., 1998). This negative control relies on cyclin-dependent kinase (CDK) activity as induction of the cellular CDK inhibitor p21WAF1/CIP1 or short-term treatment with pharmacological CDK inhibitors can overcome the blockade of IE1/IE2 expression in S/G2 cells (Zydek et al., 2010). CDK inhibition at the beginning of infection also prevents establishment of a quiescent, latent-like state in undifferentiated NTera-2 cells (Zydek et al., 2010), suggesting that the CDK-dependency of HCMV may play a role in viral latency. Unfortunately, downstream of CDK activity the cell cycle-dependent block of lytic gene expression is largely uncharacterized. It is neither known whether the block acts on a transcriptional or post-transcriptional level, nor has it been reported whether aside from IE1/IE2 other IE genes are inhibited. It is furthermore unclear if the well-documented antiviral defence mechanisms at ND10 are involved and if viral DNA can localize at all to nuclei of S/G2 cells.

Here, we show that the cell cycle-dependent block of HCMV gene expression takes place at the mRNA level and like Daxx-mediated repression (Bresnahan & Shenk, 2000) affects not only the MIE locus but also other genomic regions transcribed at IE times of infection, as the UL36–38 and the US3 locus. However, although viral genomes are nuclear in the S/G2 phase, the block proved to be Daxx and PML independent. This suggests that CDKs can act in parallel to the ND10-based intrinsic immunity to inhibit the onset of HCMV lytic cycle.

RESULTS

The block of MIE expression in the S/G2 phase occurs at the mRNA expression level

Since the initial finding by Salvant et al. (1998) that cycling cells when infected with HCMV in or near the S phase do not support the onset of MIE gene expression this cell cycle-dependent block was solely analysed at IE1 and IE2 protein levels (Fortunato et al., 2002; Wiebusch et al., 2008; Zydek et al., 2010). In order to address the question as to whether the absence of MIE proteins in the S/G2 phase is due to a lack of gene transcription we set out to analyse IE1/IE2 mRNA expression as a function of cell cycle progression. To this end, we used fluorescence activated cell sorting (FACS) to sort proliferating fibroblasts shortly (3 h) after HCMV infection according to their DNA content in to G0/G1, S and G2/M fractions. Owing to the large size of primary human fibroblasts and their tendency to aggregate during the separation process the sorted fractions were not absolutely pure and contained about 15% contaminants from other cell cycle phases (Fig. 1a). Nevertheless, subsequent RNase protection assay (RPA) analysis revealed pronounced differences between IE1/IE2 mRNA levels in the G1 and other cell cycle phases (Fig. 1b). Both IE1 and IE2 mRNAs were only barely detectable in the S and G2/M-phase fractions and the residual amounts of IE1/IE2 mRNAs in these fractions are most probably attributable to the proportion of contaminating G1 cells. In contrast, MCMV-infected cells that are characterized by cell cycle-independent MIE protein expression (Wiebusch et al., 2008) showed only slightly reduced expression of MIE mRNAs in the S/G2/M phase (Fig. 1c, d). Thus, the known virus-specific differences in cell cycle-dependent control of their IE gene expression are reflected by mRNA expression level, suggesting that in S/G2 cells transcription from the HCMV-MIE gene region is subject to negative regulation.

S/G2-dependent IE1/IE2 repression is not mediated by cis-acting elements in the MIE gene locus

As the MIEPE is the main control unit of IE1 and IE2 transcription (Stinski & Isomura, 2008) we asked whether the negative effects on IE1/IE2 mRNA expression in the S/G2 phase are mediated via this potent cis-acting region. If this were the case, the HCMV-MIEPE should confer cell cycle dependency on MCMV when placed in front of the ie1/ie3 coding region. To test this prediction, we made use of two independently created MCMV swap mutants where the MIEPE region of MCMV has been exchanged for HCMV-MIEPE (Angulo et al., 1998; Grzimek et al., 1999).

The fact that human cells support the initiation of MCMV-IE gene expression in a cell cycle-independent manner and, vice versa, the onset of HCMV-IE gene expression is restricted to G1 also in murine fibroblasts (Wiebusch et al., 2008), allowed us to compare the MCMV swap mutants and HCMV in both cellular systems. At 4 h post-infection (p.i.), the MCMV-ie1 protein was indeed well expressed in mouse and human fibroblasts, however, both swap mutants showed the same cell cycle-independent ie1 expression pattern as MCMV wild-type (WT) and the parental recombinant viruses (Fig. 2a). This was even the case when we co-infected cells with MCMV swap mutants and HCMV to provide all the trans-acting factors present at pre-IE times during the infection with HCMV. Thus, the HCMV-MIEPE neither alone nor in combination with incoming HCMV virion proteins can exert cell cycle-dependent repression on viral gene expression. This suggests that the block of MIE
expression in the S/G2 phase is not mediated by promoter/enhancer-specific elements. Furthermore, an analysis of IE1/IE2 expression after HCMV/MCMV-coinfection revealed that MCMV cannot rescue the defective HCMV transcription in the S/G2 phase (Fig. 2b). This was also not the case when MCMV-IE gene expression was prevented by UV-irradiation and clearly argues against a model where MCMV but not HCMV has evolved a virion factor counteracting an S/G2-specific antiviral activity (see Discussion).

Upstream of the MIEPE further cis-regulatory regions are located, namely the ‘unique region’ (UR) and the modulator (Fig. 3a). Both were reported to contain binding sites for transcriptional repressors (Bain et al., 2003; Huang et al., 1996; Lashmit et al., 2004) and may therefore be required for the S/G2-specific block of IE1/IE2 transcription. To test this possibility we constructed HCMV mutants where either the modulator alone or together with the UR and parts of the 5’-flanking UL128 gene locus was deleted and substituted by a kanamycin-resistance cassette (Fig. 3a). As expected from published data (Meier & Stinski, 1997), the mutant viruses (AD169-DMod, AD169-DMod/UL128 and AD169-DMod/UL128/UR) maintained their ability to initiate MIE expression (Fig. 3b) and to replicate to high titres (data not shown). Importantly, the characteristic S/G2-dependent block of IE1/IE2 expression was still preserved and occurred with similar efficiency in mutant and parental virus infections (Fig. 3b). This argues against a specific role of the modulator and adjacent regions in the cell cycle-dependent regulation of MIE gene expression.

We next wanted to rule out that instead of the 5’-flanking UTR sequence elements within the UL122/123 gene body or the 3’-UTR are needed for suppression of IE1 and IE2 gene expression in S/G2. We therefore subcloned the whole MIE region including 4 kb of 3’-UTR (Fig. 3c) via homologous recombination into pcDNA3 and introduced the recombined plasmid into proliferating cells by electroporation. The Nucleofector technology we used for this purpose led to rapid induction of transgene expression allowing detection of MIE gene products as early as 3 h post-transfection. Intriguingly, in this setting IE1/IE2 expression was initiated in a completely cell cycle-independent manner (Fig. 3d). G0/G1 and S/G2 cell cycle compartments were almost equal for both, percentage of MIE-positive cells and average MIE protein level. This was in clear contrast to virus-infected cells and demonstrates that the MIE locus per se, i.e. uncoupled from the context of viral infection, acts as a strong cell cycle-independent transcription unit. Taken together, our data make it rather unlikely that cis-acting sequence elements exist within the MIE region that are responsible for the cell cycle dependency of IE1/IE2 expression in the initial phase of infection.

**General repression of HCMV-IE genes in the S/G2 phase**

The fact that during virus infection the inherent cell cycle-independent nature of the MIE gene region is counteracted...
by a dominant S/G2-specific block raised the question as to whether other IE genes were also subject to transcriptional repression. To address this point we extended the analysis of viral RNAs from sorted cell cycle fractions (see Fig. 1) to transcripts of the US3 and the UL36–38 locus, two other well-established sites of IE gene expression (Colberg-Poley, 1996; Wathen & Stinski, 1982). At 3 h p.i., US3, UL36 and UL37 mRNAs were like IE1 mRNA well detectable in G1 and below the detection limit in S-phase cells (Fig. 4). Except for UL37, which was only moderately affected, the same level of repression was also observed in G2 cells. Thus, the cell cycle-dependent block of lytic gene expression

![Fig. 2. The HCMV MIE enhancer does not confer cell cycle dependency on the MIE locus of MCMV. (a) Proliferating fibroblasts of human and mouse origin were mock-infected or infected with the indicated MCMV variants (see Table). This was done as a single infection (upper rows) or as co-infection with HCMV (lower rows). Cells were harvested 4 h p.i. and analysed for cell cycle distribution and MIE gene expression by flow cytometry. For detection of MCMV gene expression, an ie1-specific antibody was used. An antibody recognizing the common N terminus of IE1 and IE2 was used in the case of HCMV. Shown are dot plots (n=haploid number of chromosomes), where cells were divided into four subpopulations: G0/G1-MIE- (lower left quadrant), G0/G1-MIE+ (upper left quadrant), S/G2/M-MIE- (upper right quadrant) and S/G2/M-MIE+ (lower right quadrant). The proportion of each subpopulation is given as a per cent of total cells. (b) Proliferating human fibroblasts were infected with HCMV and co-infected with normal MCMV or with MCMV that was UV-irradiated before infection (MCMVUV). Cells were harvested at 4 h p.i. and analysed as described in (a).]
Fig. 3. The S/G2-specific block of HCMV MIE gene expression is not mediated by cis-regulatory sequences but requires the context of viral infection. (a) Schematic representation of the upstream control region of the MIE locus (UR, unique region; Ex1, exon 1 of the IE1/IE2 transcript and +1, transcription start site) in the genomes of HCMV-AD169 WT and of the indicated AD169 derivatives. The sections that were deleted by BAC recombineering and substituted by a kanamycin-resistance cassette are indicated by dashed lines. (b) Proliferating HEL fibroblasts were infected with the reconstituted viruses as indicated and analysed at 3 h p.i. for IE1/IE2 gene expression and cell cycle distribution as described in the legend to Fig. 2. (c) The depicted subgenomic fragment of HCMV ('SubMIE'), ranging from nt −1338 to +8409 relative to the start site of MIE gene transcription, was cloned into the plasmid backbone of pcDNA3. (d) The resulting plasmid pSubMIE and pcDNA3 as an empty vector control were transfected into proliferating HEL fibroblasts by nucleofection. Cells were harvested 3 h post-transfection and analysed for IE1/IE2 expression and DNA content by flow cytometry.
is not restricted to the MIE locus but pertains to other known IE genes of HCMV, pointing towards a general rather than a gene-specific mechanism of repression.

**Nuclear localization of viral DNA and tegument proteins is not compromised in HCMV-infected S-phase cells**

Impaired nuclear entry of viral DNA or tegument proteins could be possible reasons for such a general block of de novo viral gene expression. For instance, inefficient translocation of incoming viral genomes to the nucleus of infected cells was shown to be responsible for the inability of fibroblast-adapted HCMV strains to initiate IE gene expression in endothelial and monocytic cells (Sinzer et al., 2000; Straschewski et al., 2011). And cytoplasmic localization of pUL82 (pp71) contributes to the block of HCMV-IE gene expression in haematopoietic progenitor and undifferentiated NT2 cells (Saffert & Kalejta, 2007; Saffert et al., 2010). This led us to analyse the subcellular localization of viral DNA and selected tegument factors in S/G2 cells.

To facilitate detection of HCMV genomes we used the BrdU-labelling technique (Rosenke & Fortunato, 2004). First, we ensured that BrdU incorporation has no influence on the cell cycle-dependent onset of MIE gene expression after de novo infection (Fig. 5a). We then analysed whether cells that were IE-positive at 3 h p.i. (90% of them residing in the G1 phase – see Fig. 5a) differed from the remaining fraction of IE-negative cells (70% of them in the S/G2 phase) in their ability to support nuclear entry of viral DNA. Confocal imaging permitted a clear distinction between nuclear and cytoplasmic viral DNA (Fig. 5b). By scanning several hundred cells we found that the number of IE-negative cells with nuclear-localized HCMV genomes almost reaches that of IE-positive cells (Fig. 5c). This suggests that the process of nuclear entry as well as the preceding steps of virus penetration, cytoplasmic transport and uncoating are not affected by the cell cycle and therefore cannot account for the general blockade of IE gene expression in the S/G2 phase.

We then proceeded to examine the localization of pp65 and pp71, the best investigated antagonists of intrinsic immune responses during the pre-IE phase of infection (Abate et al., 2004; Browne & Shenk, 2003; Cantrell & Bresnahan, 2006; Cristea et al., 2010; Lukashchuk et al., 2008; Saffert & Kalejta, 2006; Tavalai & Stamminger, 2011). This time, S-phase cells were distinguished from non-S-phase cells by 5-ethynyl-2'-deoxyuridine (EdU) labelling. At the end of the absorption period (1 h p.i.) both EdU-positive and -negative cells showed a clear and specific nuclear staining of pp65 and pp71 (Fig. 6a). This demonstrates that nuclear translocation of tegument proteins is not disturbed in the S phase. The detection of nuclear pp65 in S-phase cells was also consistent with previously published evidence (Arcangeletti et al., 2011; Salvant et al., 1998). Interestingly, the report by Arcangeletti et al. (2011) contained further data showing a reduced nucleolar accumulation of pp65 in S/G2 cells. As the same authors had suggested that nucleolar targeting of pp65 might be important for the regulation of IE gene expression (Arcangeletti et al., 2009), we decided to test whether the cell cycle-dependent pattern of IE gene expression is altered in the absence of pp65. However, when comparing AD169 WT virus with a pp65-deleted derivative (Schmolke et al., 1995) we were unable to observe any difference in cell cycle-dependent repression (Fig. 6b). This finding argues against a role for pp65 in the S/G2-specific block of HCMV gene expression.

We also considered it to be important to take a closer look at pp71 because the Daxx protein, which is required for correct subnuclear localization and function of pp71 (Hofmann et al., 2002; Ishov et al., 2002), was reported to relocalize during the S phase from ND10 structures to heterochromatin (Ishov et al., 2004). Consequently, we employed confocal microscopy to analyse whether in HCMV-infected S-phase cells pp71 can still accumulate at ND10 and exert one of its earliest functions, the displacement of the Daxx-interacting protein ATRX from

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**Fig. 4.** The S/G2-specific block of HCMV gene expression is not restricted to the MIE region. HCMV-infected HEL fibroblasts were FACS-sorted into G0/G1, S and G2/M populations as described in the legend to Fig. 1. The mRNA expression levels of the indicated IE genes were analysed by RPA as described in the legend to Fig. 1. RNA of non-infected fibroblasts (non-inf.) was used to control the viral origin of RPA signals.
We again compared EdU-positive and -negative cells and in both we observed at 2 h p.i. a clear focal co-localization of pp71 and PML, the most prominent ND10 marker protein (Fig. 7a). At the same time, co-localization of ATRX with PML was disturbed by the virus regardless of cell cycle position (Fig. 7b). This suggests that pp71 localization is not a target of cell cycle-dependent regulation and that pp71 functions properly in S/G2 cells.

Cell cycle-dependent repression of HCMV lytic gene expression is PML and Daxx independent

Both, PML and Daxx independently contribute to the intrinsic antiviral defence that is orchestrated at ND10 structures (Tavalai et al., 2008) and in the absence of pp71 leads to a global repression of IE genes at low input m.o.i. (Bresnahan & Shenk, 2000). As the CDK-dependent block in the S/G2 phase similarly appears to target IE gene transcription in a general and not gene-specific fashion (see above), we asked whether both mechanisms can be genetically separated. To address this question we took advantage of previously published fibroblasts with stable shRNA-mediated Daxx and PML knock-down (Tavalai et al., 2008). The knock-down was very efficient and in the case of PML pertained to all PML isoforms (Fig. 8a). Using high m.o.i. infections – as in all experiments – both PML and Daxx knock-down resulted, compared with control cells, only in a slight increase of MIE-positive cells (Fig. 8b). This effect was cell cycle independent as it applied to all cell cycle phases. The large majority of S/G2 cells was still inhibiting IE gene expression, suggesting that the Daxx/PML-dependent intrinsic immune defence is not involved in the cell cycle-dependent blockade of the HCMV lytic cycle. This was further supported by the observation that histone deacetylase (HDAC) activity known to exert Daxx-dependent repression at the level of chromatin structure (Hollenbach et al., 2002; Woodhall et al., 2006) was not required for the S/G2-specific repression of viral genes (Fig. 8c).
**Fig. 6.** The S/G2-specific block of IE gene expression is pp65 independent. (a) Proliferating HEL fibroblasts were infected with HCMV-AD169 or left uninfected. During the period of virus absorption (1 h), the cell culture medium was supplemented with EdU to label S-phase cells. At 2 h p.i., cells were analysed for EdU incorporation as well as subcellular localization of pp65 and pp71 by fluorescence microscopy. Nuclei were counterstained by DAPI. Merged images of EdU and pp65/pp71 staining are shown in the lowest row. (b) Proliferating HEL fibroblasts were infected with WT or a pp65-negative recombinant (RVAd65) AD169 virus. At 4 h p.i., cells were harvested and analysed for cell cycle distribution and MIE expression by flow cytometry (dot blots on the left). In addition, pp65 and GAPDH (loading control) expression was determined by immunoblot analysis (right-hand panel).
DISCUSSION

It is remarkable that the strong MIEPE of HCMV that is used as a constitutively active, cell cycle-independent promoter unit in many eukaryotic expression vectors can so efficiently be dominated in the context of lytic infection by repressive activities. Several lines of evidence suggest that the cell cycle-dependent repression is not promoter-specific but acts in a general, possibly genome-wide manner. First, when swapped into MCMV the MIEPE does not confer cell cycle dependency on ie1, even in HCMV co-infection experiments (Fig. 2a). Second, further upstream regulatory regions of the

Fig. 7. Recruitment of pp71 to nuclear domain-10 (ND10) and displacement of ATRX are cell cycle-independent. Proliferating fibroblasts were HCMV-infected and labelled with EdU as described in the legend to Fig. 6. At 2 h p.i., cells were analysed for EdU incorporation and subcellular localization of pp71 (a), PML (a, b) and ATRX (b) by confocal fluorescence microscopy. To control for protein co-localization merged images of pp71 and PML immunofluorescence were created and the z dimension was analysed as depicted.
MIE locus including the modulator are dispensable for repression in the S/G2 phase (Fig. 3b). And third, distant gene loci (US3 and UL36–38) with IE characteristics are repressed with similar efficiency as the MIE locus (UL122–123) – see Fig. 4. This of course raises the question of how such general repression can be achieved. It is known that HCMV DNA becomes histone-associated shortly after infection (Nitzsche et al., 2008) and both negative- and positive-regulation of IE transcription is mediated by structural changes at the chromatin level (Sinclair, 2010). Given the intranuclear localization of viral genomes in the S/G2 phase (Fig. 5), CDK-dependent formation of repressive HCMV chromatin would represent a plausible explanation for the observed general block of IE gene expression. However, our finding that this block works independent of HDAC activity (Fig. 8c) excludes a central mechanism of histone-mediated gene repression, which has been shown to contribute to the silencing of lytic transcription during quiescent and latent HCMV infection (Murphy et al., 2002; Saffert et al., 2010).

To test a possible role of HDAC-independent chromatin modifications more thoroughly by chromatin analysis of homogeneous, HCMV-infected S/G2 populations will be technically demanding. Primary fibroblasts, the preferred cell type for this analysis, do not accumulate to more than 70% in the S/G2 phase when using gentle synchronization procedures such as replating of contact-inhibited or restimulation of serum-starved cells. Other protocols using for example chemical inhibitors of DNA replication are not suitable because stress pathways that are induced affect the CDK-dependent control of IE gene expression (Zydek et al., 2010). Purification of S/G2 fractions by FACS as done in this paper on a smaller scale for the analysis of viral RNAs (see Fig. 1), cannot produce the required quantities of high quality cell fractionations needed for chromatin precipitation experiments. Thus, the main focus when studying the mechanism of the cell cycle-dependent control of HCMV relies on the identification of the CDK substrate responsible for the repressive effects on lytic gene expression (Zydek et al., 2010).

Disregarding the mechanism of IE gene repression, in this work we were able to rule out the functional importance of a well-recognized physiological pathway to restrict viral gene expression programmes. HCMV, as many other viruses, has to deal with the intrinsic nuclear defence

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**Fig. 8.** The S/G2-specific block of IE gene expression is independent of PML, Daxx and HDAC activity. (a, b) Proliferating HF fibroblasts expressing either Daxx-specific (shDaxx1), PML-specific (siPML2), functionally inactive (siC) or no exogenous (vector) shRNA were infected with HCMV-AD169 or left uninfected. (a) Uninfected cells were analysed for Daxx and PML knock-down efficiency by immunoblot analysis. The expression of β-actin was determined to control for equal loading. (b) At 4 h p.i., cells were analysed for DNA content and IE1/IE2 gene expression by flow cytometry as described in the legend to Fig. 2. (c) Proliferating HEL fibroblasts were infected with HCMV-AD169. Where indicated, CDK inhibitors (10 μM SU9516 and 50 μM Roscovitine) or HDACs inhibitors (10 mM sodium butyrate and 100 ng Trichostatin A ml⁻¹) were added to the cells 30 min before infection. CDK inhibitors were removed at 2 h p.i., HDAC inhibitors were left on the cells until harvest at 5 h p.i. Cells were analysed for DNA content and IE1/IE2 expression as described above.
that is organized at ND10 domains by Daxx, ATRX, PML and other proteins, acting towards a global silencing of incoming viral genomes (Everett & Chelbi-Alix, 2007; Tavalai & Stamminger, 2008). However, we provide evidence that this pathway is not responsible for the cell cycle restricted expression of viral IE genes. Rather the cell cycle exerts an additional level of nuclear control in the pre-IE phase of an HCMV infection, which is also characterized by a general suppression of viral mRNA expression but is independent of known ND10-based antiviral mechanisms. The latter conclusion follows from several lines of evidence: (i) the major ND10 components Daxx and PML are dispensable for repression of HCMV in the S/G2 phase (Fig. 8b); (ii) a role for ATRX appears unlikely as the antiviral activity of this chromatin remodelling factor depends on its Daxx-mediated recruitment to ND10 (Ishov et al., 2004; Lukashchuk et al., 2008); (iii) the Daxx/ATRX-antagonist pp71 localizes normally in S-phase cells (Fig. 7a) and is not retained to the cytoplasm as in quiescently or latently infected cells (Saffert & Kalejta, 2007; Saffert et al., 2010); (iv) moreover, pp71 seems to be functional in infected S-phase cells as ATRX was found dislocated from ND10 in a virus-dependent manner (Fig. 7b). Finally, an ND10-independent mechanism of the block in the S/G2 phase is consistent with our recent finding that CDK inhibition rescues IE gene expression after quiescent infection without changing the cyttoplasmic localization of pp71 (Zydek et al., 2010). Interestingly, a partial redistribution of Daxx from ND10 domains to heterochromatin was reported to occur in S/G2 cells (Ishov et al., 2004). Considering the antiviral function of Daxx at ND10 in the pre-IE phase of infection (Cantrell & Bresnahan, 2006; Saffert & Kalejta, 2006; Tavalai et al., 2008; Woodhall et al., 2006) this redistribution might weaken the ND10-based defence and could be a further argument for an ND10-independent pathway of IE gene repression in the S/G2 phase.

A notable difference between the ND10- and the CDK-dependent pathways restricting HCMV gene expression is that the intrinsic immunity can rapidly be overcome in lytically infected cells by numerous viral countermeasures. In contrast, the CDK-dependent block in the S/G2 phase is fully in place until completion of cell division and hence causes a delay of HCMV replication of up to 20 h (Fortunato et al., 2002; Zydek et al., 2010). Also, this block withstands even high m.o.i., whereas altering the balance between the ND10-based defence and its viral antagonists under lytic conditions becomes apparent only at low m.o.i. as shown by the m.o.i.-dependent growth of IE1 (the PML-antagonist of HCMV) and pp71-deficient viruses (Bresnahan & Shenk, 2000; Mocarski et al., 1996). What can account for the high viral sensitivity to the blockade of IE gene expression in S/G2? Two different concepts are conceivable: either HCMV is lacking counteracting factors against an existing CDK-dependent antiviral activity of the host cell. However, since HCMV has been co-evolved with its host and the blockade cannot be relieved by co-infection with a close, cell cycle-independent relative of HCMV, i.e. MCMV (Fig. 2b), this option appears somewhat unlikely. Alternatively, HCMV has specifically developed mechanisms making it susceptible to a strictly negative regulation by a CDK-dependent activity in S/G2 cells. Possibly, because from a viral viewpoint repression in the S/G2 phase is desirable or in other words linking HCMV gene expression to distinct cell cycle phases may be advantageous for (certain aspects of) viral physiology. Support for this view comes from studies showing that – once lytic gene expression in G1 has started – HCMV gene products IE2 and pUL117 block entry into the S phase by interfering with cellular DNA synthesis (Wiebusch & Hagemeier, 2001; Qian et al., 2010) and that a loss of these functions impairs the efficiency of virus replication (Petrík et al., 2006; Qian et al., 2008).

METHODS

Cells. Human embryonic lung (HEL) fibroblasts (Fi301, obtained from the Institute of Virology, Charité, Berlin, Germany) and 3T3 mouse fibroblasts were maintained as described previously (Wiebusch et al., 2008; Zydek et al., 2010). Human foreskin (HF) fibroblasts with stable knock-down of PML (siPML2 cells) or Daxx (siDaxx1 cells) as well as control fibroblasts stably transduced with a non-functional half-side shRNA sequence (siC cells) or the empty shRNA expression vector (vector cells) were generated and cultured as described previously (Tavalai et al., 2008). Where indicated, cells were incubated for 60 min with 10 mM EdU (Invitrogen) prior to infection to label S-phase cells. Non-incorporated EdU was removed by several washes with normal growth medium.

Viruses. HCMV strain AD169 was obtained from ATCC. RVAd65, a UL83-deleted AD169 derivative (Schmolke et al., 1995), was obtained from Bodo Plachter (University of Mainz, Germany). MCMV swap mutants mCMVhMIEPE (Grzimek et al., 1999) and hMCMV-ES (Angulo et al., 1998) and the corresponding parental viruses (Fig. 2a) were kindly provided by Natasha Grzimek (University of Mainz, Germany) and Ana Angulo (IDIBAPS, Barcelona, Spain), respectively. Viruses were grown on HEL (HCMV) or 3T3 (MCMV) fibroblasts according to standard procedures. Virus titres of the infectious cell culture supernatants were determined by IE1/IE2 immunofluorescence analysis as described previously (Zydek et al., 2010). Unless otherwise stated an m.o.i. of 10 IE protein forming units per cell was used for infection experiments. Where indicated, MCMV was exposed to 1000 J/m2 UV-C radiation prior to infection (Wiebusch et al., 2008).

Mutagenesis of HCMV and subcloning of the MIE locus. HCMV deletion mutants were generated in Escherichia coli DY380 by bacterial artificial chromosome (BAC) recombineering (Lee et al., 2001). pHB15, derived from HCMV-AD169, was used as the parental BAC (Hobom et al., 2000). First, a kanamycin-resistance gene was PCR amplified from the plasmid pSLFRTKn (Atalay et al., 2002) using primers containing 50 nt overhangs, identical to the flanking sequences of the genomic region to be deleted (see Supplementary Table S1, available in JGV Online). This recombination cassette was then electroporated into pHB15 containing DY380 with prior induction of λ-Red recombinases by heat shock. Thereafter, bacteria were selected on LB agar plates containing 12.5 µg chloramphenicol ml-1 and 25 µg kanamycin ml-1. Positive clones were identified by PCR analysis and EcoRI digestion. To reconstitute recombinant viruses HEL fibroblasts were transfected with the purified bacmid DNA, the pp71 expression plasmid pcDNA-pp71-flag (provided by Bodo Plachter) and the CRE
expression plasmid pBRep-CRE (provided by Wolfram Brune, HPI, Hamburg, Germany). Exgen-500 (Thermo Fisher-Fermentas) was used as the transfection reagent following the manufacturer’s instructions.

The MIE locus of HCMV (see Fig. 3c) was cloned by homologous recombination into pcDNA3 (Agilent-Stratagene), thereby eliminating the CMV expression cassette of pcDNA3. PCR primers were designed in a way that the amplified recombination cassette contained the bacterial origin of replication and the ampicillin expression unit of pcDNA3 as well as 5'– and 3'–termini identical to the borders of the MIE locus (see Supplementary Table S1 for primer sequences). Positive clones were selected by ampicillin resistance.

**Nucleofection.** An Amaxa Nucleofector (Lonza) was employed according to the manufacturer’s basic nucleofection protocol for primary mammalian fibroblasts. Plasmid (1 μg) was used to transfect 5 × 10⁵ cells applying the Nucleofector program U-23.

See Supplementary Methods (available in JGV Online) for detailed methodology of FACS, RPA, flow cytometry, fluorescence microscopy, subcellular localization of viral genomes and immunoblot analysis.

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