Review

Viral and cellular subnuclear structures in human cytomegalovirus-infected cells
Blair L. Strang

In human cytomegalovirus (HCMV)-infected cells, a dramatic remodelling of the nuclear architecture is linked to the creation, utilization and manipulation of subnuclear structures. This review outlines the involvement of several viral and cellular subnuclear structures in areas of HCMV replication and virus–host interaction that include viral transcription, viral DNA synthesis and the production of DNA-filled viral capsids. The structures discussed include those that promote or impede HCMV replication (such as viral replication compartments and promyelocytic leukaemia nuclear bodies, respectively) and those whose role in the infected cell is unclear (for example, nucleoli and nuclear speckles). Viral and cellular proteins associated with subnuclear structures are also discussed. The data reviewed here highlight advances in our understanding of HCMV biology and emphasize the complexity of HCMV replication and virus–host interactions in the nucleus.

Introduction

The betaherpesvirus human cytomegalovirus (HCMV) is a widespread opportunistic pathogen that severely affects immunocompromised and immunodeficient populations (Mocarski et al., 2007). Like all herpesviruses, HCMV undergoes either productive or latent infection. During productive infection, infectious virus is produced, while in latent infection the virus becomes largely transcriptionally quiescent (Mocarski et al., 2007). Like other herpesviruses, many events that are critical for productive HCMV replication take place within the nucleus. These include essential steps in viral replication, such as: the transcriptional cascade of immediate-early (IE), early and late viral RNA transcripts, synthesis of viral DNA and the production of DNA-containing capsids (Mocarski et al., 2007). Compared with the prototype human herpesvirus, the alphaherpesvirus herpes simplex virus (HSV), certain features of productive HCMV infection are not well characterized. However, it is clear that several subnuclear structures are involved in HCMV genome replication and virus–host interactions. Uncovering the roles of these structures has led to significant advances in our understanding of HCMV biology. This review summarizes the involvement of several viral and cellular subnuclear structures in productive HCMV infection. The participation of each structure in HCMV replication and virus–host interactions is described from entry of the viral genome into the nucleus to the egress of viral capsids through the nuclear membrane. The data discussed highlight recent advances in our understanding of subnuclear structure function, introduce viral and cellular factors associated with subnuclear structures and indicate what questions have yet to be answered.

Entry of the HCMV genome into the nucleus: the nuclear pore complex, nuclear speckles, promyelocytic leukaemia protein nuclear bodies and pp150 bodies

HCMV genome entry into the nucleus is poorly defined but may be similar to movement of the HSV DNA genome through the nuclear pore complex (NPC) (Kobiler et al., 2012) (Fig. 1a). HCMV capsid and tegument proteins (potentially HCMV UL48 and UL77, homologues of HSV UL36 and UL25, respectively) probably interact with proteins on the cytoplasmic face of the NPC (potentially including Nup214, Nup358 and hCG1) (Table 1). This leads to opening of the capsid. The pressure of viral DNA on the capsid wall leads to release of a linear DNA form of the HCMV genome. Viral DNA travels out of the capsid via a portal on a unique vertex of the capsid comprised of UL104 (HSV UL6 homologue) (Kobiler et al., 2012). The HCMV genome moves through the central channel of the NPC into the nucleus (Kobiler et al., 2012) (Fig. 1a).

The linear HCMV DNA genome circularizes upon entry into the nucleus via fusion of the genomic termini (McVoy & Adler, 1994) (Fig. 1b). This could be mediated by either homologous or non-homologous end-joining DNA repair mechanisms. The circular form of the genome is utilized as a template for viral RNA transcription and DNA synthesis. Very early in infection, viral genomes can be found at the periphery of the nucleus where transcription from the viral genome takes place (Ishov et al., 1997). At the periphery of the nucleus, the major viral IE transcriptional transactivator IE2 co-localizes with cellular RNA polymerase II transcription factors TATA-binding protein (TBP) and
transcription factor IIB (TFIIB) (Ishov et al., 1997) (Fig. 1b). It is possible that, like HSV RNA transcription, HCMV RNA transcription involves the nuclear lamina. The nuclear lamina is a dense fibrillar protein network at the nuclear membrane composed of lamin proteins and lamin-associated proteins (Fig. 1a). In HSV-infected cells, the lamina component lamin A/C is important for genome targeting to the lamina, and at the lamina hetero- or euchromatin on viral promoters modulates HSV RNA transcription (Silva et al., 2008).

The majority of HCMV genomes localize with promyelocytic leukaemia protein nuclear bodies (PML-NBs, also referred to as ND10 bodies) and SC35-containing structures within intrachromosomal space. IE2 bridges PML-NBs and SC35-containing structures (Ishov et al., 1997) (Fig. 1b). As transcription progresses, viral IE RNA transcripts co-localize with SC35-containing bodies but not PML-NBs (Ishov et al., 1997). SC35 is a major protein component of nuclear speckles, which are dynamic nuclear structures composed of RNA and protein and enriched in pre-mRNA splicing factors (Lamond & Spector, 2003). A role for nuclear speckles in HCMV replication, or that of any other human virus, is not often commented on. It is likely that nuclear speckles are involved in HCMV IE gene transcription, but it is unknown what transcriptional events they might facilitate. In HSV-infected cells, it has been reported that nuclear speckles are involved in export of viral RNA transcripts (Chang et al., 2011). Thus, nuclear speckles may serve in the processing and export of HCMV RNA transcripts early in infection. These RNAs probably include the viral RNAs transcribed from the UL122–123 locus of the HCMV genome that are multiply spliced to produce mRNA transcripts encoding the IE proteins IE1 and IE2.

The role of PML-NBs in HCMV replication is one of the most studied and debated facets of HCMV biology. The complex relationship between PML-NBs and HCMV cannot be fully summarized here but is discussed in detail elsewhere (Everett et al., 2013; Tavalai & Stamminger, 2009, 2011). PML-NBs are dynamic structures that comprise at least 70 constitutively or transiently present proteins, including PML protein (which acts a scaffold for PML-NB formation) (Ishov et al., 1999). The majority of, if not all, PML-NB proteins are post-translationally modified with the addition of small ubiquitin-related modifier (SUMO) moieties. PML-NBs contain several proteins required for protein SUMOylation and the relationship between protein function and post-translational modification by SUMO is a rapidly developing topic in HCMV biology and elsewhere (Everett et al., 2013).

PML-NBs are found throughout the nucleus. Upon entry of the viral genome into the nucleus, PML-NBs form on SUMO-modified PML proteins juxtaposed to incoming viral genomes. PML-NBs act to repress viral transcription via the co-operative function of the PML-NB proteins PML, Sp100 and hDaxx (Glass & Everett, 2013). PML protein is one of the best characterized PML-NB proteins involved in HCMV transcriptional repression and acts to facilitate assembly of PML-NBs containing transcriptionally repressive proteins at HCMV genomes (Tavalai & Stamminger, 2009, 2011). Sp100 promotes transcriptional repression through its interaction with the transcriptional repressor heterochromatin protein (HP1) (Seeler et al., 1998). SUMO modification of Sp100 promotes interaction with HP1 (Seeler et al., 2001) but is not required for localization to PML-NBs (Sternsdorf et al., 1999). hDaxx is recruited to PML-NBs via an interaction with SUMOylated PML (Everett et al., 2013) and acts as a transcriptional co-repressor via its interaction with the chromatin remodeller ATRX (Ishov et al., 2004; Xue et al., 2003). PML-NBs contain other transcriptionally repressive cellular proteins (Tavalai & Stamminger, 2011), and it is possible other PML-NB proteins may antagonize HCMV replication.

Crucially, HCMV encodes antagonists of PML-NB function (Table 1) whose actions lead to removal of proteins from PML-NBs and the dispersal of PML-NBs (Everett et al., 2013; Tavalai & Stamminger, 2009, 2011).
<table>
<thead>
<tr>
<th>Nuclear structure</th>
<th>HCMV associated proteins</th>
<th>Kinetic class of HCMV protein*</th>
<th>HCMV protein function†</th>
<th>Requirement for HCMV replication‡</th>
<th>Known or proposed cellular partner(s)</th>
<th>References</th>
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<tr>
<td>Nuclear pore</td>
<td>UL48 L</td>
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<td>Nup214, Nup358 hCG1</td>
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<td>Nup214, Nup358 hCG1</td>
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<td></td>
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<td></td>
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<td>Kalejta &amp; Shenk (2003), Lukashchuk et al. (2008)</td>
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<td>SC35</td>
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<td>A2-CDK</td>
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<td>E</td>
<td>Nucleolin</td>
<td>Ahn et al. (1999), Penfold &amp; Mocarski (1997), Strang et al. (2012b)</td>
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<td>Penfold &amp; Mocarski (1997)</td>
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<td>UL84 E–L</td>
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<td>E</td>
<td>hnRNP K</td>
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<td></td>
<td>TRL9 L</td>
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<tr>
<td></td>
<td>UL29 L</td>
<td>Temperance factor</td>
<td>A</td>
<td>Unknown</td>
<td>Salsman et al. (2008)</td>
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</table>

HCMV and subnuclear structures
In HCMV-infected cells, two major antagonists of PML-NB function are pp71 and IE1. pp71 degrades hDaxx by a proteasome-dependent, ubiquitin-independent mechanism (Kalejta & Shenk, 2003) and displaces ATRX from PML-NBs (Lukashchuk et al., 2008). The presence of IE1 promotes the disruption of PML-NBs, induces the proteasome-independent loss of SUMOylated PML and Sp100 proteins (Ahn & Hayward, 1997; Kang et al., 2006; Kim et al., 2011; Korioth et al., 1996; Lee et al., 2004; Tavalai et al., 2011; Wilkinson et al., 1998; Xu et al., 2001) and potentially induces the proteasome-dependent degradation of unSUMOylated Sp100 late in infection (Tavalai et al., 2011). Importantly, IE1 does not act like its well-studied counterpart expressed by HSV, ICP0. ICP0 possesses an E3 ubiquitin ligase activity directly involved in the proteasome-dependent degradation of PML protein (Boutell & Everett, 2013). IE1 has no known ubiquitin ligase or SUMOylase activity (Kang et al., 2006), but is able to exert an effect that leads to PML-NB disruption, loss of SUMOylated PML and an increase in the abundance of unmodified PML. This would suggest that IE1 can recruit a cellular protein to deSUMOylate PML within PML-NBs or SUMO is removed from PML by a cellular deSUMOylase after PML-NBs have been dispersed in the presence of IE1. The role of IE1 in Sp100 degradation is unclear. IE1 binding to Sp100 (Kim et al., 2011) may result in Sp100 degradation by an as-yet-unknown mechanism. Alternatively, Sp100 degradation may be an indirect result of PML-NB modification by IE1.

Other HCMV proteins may be involved in PML-NB destruction or modification (Table 1). Expression of epitope-tagged versions of US25, UL29, UL30, UL69, UL76, UL98 or TLR9 is sufficient to modestly reduce the number of PML-NBs in uninfected cells (Salsman et al., 2008). These proteins are expressed in different kinetic classes of viral transcription and have different effects on HCMV replication, plus the function of several of these proteins is unknown (Table 1). In transfection experiments epitope-tagged HCMV proteins US32, UL80a and UL35 co-localize with, but do not obviously degrade, PML-NBs in uninfected cells (Salsman et al., 2008). Epitope-tagged US32 and UL35 alter the shape and size of PML-NBs in uninfected cells. The localization and function of these proteins in relation to PML-NBs in HCMV-infected cells has yet to be elucidated. UL80a is a capsid protein. UL80a association with PML-NBs is reminiscent of PML cages trapping alphaherpesvirus varicella-zoster virus (VZV) capsids in infected cells via physical interaction between PML-NB proteins and VZV capsids (Reichelt et al., 2011). This mechanism of cellular defence to infection has not yet been observed in HCMV-infected cells. An epitope-tagged version of the putative HCMV protein UL3 has been reported to alter PML-NBs (Salsman et al., 2008). However, it is now thought that the HCMV genome does not contain an ORF encoding UL3 (Gatherer et al., 2011). A final protein

Table 1. cont.

<table>
<thead>
<tr>
<th>Nuclear structure</th>
<th>HCMV associated proteins</th>
<th>Kinetic class of HCMV protein</th>
<th>HCMV protein function†</th>
<th>Requirement for HCMV replication‡</th>
<th>Known or proposed cellular partner(s)</th>
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<td>E</td>
<td>E–L</td>
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</tr>
</tbody>
</table>

*From Chambers et al. (1999). E, Early; IE, immediate early; L, late; E–L, early to late.
†Protein functions described in references indicated in the table and/or Fields Virology (Mocarski et al., 2007).
‡From Yu et al. (2003). E, Essential; NE, non-essential; A, augments replication.
to consider in the alteration of PML-NBs in HCMV-infected cells is the HCMV viral kinase UL97. UL97 phosphorylates and inactivates the PML-NB protein retinoblastoma (Rb) (Hume et al., 2008) and expression of epitope-tagged UL97 in uninfected simian cells (Prichard et al., 2010), notably reduces the number of PML-NBs in the cell. Clarification of the roles, if any, of the UL97 and Rb proteins in PML-NB function in HCMV-infected cells is required.

Future consideration of PML-NB function in HCMV-infected cells will benefit from further exploration of the interaction of PML-NBs with other viruses, including other herpesviruses. Indeed, most detailed information concerning the interaction of PML-NBs with viral DNA genomes comes from the study of HSV infection and analysis of PML-NB antagonism by the HSV protein ICP0 (Boutell & Everett, 2013). Interaction of PML-NBs with adenovirus and papillomaviruses has been reported but is less well understood. Prominent findings include the ability of the adenovirus proteins E1B and E4orf5 to antagonize PML and hDaxx functions (Schreiner et al., 2010; Ullman & Hearing, 2008) and the repression of papillomavirus RNA transcription by Sp100 (Stepp et al., 2013). Future studies of adenovirus and papillomavirus replication may illuminate important features of herpesvirus interactions with PML-NBs.

Subnuclear structures other than PML-NBs could also be involved in repression of viral transcription. These include bodies such as the HCMV tegument protein pp150 (UL32), pp150 is a substrate of cyclin A2-CDK (Table 1) and is a sensor for cell-cycle progression during HCMV infection. By an unknown mechanism, pp150 is involved in blocking viral transcription when levels of cyclin A2-CDK are high (Bogdanow et al., 2013). It has been suggested that pp150 nuclear bodies are sites of pp150-mediated transcriptional repression and are the destination of HCMV genomes not associated with PML-NBs (Bogdanow et al., 2013; Ishov et al., 1997) (Fig. 1b).

Therefore, upon engagement of the viral capsid with proteins on the cytoplasmic face of the NPC, the HCMV genome is delivered into the nucleus through the nuclear pore. Once in the nucleus, transcription from the viral genome involves cellular transcription factors and may be facilitated by interaction of the genome with the nuclear lamina at the periphery of the nucleus. At the periphery of the nucleus, the genome encounters nuclear speckles, which probably facilitate the splicing and export to the cytoplasm of viral RNA transcripts, and PML-NBs or pp150 bodies that restrict viral transcription. Restriction of viral transcription by PML-NBs involves the recruitment of transcriptional repressors to the viral genome and is antagonized by virally encoded proteins. Restriction of viral transcription by pp150 bodies is less well defined and is associated with progression of the infected cell through the cell cycle.

Replication of the viral genome: HCMV replication compartments, chromatin partitioning, protein quality-control compartments and nuclear speckles

Once transcription of IE and E RNA transcripts is under way, viral proteins that replicate HCMV genomic DNA are produced. Viral DNA synthesis and several early and late transcriptional events occur within non-membrane-bound compartments created by the virus composed of protein and nucleic acids. The formation of these ‘viral replication compartments’ poorly understood but allows the recruitment and concentration of factors required for viral replication to specific areas of the infected cell nucleus. Analysis of alphaherpesvirus infection implies that a herpesvirus replication compartment can develop from a single incoming viral genome (Kobiler et al., 2011). In HCMV-infected cells, replication compartments form in the vicinity of PML-NBs (Ahn et al., 1999). As compartments develop, they contain viral transcriptional transactivators (including IE2 and UL112–113 proteins), viral proteins involved in viral DNA synthesis (including UL44 and UL57) and viral DNA (Ahn et al., 1999; Penfold & Mocarski, 1997; Strang et al., 2012b) (Table 1). Early in the formation of replication compartments, factors involved in the DNA damage response, including γH2AX and ATM, localize to replication compartments (E et al., 2011) and may recognize viral genomes within compartments. It is thought that viral transcriptional transactivators drive E2F1-mediated activation of ATM signalling, which activates DNA damage response factors such as γH2AX and p53 (E et al., 2011). Activation of this pathway may be required for modulation of cell-cycle-related functions that promote viral replication. ATM signalling promotes the development of replication compartments (E et al., 2011), but it is unclear if this is an effect that directly relates to replication compartment development or an indirect effect related to events that promote viral RNA transcription or viral DNA synthesis.

During HCMV replication, several compartments can form and then coalesce to form a single compartment that occupies nearly all the nucleus but does not encroach into nucleoli (Penfold & Mocarski, 1997; Strang et al., 2012a, b) (Fig. 2a, b). The mechanisms driving coalescence of HCMV replication compartments have yet to be defined. Replication compartment coalescence in HSV-infected cells occurs via non-random movement and is dependent upon nuclear actin, myosin and viral transcription (Chang et al., 2011).

To provide areas in the nucleus for replication compartments to develop, the compaction and movement of chromatin is observed (O’Dowd et al., 2012; Strang et al., 2012b). This process is termed ‘chromatin partitioning’. It is unknown what drives this process, but UL98 and UL76 may be involved (Table 1). UL98 is an alkaline nuclease capable of digesting DNA (Sheafer et al., 1997). UL76 is member of the UL24 gene family, all of which contain a
conserved putative PD-(D/E)XK endonuclease motif (Knizewski et al., 2006). Although endonuclease activity of UL76 has yet to be demonstrated, indirect evidence points to a role for UL76 in the chromosome breakage required for chromatin partitioning. For example, the presence of UL76 in uninfected cells is sufficient to stimulate chromosomal aberrations (Siew et al., 2009), and in HCMV-infected cells, UL76 is involved in stimulating a DNA damage response (Costa et al., 2013).

Several facets of HCMV replication compartment organization and function late in infection have recently been described, each involving the localization of HCMV protein UL44. UL44 is the putative processivity subunit of the HCMV DNA polymerase (Ertl & Powell, 1992), and several different phosphorylated forms of UL44 are present in the infected cell (Silva et al., 2011). At least one form of UL44 accumulates at the periphery of replication compartments throughout HCMV replication, and UL44 is also found at considerably lower levels within certain domains inside replication compartments, plus in the nucleus outside replication compartments and also in the cytoplasm (Hamirally et al., 2009; Penfold & Mocarski, 1997; Strang et al., 2012b). Viral DNA synthesis occurs at foci within the layer of UL44 at the periphery of replication compartments (Fig. 2b), and newly synthesized viral DNA moves into the interior of replication compartments (Strang et al., 2012b).

The viral ssDNA-binding protein UL57 co-localizes with UL44 at the periphery of replication compartments and is found throughout the interior of compartments (Penfold & Mocarski, 1997; Strang et al., 2012b). UL57 at the periphery of replication compartments is likely to be directly involved in viral DNA synthesis. UL57 protein in the interior of replication compartments is associated with viral DNA (Penfold & Mocarski, 1997) and is possibly involved in UL57-mediated DNA strand exchange events, including recombination and repair of newly synthesized HCMV DNA.

As well as UL44 and UL57, other factors required for viral DNA synthesis have been reported. These include the viral DNA polymerase subunit UL54, replication factor UL84 and the helicase DNA complex (UL70, UL102 and UL105) (Pari & Anders, 1993; Pari et al., 1993). However, the full repertoire of viral and cellular proteins acting in concert during DNA synthesis has yet to be described. These proteins probably include those viral and cellular proteins that associate with UL44 and/or UL84 (Gao et al., 2008; Strang et al., 2009, 2010a, b), several proteins that have ill-defined roles in viral DNA synthesis (for example, UL36, IRS1 and IE2; Pari & Anders, 1993; Pari et al., 1993; Sarisky & Hayward, 1996), plus viral and cellular proteins involved in DNA damage responses and viral DNA repair (E et al., 2011; O'Dowd et al., 2012; Strang & Coen, 2010). How these and other proteins are organized within HCMV replication compartments is unclear but may involve UL44. UL44, a homodimer, is a structural homologue of the cellular proliferating cell nuclear antigen (PCNA), which exists as a trimer (Appleton et al., 2004, 2006; Loregian et al., 2004a, b). PCNA binds multiple proteins of multiple functions at the DNA replication fork (Moldovan et al., 2007). UL44 could play a similar role and bind multiple proteins in replication compartments. Proteomic studies have indicated that UL44 associates with viral and cellular proteins found in replication compartments, including UL84 (Strang et al., 2010a). The binding of proteins to UL44 is increasingly well characterized. Studies investigating the binding of UL54 to UL44 have revealed that, like PCNA, UL44 is able to bind proteins within ‘hydrophobic pockets’; crevices located in the N termini of UL44 containing several hydrophobic amino acids into
which proteins binding UL44 insert their binding domains (Appleton et al., 2004, 2006; Loregian et al., 2004a, b). Unexpectedly, UL44 can also associates with proteins outside the hydrophobic pockets, as it has been demonstrated that the association of UL84 with UL44 is not dependent upon binding in the hydrophobic pocket (Strang et al., 2009). Rather, association of UL84 with UL44 takes place elsewhere in the N-terminal domain of UL44 and is not dependent upon homodimerization of UL44 (Strang et al., 2009). It is unclear what drives selection of proteins for binding to UL44, but there are likely determinants of protein binding in both UL44 and its interacting proteins. Indeed, it has been demonstrated that the HCMV proteins IRS1 and TRS1, which have identical N termini that are required for association with UL44, compete for UL44 binding (Strang et al., 2010b).

UL44 is also indirectly involved in the recruitment of viral proteins to replication compartments. The viral uracil DNA glycosylase UL114 is required for efficient viral DNA synthesis and base excision repair (Prichard et al., 1996). Base excision repair maintains genome integrity by removing uracil from the HCMV genome, which is the result of either misincorporation of uracil during viral DNA synthesis or the spontaneous deamination of cytosine in the viral genome. Although UL114 localizes with UL44 within replication compartments (Prichard et al., 2005), it does not bind UL44, as first thought (Ranneberg-Nilsen et al., 2008). Rather, UL114 associates with the catalytic subunit of the viral DNA polymerase UL54, and UL114, UL54 and UL44 form a protein complex (Strang & Coen, 2010). Thus, UL44 indirectly recruits UL114 to replication compartments via the interaction of UL114 with UL54.

There are few reports of cellular proteins localizing to HCMV replication compartments, although it is known that both p53 and replication protein A are recruited to the interior of replication compartments (Fortunato & Spector, 1998). Their roles within replication compartments are unknown but may involve modulation of cellular responses to infection and promoting efficient viral DNA synthesis.

Several factors influence the architecture of HCMV replication compartments. While the N terminus of UL44 is required for binding of UL44-associated proteins, the C terminus of UL44 is required for the development of replication compartments and correct localization of UL44 in the nucleus (Silva et al., 2010). Although the C terminus of UL44 can be extensively phosphorylated by both the viral kinase UL97 and the cellular kinase cdk1, it is unlikely that these post-translational modifications affect replication compartment function, as mutational analysis of phosphorylated residues in the UL44 C terminus has little effect on viral DNA synthesis and viral replication (Silva et al., 2011). Efficient viral DNA synthesis is also required to maintain UL44 localization and compartment structure (E et al., 2011; Penfold & Mocarski, 1997; Strang et al., 2012a, b). Unexpectedly, the multifunctional cellular nucleolar protein nucleolin (Ginisty et al., 1999) is involved in maintaining replication compartment architecture. UL44 and nucleolin associate via the N terminus of UL44 (Strang et al., 2010a) and in infected cells nucleolin co-localizes with UL44, but not viral DNA, on the exterior of replication compartments (Strang et al., 2010a, 2012a). The absence of nucleolin in the infected cell causes mislocalization of UL44 at the periphery of replication compartments and compromises efficient viral DNA synthesis (Strang et al., 2012a). Nucleolin is also required for UL84 localization with UL44 at the periphery of replication compartments, although in the absence of nucleolin, UL84 does not redistribute throughout the nucleus but relocates to the cytoplasm (Bender et al., 2014). Thus, nucleolin is required for both proper nuclear and subnuclear localization of HCMV replication compartment proteins. Furthermore, it is possible that, in infected cells, nucleolin, UL44, UL84 and either IRS1 or TRS1 interact within a large multiprotein complex (Strang et al., 2010a, b). Therefore, nucleolin may have an effect on the localization of both IRS1 and TRS1 in the infected cell.

Although the organization of factors required for viral DNA synthesis in replication compartments is increasingly well understood, perhaps the most important unanswered questions involving the architecture of replication compartments involve the localization of factors required for RNA transcription from the viral genome. The location of transcriptionally active genomes within replication compartments is unknown. There is little information on the localization of viral and cellular proteins required for viral RNA transcription. In infected cells, both phosphorylated (transcriptionally active) and unphosphorylated (transcriptionally inactive) forms of RNA polymerase II concentrate within nuclear structures thought to be replication compartments (Feichtinger et al., 2011). Within these structures, unphosphorylated and phosphorylated forms of RNA polymerase II localize with the viral protein UL69 (Feichtinger et al., 2011), which is known to be required for viral RNA processing and export to the cytoplasm. The localization of RNA polymerase II in relation to UL44 has yet to be described; however, viral proteins involved in transcription and RNA metabolism, for example IE1, IE2 and UL84 (Bender et al., 2014; Silva et al., 2010), are found throughout the nucleus and co-localize with UL44 in viral replication compartments. Therefore, it is likely that viral transcription occurs throughout the interior of replication compartments and, unlike viral DNA synthesis, is not restricted to specific areas of replication compartments such as the layer of UL44 that accumulates at the periphery of replication compartments.

The presence of subcompartments within HCMV replication compartments has been reported. Components of the ubiquitin–proteasome system (UPS) assemble into domains at the periphery of replication compartments (Fig. 2b), where they co-localize with UL57 (Tran et al., 2010). The accumulation of UPS proteins at the periphery of HCMV replication compartments is analogous to the formation of VICE (virus-induced chaperone enriched)
domains at the periphery of HSV replication compartments. VICE domains degrade misfolded proteins in a proteasome-dependent fashion to promote efficient virus replication (Livingston et al., 2009). Thus, compartments containing UPS components at the periphery of HCMV replication compartments may act as quality-control areas for HCMV proteins. It is unclear which HCMV proteins other than UL57 localize to UPS compartments. However, HCMV UL76 co-localizes with the UPS component Rpn10/S5a in discrete areas of infected cell nuclei termed aggressomes (Lin et al., 2013). Rpn10/S5a is found in UPS compartments (Tran et al., 2010). Thus, some aggressomes may be UPS compartments associated with replication compartments that contain both UL76 and Rpn10/S5a. It is unknown what role UL76 might have in UPS compartments. Subcompartments within the interior of replication compartments that do not contain UL44 or viral DNA have been observed (Strang et al., 2012b). It is possible that these subcompartments are the aforementioned UPS compartments.

The presence of SC35-containing structures on the exterior of HCMV replication compartments has been reported (Rechter et al., 2009) (Fig. 2b). It is likely that, as in HSV-infected cells (Chang et al., 2011), these are nuclear speckles that remain associated with HCMV replication compartments throughout virus replication. The directed movement of nuclear speckles with HSV replication compartments, or vice versa, is associated with efficient viral RNA transcription in HSV-infected cells, in particular promoting the export of late HSV RNA transcripts to the cytoplasm (Chang et al., 2011). The function of nuclear speckles associated with replication compartments in HCMV-infected cells has yet to be described, although it is likely that they are involved in viral RNA transcription.

In summary, HCMV replication compartments are necessary for the concentration and organization of factors required for viral replication. The organization of factors required for viral DNA synthesis in replication compartments is increasingly well defined and involves UL44. Viral replication compartments are dynamic structures whose architecture is influenced by UL44 localization, viral DNA synthesis and the cellular nucleolar protein nucleolin. Nucleolin is not only required to maintain replication compartment architecture but is also involved in the subcellular localization of at least one viral protein found in replication compartments. The organization of factors required for RNA transcription from the HCMV genome in replication compartments is not well defined. However, it can be suggested that viral transcription takes place throughout the interior of replication compartments, in contrast to viral DNA synthesis, which takes place within a layer of UL44 at the periphery of replication compartments. Within replication compartments, there are subcompartments, some of which are associated with protein quality control. Nuclear speckles are associated with viral replication compartments where they probably enable viral transcription, possibly by facilitating the export of viral transcripts to the cytoplasm.

**Structures associated with HCMV replication: nucleoli, Cajal bodies and perinucleolar compartments**

To date, little attention has been paid to the role of nucleoli and associated subnuclear structures such as Cajal bodies and perinucleolar compartments in HCMV-infected cells. It is increasing clear, however, that these subnuclear structures may have important roles in HCMV genome replication and virus–host interactions.

Nucleoli are dynamic non-membrane-bound protein and RNA structures that form on rRNA genes. A nucleolus has a tripartite structure in which the fibrillar centre is surrounded by a dense fibrillar component (containing the majority of nucleolin found in nucleoli) that is surrounded by the granular component. In HCMV-infected cells, nucleoli remain intact (Strang et al., 2012c), and visualization of nucleolin in HCMV-infected cells suggests changes in the size of nucleoli upon infection (Strang et al., 2012a). Also, striking changes to nucleolar composition and architecture occur, as, following infection, nucleolin levels in nucleoli increase and nucleolin accumulates at the periphery of nucleoli (Strang et al., 2012a) (Fig. 2c). These changes in nucleolar composition and architecture may relate to the recruitment of nucleolin to replication compartments, a nucleolar stress response to infection or the production of ribosomes in the HCMV-infected cell. The recruitment of nucleolin to replication compartments may be related to the interaction of nucleolin with viral proteins found in replication compartments. It has been observed that the HCMV protein UL84 co-localizes with nucleolin in nucleoli and at the periphery of replication compartments (Bender et al., 2010). Therefore, it is possible that UL84 recruits nucleolin to replication compartments from nucleoli, which results in changes to nucleolar composition and architecture.

Nucleoli can act as sensors of environmental, genotoxic or osmotic stress, and pathogen invasion (Boisvert et al., 2007; Boulon et al., 2010; Hiscox, 2002; Hiscox et al., 2010). These stresses result in a variety of outcomes including changes in nucleolar morphology and the nucleolar proteome, plus pleiotropic effects of p53 activation (Boulon et al., 2010). The changes in the nucleolar proteome and architecture observed in HCMV-infected cells could relate to a nucleolar stress response. The nucleolar localization of nucleolin in HCMV-infected cells is akin to that seen in coronavirus-infected cells. It has been suggested that localization of nucleolin in coronavirus-infected cells is related to ‘nucleolar capping’ (Dove et al., 2006). Capping is associated with a nucleolar stress response caused by factors such as transcriptional inhibition or DNA damage. These stresses cause condensation and segregation of the fibrillar centre and the granular component. This is accompanied by the formation of ‘caps’ of nucleoplasmic RNA-binding proteins on the body of nucleoli (Boulon et al., 2010; Shav-Tal et al., 2005). It has been reported that nucleolar capping is observed in HCMV-infected cells (Gaddy et al., 2010). However, it is
unclear if the reported nucleolar caps are actually associated with nucleoli. Furthermore, analysis of nucleoli in HCMV-infected cells by electron microscopy does not suggest the presence of nucleolar caps (Strang et al., 2012c). It is unknown whether HCMV infection stimulates p53-mediated nucleolar stress responses, which include specific changes to the protein translational profile of the cell (Boulon et al., 2010). Finally, the principal role of nucleoli in uninfected cells is to produce ribosomes. Levels of certain rRNAs increase in infected cells (Tanaka et al., 2000). Therefore, ribosome production during HCMV replication may not be compromised. However, the increase in rRNA production in infected cells could be related to changes in nucleolar composition and architecture.

An integral part of any of the possibilities discussed could be the action of HCMV proteins in nucleoli. Several viral proteins can localize to nucleoli. It has been reported that HCMV pp65 and UL84 proteins are a nucleolar antigens (Arcangeletti et al., 2009; Bender et al., 2014). Also, transfection experiments have indicated that several epitope-tagged versions of HCMV proteins (TLR5, TLR7, US33, UL29, UL31 and UL76) localize to the nucleolus in uninfected cells (Salsman et al., 2008). These proteins are expressed in different kinetic classes of viral RNA transcription and have different effects on HCMV replication, plus the function of several of these proteins is unknown (Table 1). Also, it has been reported that epitope-tagged versions of the putative HCMV proteins TLR7 and UL108 localize to nucleoli in uninfected cells (Salsman et al., 2008). However, it is now thought that the HCMV genome does not possess ORFs encoding TLR7 or UL108 (Gatherer et al., 2011).

Cajal bodies and perinucleolar compartments are sub-nuclear structures associated with nucleoli (Fig. 2c) and both may be involved in viral RNA production. Cajal bodies are non-membrane-bound protein and RNA structures involved in transcription and maturation of ribonuclear particles (Ogg & Lamond, 2002). A role for Cajal bodies in HCMV replication has yet to be described. However, transfection of epitope-tagged HCMV UL30 leads to disruption and/or loss of Cajal bodies in uninfected cells (Table 1). Thus, it has been suggested (Salsman et al., 2008) that UL30 may allow HCMV to inhibit transcription and ribonuclear particle maturation. Cajal bodies are thought to play a role in adenovirus replication, wherein adenovirus infection results in fragmentation of Cajal bodies (James et al., 2010). Also, Cajal bodies are required for efficient production of several late adenoviral RNA transcripts (James et al., 2010). Thus, it is possible, as in adenovirus-infected cells, that Cajal bodies are disrupted upon HCMV infection and have a role in HCMV RNA transcription. However, Cajal bodies are generally absent in slowly dividing or non-dividing cells (Ogg & Lamond, 2002) and may not be present in the fibroblast, vascular and neuronal cell types within which HCMV replicates during productive infection. It has been suggested that the putative HCMV protein UL3 is also involved in modification of Cajal bodies (Salsman et al., 2008). However, as mentioned above, it is now thought that an ORF encoding UL3 is not present in the HCMV genome (Gatherer et al., 2011).

Perinucleolar compartments are thought to be involved in RNA polymerase II and III mediated RNA transcription. In HCMV-infected cells, at least one perinucleolar compartment protein, polyryrimidine tract-binding protein (PTB), is relocated from the nucleus to the cytoplasm or degraded in the nucleus (Gaddy et al., 2010). It has been suggested (Gaddy et al., 2010) that loss of PTB from perinucleolar compartments is related to negative regulation of viral splicing by PTB (Cosme et al., 2009).

In summary, upon HCMV infection of the cell, nucleoli remain intact but changes to nucleolar composition and architecture are observed. These changes may be related to a cellular nucleolar stress response, the recruitment of nucleolin to HCMV replication compartments or the presence of viral proteins within nucleoli. Subnuclear structures associated with nucleoli, including Cajal bodies and perinucleolar compartments, may be involved in viral RNA production in HCMV-infected cells.

Production of DNA-filled capsids: a viral structure putatively involved in capsid assembly and genome packaging

Late viral transcription produces proteins required to form viral capsids. The assembly of HCMV capsids and packaging of genomes into them is increasingly well understood (Gibson, 2008; McVoy et al., 2000; Scheffczik et al., 2002; Wang et al., 2012). However, it is unclear where within viral replication compartments capsid assembly and genome packaging take place. These questions have not been extensively investigated.

There are few data regarding the localization of HCMV capsid proteins within the infected cell nucleus. However, it has been reported that a ‘sponge-like’ web of UL80 proteins forms throughout the infected cell nucleus, dependent upon UL80 self-interaction (Loveland et al., 2007; Nguyen et al., 2008). UL80 proteins include the capsid assembly protein precursor (UL80.5) and the maturational protease precursor (UL80a), which interact with the major HCMV capsid protein UL86 (Wood et al., 1997). It is possible that the web of UL80 proteins could act as a scaffold for capsid assembly and genome packaging within viral replication compartments.

Packaging of viral DNA into nascent viral capsids probably takes place within replication compartments. The viral protein responsible for DNA binding during the packaging process, UL56, is found within replication compartments. UL56 localization to replication compartments is dependent on viral DNA synthesis, and UL56 may interact with UL44 within replication compartments (Giesen et al., 2000). Furthermore, it has been speculated that packaging of viral DNA into capsids is responsible for the movement of newly synthesized viral DNA from the periphery of
replication compartments to the interior of replication compartments (Strang et al., 2012b).

Therefore, further study is required to understand the relationship between the production of DNA-containing viral capsids and viral replication compartments. It can be proposed, however, that viral capsid proteins play a role in organizing capsid production by creating a subnuclear structure on which capsid assembly and DNA packaging might take place.

Exit of capsids from the nucleus: the nuclear lamina

To escape the nucleus, DNA-containing capsids must first travel from within viral replication compartments to the nuclear periphery. It is unknown what facilitates this process in HCMV-infected cells. In HSV-infected cells, directed capsid movement in the nucleus is dependent upon myosin and actin (Forest et al., 2005). It is possible that HCMV also utilizes these cellular cargo transporters to facilitate capsid movement. Recently, it has been reported that mutation of HCMV UL84 leads to a defect in virus replication associated with a defect in nuclear egress of capsids and the clustering of capsids around nucleoli (Strang et al., 2012c). This would suggest that UL84 is, directly or indirectly, involved in the efficient movement of HCMV capsids within the nucleus. It has yet to be determined what relevance the mislocalization of capsids to nucleoli has on the movement of capsids in the nucleus.

At the periphery of the nucleus, capsids must traverse the physical barrier of the nuclear lamina and pass though the nuclear membrane. There have been several recent advances in our understanding of these processes. HCMV proteins mediate events at the nuclear lamina required for nuclear egress. The viral nuclear egress complex (UL50 and UL53) recruits the viral kinase UL97 to the nuclear lamina in HCMV-infected cells (Sharma et al., 2014) (Table 1). Phosphorylation of the lamina component lamin A/C by UL97 promotes lamina disruption, akin to that observed during cell division, resulting in thinning and the appearance of gaps in the lamina (Hamirally et al., 2009). This should facilitate capsid movement through the lamina to the nuclear membrane. The requirement for other viral (including c-ORF29 and UL45) and cellular (including emerin, p32 and protein kinase C) proteins implicated in nuclear egress in this model remains unclear (Milbradt et al., 2014; Miller et al., 2010). It is unknown what mechanism selects only DNA-containing capsids for nuclear egress in HCMV-infected cells. In HSV-infected cells, this process is governed by the interaction of the nuclear egress complex and capsid proteins (Yang & Baines, 2011). A similar mechanism may operate in HCMV-infected cells.

Once through the lamina, capsids must traverse the nuclear membrane in a process now thought to be similar to the passage of large ribonucleoprotein particles from the nucleus to the cytoplasm (Jokhi et al., 2013; Speese et al., 2012). Briefly, capsids must bud through the inner leaflet of the nuclear membrane into the intramembrane space. A fusion event with the outer leaflet of the membrane grants capsids access to the cytoplasm. Capsids then engage the cytoplasmic membrane envelopment processes via a cytoplasmic viral assembly compartment (Alwine, 2012), which leads to virion exit from the cell. It is thought that the development of the cytoplasmic assembly compartment next to the nucleus deforms the nuclear membrane. This creates a concave surface on the nucleus, giving it a ‘kidney bean’ shape (Azzeh et al., 2006). To gain direct access to the cytoplasmic assembly compartment, capsids probably exit the nucleus through the concave face of the nuclear membrane.

In summary, the process that facilitates the movement of DNA-containing capsids to the nuclear periphery is ill defined but probably involves cellular cargo transporters. Once at the nuclear membrane, a virally encoded nuclear egress complex possibly selects DNA-containing capsids for nuclear egress. This nuclear egress complex then facilitates capsid movement through the nuclear lamina by promoting modification of lamina components which results in thinning and the appearance of gaps in the lamina. The capsid moves through the inner and outer leaflets of the nuclear membrane via the intramembrane space. Once in the cytoplasm, capsids participate in a complex membrane envelopment process via a viral cytoplasmic assembly compartment that leads to virion exit from the cell. There may be a relationship between the construction of the viral cytoplasmic assembly compartment, deformation of the nucleus and exit of capsids through the nuclear membrane proximal to assembly compartments.

Further questions and future perspectives

As discussed here, there have been notable advances in our understanding of HCMV replication and virus–host interactions by examining subnuclear structures. However, a full examination of the questions that remain is limited by several factors. These include our inability to fully dissect viral gene expression or protein function and explore the composition of viral and cellular subnuclear structures. To address these issues, technological development is required. Of particular importance to interrogating the role of virally encoded factors will be the continued development of robust systems to produce recombinant HCMV viruses, including viruses with lethal mutations in their genomes. The study of viral genomes containing lethal mutations can currently be achieved by studying cells transfected with bacmids containing mutated HCMV genomes, for example Silva et al. (2010), although this methodology has its limitations. Examination of viral cellular subnuclear structure composition requires the development of well-characterized antibodies recognizing unmodified and post-translationally modified forms of viral and cellular proteins. Recombinant viruses and antibodies can then be coupled with increasingly sophisticated
molecular and cellular techniques. These include live-cell imaging, super-resolution microscopy, correlative light electron microscopic analysis (Sharma et al., 2014), chemical biology techniques (for example, ‘click chemistry’; Strang et al., 2012b) and proteomic analysis of subnuclear structures (Andersen et al., 2002; Hiscox et al., 2010).

A number of the observations discussed above rely on the study of transfection experiments using epitope-tagged versions of viral proteins in uninfected cells (Kuny et al., 2010; Prichard et al., 2008; Salsman et al., 2008). It is unclear what effect overexpression during transfection or epitope tagging might have on protein function. Thus, data based on transfection experiments should be interpreted cautiously. Furthermore, certain aspects of this review rely on the study of HSV. However, several features of HSV replication differ from HCMV replication, probably due to evolutionary divergence among herpesviruses. These include antagonism of the PML response (Everett et al., 2013), nucleolar organization (Lymberopoulos & Pearson, 2007; Strang et al., 2012a), the localization of nucleolin (Lymberopoulos & Pearson, 2007; Strang et al., 2012a) and replication compartment organization (Liptak et al., 1996; Strang et al., 2012b). There are areas where examination of HSV, or other herpesviruses, may not benefit HCMV research. These points underscore the need to generate reagents and methodologies to examine events that occur in HCMV-infected cells.

The use of laboratory viral strains, the cell types studied and virus latency must be considered in future experiments. Most of the data outlined in this review were derived from the study of HCMV laboratory strains. The genomic complexity of HCMV is still being addressed. Primary strains of HCMV contain several more ORFs than laboratory strains of HCMV (Dolan et al., 2004). Also, recent high-resolution transcriptome mapping (Gatherer et al., 2011) and ribosome profiling (Stern-Ginossar et al., 2012) have revealed the presence of many previously unrecognized viral RNA transcripts and non-canonical ORFs. Further investigation is required to discover how recently identified HCMV factors might influence viral or cellular subnuclear structures. These factors include newly identified transcripts from the US33 and TRL9 loci (Gatherer et al., 2011), which may localize to the nucleolus of HCMV-infected cells (Salsman et al., 2008) (Table 1). Also, the HCMV transcriptome contains a number of long (Gatherer et al., 2011) and short (Grey et al., 2005) non-coding RNAs. As yet, there are no data indicating that HCMV non-coding RNAs are involved in the function of viral or cellular subnuclear structures.

The HCMV transcriptome varies between cell types (Towler et al., 2012), suggesting cell-type-specific requirements for viral factors. Transcripts differentially expressed between cell lines tested include those encoding UL44 (involved in viral replication compartment architecture), UL98 (suggested to be involved in chromatin partitioning), TRL5 (thought to be localized to the nucleolus), UL80 proteins (thought to be involved in capsid assembly within replication compartments) plus UL97 and UL50 (involved in capsid egress through the nuclear lamina) (Towler et al., 2012) (Table 1). Thus, the function of viral and cellular subnuclear structures could vary among cell types.

Virus latency is a hallmark of herpesvirus infection. However, our understanding of the establishment, maintenance and reactivation from latency of HCMV is incomplete. Recent in vitro models of virus infection in haematopoietic cells (for example, CD14+ and CD34+ cells) have illuminated important aspects of HCMV latency. The loss of hDaxx in CD34+ cells is required for transcription of IE viral genes upon infection by laboratory, but not primary, strains of HCMV (Saffert et al., 2010). This would argue that PML-NBs repress HCMV transcription from laboratory HCMV strains and that primary HCMV strains possess as-yet-unidentified functions that control viral transcription during latency. Co-infection of CD34+ cells with both laboratory and primary strains of HCMV blocks the ability of histone deacetylases to activate IE gene transcription from the laboratory strain of HCMV (Saffert et al., 2010). Thus, transcriptional repression of primary HCMV strains may relate to chromatin modification by histone deacetylases. Also, it is unclear if hDaxx has a major role in repression of HCMV gene transcription during latency. In the embryonic carcinoma cell line NT2D1, which can recapitulate the differentiation-dependent regulation of IE gene expression observed during latency, repression of IE gene expression has been reported in the presence (Saffert & Kalejta, 2007) and absence (Groves & Sinclair, 2007) of hDaxx in undifferentiated NT2D1 cells. This may be due to differences in the virus strains used in each study. Further experiments are required to ascertain the relationship between virus strain, cell type, cell differentiation and gene expression during HCMV latency.

There are few other data regarding the involvement of other subnuclear structures in HCMV latency. Furthermore, the latent HCMV transcriptome in either CD14+ or CD34+ cells (Rossetto et al., 2013) does not produce the high levels of transcripts encoding HCMV proteins thought to be localized to cellular subnuclear structures during productive HCMV replication. Thus, if cellular subnuclear structures are involved in HCMV latency, different repertoires of viral factors interact with subnuclear structures during productive and latent HCMV infections.

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Roles of...


