INTRODUCTION

Herpes simplex virus type 1 (HSV-1) virions have a multi-layered structure composed of an icosahedral capsid that protects a DNA genome, a proteinaceous layer called the tegument and a host-derived lipid envelope. These constituents are acquired sequentially during the maturation of HSV-1 through three major intracellular compartments: the nucleus, the cytoplasm and the trans-Golgi network (TGN) (Mettenleiter, 2002; Mettenleiter et al., 2009; Turcotte et al., 2005). Although the details by which the virus acquires its tegument are still unclear, recent reviews highlight the importance of the tegument during the viral life cycle (Guo et al., 2010; Kelly et al., 2009).

Following virus entry, most of the tegument proteins dissociate from incoming capsids (Kelly et al., 2009; Mettenleiter et al., 2009) and this early release seems important for efficient virus infection. For example, the viral protein VP16, encoded by the UL48 gene, trans-activates the viral immediate-early genes and triggers the cascade of viral gene expression necessary for the subsequent stages of the HSV-1 life cycle (Kelly et al., 2009; Roizman et al., 2005; Wysocka & Herr, 2003). In contrast, the UL36 and UL37 viral proteins remain associated with incoming HSV-1 capsids and contribute to capsid transport towards the nucleus and injection of the viral DNA into the nucleus through the nuclear pores (Döhner et al., 2002; Liashkovich et al., 2011; Ojala et al., 2000; Shanda & Wilson, 2008; Smith & Enquist, 2002; Sodeik et al., 1997; Wolfstein et al., 2006). The presence of a protein within the tegument is therefore not fortuitous and may reflect an early role in the viral life cycle. However, the tegument is also implicated during virus egress (Bjerke & Roller, 2006; Mou et al., 2007; Scholtes et al., 2010; Skepper et al., 2001; Stackpole, 1969) as well as in immune evasion and apoptosis (Hwang et al., 2009; Leopardi et al., 1997; Ogg et al., 2004; Orvedahl et al., 2007; Smiley, 2004; Trgovcich et al., 2002). It additionally forms a bridge between the capsid proteins and glycoproteins of the viral envelope (Mettenleiter, 2004; Mettenleiter et al., 2006, 2009). Despite the important and multifunctional roles of the tegument, its precise composition has only recently been elucidated by proteomics (Loret et al., 2008). That study confirmed many known virion components, but also identified UL7, UL23, UL50 and UL55 as novel virion proteins. UL50 encodes a dUTPase that was also detected by proteomics in the related pseudorabies virus (PRV) (Kramer et al., 2011) and plays an important role in nucleotide metabolism, but is otherwise poorly characterized (Bergman et al., 1998; Björnberg et al., 1993; Jöns &
The function of the UL55 protein is totally unknown. In contrast, two studies demonstrated the involvement of UL7 in the maturation and egress of HSV-1 and PRV and its interaction with the mitochondrial adenine nucleotide translocator 2 (ANT2) (Fuchs et al., 2005; Tanaka et al., 2008). Finally, UL23 encodes a viral kinase involved in nucleotide metabolism (Boehmer & Lehman, 1997; Wurth et al., 2001). Interestingly, this kinase has been detected in Kaposi’s sarcoma-associated herpesvirus, Epstein–Barr virus and alacphan herpesvirus-1 virions (Rechtel et al., 2005; Dry et al., 2008; Johannsen et al., 2004; Zhu et al., 2005), although it was undetected in the aforementioned PRV proteomics study (Kramer et al., 2011). Further work is clearly needed to define where in the virus these proteins reside and what function they have.

Infected cell polypeptide (ICP)0 and ICP4 are extensively studied immediate-early proteins that regulate gene expression and transactivation of early and late viral genes (Everett, 2000; Kalamvoki & Roizman, 2010). Early studies suggested that ICP0 and ICP4 might be packaged into mature HSV-1 particles (Yang & Courtney, 1995; Yao & Courtney, 1989, 1991), but ICP0 was not detected in the virions by immuno-electron microscopy (EM) (Kalamvoki et al., 2008). More recently, reports from various laboratories reignited the debate about the incorporation of ICP0 and ICP4 into mature virions (Delboy et al., 2010; Elliott et al., 2005; Loret et al., 2008; Maringer & Elliott, 2010; Radtke et al., 2010; Sedlackova & Rice, 2008). Finally, some studies suggested that ICP0 and ICP4 might be absent on HSV-1 intranuclear capsids (Maringer & Elliott, 2010; Radtke et al., 2010; Yao & Courtney, 1992), despite the presence of the two proteins in the nucleus. Given the role of these proteins, it was thus important to clarify whether these proteins are incorporated in the viroms and intranuclear capsids.

The process of tegument acquisition is poorly understood and involves a highly ordered network of protein–protein interactions (Lee et al., 2008; Mettenleiter, 2006; Vittone et al., 2005). Cysteines are often involved, given their highly reactive thiol side chains. The presence of LEA domains in the tegument proteins suggests that they may interact through disulfide bonds. Disulfide bonds are important in the organization of the viral capsid and tegument proteins. The present work focuses on the HSV-1 virion proteins UL7, UL23, ICP0 and ICP4. A detegumentation assay was used to confirm that they are virion components and to analyse their biochemical properties. In addition, pre-treatment of extracellular HSV-1 virions with NEM was employed to examine the importance of reduced cysteines in the organization of these viral molecules. The data show that these four proteins have rather different biochemical characteristics. UL7 and UL23 are both salt-extractable tegument proteins whose interaction with the virions was affected strongly by NEM. In contrast, ICP0 remained tightly associated with the capsid during biochemical detegumentation, as reported previously (Delboy et al., 2010; Maringer & Elliott, 2010; Radtke et al., 2010), but this association was completely resistant to NEM. These properties extended to ICP4, suggesting that both of these proteins are recruited to capsids via different types of interactions from those for UL7 and UL23. Unexpectedly, ICP0 and ICP4 were detected on intranuclear capsids isolated by two independent protocols, a finding suggesting that these proteins could at least partially be recruited to the virus particle at an early stage of HSV-1 egress.

RESULTS

Characterization of newly identified HSV-1 extracellular virion components

We recently reported the detection by mass spectrometry of the viral proteins UL7, UL23, UL50, UL55, ICP0 and ICP4 on mature HSV-1 particles (Loret et al., 2008). This is in line with some studies demonstrating the tight association of ICP0 with the capsid and the presence of salt-extractable ICP4 in the tegument (Delboy et al., 2010; Maringer & Elliott, 2010; Radtke et al., 2010), but in disagreement with another paper (Kalamvoki et al., 2008), thus warranting further study. In addition, the tentative assignment of UL7, UL23, UL50 and UL55 to the tegument layer remained to be demonstrated experimentally. A biochemical strategy was therefore employed. Whilst envelope proteins are extracted by detergents and tegument proteins are released gradually from virus particles with increasing salt concentrations, capsid-associated proteins are resistant to such treatment (Delboy et al., 2010; Maringer & Elliott, 2010; Ojala et al., 2000; Radtke et al., 2010; Wolfstein et al., 2006). It is thus possible to distinguish the main components of the virus biochemically. Highly purified virions were therefore prepared as reported previously (Loret et al., 2008) and lysed with a non-ionic detergent to remove the viral envelope in the presence of increasing salt concentrations to strip the tegument (0.1, 0.5 or 1.0 M NaCl). To prevent the release of a tegument by the detergent through a strong covalent disulfide bond with an envelope protein, DTT was present during the detegumentation in all buffers to clearly

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separate each viral layer (envelope, tegument and capsid). Virions were subsequently fractionated through a 20% (w/v) sucrose cushion to dissociate the released viral proteins (supernatant) from those that remained capsid-associated (pellet). As a control, the envelope was left intact by omitting the detergent, hence preventing the salt from releasing any tegument. Pellets and supernatants were examined by Western blot analysis (Fig. 1a) and quantified (Fig. 1b). Markers of the capsid (VP5), tegument (VP16) and envelope glycoprotein D (gD) were first analysed. As expected, VP5 was not affected by the detergent and at least 97 ± 4% of VP5 remained in the capsid pellet, even in high NaCl concentrations. In contrast, at least 72 ± 8% of gD was found in the supernatant irrespective of the salt concentration when the detergent was present. Finally, the tegument protein VP16 was gradually extracted from the virus by increasing salt concentrations in the presence of detergent (up to 86 ± 8% at 1.0 M salt). These controls behaved as reported previously (Delboy et al., 2010; Maringer & Elliott, 2010; Radtke et al., 2010; Wolfstein et al., 2006), thus confirming the relevance of the approach.

Given the clear capsid, tegument or glycoprotein fingerprint obtained for well-defined virion proteins, we next analysed our proteins of interest (Fig. 1). Unfortunately, the absence of antibodies for UL50 and UL55 prevented us from examining these two particular proteins. However, the results revealed that UL7 and UL23 were progressively stripped from the capsids by salt in the presence of detergent (up to 70 ± 3% of total UL7 and 97 ± 2% of total UL23). Unlike previous reports (Delboy et al., 2010; Radtke et al., 2010), ICP4 behaved like the major capsid protein VP5 and was not released from the capsid under high-salt conditions (97 ± 6% in the pellet). Finally, ICP0 was found exclusively in the pellet even in the presence of 1.0 M salt, as reported previously (Delboy et al., 2010; Maringer & Elliott, 2010; Radtke et al., 2010). These results indicated
strongly that ICP0 and ICP4 biochemically behave very differently from UL7 and UL23.

**Effect of NEM on UL7, UL23, ICP0 and ICP4**

Accumulating evidence suggests that reduced cysteines play a role in virion protein–protein interactions (Meckes & Wills, 2007, 2008; Yeh *et al.*, 2008). NEM is a common tool for studying these interactions, given its ability to alkylate reduced cysteines and prevent them from interacting with other residues. As it is membrane-permeable, it also prohibits the formation of novel irrelevant bonds when added before virion disruption (Meckes & Wills, 2008; Szczepaniak *et al.*, 2011). It was therefore used to probe the importance of reduced cysteines within HSV-1 particles. Highly purified extracellular HSV-1 virions were thus pre-treated with NEM prior to biochemical detegumentation. The capsid-associated and free viral proteins were again analysed by Western blotting for the three control proteins VP5, gD and VP16. Representative blots, as well as the quantification of three independent experiments (Fig. 2), revealed that the VP5 and gD salt-extraction patterns were unaltered by NEM. Essentially, all VP5 remained capsid associated (95 ± 8%), whereas >86 ± 5% of gD was released in the supernatant during lysis. In contrast, VP16 was now mostly associated with the capsid pellet (>96% in the pellet) instead of being gradually stripped by salt. These results indicate that NEM altered the organization of VP16 with the virus and favoured the interaction of VP16 with the capsid. We next sought to determine the behaviour of UL7, UL23, ICP0 and ICP4 in the presence of NEM (Fig. 2). Interestingly, UL7 and UL23 were also affected by NEM and followed the same pattern as VP16 by remaining in the capsid pellet despite high salt concentrations (>91% for UL7 and >94% for UL23), hinting once again at the participation of reduced cysteines in the organization of these two tegument proteins. Surprisingly, NEM did not alter the biochemical behaviour of ICP0

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**Fig. 2.** Western blot analysis of detegumented extracellular HSV-1 virions treated with NEM. (a) Highly purified virions (20 µg) were pre-treated with 10 mM NEM prior to biochemical detegumentation with or without detergent (‘no lysis’) and analysed by Western blotting as in Fig. 1. A mock-infected cell lysate (10 µg) was again included as the control. (b) The relative distribution of each protein in the pellet and supernatant was quantified from three independent experiments as in Fig. 1. Values are means ± SD.
and ICP4. In fact, both remained tightly associated with the capsid, suggesting a type of interaction with the virus particles distinct from that for UL7, UL23 and VP16.

**Presence of ICP0 and ICP4 on HSV-1 intranuclear capsids**

Unlike UL7 and UL23, ICP0 and ICP4 associated strongly with the capsid. This raised the possibility that ICP0 and ICP4 might already be associated with capsids in the nucleus. This is relevant because these proteins, much like VP16 and VP13/14, are present in the nucleus early in infection (Donnelly & Elliott, 2001; Wysocka & Herr, 2003; Yedowitz et al., 2005), where capsid assembly takes place.

To explore this possibility, two distinct protocols were employed. In the first case, we took advantage of our previously reported preparation protocol for nuclei devoid of cytoplasmic capsids (Rémillard-Labrosse et al., 2006). Hence, nuclei purified from infected cells were subsequently lysed and cleared by low-speed centrifugation to remove unbroken nuclei. The nuclear extract was then filtered through a 0.45 μm filter to remove large debris and centrifuged through a 35% (w/w) sucrose cushion to pellet all nuclear capsids. These enriched nuclear capsids were then negatively stained and examined by EM. As shown in Fig. 3(a), the capsid preparations consisted exclusively of naked capsids and no major contamination by cellular debris was observed. To probe the efficacy of this

**Fig. 3.** Evidence for the presence of ICP0 and ICP4 on HSV-1 nuclear capsids. (a) Total nuclear capsids isolated from purified infected nuclei were negatively stained and analysed by EM. The images show various typical views of the samples. All were naked capsids and exempt of obvious cellular debris, cellular organelles and cytoplasmic enveloped capsids. Bars, 100 nm. (b) Five micrograms of mock-infected or infected cell lysates, infected nuclei or total nuclear capsids were silver-stained (top panel) or analysed by Western blotting with a pan-HSV-1 antibody (bottom panel). Note the pattern of major capsid components, which are listed on the right of the silver stain. Viral enrichment of the nuclear capsids was also evident by Western blot analysis (see arrows). The molecular masses (kDa) of the protein markers (M, top panel) are indicated at the left of the gels. (c) Total nuclear capsids (100 μg) and infected nuclei (25 μg) were immunoblotted for the viral proteins VP5, ICP0, ICP4, the nuclear viral proteins VP16 and ICP8, the cytoplasmic UL7 and UL13 viral teguments, the nuclear host proteins PCNA, lamin A/C and nup62 or the cytoplasmic host protein γ-tubulin. As controls, mock-infected or infected cell lysates (25 μg each) were also included. ICP0 and ICP4 were reproducibly detected on the nuclear capsids.
non-classical purification method, the nuclear capsids were monitored by silver staining and by Western blotting using a pan-HSV-1 polyclonal antibody (Fig. 3b). The silver stain revealed a protein pattern consistent with an enriched virus preparation where the main capsid proteins are present (Maringer & Elliott, 2010; Ren et al., 2012; Salmon et al., 1998; Yu & Weller, 1998). Furthermore, the anti-HSV-1 blot highlighted a strong enrichment of numerous viral proteins compared with the infected cell lysate. We also probed VP5 as a loading control and several nuclear [host proliferating cell nuclear antigen (PCNA), lamin A/C, nucleoporin 62 (Nup62), viral VP16 or ICP8], or cytoplasmic (host γ-tubulin, viral UL7 and UL13 teguments) markers to examine the potential contamination by host and viral proteins or cytoplasmic virus particles. As expected, all proteins were present in infected cell lysates and the cytoplasmic UL7, UL13 and γ-tubulin were absent in infected nuclei (Fig. 3c). Most important, aside from VP5, all markers were undetectable on nuclear capsids, hinting at the purity of our preparations. In contrast, when probing for ICP0 and ICP4 with antibodies previously shown to be highly specific and non-reactive in ICP0− and ICP4− deletion mutants, respectively (Geoffroy et al., 2004), both proteins were consistently detected on intranuclear capsids. To confirm the above findings and to determine which of the nuclear capsids are ICP0 and/or ICP4 positive, a second independent protocol was used to purify the nuclear capsids by classical sucrose-gradient sedimentation, which allows separation of A, B and C capsids from one another and from cytoplasmic capsids (Bucks et al., 2007; Lamberti & Weller, 1998; Newcomb et al., 2006; Roller et al., 2000; Salmon et al., 1998; Sheaffer et al., 2000; Tatman et al., 1994). Silver staining of these intranuclear capsids (Fig. 4a) revealed a pattern similar to that observed previously (Fig. 3b). Most importantly, Western blotting confirmed the presence of ICP0 and ICP4 on C capsids and the absence of nuclear or cytoplasmic contaminants (Fig. 4b), in agreement with our previous findings. Interestingly, ICP0, but not ICP4, was also detectable on B capsids. However, neither protein was present on A capsids, even with prolonged exposures of the blots (data not shown). Taken together, these two independent methods clearly hinted at the presence of ICP0 and ICP4 on intranuclear capsids.

**DISCUSSION**

UL7 and UL23 are salt-extractable tegument proteins

The experiments presented in this paper biochemically confirm our previous mass spectrometry findings (Loret et al., 2008) that UL7 and UL23 proteins are indeed present in mature extracellular virions. Moreover, these two proteins share properties with the VP16 tegument (Fig. 1), suggesting strongly that UL7 and UL23 are true components of the HSV-1 viral tegument. Based on their
similar salt extractability, it would be interesting to know whether UL7 and UL23 are released quickly upon entry and whether they share with VP16 an early role upon infection. This is plausible as UL7 interacts with the pro-apoptotic mitochondrial protein ANT2, thereby modulating virus propagation (Tanaka et al., 2008). In the same way, an early release of UL23 in the cytoplasm would be logical, as this is where nucleotide metabolism occurs and UL23 facilitates replication of the viral genome (Coppack & Pardee, 1987; Munch-Petersen et al., 1995). However, these hypotheses need further work to clarify the role of UL7 and UL23 as components of the mature virions.

The biochemical analysis of protein–protein interactions in the virions revealed that the association of UL7, UL23 and VP16 with the capsid was modulated by NEM, which prevented their release from the capsids despite high salt concentrations (Fig. 2). The impact of NEM on the tegument is not surprising because VP16, UL7 and UL23 contain several cysteine residues (six, 12 and five, respectively). Thus, NEM alters the organization of the tegument and favours the association of these proteins with the capsid, as for UL16 and UL11 (Meckes & Wills, 2008). Given the presence of DTT throughout our detegumentation assay, this NEM phenotype is not due to the alteration of pre-existing or newly formed disulfide bonds, but rather highlights the contribution of reduced cysteines in the organization of the tegument. This may be particularly relevant in the context of the dynamic nature of the tegument during entry and post-entry steps (Meckes & Wills, 2008), its acquisition and maturation at the TGN (Meckes & Wills, 2007), as well as its reported maturation in extracellular viruses (Newcomb & Brown, 2009). Therefore, pre-existing reduced cysteines appear as determinants that are important for the interaction of UL7 and UL23 with other components of the virus particle and necessary for their release during detegumentation. However, the mechanism that governs this phenotype is not clear and it is difficult at this point to define the precise targets on which NEM acts, given the multitude of proteins present in the virus, including the potential 49 host proteins reported previously (Loret et al., 2008). Two explanations come to our mind. Firstly, NEM may induce a conformational change that forces these proteins to reassociate with components of the capsid. Alternatively, NEM could block a cysteine-exchange reaction that is needed for release of the tegument protein, reminiscent of the exchange reaction reported for the SU (surface) and TM (transmembrane) proteins of murine leukemia virus upon binding to its receptor (Pinter et al., 1997). Only further work will resolve this important issue.

**Tight association of ICP0 and ICP4 with the HSV-1 capsid**

The present study is consistent with the incorporation of ICP0 and ICP4 in mature virions (Delboy et al., 2010; Elliott et al., 2005; Loret et al., 2008; Maringer & Elliott, 2010; Radtke et al., 2010; Sedlackova & Rice, 2008; Yang & Courtney, 1995; Yao & Courtney, 1989, 1991, 1992). Biochemical analysis of these proteins further revealed that they do not behave as UL7, UL23 and VP16 and were never released from the capsids by detergent and salt (Fig. 1). These results are in agreement with recent studies reporting that ICP0 remains associated with the capsid under high-salt conditions (Delboy et al., 2010; Maringer & Elliott, 2010; Radtke et al., 2010). In contrast, the tight association of ICP4 with the capsid found here contrasts with reports that ICP4 is readily extracted by salt (Delboy et al., 2010; Radtke et al., 2010). This apparent discrepancy may be explained by the different methods employed to achieve detegumentation. In fact, many distinct parameters were noted, including m.o.i., time of harvesting, cell lines, temperature, density cushion, duration of centrifugation, sonicnation and presence of DTT. It thus remains to be seen how these factors affect ICP4 individually. In the scenario where ICP0 and ICP4 remain associated with the capsid, it would be interesting to know whether they partially travel to the nucleus with incoming capsids. This process could ultimately allow these proteins to translocate into the nucleus and quickly transactivate HSV-1 gene expression. Consistent with this idea, a role for ICP0 in capsid targeting to the nucleus was recently suggested by Delboy & Nicola (2011).

Although NEM altered the release of VP16, UL7 and UL23 from the capsid by salt, it had no detectable impact on ICP0 and ICP4 (Fig. 2). This is rather surprising given the many cysteines present in the two proteins (14 and 15, respectively). Thus, either all cysteines are already involved in intra-/intermolecular interactions or the available reduced cysteines do not appear as key players in the interaction of ICP0 and ICP4 with the virus particles.

**ICP0 and ICP4 are present on intranuclear HSV-1 capsids**

The strong association of ICP0 and ICP4 with the capsid is puzzling and unlike the other tegument proteins analysed in this study. Given the strong association and the role of ICP0 and ICP4 in the nucleus, it raised the possibility they may be recruited, at least partially, to newly assembled capsids before they exit that intracellular compartment. The work presented here is the first evidence that this might be the case. Although it is impossible to formally rule out that ICP0 and ICP4 are co-purifying contaminants or that the capsids are not sufficiently pure, we favour the scenario that the two proteins are indeed coating nuclear capsids. First, enriched nuclear preparations isolated by two independent methods were positive by Western blot analysis with highly specific antibodies. Secondly, both nuclear capsid preparations were devoid of visible cellular debris (Fig. 3 and data not shown). Thirdly, all tested potential nuclear and cytoplasmic contamination markers were absent from the samples (Figs 3 and 4). Finally, we only detected ICP0 and ICP4 on some nuclear capsids and
not others. For instance ICP0 was found on B and C capsids but not A capsids, while ICP4 was only present on C capsids. This feature is most consistent with the specific addition of these proteins to some nuclear capsids, rather than simply being non-specific contaminants. The data are thus fully consistent with our proposal that these proteins coat nuclear capsids. This seemingly contradicts previous reports (Maringer & Elliott, 2010; Radtke et al., 2010; Yao & Courtney, 1992). However, it is conceivable that lower-affinity antibodies and/or the amount of loaded sample previously hampered their detection. We thus believe that ICP0 and ICP4 indeed coat some nuclear capsids specifically. It is worth noting that an initial recruitment of ICP0 and ICP4 onto nuclear capsids is fully compatible with the subsequent cytoplasmic recruitment of these two viral proteins in a VP22- and/or ICP27-dependent manner (Elliott et al., 2005; Maringer & Elliott, 2010; Sedlackova & Rice, 2008). This therefore suggests that ICP0 and ICP4 may be recruited to the virus particles at multiple sites.

In conclusion, this study sheds light on the composition of the mature HSV-1 virions and confirms that the two new proteins UL7 and UL23, identified by mass spectrometry, are indeed incorporated into extracellular HSV-1 virions and are bona fide components of the tegument. This biochemical analysis also suggests the implication of reduced cysteines in the organization of the tegument in agreement with its dynamic nature. The data additionally support the presence of the two controversial proteins ICP0 and ICP4 into the mature extracellular particles and their tight association with the capsid, which is insensitive to thiol alkylation by NEM. Finally, but not least, we present the first evidence of the presence of ICP0 and ICP4 on purified HSV-1 intranuclear capsids, consistent with their partial early recruitment during egress.

**METHODS**

**Cells and viruses.** HeLa cells were grown at 37°C in 5 % CO2 in Dulbecco's modified Eagle's medium (Sigma Aldrich) supplemented with 10 % FBS (Medicorp), 2 mM l-glutamine (Invitrogen) and antibiotics (100 U penicillin ml\(^{-1}\) and 100 µg streptomycin ml\(^{-1}\); Invitrogen). For nuclear isolation, HeLa cells adapted to culture in suspension were grown at 37°C in Joklik’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 5 % FBS, 0.1 mM MEM non-essential amino acids solution (Invitrogen), 100 U penicillin ml\(^{-1}\) and 100 µg streptomycin ml\(^{-1}\). The wild-type HSV-1 strain F, generously provided by Beate Sodeik, was propagated on BHK (baby hamster kidney) cells and titrated on Vero cells as described previously (Remillard-Labrosse et al., 2006). They were then broken in lysis buffer [1 % IGEpal CA-630 (Sigma-Aldrich), 500 U DNase ml\(^{-1}\) (Roche) and chymostatin, leupeptin, aprotinin, pepstatin A (CLAP)] for 30 min at 4°C, briefly sonicated and cleared by 5 min at 300 g at 4°C. The supernatant was then filtered through a 0.45 µm filter, top-loaded onto a 35 % (w/w) sucrose cushion (top-loaded in MNT buffer and 10 mM DTT). Finally, the pellets were resuspended in 10 µM MNT buffer while the proteins in the supernatants were precipitated at 95°C for 15 min, centrifuged for 3 min at 18,000 g, resuspended in 10 µM MNT buffer and analysed by Western blotting. When indicated, the extracellular virions were pre-treated with 10 mM NEM (Sigma-Aldrich) for 30 min at 37°C before detegumentation.

**Nuclear capsids.** HeLa cells grown in suspension were infected with HSV-1 strain F at 37°C for 8 h at an m.o.i. of 5 and the nuclei were isolated as described previously (Remillard-Labrosse et al., 2006). They were then broken in lysis buffer [1 % IGEpal CA-630 (Sigma-Aldrich), 500 U DNase ml\(^{-1}\) (Roche) and chymostatin, leupeptin, aprotinin, pepstatin A (CLAP)] for 30 min at 4°C, briefly sonicated and cleared by 5 min at 300 g at 4°C. The supernatant was then filtered through a 0.45 µm filter, top-loaded onto a 35 % (w/w) sucrose cushion and the virions were pelleted for 1 h at 100,000 g at 4°C. The nuclear capsid pellet was finally resuspended in MNT buffer and stored at −80°C.

**Silver staining.** Following electrophoresis, gels were fixed for 2 h in a 1 % acetic acid/50 % methanol solution. Gels were then rinsed extensively in distilled water and incubated in 0.02 % sodium thiosulfate solution for 1 min. Gels were then briefly washed in distilled water and incubated in reaction buffer (0.04 % formaldehyde and 2 % carbonate sodium). The reaction was stopped by the addition of 5 % acetic acid solution.

**Separation of nuclear A, B and C capsids.** Capsids from the nuclear fraction were isolated as described previously with slight modifications (Bucks et al., 2007; Lamberti & Weller, 1998; Newcomb et al., 2006; Roller et al., 2000; Sheaffer et al., 2000). Approximately 2.5 x 10^6 HeLa were infected with HSV-1 strain F at an m.o.i. of 5. At 24 h post-infection, cells were scraped in ice-cold PBS and pelleted at 300 g for 10 min at 4°C. Cells were disrupted in NP-40 lysis buffer [1 % NP-40 (Sigma-Aldrich), 10 mM Tris/HCl pH 7.4, 150 mM NaCl, 2 mM MgCl2, 5 mM EDTA, 150 mM DTT and 1 % NP-40] and incubated on ice for 30 min. The nuclei were pelleted from the suspension at low speed. The nuclear pellet was resuspended in MNT, lysed by three cycles of freeze/thaw and briefly sonicated in the presence of CLAP. The
nuclear lysate was treated with 500 U DNase ml⁻¹ at 37 °C for 10 min and the debris was cleared by low-speed centrifugation. Nuclear capsids present in the supernatant were purified by two centrifugation steps. In the first step, capsids were pelleted through a 35 % (v/v) sucrose cushion prepared in MNT buffer (Beckman SW41 rotor, 100 000 g for 1 h at 4 °C). In the second step, the capsid pellet was carefully resuspended in MNT buffer, layered onto a 20–50 % (v/v) linear sucrose gradient (prepared in MNT buffer) and centrifuged at 100 000 g for 1 h at 4 °C in an SW41 rotor. A, B and C capsids were visualized as light-scattering bands and collected from the gradient by pipetting and analysed by Western blotting.

EM. Previously described procedures were used to evaluate sample purity by negative staining (Loret et al., 2008). Briefly, a drop (5 µl) of purified nuclear capsids was deposited on a Formvar-coated and carbonated 150-mesh copper grid (Canemco and Marivac), stained with 2 % uranyl acetate (Canemco and Marivac), washed in distilled water, allowed to air-dry on filter paper and finally observed with a Philips 300 transmission electron microscope.

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