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The abundance of the herpes simplex virus type 1 UL37 tegument protein in virus particles is closely controlled

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The tegument region of herpes simplex virus type 1 virus particles contains approximately 15 virus-encoded polypeptide species. One of the less abundant species is a 120 kDa protein specified by gene UL37. The abundance of the UL37 protein in infected cells was increased about 20-fold by replacing the native promoter for gene UL37 with the strong immediate early promoter of human cytomegalovirus. This rise in abundance did not induce any detectable increase in the amount of UL37 protein incorporated into virus particles. These data contrast with those previously obtained for a second tegument protein VP22 whose level of incorporation was elevated by increasing its abundance in infected cells. Thus, for the UL37 protein, a mechanism exists to control its abundance in the tegument. Moreover, data obtained with light particles which lack the viral capsid suggest that this controlled incorporation is not directed by interaction with the capsid structure.

The tegument is the structure within infectious herpesvirus particles which lies between the nucleocapsid and the outer envelope (Wildy *et al.*, 1960). In herpes simplex virus type 1 (HSV-1) virions, it is estimated that there are at least 15 virus-encoded polypeptides located in the tegument (Heine *et al.*, 1974; reviewed in McGeoch *et al.*, 1993). Most of these polypeptides are also present in L-particles, which are non-infectious particles released from HSV-1-infected cells that contain tegument and envelope proteins but lack a nucleocapsid (Szilágyi & Cunningham, 1991; McLauchlan & Rixon, 1992). While the genes which encode most of the tegument components have been identified, very little is known about the pathway of tegument assembly and the mechanisms that control which proteins are incorporated and their abundance in mature virus particles. To date, the only HSV-1 tegument protein shown to be essential for virus assembly is VP16, which is encoded by gene UL48 (Ace *et al.*, 1988; Weinheimer *et al.*, 1992). Studies on deletion mutants have revealed that in the absence of other tegument components, including the abundant species VP11/12 and VP13/14, virus assembly can continue and viable virus is produced (Fenwick & Everett, 1990; Coulter *et al.*, 1993; Zhang & McKnight, 1993). Thus, the tegument would appear to be capable of accommodating significant changes in composition without disabling virus production. Recently, we have shown that increased levels of expression of the abundant tegument protein VP22, which is encoded by UL49 (Elliott & Meredith, 1992), induce a rise in its abundance in virions and L-particles (Leslie *et al.*, 1996). These studies suggested that, at least for VP22, there is a correlation between level of expression and the abundance of the protein in virus particles.

To determine whether this property is shared by other tegument proteins, the effect of increasing the synthesis of another component of the tegument, that encoded by gene UL37, on the composition of virus particles has been analysed. UL37 encodes a 120 kDa protein which is present as a minor component of the tegument (McLauchlan *et al.*, 1994; Schmitz *et al.*, 1995). This polypeptide is common to both virions and L-particles and appears to be present in similar quantities in the two types of virus particle (McLauchlan *et al.*, 1994). The protein is phosphorylated from early stages of virus infection (Albright & Jenkins, 1993) and has a diffuse distribution within infected cells, but is more abundant in the cytoplasm than the nucleus (McLauchlan *et al.*, 1994; Schmitz *et al.*, 1995).

In a previous report, a virus recombinant, vUL37, was constructed that expressed an epitope-tagged version of the UL37 protein under the control of the native UL37 promoter; the epitope tag was derived from the human cytomegalovirus (HCMV) UL83 gene (McLauchlan *et al.*, 1994). To create a virus recombinant which expressed high levels of UL37 protein, a plasmid, pCMV3710, was constructed (Fig. 1). This plasmid contains epitope-tagged UL37 coding sequences under the control of the HCMV immediate early (IE) promoter, flanked by sequences from the UL36 and UL38 genes. As a consequence of the cloning strategy, the UL37 gene was also flanked by *Spe*I restriction enzyme sites (Fig. 1*d*). Plasmid

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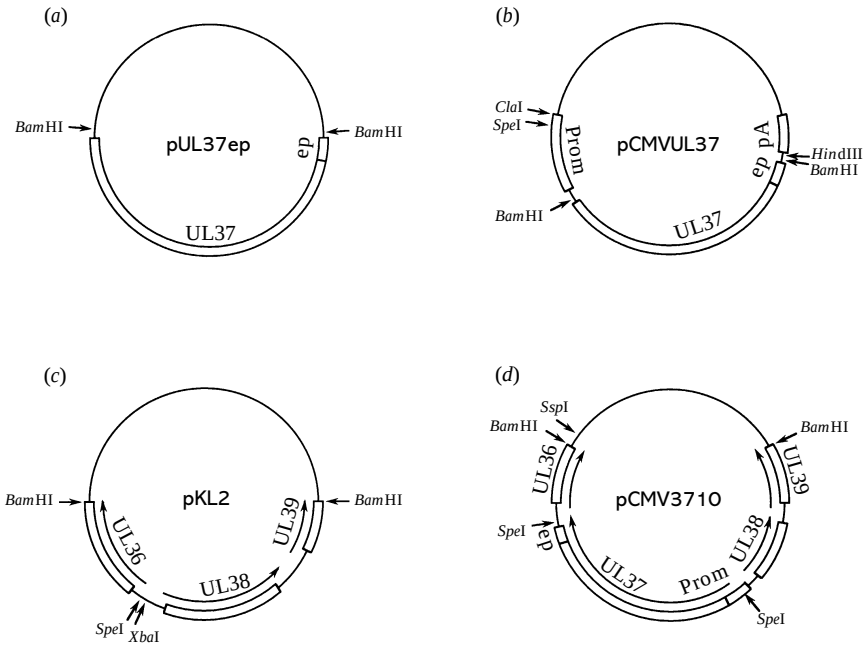

Fig. 1. Construction of plasmid pCMV3710. (*a*) Plasmid pUL37ep was derived from construct pUL371 which consisted of a 3·5 kbp *Cla*I–*Hin*dIII fragment (residues 84171–80707; McGeoch *et al*., 1988) from the HSV-1 strain 17 genome inserted into the *Acc*I–*Hin*dIII sites of pGEM-1 (Promega). The *Cla*I site lies 90 bp upstream from the 5′ terminus of the UL37 ORF and the *Hin*dIII site is 8 bp downstream from its 3′ terminus. Plasmid pUL371 was modified by inserting an oligonucleotide at the *Hin*dIII site, which destroyed this site but created a novel *Bam*HI site (plasmid pUL373), followed by inserting an oligonucleotide containing sequences encoding the epitope tag (ep) into a unique *Spe*I site at the 3′ terminus of the UL37 ORF; the product of these cloning steps was pUL37ep. Thus, pUL37ep lacks the UL37 promoter sequences (Flanagan *et al*., 1991) and the tagged UL37 ORF is flanked by *Bam*HI sites; the *Bam*HI site at the 5′ end of the UL37 sequences is derived from the multiple cloning site in pGEM-1. The nucleotide sequence for the oligonucleotide encoding the tag has been published previously and its insertion disrupts the *Spe*I site (McLauchlan *et al*., 1994). (*b*) Plasmid pCMVUL37 was created by inserting the *Bam*HI fragment from pUL37ep into the *Bam*HI site of plasmid pCMV10. The position of a naturally occurring *Spe*I site in the HCMV IE promoter sequences (Prom) is indicated. (*c*) Plasmid pKL2 is derived from a construct pGX197 which consists of *Bam*HI fragment h (residues 79441–86980; McGeoch *et al*., 1988) from the HSV-1 strain 17 genome inserted into the *Bam*HI site of pAT153. Plasmid pKL2 was made by removing an *Hpa*I–*Afl*II fragment (residues 80725–84250) from pGX197 and inserting a linker containing an *Xba*I site. Removal of these sequences deleted almost the entire UL37 sequences including the TATA box and 5′ terminus for UL37 mRNA (Flanagan *et al*., 1991); the UL37 coding sequences which remain in pKL2 are 16 residues at the 3′ terminus of the ORF which include the *Spe*I site at position 80721. The regions of the UL36, UL38 and UL39 ORFs present in pKL2 are indicated. (*d*) Plasmid pCMV3710 was constructed by inserting the *Cla*I–*Hin*dIII fragment from pCMVUL37 into the *Xba*I site in pKL2; the sites in both the fragment and plasmid were end-filled prior to ligation. The *Ssp*I site used to linearize the plasmid prior to transfection with HSV-1 strain 17 DNA is shown.

pCMV3710 was linearized with *Ssp*I (Fig. 1*d*) and co-transfected into BHK C13 cells with wild-type HSV-1 strain 17 DNA which had been digested with *Spe*I. The *Spe*I site is unique in the HSV-1 genome and lies 11 bp upstream from the 3′ terminus of the UL37 ORF (McGeoch *et al*., 1988). Progeny virus from the transfection was harvested and titrated on BHK C13 cells. A total of 48 individual plaques were selected and grown, and the infected cells from each isolate were screened for the presence of epitope-tagged protein by Western blot analysis. Three plaques which expressed tagged UL37 were then selected for further plaque purification. Finally, one virus isolate which had been purified to homogeneity was selected for further study and called vUL37IEP. Southern blot analysis verified that the HCMV IE promoter was linked to the UL37 ORF in vUL37IEP viral DNA and the modified UL37 gene was flanked by *Spe*I restriction enzyme sites (data not shown). Hence, the native UL37 coding sequences and promoter had been removed from wild-type DNA and vUL37IEP expressed only the modified version of the UL37 gene.

Comparison of the polypeptides made by vUL37IEP with those made by vUL37 revealed that there were few differences in the patterns obtained from infected cell extracts except for the presence of an abundant protein of approximately 120 kDa in the vUL37IEP samples (Fig. 2*a*, compare lanes 2 to 6 with lanes 7 to 11). This polypeptide was made in high quantities throughout the course of infection relative to other virus-encoded proteins (Fig. 2*a*, lanes 7 to 11). Western blot analysis confirmed that this species corresponded to epitope-tagged UL37 protein (data not shown). Thus, high levels of expression of UL37 protein can be achieved under the control of the HCMV IE promoter.

The amount of UL37 protein present in vUL37IEP virus

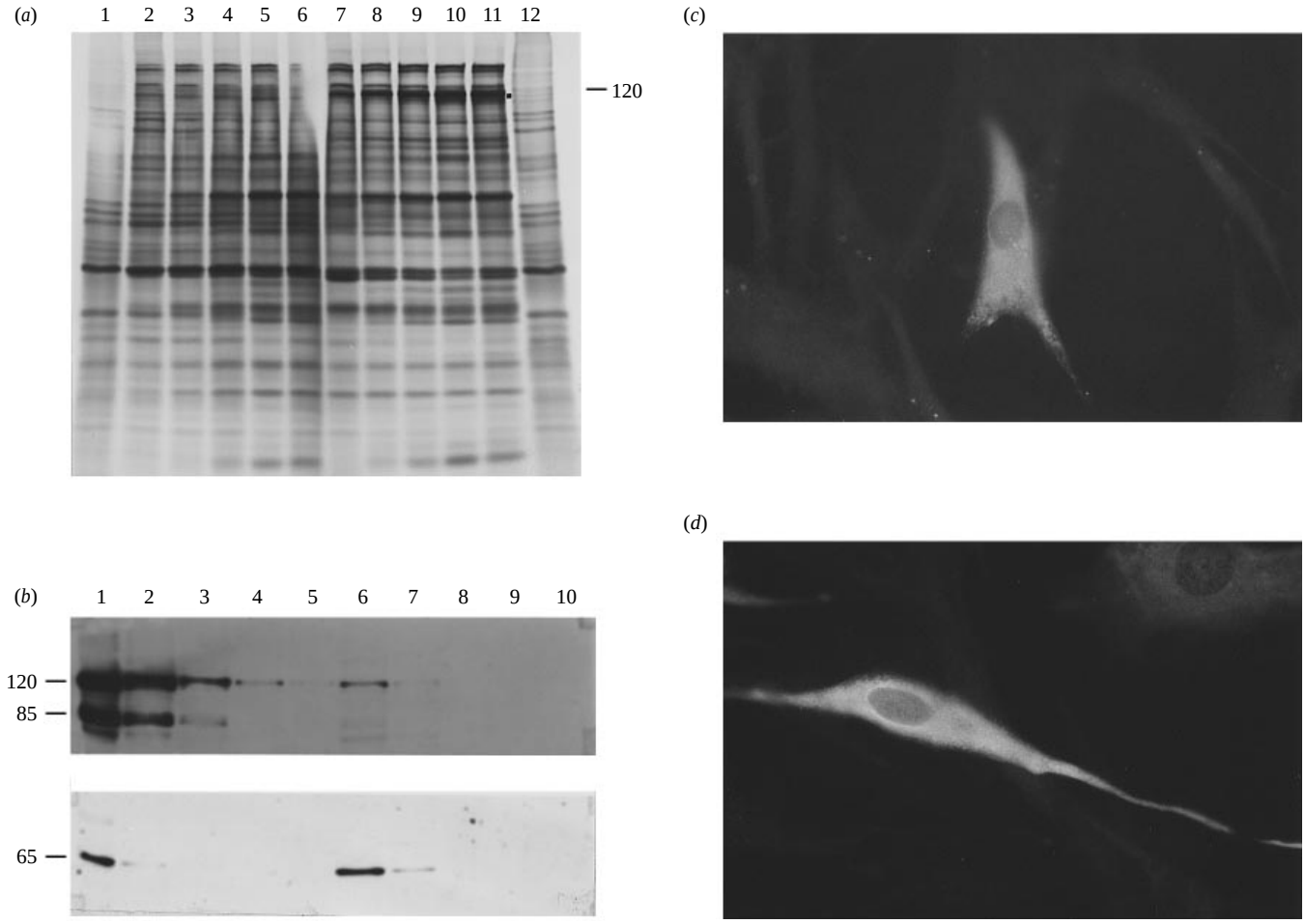

Fig. 2. Comparison of the abundance and intracellular distribution of UL37 protein in vUL37- and vUL37IEP-infected cells. (*a*) BHK C13 cells were either mock-infected or infected with virus at an m.o.i. of 5 p.f.u. per cell at 37 °C and radiolabelled with [^{35}S]methionine from 4 h after infection; in the case of samples harvested at 4 h, cells were radiolabelled from 2 h post-infection (p.i.). Whole cell extracts were prepared at 4 h intervals p.i. and samples were electrophoresed on a 10% polyacrylamide gel (Marsden *et al*., 1978). Lanes 1 and 12, mock-infected cells; lanes 2 and 7, 4 h p.i.; lanes 3 and 8, 8 h p.i.; lanes 4 and 9, 12 h p.i.; lanes 5 and 10, 16 h p.i.; lanes 6 and 11, 20 h p.i. Lanes 2 to 6 contain extracts from vUL37-infected cells, and lanes 7 to 11 contain extracts from vUL37IEP-infected cells. The 120 kDa polypeptide corresponding to UL37 protein, which is over-expressed in vUL37IEP-infected cells is indicated (■). (*b*) Four 2 litre roller bottles of confluent BHK C13 cells were infected with either vUL37IEP or vUL37 at an m.o.i. of 1:300 at 31 °C for 4 days. Cells were recovered and whole cell extracts were prepared. Dilutions of these extracts were electrophoresed on 10% polyacrylamide gels and then transferred to nitrocellulose membranes (Towbin *et al*., 1979). Membranes were probed with monoclonal antibody 9220 (1:1000 dilution; upper panel). After detection of the epitope-tagged UL37 protein, followed by removal of the bound antibody (McLauchlan *et al*., 1994), the membrane was re-probed with the VP16-specific antibody LP1 (1:1000 dilution; lower panel). The infected cell equivalents present in each lane are as follows; lanes 1 and 6, 6×10^4; lanes 2 and 7, 2×10^4; lanes 3 and 8, $6{\cdot}6 \times 10^3$; lanes 4 and 9, $2{\cdot}2 \times 10^3$; lanes 5 and 10, $7{\cdot}4 \times 10^2$. Lanes 1 to 5 and 6 to 10 contain vUL37IEP and vUL37 extracts, respectively. The 120 kDa protein encoded by UL37, a 85 kDa breakdown product of this polypeptide and the 65 kDa species representing VP16 are indicated. (*c* and *d*) Intracellular distribution of UL37 protein. BHK C13 cells were infected at an m.o.i. of 1:10 with vUL37 (*c*) and vUL37IEP (*d*). At 16 h after infection, cells were fixed by incubation in methanol at −20 °C for 20 min. Following rehydration and washing in PBS containing 2% newborn calf serum, cells were incubated with antibody 9220 (1:300 dilution) for 2 h at room temperature. Detection of primary antibody was performed with fluorescein conjugate (1:100 dilution) for 2 h at room temperature. After washing, cells were mounted and examined under a Nikon microphot-SA fluorescence microscope.

particles was analysed by purifying, on 5–15% Ficoll gradients (Szilágyi & Cunningham, 1991), virus preparations from four roller bottles of BHK C13 cells which had been infected with the virus at an m.o.i. of 1:300 p.f.u. per cell for 4 days at 31 °C. From these gradients, virions and L-particles were collected and concentrated for further analysis. For comparative purposes, virions and L-particles were made in parallel from an identical number of roller bottles which had been infected with

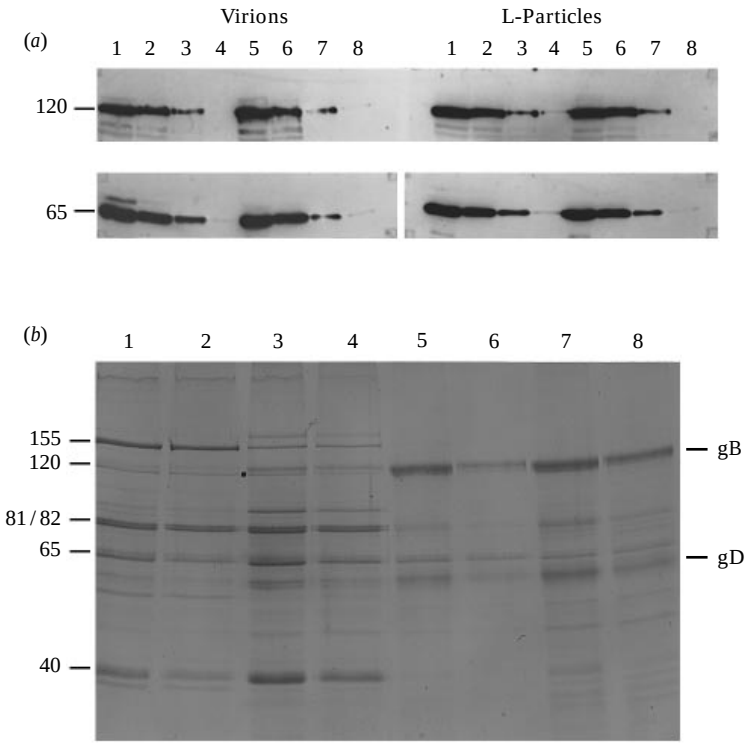

Fig. 3. Abundance of UL37 protein in vUL37IEP and vUL37 virus particles. Virion and L-particles, prepared from the media of infected cell preparations described in the legend to Fig. 2*b*, were purified on 5–15% Ficoll gradients (Szilágyi & Cunningham, 1991). Numbers of virus particles were estimated by comparison with a standard preparation of latex beads (Rixon *et al*., 1990). (*a*) Dilutions of purified particle preparations were separated on 10% polyacrylamide gels and then transferred to nitrocellulose membranes. Membranes were probed with monoclonal antibody 9220 (1:1000 dilution; upper panel). After detection of the epitope-tagged UL37 protein, followed by removal of the bound antibody, the membrane was re-probed with antibody LP1 (1:1000 dilution; lower panel). The numbers of virus particles present in each lane are as follows; lanes 1 and 5, 3×10^9; lanes 2 and 6, 1×10^9; lanes 3 and 7, $3{\cdot}3 \times 10^8$; lanes 4 and 8, $1{\cdot}1 \times 10^8$. Lanes 1 to 4 and 5 to 8 contain vUL37IEP and vUL37 virus particles, respectively. The polypeptides corresponding to epitope-tagged UL37 (120 kDa) and VP16 (65 kDa) are indicated. (*b*) 3×10^9 virions or L-particles were treated with 1% NP40 and soluble and insoluble fractions were prepared (McLauchlan & Rixon, 1992). Samples were electrophoresed on a 10% polyacrylamide gel and proteins were visualized by staining with Coomassie brilliant blue. Lanes 1 to 4 contain insoluble fractions and lanes 5 to 8 contain soluble fractions. Samples in each lane were as follows: lanes 1 and 5, vUL37IEP virions; lanes 2 and 6, vUL37 virions; lanes 3 and 7, vUL37IEP L-particles; lanes 4 and 8, vUL37 L-particles. The apparent molecular masses of the major structural species VP5 (155 kDa), VP13/14 (81/82 kDa), VP16 (65 kDa) and VP22 (40 kDa) are indicated. Also shown are the bands corresponding to the major glycoproteins gB and gD. The 120 kDa species representing the UL37 protein is indicated (■).

vUL37. To estimate the relative amounts of UL37 protein present in the cells from which the virus preparations were made, the cells were retained and whole cell extracts were made. Dilutions of these extracts were electrophoresed on polyacrylamide gels and the proteins were transferred to nitrocellulose membranes and then probed with monoclonal antibody 9220, which recognizes the epitope tag linked to the UL37 coding sequences (Fig. 2*b*; McLauchlan *et al.*, 1994). This revealed that tagged UL37 protein could be readily detected in the vUL37IEP extracts down to $2{\cdot}2 \times 10^3$ cell equivalents (lanes 1 to 5). In the vUL37 sample, UL37 protein was recognized at 6×10^4 cell equivalents, but barely detected in subsequent dilutions of the extract (lanes 6 to 10). To verify that these dilutions of the extracts contained similar quantities of other infected cell proteins, the membranes were also probed with LP1, a monoclonal antibody which recognizes the abundant tegument protein VP16 (McLean *et al.*, 1982). This antibody detected similar amounts of VP16 in equivalent dilutions of the vUL37IEP and vUL37 samples (Fig. 2*b*). From these data, it was concluded that there is an approximately 20-fold increase in the amount of UL37 protein in vUL37IEP-infected cells as compared to that in vUL37-infected cells. A similar relative rise was found in comparisons of extracts which had been prepared from cells infected with vUL37IEP and vUL37 at a high m.o.i. for 18 h (data not shown). For estimating the relative amount of tagged UL37 protein in virions and L-particles, Western blot analysis was performed on various quantities of virus particles (Fig. 3*a*). Results indicated that similar quantities of UL37 protein could be detected in equivalent numbers of vUL37IEP and vUL37 virus

particles. Thus, the protein can be recognized in $3{\cdot}3 \times 10^8$ particles, but not on further dilution of either virion or L-particle samples for both viruses (Fig. 3*a*, top panel). Again, the membranes were probed with LP1 and this revealed similar quantities of VP16 protein in equivalent dilutions of the virus particles (Fig. 3*a*, bottom panel).

The quantity of UL37 protein in these samples was further examined by treating virions and L-particles with detergent, which solubilizes the envelope components and allows their separation, by centrifugation, from the capsid and tegument proteins. Under such circumstances, UL37 protein (which co-migrates with the major envelope species gB, gC and gH) sequesters almost exclusively with the insoluble material and thus can be visualized (McLauchlan *et al.*, 1994). Following detergent treatment, both soluble and insoluble fractions were separated on a polyacrylamide gel and the bands were stained with Coomassie brilliant blue (Fig. 3*b*). This revealed that the intensity of the 120 kDa band in the insoluble fraction of vUL37IEP virions and L-particles, which contains UL37 protein, did not increase relative to the corresponding band in the insoluble fraction from detergent-treated vUL37 virus particles (Fig. 3*b*, compare lanes 1 and 3 with lanes 2 and 4, respectively). Two other preparations of virus particles purified from vUL37IEP- and vUL37-infected cells, which were again grown in parallel, gave identical results to those shown in Figs 2(*b*) and 3. Thus, it was concluded that the stimulation of expression of UL37 to very high levels did not induce an increase in its abundance in either virions or L-particles.

In this report, it has been shown that replacing the natural promoter of the UL37 gene in the HSV-1 genome with the HCMV IE promoter stimulates the expression of UL37 protein by approximately 20-fold. However, the elevated levels of this protein in infected cells do not result in any detectable rise in the amount of UL37 protein which is incorporated into the tegument of either virions or L-particles. From immunofluorescence studies of cells infected with vUL37IEP, there is no apparent difference in the distribution of UL37 protein as compared to that in vUL37-infected cells (Fig. 2*c*, *d*). Thus, it is unlikely that the lack of any change in the abundance of the protein in virus particles is due to mechanisms such as sequestration or formation of insoluble aggregates, which would render the protein unavailable for incorporation into the tegument.

The mechanisms that regulate the assembly and composition of the tegument are poorly understood. Many of the components of the tegument are not essential for assembly and, in certain cases, virus particles which lack major tegument proteins contain higher levels of other tegument and envelope components (Zhang & McKnight, 1993). More recently, we have shown that increasing the level of expression of VP22, one of the most abundant tegument proteins, results in a 3-fold rise in its abundance in both virions and L-particles (Leslie *et al.*, 1996). Taken together, these data suggest that there is inherent flexibility in the composition of the tegument and that the amount of protein which is incorporated can be modified by altering the level of expression of certain components. By contrast, the results presented here demonstrate that the abundance of UL37 protein within virus particles is tightly controlled.

One possibility is that there are only a limited number of sites within the tegument which can accommodate the UL37 protein. Limitations on the amount of UL37 protein which can be incorporated could result from interaction of this polypeptide with a component of the capsid. The stoichiometry of the proteins present in the capsid is essentially invariant (Newcomb *et al.*, 1993) and this in turn could impose limits on the abundance of tegument components which interact with the capsid. However, the UL37 protein is present in equal abundance in vUL37IEP virions and L-particles and thus, while the UL37 protein could interact with the capsid, any such interaction cannot be the primary determinant which regulates the abundance of this protein in virus particles. Previous studies have indicated that the protein binds to the major DNA binding protein, ICP8, which is not a structural component (Albright & Jenkins, 1993). Although it is possible that this interaction plays a role in UL37 protein incorporation into the tegument, further studies are required to determine whether the protein interacts with other species within the infected cell, in particular, with other structural components. Recent studies have demonstrated that interactions occur between different tegument proteins and between tegument proteins and envelope proteins (Smibert *et al.*, 1994; Zhu & Courtney, 1994; Elliott *et al.*, 1995). Characterization of those interactions involving the UL37 protein would enable a clearer picture to emerge of the pathways which generate the tegument.

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