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Assembly and egress of human cytomegalovirus particles.

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ASSEMBLY AND EGRESS OF HUMAN CYTOMEGALOVIRUS PARTICLES

by

VERONICA SANCHEZ

A DISSERTATION

Submitted to the graduate faculty of the University of Alabama at Birmingham in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT OF DISSERTATION
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Title Assembly and Egress of Human Cytomegalovirus Particles

Human cytomegalovirus (HCMV) is the largest member of the herpesvirus family. This virus is an ubiquitous agent in humans and an important pathogen for immunocompromised patients. Several approaches have been previously used to understand the complex assembly of the infectious particle; however, few studies have concentrated on the morphogenesis of the viral tegument as a means to study both nuclear egress and envelopment of the maturing virion.

In this study, we have examined the distribution of several key tegument proteins in HCMV-infected cells. Our observations suggested that, unlike the prototypical herpesvirus, herpes simplex virus type 1, HCMV incorporated its tegument proteins in nuclear and cytoplasmic compartments. These results implied that envelopment must also occur in the cytoplasm of the infected cell. Our data supported a model of nuclear egress in which the partially-tegumented

subviral particles exit the nucleus by a process of envelopment/deenvelopment at the nuclear membrane.

We have also exmained the association of HCMV structural components with the nuclear matrix of infected cells. Our data suggested that HCMV replication and assembly were linked to this subnuclear structure which could, in turn, provide spatial coordinattion to these temporally-regulated processes. pp65 was observed to associate with the nuclear lamina of the infected cell, suggesting that this protein could play a role in egress of particles from the nucleus.

An examination of the signals which mediated nuclear localization of pp65 showed that the protein sequence contained multiple signals which modulated the accumulation of this protein in the nucleus. The signals exhibited cell-type specificity as a newly described nuclear targeting signal was only effective in proliferating cells. These observations emphasized the importance of selecting an appropriate cell type to study the trafficking of viral proteins in recombinant systems.

Taken together, our data indicated that our approach of using a combination of biochemical an imaging techniques to study the composition and assembly of HCMV tegument can provide a new understanding of virion morphogensis.

DEDICATION

For Al.

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gratitude for their strength, advice, and friendship. Each of you has been an inspiration and I was truly blessed to have met you.

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LIST OF ABBREVIATIONS

aa	amino acid
Ab	antibody
°C	degrees centigrade
DBs	dense bodies
cdk	cyclin-dependent kinase
CMV	cytomegalovirus
C-terminal	carboxy-terminal
CTL	cytotoxic T-lymphocyte
EBV	Epstein-Barr virus
EM	electrom microscopy
ER	endoplasmic reticulum
FW	far-Western blot
gB	glycoprotein B
gC	glycoprotein complex
gD	glycoprotein D
gE	glycoprotein E
gH	glycoprotein H
gI	glycoprotein I
gL	glycoprotein L

gM	glycoprotein M
gp	glycoprotein
HC	heavy chain of immunoglobulin
HCMV	human cytomegalovirus
HHV-6	human herpesvirus 6
HHV-7	human herpesvirus 7
HHV-8	human herpesvirus 8
IC	intermediate compartment
IE	immediate early
INM	inner nuclear membrane
Ig	immunoglobulin
kb	kilobase
kDa	kilodaltons
mAb	monoclonal antibody
mM	millimolar
MPR^{ci}	cation-independent mannose-6-phosphate receptor
Mr	relative molecular weight
MW	molecular weight
NIEP	non-infectious enveloped particle
NM	nuclear matrix
NME	nuclear matrix extracts

orf	open reading frame
ONM	outer nuclear membrane
PBS	phosphate-buffered saline
pp	phosphoprotein
PrV	pseudorabies virus
RER	rough endoplasmic reticulum
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TGN	trans-Golgi network
UL	unique long
US	unique short
VZV	varicella-zoster virus

INTRODUCTION

Human cytomegalovirus (HCMV) is the largest member of the herpesvirus family. HCMV is the prototype virus of the beta-herpesvirus genus which is composed of several closely related viruses that infect a wide range of animals but demonstrate exquisite host specificity. Cytomegaloviruses of mice, guinea pigs, rats, rhesus macaques, and other animals have been described (51).

The importance of HCMV as a human pathogen is underscored by its prevalence in the population. It is estimated that 50-90% of the population is infected with HCMV (8, 29). HCMV remains the leading cause of congenital viral infection in humans with an incidence of 0.2-2.2% of live births in the United States (73). Among these intrauterine infections, the incidence of severe infection is estimated to be at least 10%. Several of these symptoms result from damage to the CNS and can include permanent sequelae of hearing and visual impairment, learning disabilities, and, in the most severe forms, psychomotor retardation and death (8). The risk of symptomatic HCMV infection in immunocompetent adults

is minimal; however, HCMV infection is a relevant health issue to immunocompromised hosts such as allograft recipients and others treated with immunosuppressive drugs as well as to people infected with HIV (8, 57, 72). Therefore, many studies of HCMV have focused on development of antiviral agents or vaccines to generate protective responses in susceptible individuals. Development of these therapies has been dependent on our understanding of the various aspects of viral replication and assembly in infected cells.

The Herpesvirus Family: Properties of Subfamilies

The herpesvirus family is subdivided into three groups, alpha, beta, and gamma, based on the biological properties of each member. Each subfamily is further subdivided into genera based on sequence homologies and similarities between viral proteins (63, 64). The prototype of the family is herpes simplex virus (HSV) of the alpha subfamily which also includes varicella zoster virus (VZV) and pseudorabies virus (PrV). These viruses have short replication cycles and variable host ranges and characteristically infect cells of the nervous system in their natural hosts. They are also capable of establishing reactivatable latency in the sensory ganglia of the infected animal (50, 63, 74, 76).

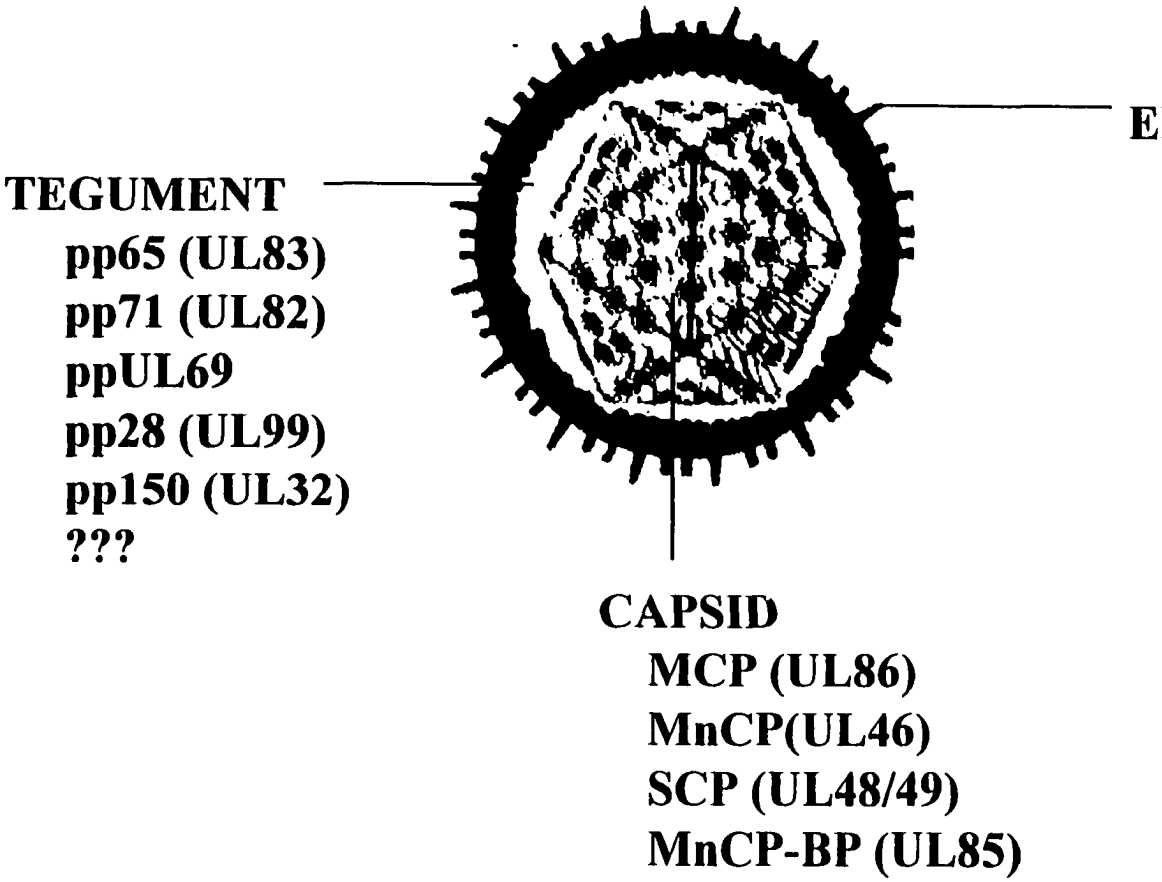
The subfamily beta-herpesviridae contains all of the cytomegaloviruses including HCMV and the recently isolated human herpesviruses 6 and 7 (HHV-6 and HHV-7). In general, these viruses exhibit restricted host range and long replication cycles. Latency is established in secretory epithelium and probably also in hematopoietic progenitor cells (16, 25, 63, 69).

The gamma herpesviruses, divided into lymphocryptoviruses and rhadinovirus, are lymphotropic viruses which have limited host ranges. All replicate to low titers in either B- or T-cells in culture, and latency is established in these same cells. Only two members of the subfamily are known to infect humans. Epstein-Barr virus (EBV) is the best studied virus in this group; however, much recent work has focused on the characterization of the newly described HHV-8 or Kaposi's sarcoma associated virus (52, 63, 83, 84).

HCMV Genome and Virion Structure

HCMV is structurally similar to other herpesviruses in that the infectious virion consists of an icosahedral capsid, an amorphous tegument, and a complex envelope containing several virally-encoded glycoproteins (Fig. 1). The 240 kbp genome contains greater than 200 open reading frames

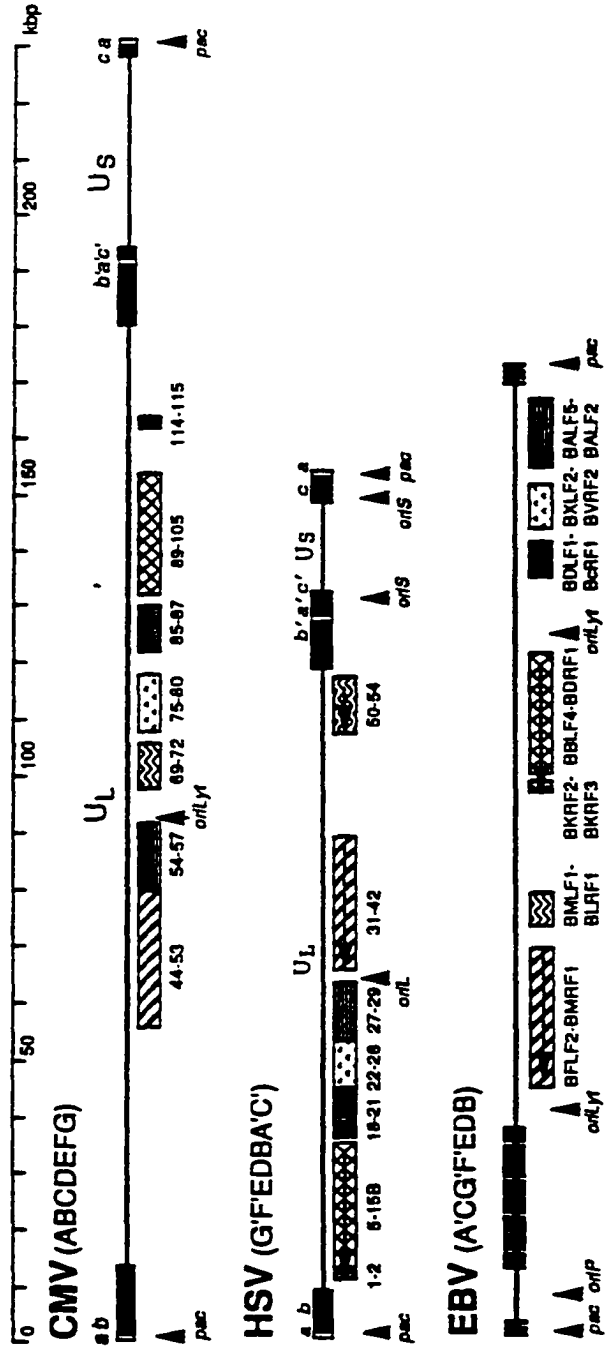
FIG. 1. Schematic of HCMV virion structure (adapted from reference 63). The virion consists of three structures: an icosahedral capsid, a lipid envelope containing virally-encoded glycoproteins, and an amorphous tegument. Experimentally defined components of each structure are listed.



(ORFS) and at least 30 proteins have been identified (15, 51).

A schematic of the HCMV genome is shown in Fig. 2. Like herpes simplex viruses, the double-stranded DNA can exist in one of four isomers based on the arrangement of long (L) and short (S) segments of the genome (51). HCMV is the only cytomegalovirus that exhibits this isomeric polymorphism. The genome has been subdivided into seven conserved sequence blocks (A-G) relative to the genomes of the other herpesviruses. The organization of ORFS into nine families containing related genes has suggested that many of the genes arose from duplication. The functions of several HCMV proteins were deduced because of sequence homology to gene products of other herpesviruses. Based on these and other similarities between members of the virus family, it has been estimated that approximately one-quarter of the HCMV genome encodes proteins necessary for viral replication and metabolism and that the other three-quarters encode for virion structural components (51). Of the 30 or so HCMV proteins identified to date, several have structural homologs in HSV; however, a few of the more abundant virion proteins have no homologs among the other herpesviruses.

FIG. 2. Conserved blocks of sequence between human CMV, HSV-1, and EBV (adapted from reference 51). The top line is a scale of kilobase pairs. The second line depicts the prototype arrangement of the CMV genome with conserved sequence blocks (A-G) shown below the UL genome segment. The third line depicts the prototype arrangement of the HSV-1 genome, and the fourth line depicts the EBV genome, each with conserved sequence blocks shown below. Numbers below the sequence blocks indicate the ORFs contained within each block. Leftward pointing arrows within blocks below the HSV-1 and EBV genomes denote the inverted orientation of the blocks relative to CMV. A lytic origin of DNA replication (oriLyt) is indicated on the HCMV genome, and packaging signals (pac) are indicated near the genomic termini.

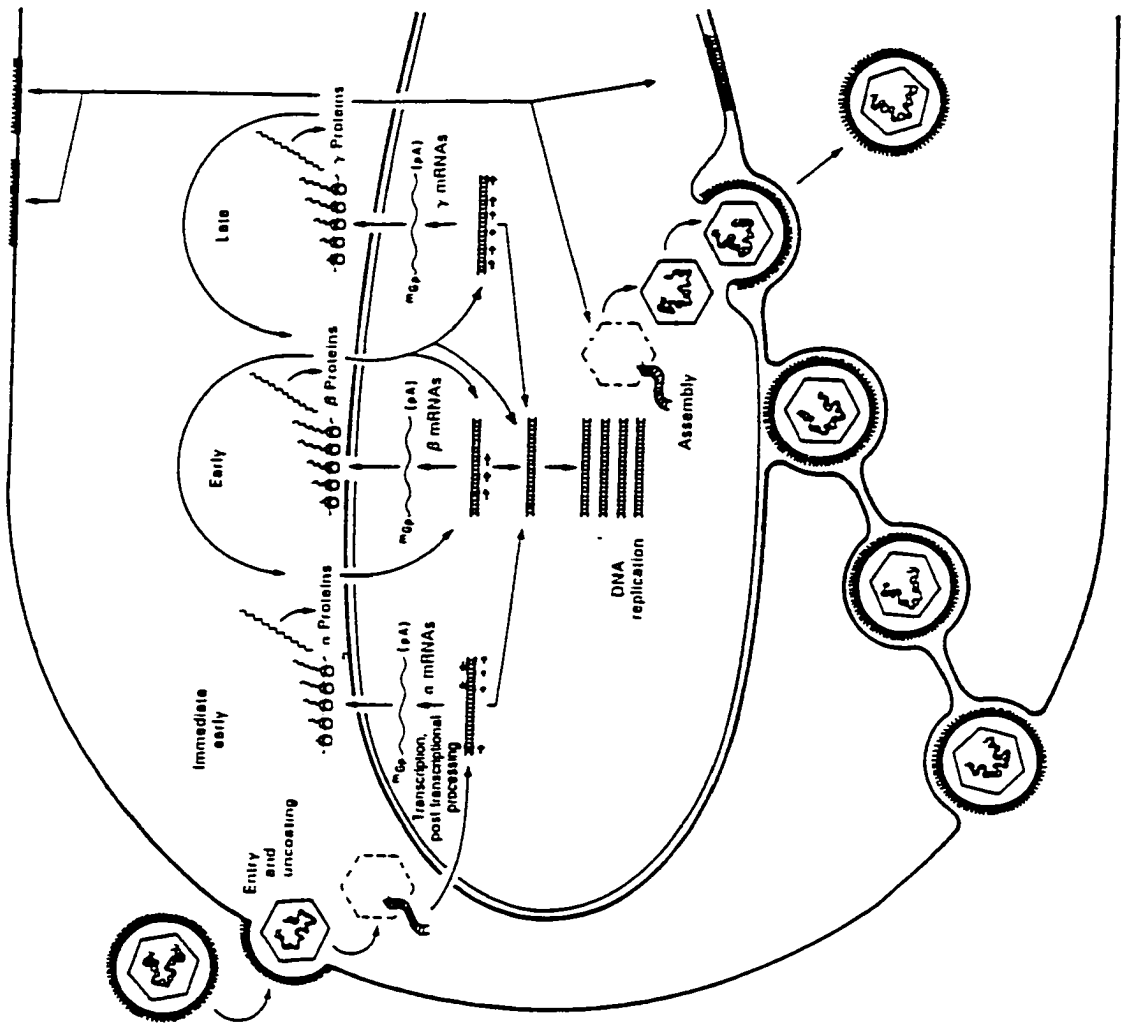


Herpesvirus Replicative Cycle: HSV-1 Transcription and Replication

The prototypic member of the herpesvirus family is herpes simplex virus type 1, and its replicative cycle has been studied most extensively (28, 64). It serves as the model to which all other herpesviruses are compared. After virion attachment to receptors on the cell surface and penetration, the deenveloped nucleocapsid containing the viral DNA is rapidly transported to the nucleus where transcription and replication of the genome takes place. In general, these processes are tightly regulated, both temporally and spatially (Fig. 3) (28, 64).

The initial event of productive infection by HSV-1 is the upregulation of the alpha gene expression by the powerful transcriptional activator VP16 (Vmw65), a structural component of the virion, which enhances transcription by host RNA polymerase II. HSV-1 encodes five alpha proteins: ICP0, ICP4, ICP22, ICP27, and ICP47. These proteins are produced in the absence of de novo viral protein synthesis and all, with the exception of ICP47, have been shown to mediate the expression of the beta genes that encode enzymes necessary for genome amplification (64). HSV-1 encodes many enzymes necessary for nucleic acid metabolism and

FIG. 3. Sequence of events in the multiplication of herpes simplex virus (adapted from reference 28). Entry of the virus into the cell occurs by fusion of the virion envelope with the membrane of the endocytic vacuole. Assembly of virions and their exit from the cell through the endoplasmic reticulum/secretory pathway is illustrated. Also illustrated are the transcription and coordinated sequential processing of mRNAs and synthesis of sets of proteins (alpha, beta, and gamma) required for DNA replication and virion structures.



replication including a ribonucleotide reductase (UL39/40), a thymidine kinase (ICP36), and its own DNA polymerase (UL30). The synthesis of the beta proteins is absolutely dependent on prior synthesis of the alpha proteins. In addition, the beta proteins accumulate in cells treated with drugs that inhibit viral DNA synthesis (64).

The replication of the HSV-1 genomic DNA occurs in the nucleus of the infected cell reportedly by a rolling-circle mechanism (35). The genome contains three origins of replication scattered between various transcriptional activation sites (64). It is speculated that transcriptional activity might enhance replication by changing local DNA structure and making the origins more accessible to the replicative machinery. The necessity of three origins is puzzling but perhaps the redundancy assures a maximal number of initiation events.

HSV-1 encodes several proteins which participate in viral genome replication (Fig. 4) (64). Seven proteins are necessary for origin-dependent, viral DNA synthesis. This group includes the viral polymerase, the single-strand-specific DNA binding protein (UL29), the origin-binding protein (UL9), a processivity factor (UL42), and a helicase/primase complex (UL5/8/52/29). The polymerase is sensitive to a few

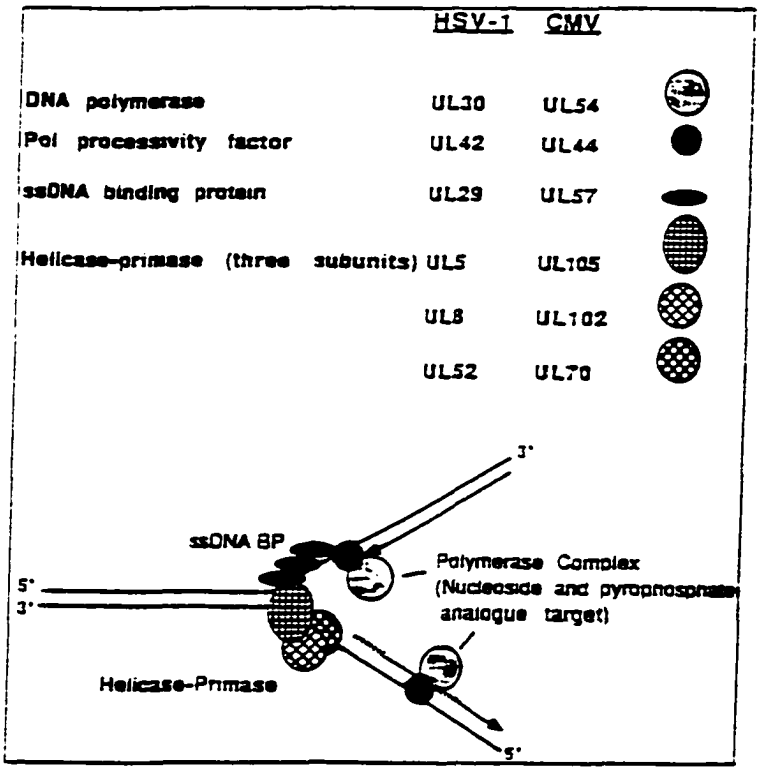


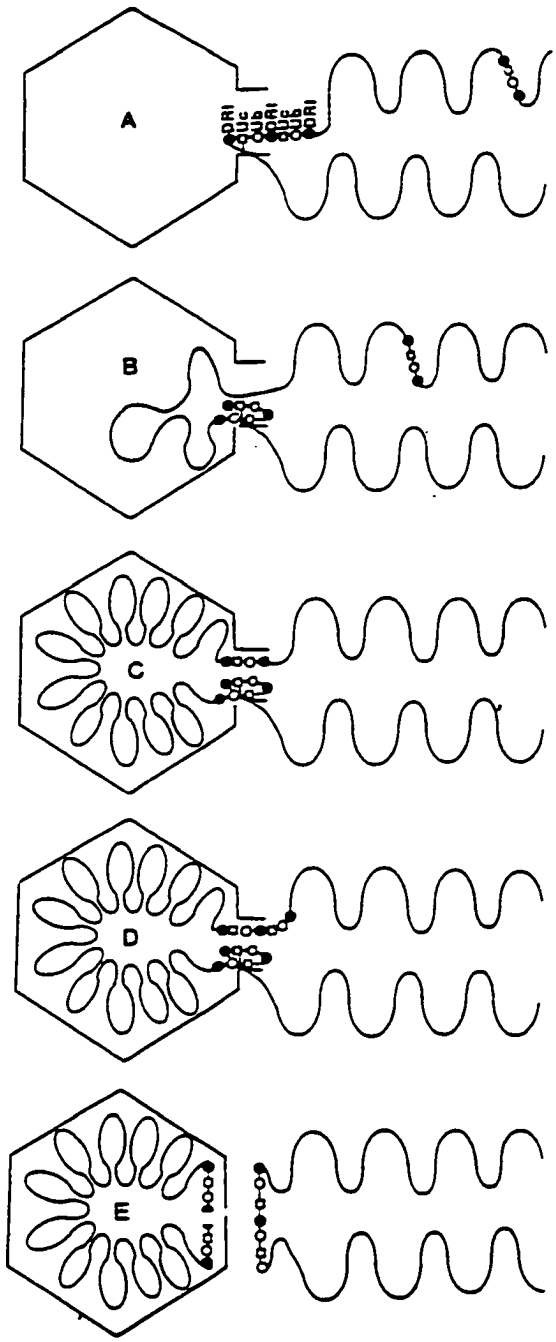
FIG. 4. DNA replication fork functions in HSV-1 and HCMV (adapted from reference 51). Top: List of polypeptides known to be a part of the HSV-1 replication fork and HCMV ORFs with functional or sequence similarity to these replication functions. All of these are contained in the set of viral *trans*-acting factors necessary to direct *oriLyt* replication. The predicted viral replication fork shows DNA strands as parallel lines. The two polypeptide polymerase complex (UL54 and UL44) is depicted in its expected roles carrying out leading (upper) strand and lagging (lower) strand synthesis and as an RNAseH in removing RNA primers (hatched lines) from the lagging strand. The three polypeptide helicase-primase is depicted in its likely roles of unwinding DNA ahead of the polymerase and synthesizing RNA primers to prime lagging strand DNA synthesis.

drugs, including phosphonoacetic acid (PAA), phosphonoformate, and acycloguanosine. HSV-1 also encodes for enzymes which influence nucleic acid metabolism but these are not essential for replication in vitro. These proteins provide pools of substrates for DNA synthesis. Perhaps the best known of these proteins is the HSV-1 thymidine kinase (TK) which has served as a potential anti-viral drug target. Studies on HSV TK have also focused on its potential use in gene therapy. In addition to the thymidine kinase and the ribonucleotide reductase activities already mentioned, HSV-1 also produces a uracil DNA glycosylase (UL2) and a deoxyuridine triphosphate nucleotidohydrolase (UL50).

After DNA synthesis, genome maturation and packaging of the DNA into preformed capsids takes place (Fig. 5) (19, 64). This complex process involves amplification of regions of the genome, *a* sequences, which mediate genome inversion, and, more importantly, the cleavage of the concatemeric DNA products of replication which occurs concomitantly with the encapsidation. The viral gene products directly responsible for the cleavage have not been definitively identified but they are likely to form a multifunctional protein complex.

The structural proteins of the virion are encoded by the gamma genes (28, 64). By definition, these proteins

FIG. 5. Packaging of HSV-1 DNA (adapted from reference 64). The model requires that proteins bind to components of a sequences (probably U_c) as in capsid A. The empty capsid scans concatemeric DNA until contact is made in a specific orientation with the first protein-U_c sequence as shown in capsid A. The DNA is taken in (capsid B) until a "head-full" or length is encountered or contact is made with an **a** sequence whose nucleotide arrangement is in the same orientation (i.e., one genome equivalent in length away) as shown in capsid C. The packaging signal requires nicking of both strands from signals on opposite sites of a DR1 sequence within **a**. In the absence of two adjacent **a** sequences (capsid D), the juxtaposition of the **a** sequences would result in duplication of the **a** sequence (capsid E).



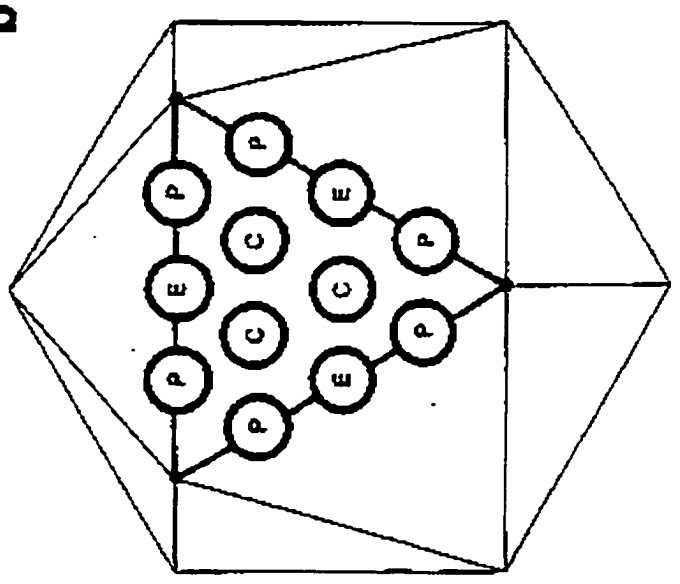
should not be expressed in the presence of inhibitors of viral DNA synthesis; however, there are exceptions. Thus, the gamma genes of HSV-1 are subgrouped into two classes, gamma-1 and gamma-2 proteins. The expression of gamma-1 genes is minimally affected by blocks to replication and some examples of these are glycoproteins B and D as well as gamma34.5. Most other structural proteins fall into the second category. Production of the late proteins is tightly coupled to the replicative process.

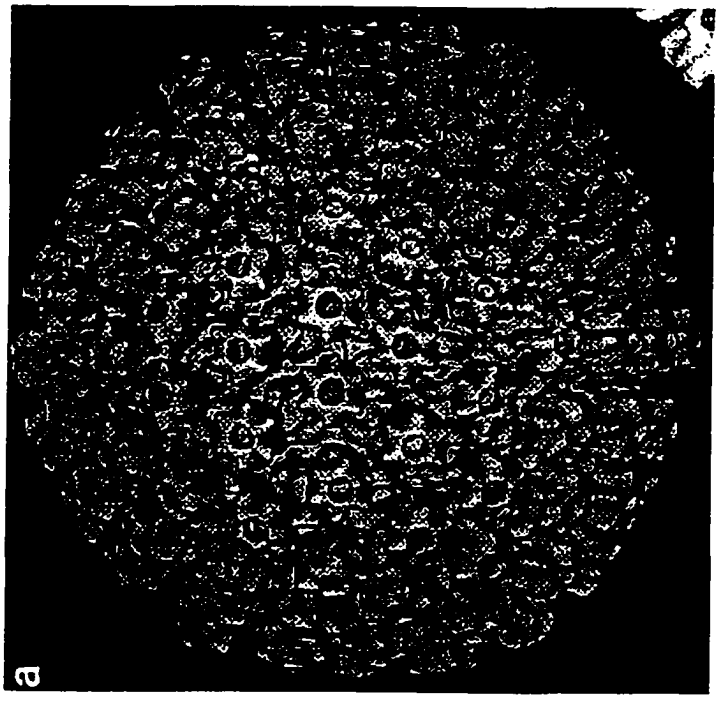
HSV-1 Assembly: Nuclear vs. Cytoplasmic Phases

As previously described, the prototypic herpesvirus particle consists of a capsid, tegument, and envelope (63). The assembly of each of these structures has been addressed in numerous reports (20, 38, 61, 64, 71). The dogma for HSV-1 assembly states that capsid and tegument assembly take place within the nucleus of the infected cell (64, 75). The initial envelopment of the subviral particles is believed to occur at the inner nuclear membrane (INM)(38). Transport of these virions through the secretory pathway then follows. Processing of the viral glycoproteins occurs in the Golgi complex (38). The mechanism by which this process occurs will be discussed in later sections.

The capsid. The ultrastructure of the herpes simplex virus-1 capsid has been established by cryo-electron microscopy and biochemical studies (54, 75). The capsid consists of 150 hexons and 12 pentons, an arrangement consistent with T = 16 icosahedral symmetry. The core within the capsid is composed primarily of VP22a which serves as the scaffold around which the capsid shell is assembled. The most abundant protein in the capsid by mass (72%) is the major capsid protein, VP5, which makes up the basic matrix of the icosahedral shell. This protein has been shown to constitute the pentamers and hexamers of the capsid (Fig. 6). Heterotrimers consisting of VP19c and VP23 at a molar ratio of 1:2 form the triplexes that occupy the vertices of each icosahedral facet and represent local areas of 3-fold symmetry. The small capsid protein VP26 decorates the outer tips of the VP5 hexon. These five proteins have been shown to be sufficient for capsid assembly in recombinant baculovirus and cell-free systems but other proteins associated with HSV-1 capsids, VP21 and VP24, have been identified (54, 75). The structural roles of these proteins in the mature capsid remains to be established.

FIG. 6. Three-dimensional reconstruction of the HSV-1 B-capsid calculated to a resolution of 25 angstroms from 56 particles (adapted from reference 75). (A) Surface rendering of the outside as viewed along an axis of 3-fold symmetry showing the expected T = 16 distribution of capsomeres. The arrangement has somewhat rounded facets and is intermediate between spherical shell and an icosahedron with planar facets. (B) Diagram marking the distribution of three classes of hexons, P, E, and C, on an icosahedral facet.





The tegument. This amorphous region of the herpesvirus virion is composed of several proteins. Most of these proteins are phosphorylated, and several functions have been assigned to them including roles in assembly, uncoating, and transcriptional regulation (64, 75). To this date, only two tegument proteins have been shown to be essential for assembly of the mature HSV-1 virion (75). Deletion of the transcriptional regulator VP16, the most abundant virion protein, results in accumulation of unenveloped capsids at the nuclear membrane (79). Similarly, a virus containing a temperature-sensitive mutation in the myristylated tegument protein UL11 is defective in both envelopment at the inner nuclear membrane and nuclear egress at the nonpermissive temperature, although the protein is defined as being dispensible in vitro (1).

Another tegument protein VP1/2 has been suggested to play a role in uncoating of the incoming virion particles as a virus containing a temperature-sensitive mutation of the protein fails to release the genome at the nonpermissive temperature (3, 75).

Finally, two kinases have been described as tegument components (75). VP18.8 (UL13) has been shown to phosphorylate VP22, another tegument protein (58). The product of

the US3 ORF is also a kinase which phosphorylates UL34, a membrane-associated protein (59).

In addition to the proteins described above, several others have been categorized as tegument proteins. All the proteins characterized thus far as tegument components have been localized to the nucleus (64, 75).

The envelope. The HSV-1 envelope contains at least 12 unique glycoproteins that have been experimentally defined, but the genome is predicted to encode many more (64, 71, 75). A major glycoprotein of the envelope is glycoprotein B, gB, which has homologs in all herpesviruses that have been studied (64). Because this protein is so highly conserved between herpesvirus families, many investigators have suggested that gB has important functions in attachment or penetration of virions or possibly mediates cell-to-cell spread of progeny virus (75). This protein forms homooligomers on the virion surface which give rise to the spikes detected by EM. gB has been shown to mediate attachment of HSV-1 to the cell surface via interactions with glucosaminoglycan (GAG) receptors (71, 75). gC has also been shown to bind GAGs and thereby enhances infectivity of the virion. Another important envelope protein is glycoprotein H which

is necessary for both viral penetration and fusion (71, 75). gL is required for transport of gH but itself is not an integral membrane protein; however, gL is detected in the virion envelope (33, 64, 75). gD is also required for penetration and fusion and appears functionally redundant to gH (75). gD has also been implicated in causing interference, a phenomenon by which intracellular enveloped virion particles are blocked from fusion with intracellular membranes (39). More recently, it has been shown that the HSV-1 gD interacts specifically with a ubiquitous cell surface molecule which has been designated HVEM for herpes virus entry mediator (81). The receptor exhibits saturable binding with gD and does appear to be the high affinity receptor for HSV-1. Two proteins, gE and gI, have roles in cell-to-cell spread of progeny virions (21, 64, 75). Several other proteins have been described, but it is important to note that to date only gB, gD, gH-gL, and gC have been shown to be required for infection and gC is only essential in certain cell types.

HCMV Structural Proteins

Early studies of HCMV capsid structure revealed the similarities to the capsids of HSV-1 (51). Homologs of the

major, minor, and small capsid proteins of HSV have now been described for HCMV as well as a homolog of the HSV-1 scaffolding protein/protease (Table 1).

TABLE 1. HCMV structural homologs of HSV capsid proteins

Protein	Major capsid	Scaffolding	Triplex	Small capsid
HSV-1	VP5	VP21, VP24	VP19c, VP23	VP26
HCMV	MCP (UL85)	UL80a UL80.5	MnCP, MnCP-BP (UL46,	SCP (UL48.5)

The HCMV envelope consists of as many as eight virally-encoded glycoproteins including at least three glycoprotein complexes (51). These glycoproteins are thought to mediate penetration, fusion, and cell-to-cell spread of virions. The most well studied and most abundant of these proteins is glycoprotein B which is a major target of neutralizing antibody and has been identified as a candidate protein for the development of a subunit vaccine (10, 12, 13, 51). Other components of the viral envelope include gH, gL, gM, gp125, gpUL4, and gp65 (Table 2) (44-46, 65).

The composition, structure, and assembly of the HCMV tegument has been poorly described. Previous reports have described the presence of at least five virally-encoded

TABLE 2. HCMV envelope glycoproteins

Protein designation	HCMV ORF	HSV homolog
gB	UL55	UL27
gH	UL75	UL22
gL	UL115	UL1
gM	UL100	UL10
gp48	UL4	-

phosphoproteins in the tegument (32, 34, 42, 47, 66, 82). The most abundant of these proteins is the lower matrix protein, pp65, which is the product of a bicistronic mRNA also encoding the upper matrix protein, pp71 (51). The tegument also includes ppUL69, pp28, and pp150, but several other potential components were recently identified by Baldick and Shenk (2). Among these are UL25, UL26, and UL49. In addition to their roles in virion structure, recent reports have suggested at least two of these proteins, pp71 and ppUL69, play a role in regulation of transcription and replication (47, 82). The distribution of these proteins in the infected cell suggests that the acquisition of the viral tegument occurs in both the nucleus and cytoplasm which implies that the tegument itself has an inherent structure (32, 42, 66). This model also requires the process of envelopment to occur in the cytoplasm of the infected cell. An investigation into the morphogenesis of the viral tegument is

therefore likely to provide clues to the pathways of envelopment as well.

HCMV Virion Assembly and Egress: Similarities and Differences Between HSV Assembly

The assembly of the HCMV virion is complex and remains poorly understood. Different approaches to the study of this process have focused on either capsid or envelope morphogenesis with little emphasis on the acquisition of the viral tegument. The conclusion from these studies will be discussed in the following sections as well as recent models of herpesvirus envelopment.

Nuclear phase: Capsid assembly. Original studies by Gibson and others documented the similarities between the capsids of HSV-1 and HCMV (51). As previously described, HCMV encodes structural homologs of all five HSV-1 capsid proteins (Table 1). Elegant studies have defined the icosahedral nature of the HSV-1 capsid and the arrangement of each of the protein components in the structure. Cell-free, in vitro assembly of structurally authentic HSV-1 capsids has been accomplished (54). Analysis of the HSV-1 capsid by

cryo-electron microscopy has permitted reconstruction of the capsid and identified substructures with the capsid (75). Similar studies of HCMV capsids are ongoing in several laboratories and will likely reveal a similar arrangement of proteins in the mature capsid.

In addition to structural studies, the protein-protein interactions that mediate capsid formation have also been investigated. In particular, the protein associations that are required for nuclear transport of HCMV capsid components have been the subject of several recent reports (4, 5). The lack of nuclear targeting signals on the major capsid protein (MCP) suggested that this protein must interact with another protein to enter the nucleus. This second protein is the UL80 gene product. It is not yet clear whether the HCMV MCP is transported as part of a bimolecular complex or whether it is translocated as part of a complex which also includes the small capsid protein (SCP). This 12 kDa protein is of a size small enough to enter the nucleus by passive diffusion but, when coexpressed in transient assays with MCP, it is retained in the cytoplasm, suggesting that the two proteins interact. Similar findings have been reported by investigators studying the HSV-1 capsid. The major capsid protein of HSV-1, VP5, interacts with the SCP

homolog VP26. Interestingly the VP26 is not required for capsid assembly but decorates the surface of the capsid. It has been proposed that modulation of nuclear transport as a result of these protein-protein interactions assures that the capsid proteins will be present in the nucleus in a proper stoichiometry to allow proper assembly of the capsid. The trafficking of the other two capsid proteins, UL85 and MnCP (UL46), is presently being investigated. Preliminary findings from the laboratory of Wade Gibson suggest that UL85 must interact with UL46 to enter the nucleus (4).

Nuclear phase: The nuclear tegument proteins. The assembly of the herpesvirus tegument has not been extensively studied. The regulatory roles of the HSV-1 tegument proteins have been previously described (64). These include UL36, UL46, UL47, UL48, UL49, US9, US10, and US11.

The UL48 gene product encodes the alpha-transinducing factor (VP16) of HSV-1 which is the primary regulator of gene transcription early in the infectious cycle of this virus (64). A homolog for this protein has not yet been described in HCMV; however, UV-irradiated HCMV virions will initiate transcription from the immediate early promoter (MIEP) of the HCMV genome, suggesting that a virion protein

can transactivate promoters within the MIEP (51). At least three HCMV tegument proteins have been described as transcription factors. The HCMV UL82 gene product, pp71, has been shown to be a weak activator of the MIE promoter (47). Similarly, ppUL69, the homolog of HSV ICP27, has been shown to regulate HCMV gene expression from early HCMV promoters (82). Homologs of the other HSV-1 tegument proteins have not been described in HCMV (Table 3).

TABLE 3. HCMV tegument proteins

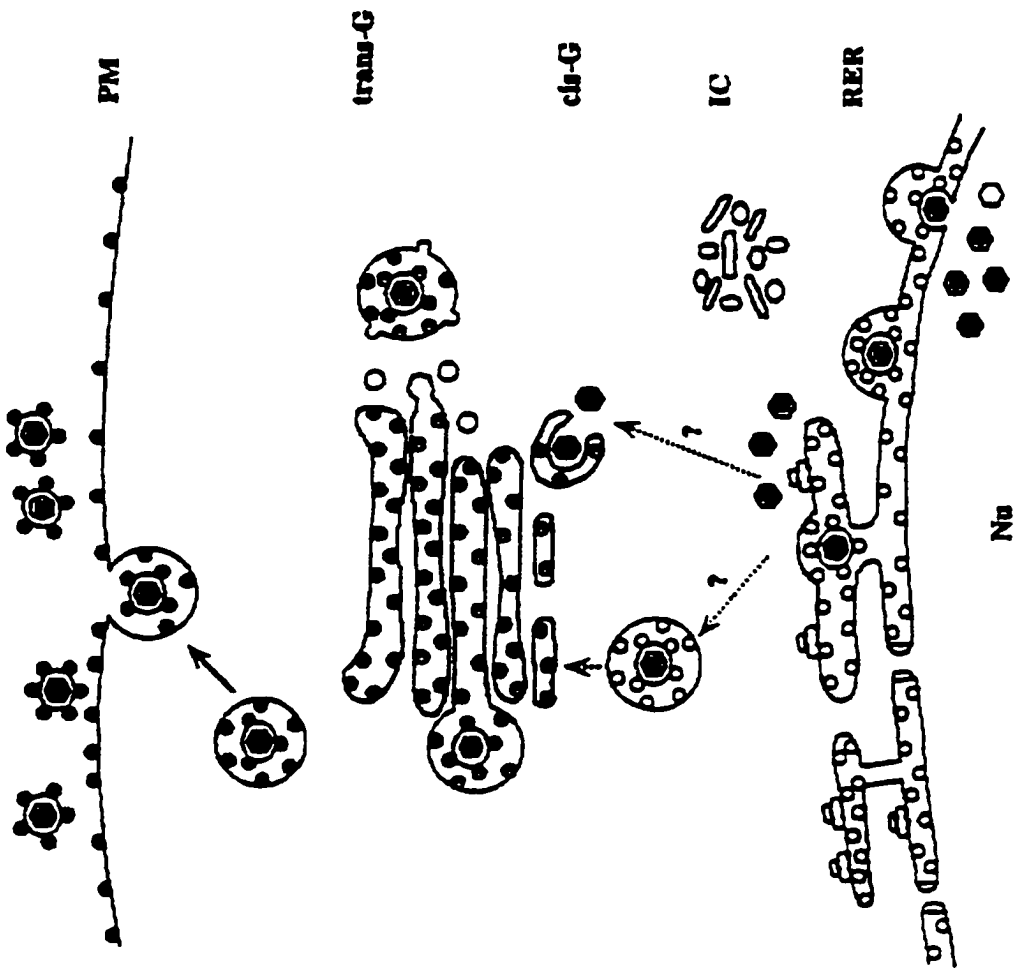
Protein designation	HCMV ORF	HSV homolog
pp28	UL99	UL11
pp150	UL32	-
ppUL69	UL69	UL54
pp65	UL83	-
pp71	UL82	-

Cytoplasmic phase: Nuclear egress and envelopment. The acquisition of the herpesvirus envelope has been an area of intense investigation for many years. The earliest model suggested that the virion acquired its envelope by budding through the inner nuclear membrane (INM), which contained immature, unprocessed viral glycoproteins, and accumulated in the perinuclear space (20). These enveloped particles

could then enter the endoplasmic reticulum (ER), which is contiguous with the nuclear envelope, where they become part of the secretory pathway (20, 38). Evidence for this mechanism of envelopment is the finding of viral glycoproteins in the INM and accumulation of HSV-1 particles within the perinuclear space. One inconsistency with this model of HCMV assembly, however, is that it does not allow for the incorporation of cytoplasmic tegument proteins into the mature particle (discussed in the next section). A second model proposes that, after the initial envelopment at the INM, the particles fuse with the outer nuclear membrane causing deenvelopment and release of tegumented capsids into the cytoplasm (61). The particles could then acquire a mature envelope by budding into a post-Golgi vesicle as shown in Fig. 7. The mature particles would then travel to the plasma membrane within a vesicle where they would be released into the extracellular space following fusion of the two membranes (Fig. 7).

Alternatively the particles in the perinuclear space could enter the cytoplasm in a nuclear membrane-derived vesicle (20). These vesicles could then fuse with the cis-Golgi and the glycoproteins of the enveloped particles could

FIG. 7. Models of possible maturation and intracellular transport pathways followed by HSV-1 in infected cells (adapted from reference 27). Virions in the perinuclear space carry immature glycoconjugates with high mannose oligosaccharides (o). Immature glycoconjugates are also present on rough endoplasmic reticulum (RER) membranes and on the inner and outer nuclear membranes (INM and ONM, respectively). Transport may proceed either by encasement of the enveloped virions from the ONM or RER (dashed arrow on the left) or deenvelopment of virions at the ONM or RER followed by reenvelopment of naked nucleocapsids by *cis*-Golgi elements (dashed arrow on the right). At the *cis* side of the Golgi complex (G), addition of GalNac residues gives rise to intermediate forms of glycoconjugates (◑), which become fully mature by substitution of GalNac with galactose and sialic acid at the level of the *trans*-Golgi (●). The viral envelopes are progressively matured as virions move along the exocytic pathway from one Golgi cisternae to the next (left side) or interact with Golgi-derived membranes originated from different cisternae (right side). Extracellular virions and the plasma membranes (PM) contain fully mature glycoconjugates. IC, intermediate compartment.



be processed as they traversed the Golgi complex (38). Evidence for these two latter pathways of envelopment includes the observation of nonenveloped, tegumented capsids in the cytoplasm of HSV-1 and HCMV-infected cells and the detection of particles budding into cytoplasmic vacuole-like structures containing viral glycoproteins. Additional evidence to support the third model is the detection of vesicles containing partially processed glycoproteins in HSV-infected cells. Another model of nuclear egress which would be consistent with the more recent models of envelopment would be focal disruptions of the nuclear membrane which would allow release of nonenveloped, tegumented particles into the cytoplasm. While there are data to support and dispute each of these models, none adequately describe experimentally the incorporation of tegument proteins.

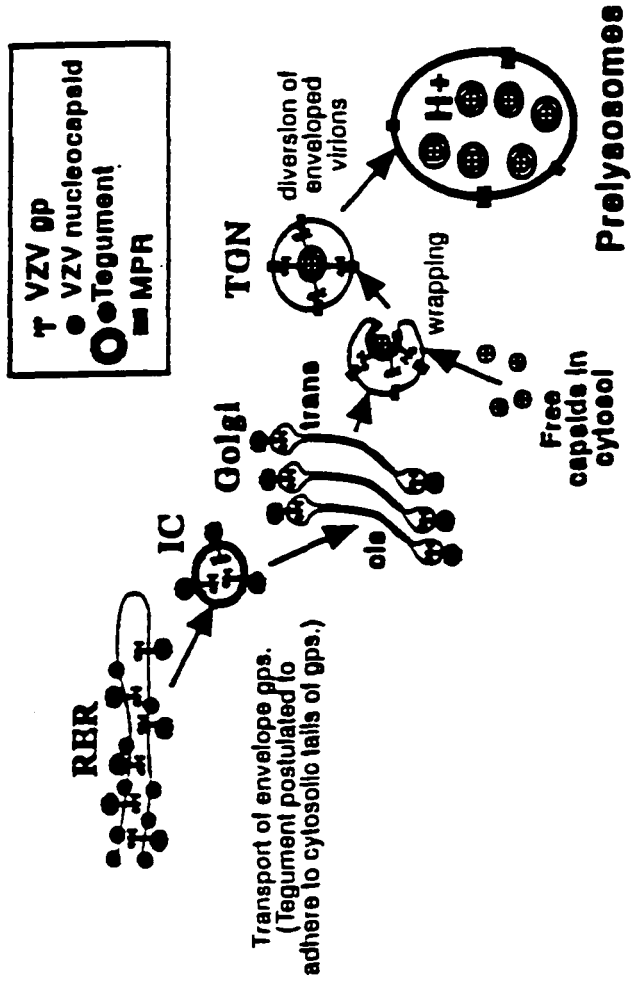
One striking contrast between the tegument proteins of HSV and HCMV is the cellular distribution of these proteins. The tegument proteins of HSV-1 have all been described as nuclear proteins, while one of the HCMV tegument proteins, pp28, is localized to the cytoplasm of infected cells (42, 64). These data alone suggest that the mechanisms of virion morphogenesis of HSV and HCMV may be inherently different. Tegument proteins of HSV-1 are acquired at areas of particle

assembly in the nucleus, termed assemblons (78). These tegumented particles then become enveloped at the INM as discussed in the previous section. The segregation of one HCMV tegument protein in the cytoplasm supports a model of envelopment/deenvelopment which results in the deposition of non-enveloped, partially-tegumented nucleocapsids into the cytoplasm where additional tegument proteins are acquired. Such a process would not be required for assembly of the HSV-1 virion since cytoplasmic tegument proteins have not been described.

Cytoplasmic sites of tegument acquisition have also been described for varicella zoster virus, human herpesvirus 6, and pseudorabies virus (27, 30, 31, 56, 62). For VZV, a model has been proposed in which cytoplasmic tegument proteins interact with the cytosolic tails of processed viral glycoproteins inserted into a cytosolic vacuole (Fig. 8) (27, 40). It has been speculated that these tegument proteins might mediate final envelopment of the maturing virion by targeting the nucleocapsid to the proper location in the infected cell.

In the case of HHV-6, tegumented particles are detected only in the cytoplasm of the infected cells, suggesting that tegument assembly and final envelopment must take place in

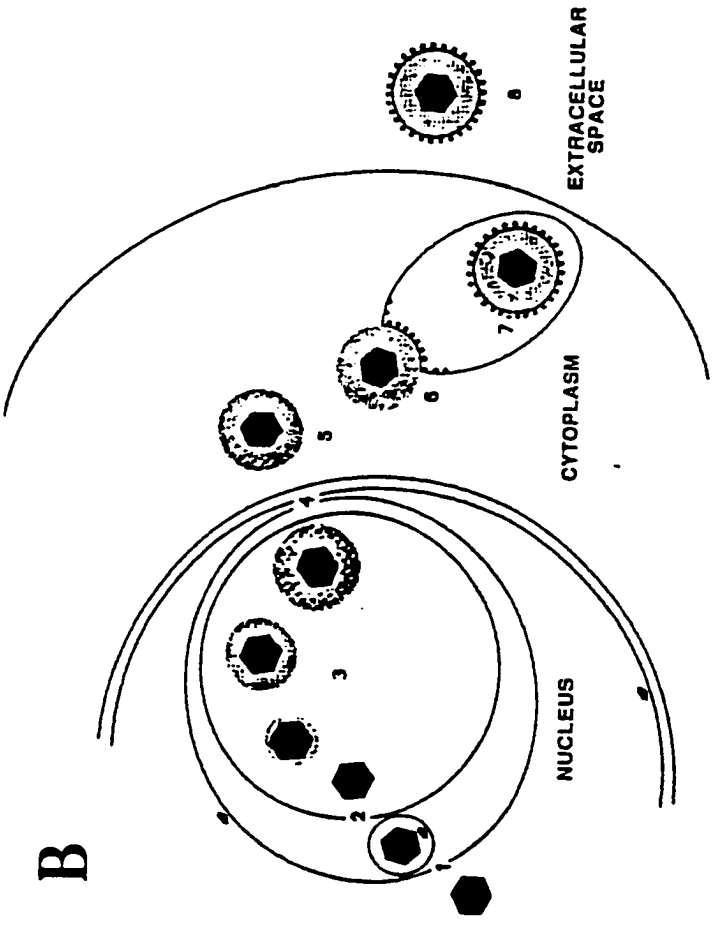
FIG. 8. Putative route of intracellular transport and maturation of VZV (adapted from reference 20). The glycoproteins (GPs) of the viral envelope are synthesized in the RER independently of the nucleocapsids and are integral membrane proteins. As is true of other such proteins, their lumenal domains are glycosylated by the addition of high-mannose sugars to asparagine residues. Tegument proteins are probably synthesized by free ribosomes. The tegument proteins are hyposthesized to interact with the cytoplasmic tails of the virion GPs. The GPs are transported through an intermediate compartment (IC) to reach the Golgi where the original high-mannose sugars are modified to complex oligosaccharides. Tegument proteins could passively follow the GPs by remaining bound to their cytoplasmic tails or be transported independently. The GPs finally are transported to the *trans*-Golgi network (TGN) where they accumulate on the concave surface of a TGN-derived sac. Tegument adheres to the cytosolic face of the concave surface. The TGN-derived sac contains the cation-independent mannose-6-phosphate receptor (MPR^{ci}). The concave surface of the TGN-derived sac becomes the definitive viral envelope. The convex surface becomes a transport vesicle which diverts the VZV virions from the secretory pathway to the lysosomal pathway, delivering the virions to acidic compartments identified as prelysosomes.



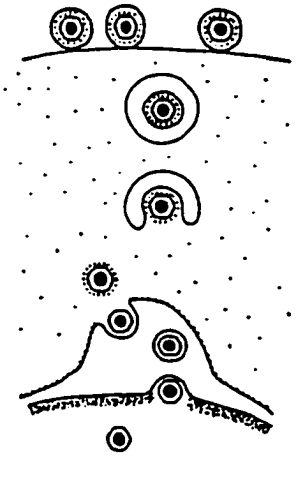
the cytoplasm (56). Another study of this virus describes tegumentation of nucleocapsids in cytoplasmic invaginations of the nuclear membrane termed tegusomes (Fig. 9) (62).

Electron microscopic studies of PrV-infected cells have described the assembly and egress of PrV particles (30). PrV nucleocapsids exiting the nucleus were observed with an electron dense coating of tegument material and a smooth envelope. These immature capsids acquired a mature envelope and another less dense layer of tegument material in the cytoplasm in association with a post-Golgi vesicle (30). Taken together, these results are consistent with a model in which the tegument is incorporated at nuclear and cytoplasmic sites of cells infected with several different herpesviruses. Such a process would imply that the tegument is not an amorphous entity but that this region of the virion has an inherent structure. Support for this hypothesis has come from studies by McLauchlan and Rixon who described the assembly of tegument-like structures in cells infected with an HSV-1 temperature-sensitive mutant incapable of producing nucleocapsids (49). These structures were morphologically similar to infectious HSV-1 virions, suggesting that the tegument proteins could coalesce into virus-like particles in the absence of other signals which could influence virion

FIG. 9. Models of HHV-6 tegumentation and assembly. (A) (Adapted from reference 56) In this model, nuclear egress is achieved by fusion of the envelope of a perinuclear virion with the outer nuclear membrane. Tegument proteins are acquired prior to reenvelopment of the cytosolic nucleocapsid at a cytoplasmic membrane. (B) (Adapted from reference 62) In this model, DNA-containing nucleocapsids undergo envelopment at a membrane surrounding a sealed cytoplamic invagination into the nucleus (step 1). A subsequent deenvelopment (step 2) releases the naked capsids into an islet of the cytoplasm within the nuclear region where they gradually acquire full tegument structures (step 3). Thereafter, fusion events with the nuclear membranes (step 4) result in release of the tegumented particles into the cytoplasm (step 5). The tegumented capsids then undergo reenvelopment in cytoplasmic vacuoles containing mature virion glycoproteins (steps 6 and 7). Fusion of the vacuole membrane with the plasma membrane releases the mature virions into the extracellular space (step 8). Arrows point at membrane sides equivalent to the nuclear side of the inner nuclear membrane.



B



A

morphology. These data alone suggest that the tegument proteins are not arranged in a random order.

Infection of permissive cells with HCMV invariably produces three types of extracellular particles-virions, noninfectious enveloped particles (NIEPs), and dense bodies (DBs) (2, 34). Dense bodies are particles overproduced by laboratory strains of HCMV which have been adapted in cell culture to produce large quantities of extracellular virus; however, DBs are also produced by clinical isolates and can be detected in the tissues of infected animals (2, 34, 51). Dense bodies have been previously reported to be structurally simple in that they were initially characterized as being composed almost entirely of the tegument protein pp65 and a small amount of the major envelope glycoprotein, gB (34). More recent studies have suggested that dense bodies are more complex in composition (2). They also contain capsid proteins and proposed tegument proteins including UL25 which is not detected in virions. Studies from our laboratory confirmed that dense bodies contained a similar set of proteins as virions including processed envelope glycoproteins. These observations suggested that DBs followed the same pathway of assembly as the infectious virion. Of particular interest to our laboratory was the finding that the

HCMV recombinant virus which lacks the UL83 gene product pp65, AdRV, does not produce dense bodies (67).

Phosphoprotein 65

To further understand the mechanism of HCMV tegument assembly, we investigated the cellular trafficking of the lower matrix protein pp65 (ppUL83). Previous studies have documented the nuclear targeting of this protein immediately after infection of permissive cells; however, late in infection when cells exhibit extensive cytopathology, pp65 is observed in the cytoplasm in association with large vacuole-like structures (26, 66). pp65 is the only tegument protein that exhibits this cellular distribution and, thus, it serves as a marker for assembly sites of the tegument in both the nucleus and the cytoplasm. Interestingly, there is not a structural or functional homolog of this protein encoded by HSV, suggesting that these two different herpesviruses may indeed assemble by different pathways (51).

Many studies have focused on the induction of cellular immune responses to pp65 (6, 8, 36, 41). This antigen has been shown to induce cytotoxic T lymphocytes in immunocompetent individuals. In addition, pp65 was found to be the most immunogenic virus-encoded protein in assays measuring

the activation of CD4+ T lymphocytes (48). The antiviral effects of this activation are not clear but are likely to involve the production of cytokines which stimulate host immune responses. In summary, pp65 is a potent inducer of T-cell responses in infected individuals and continues to be a candidate protein for an HCMV vaccine.

Other studies have focused on the intracellular distribution of pp65 and its role in the replicative cycle. The signals mediating pp65 nuclear localization have been the subject of at least two reports. Schmolke et al. reported that the pp65 protein sequence contained an efficient bipartite nuclear targeting signal (NTS) at the carboxyl-terminus of the protein (66). Like previously reported NTS, this signal could mediate nuclear localization when fused to a hetero-logous reporter protein. However, mutants of pp65 that did not contain this NLS continued to accumulate in the nucleus, indicating that other signals were present in the protein which could modulate its nuclear localization (66). Gallina et al. subsequently suggested that a second, less efficient NTS was present in a 117 amino acid sequence upstream of the previously reported signal (26). This group demonstrated that this sequence could also mediate nuclear localization by utilizing a different reporter protein and

cell system than used by the previous investigators. A putative signal within this 117 aa sequence was proposed but formal proof that this sequence was an active signal was lacking. Furthermore, forms of pp65 in which this signal was mutated by site-directed mutagenesis continued to accumulate in the nucleus, albeit less efficiently (26). Taken together, the data suggest that there are several functionally redundant signals in the pp65 protein which modulate targeting to the nucleus.

The function of nuclear pp65 is even less clear. As one of the most abundant virion components, pp65 plays an obvious role in the structural integrity of the particle. Yet, pp65 has been reported to be a dispensible gene in cell culture (67). The HCMV pp65-negative mutant virus, AdRV, reportedly replicates to similar levels as the wild type, parent strain but produces limited amounts of extracellular virus. Furthermore, the phenotype of this virus in tissue culture resembles that of early clinical isolates (which do not overexpress pp65) and not that of the parental AD169 strain (67). In contrast, a separate study in which the translation of the pp65 mRNA was inhibited by anti-sense RNAs suggested that pp65 was necessary for efficient replication (18). These observations are consistent with the

classification of pp65 as an early-late gene product which could be essential for viral replication. The role of pp65 in the replication cycle has not been extensively investigated. pp65 may be involved in the regulation of important aspects of viral replication; at least two previous studies have documented a protein kinase activity associated with this protein (9, 70). At this time, pp65 has not been conclusively shown to have intrinsic protein kinase activity but it could be speculated that pp65 acts as a shuttle that delivers cellular kinases to viral substrates. At least two cellular kinases have been shown to interact with pp65, casein kinase II and, more recently, Plk1 (68, 77). Plk1 (polo-like kinase) is related to other mitotic regulators and has a role in assembly of the mitotic spindle during cell division (43). pp65 may also play a more direct role in viral replication by associating with the replicating DNA. Michelson and coworkers have shown that pp65 binds to cellular chromatin, but a direct interaction with DNA was not shown (17).

In summary, there remains considerable controversy surrounding the function of pp65 in the HCMV replicative cycle. Although this protein has been defined as dispensable in cell culture, it is likely that pp65 does have an important

role in HCMV in vivo as it is highly conserved between all strains in spite of its immunogenicity. Several studies are presently underway to elucidate the function of pp65 in HCMV replication and morphogenesis.

The Scope of the Dissertation

The first part of my work was to examine the composition of HCMV extracellular particles in order to determine which viral proteins were incorporated into the virion and to characterize the immunological reagents which were used in further studies. We then followed the cellular distribution of the tegument proteins as markers of nuclear and cytoplasmic sites of assembly in HCMV-infected fibroblasts. The second part of my work focused on the association of HCMV proteins with the nuclear matrix of infected human fibroblasts which suggested a role for this subnuclear structure in coordination of replication and assembly of HCMV in the nucleus. The third part of this work was to characterize the signals which mediate nuclear localization of an abundant HCMV tegument protein, pp65, which is detected in the nucleus and cytoplasm of late infection. Therefore, understanding the mechanisms which drive the changes in the

cellular distribution of this protein could provide insight into nuclear egress of particles.

HUMAN CYTOMEGALOVIRUS TEGUMENT ASSEMBLY OCCURS IN THE NUCLEUS AND CYTOPLASM OF INFECTED HUMAN FIBROBLASTS

by

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ABSTRACT

The structural complexity of herpesviruses has been simplified by dividing the virion into three subviral structures, the capsid, tegument, and envelope. Each structure contains characteristic protein components which appear to be added to the subviral particle in a sequential fashion. Although there is general agreement for an intranuclear site of encapsidation and addition of the tegument during morphogenesis of herpesviruses, there continues to be debate surrounding the intracellular location of particle envelopment. Early studies have described envelopment at the nuclear membrane, whereas more recent studies have suggested that final envelopment occurs in a post-Golgi compartment. We investigated the intracellular sites of human cytomegalovirus morphogenesis by using both biochemical and imaging techniques to define the intracellular compartmentalization of several major tegument components during productive infection. Consistent with a previous report, our findings indicated that virion structural proteins of the capsid, tegument, and envelope could be found in both extracellular non-infectious dense bodies and infectious virions. These results suggested a common assembly pathway for all extracellular particles. Furthermore, we could demonstrate that two major

tegument components of the extracellular particles, ppUL99 (pp28) and ppUL32 (pp150), could be found only within the cytoplasm of infected cells. Because another tegument protein, ppUL69, was restricted to the nucleus of infected cells, our findings were most consistent with an assembly pathway in which the tegument was acquired sequentially, first in the nucleus and then in the cytoplasm. Together these findings suggested an even more complex assembly pathway for this large herpesvirus. Our results were inconsistent with an assembly pathway in which acquisition of the mature virion envelope occurred at the nuclear membrane.

INTRODUCTION

Similar to other human herpesviruses, the assembly of human cytomegalovirus (HCMV) particles is a complex, poorly understood process. Extracellular virions produced by HCMV-infected cells appear structurally similar to those of other herpesviruses in that HCMV virions consist of three major structural regions, the capsid, the tegument, and a lipid-containing envelope (25). While it is generally accepted that capsid assembly occurs in the nuclei of infected cells, the cellular compartments in which tegument assembly takes

place remain incompletely defined (25). In addition, several seemingly mutually exclusive pathways of envelopment have been proposed for herpesviruses (8, 10, 11, 14, 17, 18, 21, 23, 24, 28-32, 34). The earliest model of envelopment of herpes simplex viruses proposed that the viral particle was enveloped during passage through the inner nuclear membrane (17, 25). Glycoproteins within the envelope are then modified as the particle traverses the Golgi (8, 17). More recent models have suggested that envelopment and deenvelopment occur at the nuclear envelope and that final envelopment of the tegumented nucleocapsid occurs in the *trans*-Golgi following fusion with vesicles containing processed glycoproteins (8, 10, 11, 14, 18, 21, 23, 30-32, 34). Addition of the tegument is assumed to take place in the nucleus in both models, although formal proof of such a site of tegument acquisition is lacking. In HCMV-infected cells, the cellular distribution of several of the more characterized tegument proteins has suggested that tegument assembly might occur in two different compartments, the nucleus and the cytoplasm. Recent studies of varicella zoster virus morphogenesis have suggested that the tegument of this herpesvirus is also partially assembled in the cytoplasm of the infected cell, perhaps concurrently with final envelopment of the

maturing particle at the *trans*-Golgi network (10, 34). The presence of tegument components in different cellular compartments also implies that incorporation of these proteins into the virion occurs in a sequential and possibly ordered manner. Together, these observations suggest that any model of envelopment must account for the incorporation of the cytoplasmic tegument proteins prior to final envelopment of the particle.

It has been reported that HCMV-infected cells produce three types of extracellular particles, virions, NIEPs (non-infectious, enveloped particles), and dense bodies (2, 16). Only virions contain infectious nucleic acid; however, all three particles appear to be enveloped with a membrane containing virus-encoded glycoproteins (16). Definitive studies by Irmiere and Gibson demonstrated that the protein composition of the mature virion and NIEP were essentially the same with the exception of the presence of the scaffolding protein, UL80.5, within the NIEP (16). In contrast, dense bodies were believed to lack components of the capsid and several abundant tegument phosphoproteins (16). More recent studies have provided conflicting information and suggested that dense bodies may contain a wider array of virus-encoded proteins (2). It is of interest to note that both studies

reported that dense bodies contained an abundant nuclear tegument protein (pp65, ppUL83) and a limited number of glycoproteins, raising the possibility that the assembly of dense bodies might reflect the assembly of the mature virion. Understanding the formation of dense bodies could provide a simple model for investigating the morphogenesis of infectious virions.

In this study we examined the subcellular location of tegument acquisition using well-characterized HCMV-specific monoclonal and polyclonal antibodies to describe both the expression and cellular distribution of four different tegument proteins. As a second approach for understanding tegument acquisition and envelopment, we attempted to define the minimum number of proteins required for extracellular particle formation. Our findings indicated that tegument morphogenesis occurred in both nuclear and cytoplasmic compartments, suggesting that the final envelopment of HCMV occurred in the cytoplasm of infected cells. Furthermore, our results suggested that dense bodies and virions were composed of a similar set of virus-encoded proteins which implied that these particles followed a common pathway of assembly in the infected cell.

MATERIALS AND METHODS

Cells, Viruses, and Antibodies

Primary human foreskin fibroblast (HF) cells were prepared, propagated, and infected as has been described (4-6). Human cytomegalovirus strain AD169 was used for all experiments. Infectious stocks were prepared from supernatant of infected HF cells which exhibited 100% cytopathic effect. Human 293 cells or monkey Cos7 cells were used for transient expression of pp150 and pp28 and were transfected by following either a liposome- (Lipofectin, Bethesda Research Laboratories, Gaithersburg, Md.) or calcium phosphate-mediated transfection protocol (7).

HCMV proteins were detected with monoclonal antibodies (mAbs) as previously described (1, 4-6, 22, 26, 33). Monoclonal antibodies used in this study included those specific for IE-1 (p63-27), IE-2 (IE-2-9-5), pp65 (28-19), MCP (28-4), SCP (11-2-23), gB (7-17), pUL69 (UL69), pUL44 (28-21), and pp28 (41-18). The specificity of mAb 41-18 for pp28 was confirmed by its reactivity with pp28 (UL99) expressed in recombinant systems. The generation of the monospecific serum recognizing pp150 has been previously described (13).

Extracellular Virus and Dense Body Purification

Cell-free supernatant was prepared from eight 175 cm^2 flasks infected 8 days earlier with AD169. The supernatant was initially clarified by centrifugation at 4,000 rpm in a Sorvall GSA rotor for 20 min. Extracellular particles were then collected by layering the clarified supernatant over a 2-ml cushion of 25% sorbitol and centrifuging the material at 20,000 rpm in a SW28 rotor (Beckman, Palo Alto, Calif.) for 40 min. The pelleted material was resuspended in 1 ml of PBS (phosphate buffered saline, pH 7.4) by repeated pipetting taking care not to create foam in the virus preparation. The material was then layered on top of a preformed 15-50% sucrose gradient and centrifuged at 39,000 rpm in a Beckman SW41 rotor for 20 min. Three bands were observed corresponding to NIEPS, virions, and dense bodies as reported previously (3, 16). The virion band and dense body bands were collected by needle aspiration of the appropriate band. The sucrose gradient centrifugation procedure was repeated twice and the final dense body and virion fractions were collected by needle aspiration and analyzed. Cross-contamination of the samples was determined by both imaging and biochemical techniques.

Analysis of Virions and Dense Bodies

Virus and dense body infectivity were determined by titration on HF cells as described previously (4). Residual infectivity in the virion and dense body preparation was expressed as a percentage of the total infectivity in the clarified supernatant (post-centrifugation at 4,000 rpm) prior to pelleting of the particles by ultracentrifugation. The virion fraction contained essentially the same amount of infectious virus as the clarified supernatant.

SDS-PAGE and Immunoblotting

Electrophoresis under reducing conditions and immunoblotting were carried out essentially as described (4-6, 13). For comparison of viral protein composition of virions and dense bodies, equivalent volumes of virions and dense bodies were applied to each gel. Protein concentrations were determined by micro BCA assay (Pierce Chemical Co., Rockford, Ill.). Infected cell proteins were obtained from HCMV-infected HF cells grown in 35-mm tissue culture dishes and washed with phosphate-buffered isotonic saline (PBS, pH 7.4). The cells were lysed in sample buffer containing 5% 2-mercaptoethanol and 2% SDS and heated to 100°C. The solubilized proteins were then subjected to SDS-PAGE and

transferred to either nitrocellulose or PVDF membranes. Murine monoclonal antibodies and a 1:500 dilution of the IgG fraction of the rabbit anti-pp150 serum were used to detect specific proteins. Antibody binding was detected by ^{125}I-iodine protein A followed by autoradiography or alternatively by reaction with horseradish peroxidase conjugated goat anti-mouse or rabbit IgG, followed by enhanced chemiluminescence fluorography (ECL, Amersham, Arlington Heights, Ill).

Immunofluorescence Microscopy

HF cells were grown in 4-well glass chamber slides and infected with AD169 at an multiplicity of infection of 2-5 when the monolayer reached 90% confluency. At the indicated times after infection the monolayer was washed with isotonic HEPES buffered solution (50 mM HEPES, 150 mM NaCl, pH 7.4) and fixed by application of 4% paraformaldehhyde in isotonic buffer for 30 min at room temperature. After two washes in isotonic buffer, the infected monolayer was permeabilized with 0.5% Triton X-100 in isotonic buffer for 5 min. Some experiments included a blocking step using normal goat serum containing 3% bovine serum albumin prior to addition of the primary antibody. After removal of the blocking solution, the monolayers were incubated with a Protein-A purified IgG

fraction of the rabbit anti-pp150 serum or murine monoclonal antibody-containing tissue culture supernatants for 30 min at 37°C. The antibody-containing solution was aspirated and the monolayer was washed twice with isotonic buffer and once with 0.05% Triton X-100 containing isotonic buffer. The monolayer was then incubated with a fluorescein-conjugated goat anti-mouse IgG (Jackson Laboratories, West Grove, Penn.) or a Texas Red-conjugated goat anti-rabbit IgG (Jackson Laboratories, West Grove, PA) second antibody. In some experiments, DAPI (4',6-diamidino-2-phenylindole) (Sigma Chemical Co., St. Louis, Mo.) was included with the second antibody as a nuclear counterstain. Following a 30-min incubation at 37°C, the stained monolayers were washed as described following the primary antibody. The chambers and gaskets were removed and the glass coverslips were mounted onto slides in buffered isotonic saline containing 10% glycerol and sealed with fingernail polish. Slides were examined on a Leitz Orthoplan Fluorescence microscope (Leitz, Rockleigh, N.J.) equipped with a Vario Orthomat II photomicrography system and were photographed at a magnification of 20x or 40x using Kodak Ektachrome Elite film (ASA1600) exposed at 800 ASA.

Immunoelectron Microscopy

HF cells were grown and infected with AD169 as described above. Following fixation the cells were processed with primary antibody as described above. After washing, the monolayers were incubated with 6-nm colloidal gold particles conjugated to goat anti-rabbit IgG antibodies (Jackson Laboratories, West Grove, Penn.) diluted to 1:20 in PBS 3% BSA for 30 min. After washing with buffered isotonic saline as described above, the monolayers were post-fixed with osmium tetroxide in 100 uM sodium phosphate, pH 7.4, for 30 min at room temperature and processed for observation with an electron microscope. Briefly, the immunogold-stained monolayers were dehydrated with successive washes of increasing concentration of ethanol as follows: 50%, 70%, and 90% each for 5 min, followed by three washes with 100% ethanol, 10 min each. The dehydrated monolayers were immediately embedded in gelatin capsules containing Epon 812 (EM Sciences, Fort Washington, Penn.) at 80°C overnight. Thin sections were prepared and stained with uranyl acetate and lead citrate just prior to viewing by EM.

RESULTS

Extracellular HCMV Dense Bodies Contain Capsid, Tegument, and Envelope Proteins

Previous studies have reported that highly enriched preparations of extracellular dense bodies produced from HF cells infected with laboratory isolates of HCMV were composed of a relatively limited set of virus-encoded proteins (16). These reports suggested that dense body particles were composed primarily of a single tegument protein, pp65 (ppUL83), and the major envelope glycoprotein, gB (gpUL55) (16). Notable was the apparent absence of capsid proteins as well as other tegument proteins (16). A recent study provided conflicting results and indicated that dense bodies contained a large number of virion structural proteins (2). We characterized the proteins associated with dense bodies using well-defined monoclonal antibodies reactive with viral proteins as an initial step towards understanding the assembly of these abundant particles produced by HCMV-infected cells. An enriched population of dense bodies was prepared by repetitive rate-velocity centrifugation of extracellular particles through sucrose gradients using a similar protocol as described in previous reports (3, 16). After each centrifugation, fractions containing dense bodies or virions

were collected and sequentially rebanded in sucrose gradients. Following three rounds of centrifugation, we obtained a population of dense bodies which contained less than 1% of the original infectivity present in the clarified supernatant from the infected monolayers (Table 1). Analysis of

TABLE 1. Infectivity of purified HCMV particles

	Supernatant[a]	Virions	Dense bodies
Infectivity[b]	4.2×10^9	4.2×10^9	3×10^6
% Total[c]	100	100	<0.1

[a]Cell-free supernatant prior to high speed centrifugation was titered for infectivity and represents total infectious virus contained in the starting material.

[b]Infectivity was determined in a microtiter assay as described in a previous report (4). Infectivity represents the total number of infectious particles within the sample. The virion and dense body infectivity were titered after the third sequential centrifugation through a sucrose gradient as described in Materials and Methods.

[c]The percentage of total infectivity was derived by using the following formula:

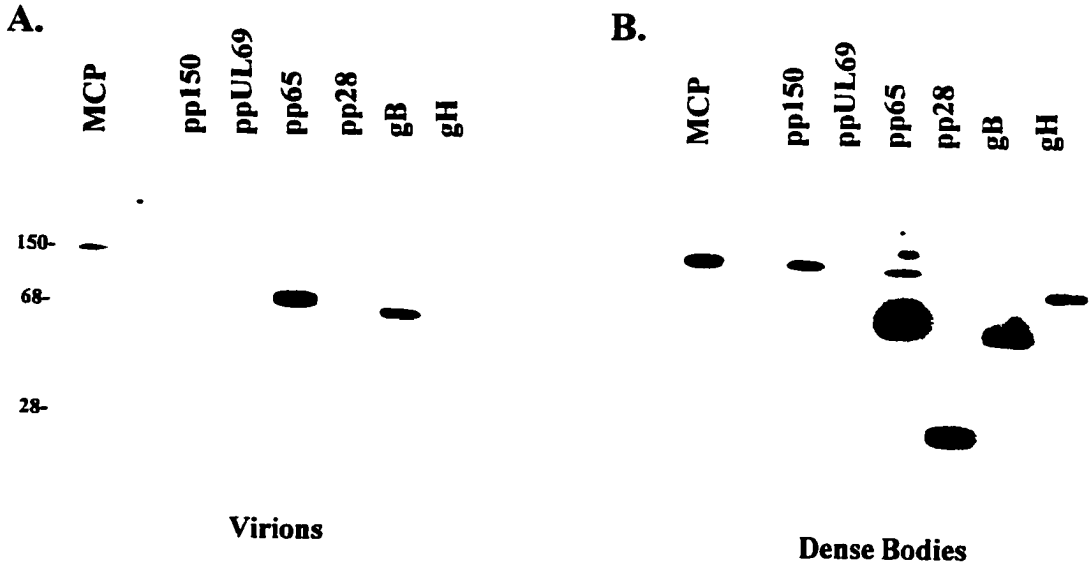
% = number of infectious units/infectivity of supernatant x 100

this material by electron microscopy (EM) revealed dense bodies in every field examined and only an occasional particle (data not shown). In addition, we could find no evidence of virions embedded into dense bodies in these electron micrographs (data not shown). These data suggested that we had achieved a significant level of enrichment of

dense bodies from extracellular particles, similar to that described in previous reports (2, 16).

The protein composition of dense bodies was next analyzed using a panel of well-characterized monoclonal antibodies (mAbs) with reactivity for envelope, tegument, and capsid structural proteins as well as two non-structural HCMV proteins. Equivalent volumes of virions and dense bodies obtained from the final gradients were solubilized by heating in SDS-containing sample buffer and subjected to SDS-PAGE. Following transfer of the separated proteins to membranes, individual proteins were detected with mAbs. We readily detected several structural proteins in the virion fraction including the major capsid protein (MCP, pUL86), the tegument proteins pp150 (ppUL32) and pp65 (ppUL83), and the envelope glycoprotein gB (gpUL55) (Fig. 1A). Upon longer exposure of the autoradiogram, we could also demonstrate the presence of the tegument proteins ppUL69 and pp28 (ppUL99) as well as glycoprotein H (gpUL75) in the virion sample (data not shown). When the membrane containing the DB proteins was developed with the same panel of antibodies, a significantly stronger signal was noted. This finding was most likely explained by at least a 5-fold greater protein content (data not shown) in the DB preparation. We detected

FIG. 1. Immunoblot analysis of virions and dense bodies. Equal volumes of the dense body and virion preparations (approximately 0.1 ug of the virion preparation and 0.5 ug of the dense body preparation) were subjected to SDS-PAGE in a 10% gel and transferred to nitrocellulose membranes as described in Materials and Methods. The separated proteins were then reacted with mab reactive with the major capsid protein (MCP, UL86), ppUL69, pp65 (UL83), pp28 (UL99), gB (UL55), gH (UL75), and a rabbit anti-peptide sera reactive with PP150 (UL32). Reactivity is shown for the virion preparation (A) and the dense body preparation (B). The migration of known molecular weight standards is shown on the left.



the MCP, as well as the tegument proteins pp150, ppUL69, pp65 and pp28 in the dense body sample (Fig. 1B). Two envelope glycoproteins gB and gH were also present (Fig. 1B). It was of interest to note that the fully-processed form of the gB was present in the DB preparation. Consistent with the findings of other investigators, we could not detect the IE-1 (ppUL123) or pp50 (ppUL44) non-structural proteins in either the virion or DB preparation (data not shown).

The finding that non-infectious dense bodies contained a similar representation of structural proteins as previously described for infectious virions suggested that these particles represented defective forms of extracellular virions and were not the products of a separate assembly pathway as had been suggested (16). Furthermore, the presence of processed gB (gpUL55) in the membrane of these particles suggested that they contained glycoproteins which had been exposed to an enzymatic activity in the Golgi. Lastly, and perhaps most interesting, was the presence of several tegument proteins which, until recently, were thought to be absent in dense body preparations (16). This was of particular interest because at least one of the tegument proteins, pp28 (ppUL99), has been shown to localize in a cytoplasmic compartment suggesting that tegument assembly of infectious

HCMV particles and DB occurred in both nuclear and cytoplasmic sites (19).

The Basic Phosphoprotein, pp150 (ppUL32), Is Expressed as a Late Protein in the Cytoplasm of HCMV-infected Cells

The finding of a cytoplasmic tegument protein in DB suggested that, during morphogenesis of extracellular HCMV particles, at least some protein components of the tegument were added to the particle in the cytoplasm. These results prompted us to reexamine the intracellular location of the tegument proteins pp28, pp150, ppUL69, and pp65 in HCMV-infected HF cells. First, we confirmed the cytoplasmic distribution of pp28 and the nuclear localization of ppUL69 and pp65. We next examined the expression of the pp150 in HCMV-infected cells using an anti-peptide serum generated against the carboxyl-terminus of the molecule (13). The kinetics of expression of pp150 in HCMV-infected cells were initially determined by immunoblotting of cell lysates harvested on days 1-6 post-infection. Additional HCMV-specific mAbs, including mAbs reactive with pp72 (IE-1) and pp65 (ppUL83), were used to detect infected-cell proteins of the immediate-early and early-late kinetic classes, respectively. As was expected, pp72 was expressed by day 1 post-infection and could be detected throughout the duration of the infection

(Fig. 2). In contrast, pp150 was first detected on day 3 and appeared to increase in amount during subsequent days (Fig. 2). At the multiplicity of infection used in this experiment, we could not detect pp65 until day 3 post-infection (Fig. 2). These studies suggested that the antiserum used to detect pp150 was specific for this protein and that pp150 was expressed with kinetics consistent with either an early-late or late protein. The lack of detectability of pp150 in infected cells treated with phosphonoformic acid (PFA) further supported the classification of pp150 as an early-late or late protein (data not shown).

Utilizing the anti-pp150 peptide antiserum we characterized the intracellular distribution of the pp150. Analysis of infected cells on day 5 post-infection by immunofluorescence revealed almost exclusive cytoplasmic localization of the protein when compared to the localization of the 72 kda IE-1 protein (Fig. 3e). The distribution of pp150 in these cells was often perinuclear in a ring- or cap-like accumulation. Furthermore, a similar distribution was noted on days 2-5 post-infection, suggesting that pp150 remained within the cytoplasm throughout the replicative cycle of HCMV (data not shown). When 293 or Cos-7 cells were transfected with the open reading frame (ORF) encoding

FIG. 2. Kinetics of pp150 expression in HCMV-infected human fibroblasts. Primary human fibroblast cells were infected with HCMV strain AD169 and harvested for immunoblot analysis as on day post-infection (p.i.) as indicated. Following SDS-PAGE and transfer to PVDF membranes, expression of pp150, IE-1 (pp72, UL123), and pp65 were detected as described in Materials and Methods.

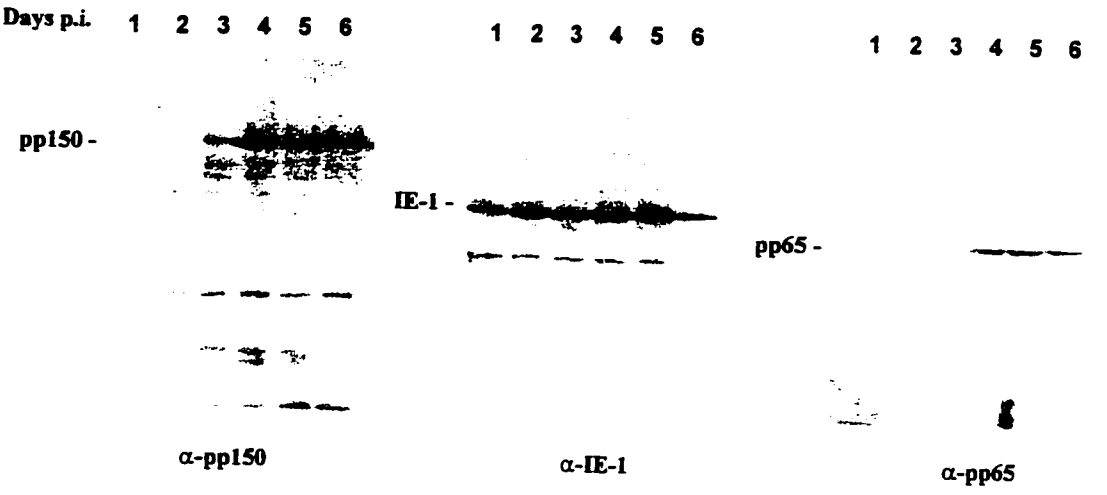
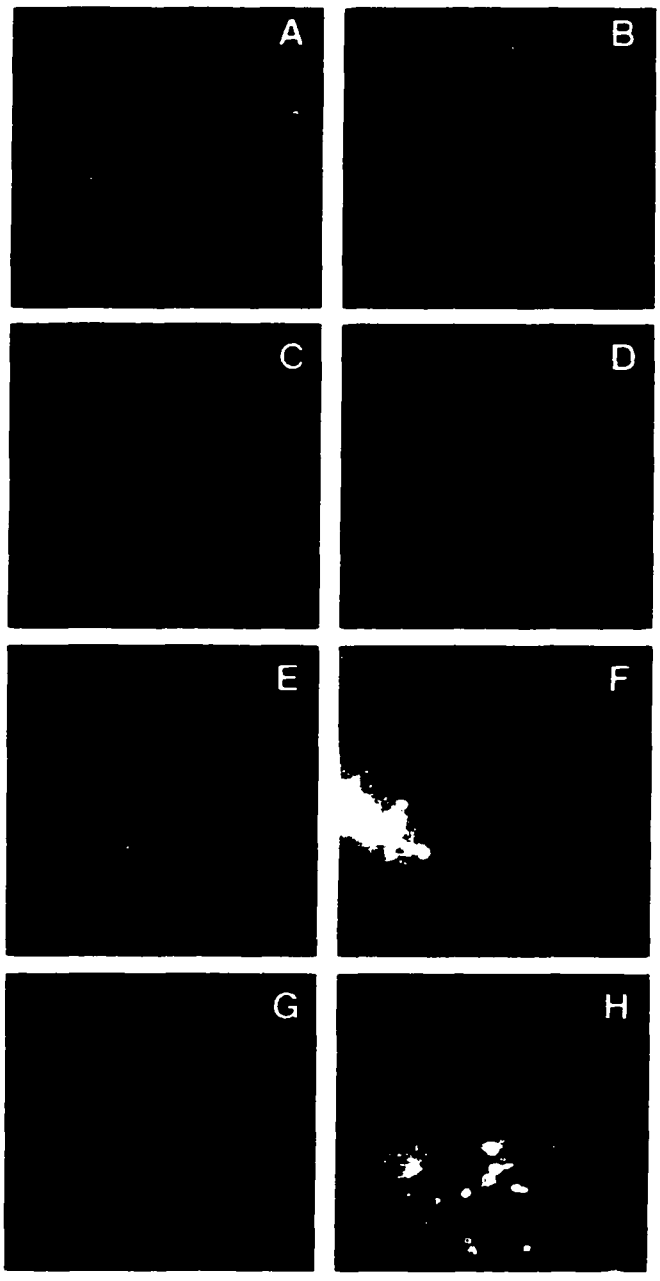


FIG. 3. Cellular location of HCMV-encoded proteins in virus infected cells. Primary human fibroblasts were infected with HCMV strain AD169 and prepared for immunofluorescence 5 days following infection as described in Material and Methods. (A) Nuclear staining of IE-1 as detected with the IE-1 specific mAb p63-27. (B) Nuclear staining of ppUL69 detected with mAb UL69. (C) Nuclear and cytoplasmic staining of pp65 detected with mAb 28-19. The nucleus of the infected cell in this panel is at the extreme lower left corner. This area can be better appreciated in panel F as the area below and to the left of the large perinuclear accumulation of pp150. (D) Cytoplasmic staining of pp28 detected with mAb 41-18. (E) Cytoplasmic staining of pp150 detected with anti-peptide serum and anti-rabbit IgG antibodies conjugated with Texas Red. This panel reveals a large perinuclear accumulation of pp150 (left) and multiple cytoplasmic vacuole-like structures (right). (F) Colocalization of pp150 and pp65 in cytoplasmic vacuoles of infected cells photographed in panels C and E. (G) Cytoplasmic distribution of pp150 as detected with anti-peptide sera described in panel E. (H) Colocalization of pp150 and pp28 in cytoplasmic vacuoles in infected cell photographed in panels G and D.

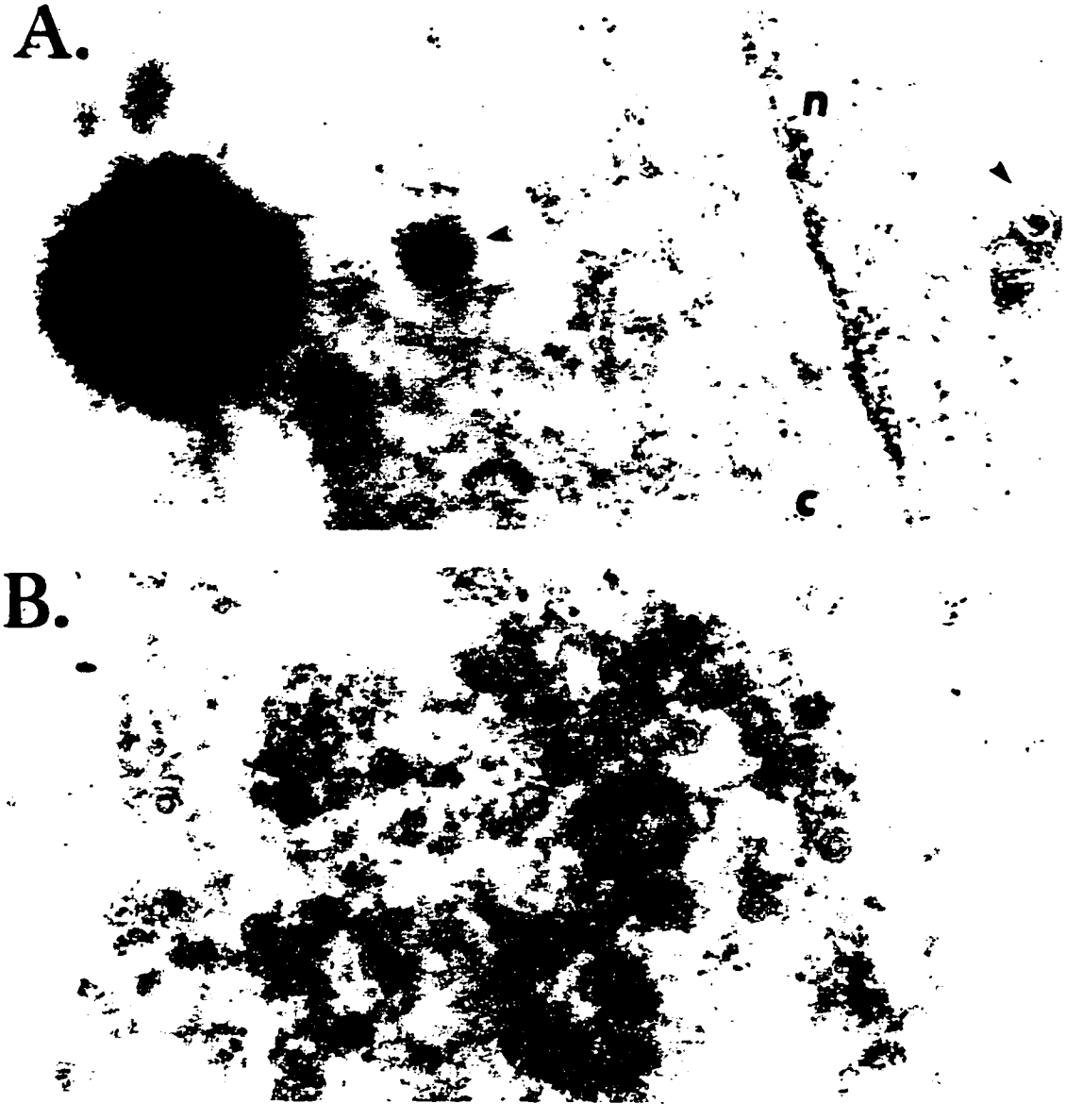


pp150 under control of the HCMV IE promoter/enhancer, the protein was distributed diffusely in the cytoplasm as demonstrated by immunofluorescence but again the protein was concentrated along the periphery of the nucleus (data not shown). This finding suggested that the cytoplasmic location of pp150 was independent of other viral proteins. Additional evidence of its cytoplasmic localization was obtained by immune electron microscopy of HCMV-infected HF cells using gold-conjugated anti-rabbit IgG antibodies to detect pp150. Gold particles were detected in the cytoplasm but not in the nucleus of infected cells (Fig. 4). In addition, we also noted the presence of gold particles on cytoplasmic virions and dense bodies, but not intranuclear subviral particles (Fig. 4). In contrast to a recent report describing the nuclear localization of pp150 (13), these results indicated that pp150 was a cytoplasmic protein which could also be detected on both cytoplasmic virions and dense bodies.

Structural Proteins of the HCMV Tegument Are Localized in Different Cellular Compartments During Permissive Infection

The availability of defined antibody reagents against two cytoplasmic and two nuclear tegument proteins allowed us

FIG. 4. Immune electron microscopic detection of pp150 in the cytoplasm, but not the nucleus, of infected cells. Primary human fibroblasts were infected with HCMV strain AD169 and fixed on day 5 post-infection as described in Materials and Methods. Following permeabilization, the monolayer was reacted with anti-pp150 peptide sera and antibody re-=activity detected with 6-nm colloidal gold particles conjugated to anti-rabbit IgG. (A) Gold particles are shown surrounding cytoplasmic dense body and a virion (c), with nuclear particles (n) exhibiting no gold particles. (B) Expanded view of nucleus from the cell photographed in panel A revealing no evidence of gold-labelled particles in the nucleus of infected cells.



to examine colocalization of these proteins during permissive HCMV infection of HF cells. As previously reported and described earlier, we detected pp28 exclusively in the cytoplasm of infected cells (Fig. 3D). The tegument protein pp150 could be detected in the cytoplasm and to a lesser extent, at the periphery of the nucleus (Fig. 3E). Both proteins were detected in large vacuole-like structures and in perinuclear caps. In contrast to these results, ppUL69 was localized to the nucleus of infected cells (Fig. 3B). A second nuclear tegument protein, pp65, was also found only in the nucleus of infected cells until late in infection when it was dispersed throughout the cell (Fig. 3C). The two nuclear tegument proteins, pp65 and ppUL69, failed to colocalize with either pp28 or pp150 until extremely late in infection when the infected HF cells exhibited extensive cytopathology (Fig. 3F, data not shown). Interestingly, pp150 and pp28 colocalized to the large vacuole-like structures in the cytoplasm (Fig. 3H). These structures were estimated by confocal microscopy to be on the order of 1000-2200 nm in diameter, suggesting that they were not cytoplasmic dense bodies but more likely virus-induced, host-derived structures. Late in infection we could also detect pp65 in similarly sized structures, suggesting that these

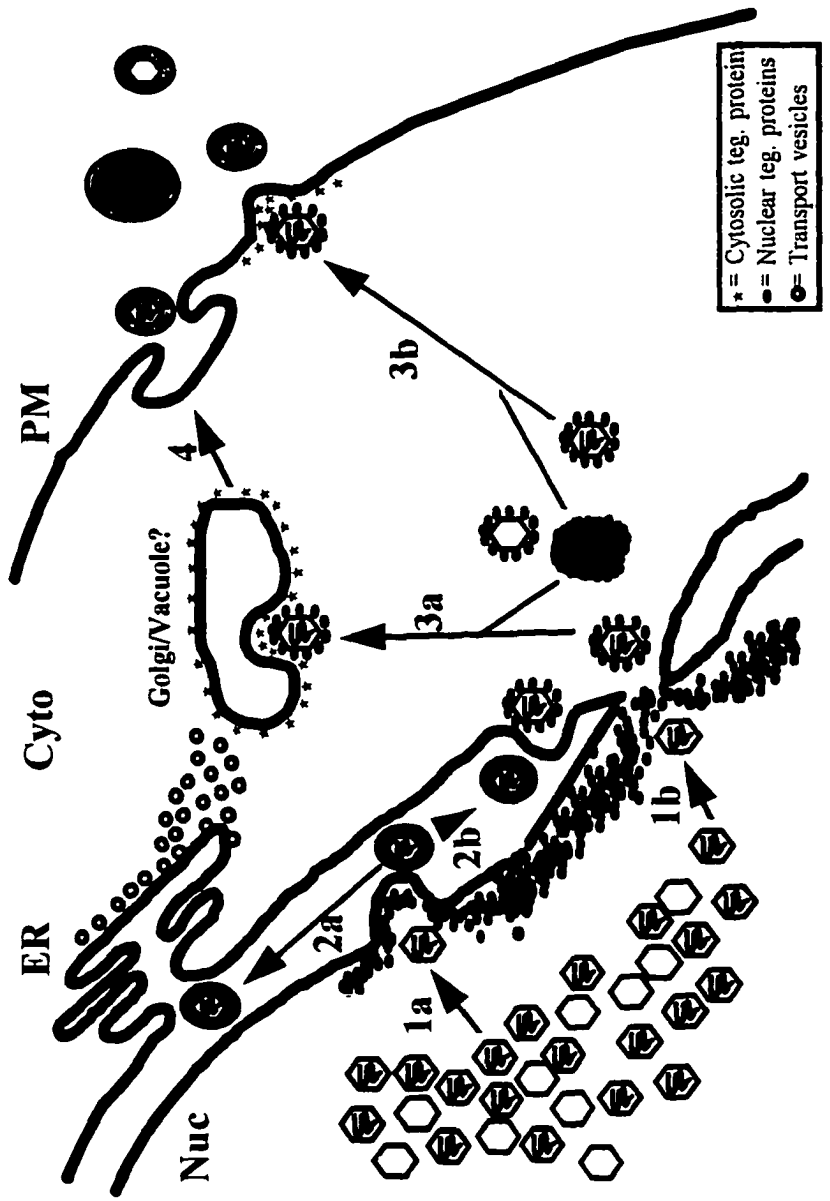
vacuoles represent areas of particle assembly (Fig. 3F). Taken together, our results indicated that proteins of the tegument of HCMV were localized to different compartments within the infected cells. Moreover, our findings suggested that viral proteins which were found in two different cellular compartments were incorporated into the tegument of extracellular particles produced by HCMV-infected cells, suggesting a complex, nuclear and cytoplasmic tegument assembly pathway for this human herpesvirus.

DISCUSSION

Over the past 10 years numerous studies have addressed the morphogenesis of the virion envelope of herpesviruses (8, 10, 11, 12, 14, 17, 18, 20, 21, 23, 24, 28-32, 34). The earliest findings from studies utilizing electron microscopy for analysis of virion assembly and viral protein distribution within infected cells suggested that herpesviruses acquired their envelope at the nuclear membrane (17, 25, 29). Non-enveloped capsids within the cytosol were thought to be defective particles arising from a default pathway of assembly. More recently, alternative models of envelopment for several other herpesviruses including varicella-zoster (VZV), pseudorabies virus (PrV), human herpesvirus 6, and

herpes simplex virus (HSV) have been presented (8, 10, 11, 12, 14, 20, 21, 23, 24, 30-32, 34). A simplified composite of many of these models is presented in Fig. 5. Common to many of the models is a primary envelopment step in which capsids or defective particles, such as DBs and NIEPs, bud through the inner nuclear membrane and accumulate in the perinuclear space (Fig. 5). At this stage the virus particle could follow a pathway suggested for HSV and enter adjacent ER which is contiguous with the nuclear envelope. From this point the enveloped particle could then travel through the secretory pathway, which in turn would provide the post-translational modifications which have been reported in the fully-processed envelope glycoproteins of mature virions (17). A recent study using gold-labelled lectins in electron microscopy documented the presence intracellular HSV virions containing both fully-modified and immature glycoproteins, a finding consistent with this assembly pathway or a pathway in which the particle acquires its envelope in the *cis*-Golgi (8). An alternative pathway of envelope assembly invokes deenvelopment of the particles within the perinuclear space following fusion with the outer nuclear membrane and eventual release of non-enveloped subviral particles into the cytoplasm (8, 10-12, 14, 18, 21, 23, 34). A

FIG. 5. Proposed pathways of herpesvirus morphogenesis. (1) Tegument containing capsids acquire additional tegument proteins and viral glycoproteins in the nucleus by budding through areas of the nuclear envelope containing viral glycoproteins and tegument proteins (a) or through tegument modified and fragmented nuclear envelope, which does not require an envelopment/development step (b). (2) Enveloped particles can remain enveloped and follow the secretory pathway through the ER and Golgi (a) or following deenvelopment bud into the cytoplasm as non-enveloped but partially tegumented particles (b). (3) Tegument containing particles, virions, NIEPS, and dense bodies can acquire their final tegument and envelope in a viral glycoprotein containing cytoplasmic vacuole (a) or less likely at the plasma membrane (b). (4) Following envelopment in a cytoplasmic vacuole, fully enveloped particles leave the cell either through an exocytic pathway or as a result of cell lysis.



third but unstudied potential mode of nuclear egress could involve virus-induced, localized modification of the nuclear membrane followed by release of tegument-coated particles into the cytosol. Once in the cytoplasm, capsids or defective particles could acquire an envelope of fully-processed glycoproteins from a cytosolic, perhaps post-Golgi, vesicle (Fig. 5, step 3a). Evidence for this process has been documented in VZV- and PrV-infected cells (10, 12, 18, 34). A less likely possibility and one that is not well supported in the literature is that the final envelopment of herpesviruses occurs by budding at the plasma membrane. While electron microscopy has provided evidence consistent with many of the these models, this technique has provided only static, often non-quantitative images of a dynamic pathway. In addition, identification of selected particles by electron microscopy provides no information about their infectivity. Thus, readily observable particles in electron micrographs may represent populations of non-infectious, intracellular particles destined for degradation. For these as well other reasons, models of virus egress from herpesviruses infected remain highly contentious and continue to be the subject of intense discussion and investigation. In an attempt to further characterize the process of envelopment,

we took a topological approach by determining the subcellular location of four tegument proteins using well-characterized antibodies. Since extracellular virions contain all of these proteins surrounded by the viral envelope, this would suggest that tegument acquisition and final envelopment may be either a sequential or coupled process.

Initially we believed that the assembly of extracellular dense bodies could provide a simple model of particle assembly because of their reported composition of a very limited set of virus-encoded proteins. However, using a panel of HCMV-specific mAbs, we readily demonstrated that an enriched population of dense bodies contained a similar set of structural proteins as has been reported in extracellular virions. Specifically we could show that dense bodies contained capsid proteins, several different tegument proteins, including tegument proteins found only in the cytoplasm, as well as two different glycoproteins, gB (gpUL55) and gH (gpUL75). The processed and cleaved form of gB was present within dense bodies, thus indicating that the dense body envelope was either acquired or modified after exposure to enzymatic activities in the *trans*-Golgi. These results, including the finding of a processed form of gB, were consistent with a recent report by Baldick and Shenk but were

somewhat different from those of Irmiere and Gibson, who reported that dense bodies contain only pp65 and gB (2, 16). We could not readily explain the differences between our findings and those of these later authors as we achieved a similar level of enrichment of dense bodies as defined by their decreased infectivity ($>3_{log}$) as compared to the virion fraction (16). Moreover, we recovered over 99% of input infectivity from the virion fraction, suggesting that the dense body preparation was not heavily contaminated with infectious virions. Perhaps the most likely explanation for the differences between our results and those of Irmiere and Gibson could be found in the methodologies employed in the two studies (16). First, we collected and analyzed the dense bodies as a pooled population and not as individual fractions from a gradient as reported by Irmiere and Gibson. Thus, we analyzed an average signal from this heterogenous population of non-infectious particles. Furthermore, we utilized HCMV-specific mAbs in an immunoblotting assay to define composition, a method which is inherently more specific and sensitive than SDS-PAGE analysis of radiolabelled protein (16). These differences could have accounted for the detection of a greater number of proteins within dense

bodies in the current study as compared to the findings of Irmiere and Gibson (16).

The finding that dense bodies contained a similar array of structural proteins as infectious virions suggested that assembly of these non-infectious particles likely was similar to that of infectious virions. Examination of the distribution of tegument proteins within the infected cell provided some insight into the assembly of HCMV. Immunofluorescence and EM localization studies of the tegument proteins pp150, pp65, and pp28 revealed that, while each of the proteins was present in extracellular virions and non-infectious particles such as dense bodies, they were distinct in their location within the infected cell. While we could show that pp28 and pp150 remained in the cytoplasm throughout the replicative cycle of HCMV, pp65 was retained within the nucleus and only appeared in the cytoplasm very late in infection when extensive cellular damage was present. Immunolocalization of recombinant expressed pp28 and pp150 demonstrated that both proteins were excluded from the nucleus (data not shown). In the case of pp28, this result confirmed an earlier report using immune electron microscopy (19). By immunofluorescence microscopy, pp150 was detected predominantly in the cytoplasm of transfected cells but a

distinct distribution along the nucleus was also observed. However, immunoelectron microscopy of HCMV-infected cells failed to reveal the presence of pp150 in the nucleus. Computer analysis of the predicted primary sequence of pp28 and pp150 failed to reveal any sequence similar to known nuclear localization signals. These results were in contrast to pp65 and ppUL69 which were both nuclear proteins. Although the nuclear localization signals of ppUL69 have not been well defined, the nuclear localization of pp65 has been the subject of at least two reports (9, 27). Studies from our laboratory have further defined the regions of pp65 which appeared responsible for nuclear retention as well as nuclear targeting (V. Sanchez, manuscript in preparation). Taken together, the finding that two abundant components of the tegument were cytosolic proteins and that at least two tegument proteins were nuclear in their distribution presented a topological contradiction for an assembly pathway which required nuclear tegumentation and final envelopment at the nuclear envelope.

A second observation that also provides insight into tegument acquisition and envelopment was the colocalization of the pp28, pp150, and pp65 tegument proteins in the cytosol of infected cells late in infection, just prior to cell

lysis. The colocalization of these proteins was consistent with an envelopment/deenvelopment of a partially tegumented particle followed by trafficking of this particle to a cytoplasmic site for the addition of remaining tegument proteins. The acquisition of pp150 and pp28 could then occur prior to final envelopment in a cytosolic vesicle. Evidence for such a site was the colocalization of the pp28 and pp150 in large vacuole-like structures very late in infection (Fig. 3).

Current models of nuclear egress of herpesviruses have in common the budding of the subviral particle through a viral glycoprotein modified nuclear membrane. An alternative mechanism for nuclear egress could involve the direct extrusion of a tegumented particle from the nucleus following a focal disruption of the nuclear envelope. Such a process could accelerate late in infection and provide a large number of cytoplasmic particles in cells with a fragmenting nucleus. Although there is no direct evidence for such a process of nuclear egress, our recent findings of a nuclear lamins within tegument-containing vacuoles are consistent with this pathway (26). If such a model of nuclear egress constitutes a productive pathway to infectious virion assembly, then a process of envelopment and deenvelopment is

not required to explain the passage of partly tegumented particles from the nucleus to the cytoplasm. Understanding the nuclear egress and tegument assembly of HCMV will likely clarify many of the issues surrounding the addition of the envelope to this large herpesvirus.

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LOCALIZATION OF HUMAN CYTOMEGALOVIRUS STRUCTURAL PROTEINS TO THE NUCLEAR MATRIX OF INFECTED HUMAN FIBROBLASTS

by

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ABSTRACT

The intranuclear assembly of herpesvirus subviral particles remains an incompletely understood process. Previous studies have described the nuclear localization of capsid and tegument proteins as well as intranuclear tegumentation of capsid-like particles. The temporally- and spatially-regulated replication of viral DNA suggests that assembly may also be regulated by compartmentalization of structural proteins. We have investigated the intranuclear location of several structural and non-structural proteins of human cytomegalovirus (HCMV). Tegument components, including pp65 (ppUL83) and ppUL69, and capsid components, including the major capsid protein (MCP, pUL86) and the small capsid protein (SCP, pUL48/49), were retained within the nuclear matrix (NM), whereas the immediate-early regulatory proteins IE-1 and IE-2 were present in the soluble nuclear fraction. The association of pp65 with the NM resisted washes with 1 M guanidine hydrochloride, and direct binding to the NM could be demonstrated by far-Western blotting. Furthermore, pp65 exhibited accumulation along the nuclear periphery and in far-Western analysis bound to proteins which comigrated with proteins of the size of nuclear lamins. A direct interaction between pp65 and lamins was demonstrated by

coprecipitation of lamins in immune complexes containing pp65. Together our findings provide evidence that major virion structural proteins localized to a nuclear compartment, the nuclear matrix, during permissive infection of human fibroblasts.

INTRODUCTION

Recent studies have indicated that the human cytomegalovirus (HCMV) virion is composed of even a larger number of proteins than previously thought, suggesting that the assembly of the infectious particle is extraordinarily complex (3). The description of the architecture of the virion has been simplified to include three distinct structures: the capsid, the envelope, and a poorly characterized region between the capsid and envelope termed the tegument (54, 55). The protein composition of the HCMV tegument has been incompletely defined, but it is thought to be composed of a large number of phosphoproteins (3, 46). Although there is general agreement that the capsid is assembled in the nucleus, considerable controversy continues to surround the identity of the cellular site of envelopment of herpesviruses (4, 19, 30, 53). The assembly pathway of the tegument region remains even less well understood. The distribution of

protein components of the HCMV tegument suggests that assembly of this virion structure takes place both in the nucleus and the cytoplasm. Tegument proteins encoded by UL82 (pp71), UL83 (pp65), and UL69 open reading frames (ORFs) appear to localize in the nucleus while the tegument protein pp28 (ppUL99) is detected in extranuclear compartments of infected cells (24, 26, 38, 58, 61). HCMV pp150 (UL32) has been reported to demonstrate both a nuclear and cytoplasmic distribution (34), although studies in our laboratory have suggested that pp150 is predominantly a cytoplasmic protein (58). This organization of tegument components suggests that these proteins are incorporated into the virion in an ordered manner and, furthermore, that understanding tegument morphogenesis could provide insight into the pathways of virion assembly and nuclear egress.

In order to further describe virion maturation, we have begun an investigation of the pathway in which the tegument is assembled around the nucleocapsid. Recent studies in herpes simplex virus (HSV) together with previous reports describing replication centers in the nuclei of infected cells have suggested that herpesviruses not only employ complex regulatory controls of transcription and replication (18, 41, 42, 56) but also possibly regulate particle

assembly by localizing structural proteins into discrete subnuclear compartments (63, 64). Recent studies by Ward and coworkers have divided the nucleus of HSV-infected cells into different compartments called assemblons based on localization of known proteins of HSV (64). These included compartments for replication and subviral particle formation (64). We have begun a series of experiments to further define the assembly and nuclear egress of human cytomegalovirus. Specifically, we have examined the distribution of several tegument proteins within the nuclear matrix of infected cells in order to define spatial relationships and potential colocalization of structural proteins late in infection. This compartment of the nucleus was examined initially because it has been defined biochemically and thus represented a nuclear compartment which could be analyzed by both biochemical and imaging techniques.

The nuclear matrix is a proteinaceous network which is tightly associated with the inner nuclear membrane. In many cell systems, the nuclear matrix has been found to be the site of active transcription and replication of cellular DNA (6, 28, 35, 48, 49, 62). Proteins involved in these processes as well as those with regulatory roles in cell division localize to this nuclear scaffold (8, 15, 22, 32, 39,

40, 43, 52). Among DNA viruses, there are numerous examples of viral gene products which associate with the nuclear matrix or nuclear matrix structures (2, 10, 14, 16, 21, 25, 31, 37, 44, 51, 60, 66). This association may serve to compartmentalize products necessary for efficient transcription and replication of the viral genome or to sequester components involved in virion maturation (5, 7, 25, 37, 44, 51, 66). The role of the nuclear matrix in the replicative cycle of herpesviruses, including HCMV, has not been extensively studied. In this report we have described the binding of several HCMV virion structural proteins including pp65 (ppUL83), ppUL69, and MCP (pUL86) to the nuclear matrix of HCMV-infected cells. The accumulation of virion components on the nuclear matrix late in infection suggested that this compartment was a potential staging site for virion structural proteins prior to their assembly into subviral particles.

MATERIALS AND METHODS

Cells, Viruses, and Antibodies

Human foreskin fibroblasts (HF) and monkey BSC-1 cells were maintained in Medium 199 with 5% newborn calf serum and

antibiotics at 37°C. For in situ nuclear matrix extractions, HF were grown on glass coverslips at 37°C in 5% CO_2. Cells were infected with the AD169 strain of HCMV at a multiplicity of infection of 0.1 to 1. The human epidermoid carcinoma cell line, HEp2, and monkey Cos7 cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and antibiotics.

The recombinant vaccinia viruses, vv-Eco V gB and vv-pp65, were propagated in BSC-1 cells. The construction and characterization of these recombinant vaccinia viruses have been reported (9, 13). The vv-Eco V gB construct differs from the vv-gB recombinant previously reported in that the Eco V construct contains the gB gene of AD169 truncated at nucleotide 1950 and the corresponding gB protein terminates at amino acid 650. This protein lacks the carboxy terminus including the transmembrane region and represents a secreted form of HCMV gB. The pp65-green fluorescent protein fusion was constructed using the EGFP-N2 plasmid (Clontech, Palo Alto, Calif.). A *Bam*HI site was generated at the 5' end of the pp65 genomic sequence by PCR using the primer 5' TTTTT-TGGATCCATGGAGTCGCGCGGT 3'. The product was fused in-frame into the EGFP-N2 vector 3' to the EGFP coding sequence.

HCMV proteins were detected with monoclonal antibodies (MAbs) previously described (1, 12, 50, 65). The MAbs used in this study included those with specific reactivity to IE-1 (p63-27), IE-2 (IE-2-9-5), pp65 (28-19, 65-8, 28-103, 28-77), MCP (28-4), SCP (11-2-23), gB (7-17), UL69 (UL69), UL44 (28-21), and pp28 (41-18). The guinea pig polyclonal serum recognizing pp65 was generated by repeated immunization of guinea pigs with pp65 purified from bacteria. The previously characterized rabbit polyclonal antibody 237 against lamins a, b, and c was a generous gift from Dr. Robert Goldman (Department of Cell and Molecular Biology, Northwestern University School of Medicine, Chicago, Ill.) (29). The rabbit polyclonal sera against lamins a and c and against lamin b (17) were a generous gift from Dr. Nilabh Chaudhary (RPI, Boulder, Colo.). The monoclonal antibody against lamin B1, NA12, was purchased from Oncogene Sciences (Boston, Mass.). FITC-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-guinea pig IgG antibodies were obtained from Cappell Laboratories (Raleigh, N.C.). Texas Red-conjugated goat anti-rabbit IgG antibody was purchased from Southern Biotechnology Associates (Birmingham, Ala.).

Preparation of Nuclear Matrix

Nuclear matrix fractions were prepared by a method similar to that described by Mirkovitch et al. (45). AD169-infected HF, vv-pp65 and vv-Eco V gB-infected BSC-1 cells, or uninfected HEp2 cells were fractionated by extraction in 0.1% or 0.2% Nonidet-P-40 (NP-40) in phosphate-buffered saline (PBS) (137 mM NaCl, 8.1 mM NaH_2PO_4-$12H_2O$, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4) to yield crude nuclei. Nuclei were resuspended in 1 ml of digestion buffer (20 mM Tris-HCl [pH 7.4], 20 mM KCl, 70 mM NaCl, 10 mM $MgCl_2$, 0.05 mM spermine, 0.125 mM spermidine) with 1 mM phenylmethylsulfonyl fluoride (PMSF) and subjected to DNase I digestion (0.05 mg/mL) for 15 min at room temperature. Nuclei were then resuspended in digestion buffer with 0.1% digitonin and incubated at room temperature for 10 min. The nuclear material was pelleted then extracted in high-salt buffer (2 M NaCl, 20 mM HEPES [pH 7.4], 20 mM EDTA) on ice for 5 min. The nuclear material was then pelleted and washed twice in digestion buffer and finally resuspended in SDS-sample buffer with 5% 2-mercaptoethanol or washed three times in 1 M guanidine hydrochloride in digestion buffer (2 min per wash) before addition of sample buffer (25). For a typical experiment, six 150 cm^2 flasks of AD169-infected HF were harvested. For

quantitative Western blots, AD169-infected HF cells from fourteen 150 cm^2 flasks were fractionated and protein content was determined using bicinchoninic acid reagent (Pierce, Rockford, Ill.). Quantitation of the radioactive signal was determined on a Molecular Dynamics Phosphorimager. For far-Western blots, nuclear matrix fractions were isolated from four 150 cm^2 flasks of HEp2 cells.

Nuclear matrix extracts for immunoprecipitation were prepared by a method similar to that described by Fey and Penman (23). Briefly, the nuclear matrix material extracted from AD169-infected cells was solubilized in disassembly buffer (8 M urea, 20 mM morpholineethanesulfonic acid [pH 6.6], 1 mM EGTA, 1 mM PMSF, 0.1 mM $MgCl_2$, 1% 2-mercaptoethanol), 16 h at 4°C. The insoluble material was removed by centrifugation. The urea was removed from the supernatant by step-dialysis against Tris-buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4) at 4°C, and the insoluble material was removed at each step. A mixture of three pp65-specific MAbs (28-19, 28-103, and 65-8) was used to precipitate the dialyzed extract. Immunoprecipitates were collected on protein A-agarose and washed extensively in radioimmunoprecipitation assay buffer (1% NP-40, 1% deoxycholate, 0.2% SDS in TBS [pH 7.4]). The samples were

subjected to SDS-polyacrylamide gel electrophoresis (PAGE) then transferred to nitrocellulose for Western blotting as described previously (12).

For in situ extraction of AD169-infected HF, a different method was used for nuclear matrix preparation. This method was similar to that described by He et al. (33). Infected monolayers grown on glass coverslips were treated with digestion buffer (described above) with 0.1 mM PMSF and 0.5% NP-40 for 3 to 5 min on ice. The monolayers were then treated with digestion buffer containing 0.05 mg/mL DNase I for 15 min at room temperature. Chromatin was removed by washing monolayers three times with 0.25 M ammonium sulfate in digestion buffer (pH 7.2) at room temperature, 10 min per wash. Cells were then extracted with high salt buffer (described above) for 5 min on ice. Cell cytoskeletons were carefully rinsed with digestion buffer then fixed in 2.5% paraformaldehyde in PBS for 20 min at room temperature.

Fluorescence Microscopy

Virus-infected HF cells grown on glass coverslips were fixed in 2.5% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS for 5 min. After rinsing, cells and extracted cytoskeletons were blocked with 30% goat serum

in PBS for 30 min at 37°C. Coverslips were incubated with primary antibody with 1% goat serum for 1 h at 37°C. Coverslips were washed three times in PBS, 5 min per wash, then incubated with FITC-conjugated and/or Texas Red-conjugated secondary antibody for 1 h at 37°C. After washing, coverslips were refixed with 0.5% paraformaldehyde in PBS for 10 min. After rinsing in PBS, coverslips were mounted with SlowFade antifade reagent (Molecular Probes, Eugene, Oreg.) and sealed with fingernail polish and were viewed on a Leitz Diavert fluorescence microscope or a Zeiss confocal microscope.

Cos7 cells grown on coverslips were transfected using either lipofectin (57) or calcium phosphate protocols (5' Prime-3' Prime, Boulder, Colo.). Cells were transfected with a modified pcDNA3 vector (Invitrogen, San Diego, Calif.) containing e pp65 genomic sequence or a vector encoding a green fluorescent protein-pp65 fusion protein (Clontech). Transfected cells were fixed 36 to 48 h post-transfection and cells expressing pp65 were reacted with MAb 28-19 followed by FITC-conjugated goat anti-mouse IgG antibody as described above.

Far-Western Blots

pp65 was purified from *Eschirichia coli* transformed with the plasmid trc/hisA (Invitrogen) which contained the complete pp65 ORF. After induction with isopropyl-thio-B-D-galactopyranoside (IPTG), cultures were collected and the bacterial pellet was resuspended in denaturing lysis buffer (20 mM Tris-HCl, 100 mM NaCl, 8 M urea [pH 8.0]). The suspension was sonicated on ice until translucent. Insoluble material was removed by centrifugation at 10,000 x g for 10 min. The lysate was incubated with Talon metal affinity resin (Clontech) at room temperature with gentle rocking for 30 min. The resin was collected by centrifugation and washed with denaturing lysis buffer three times, 10 min per wash. pp65 was eluted by incubating the resin with denaturing lysis buffer containing 75 mM imidazole four times, 10 min per elution. Fractions were pooled and the urea was removed by dialysis against TBS. Protein concentration was determined using bicinchoninic acid reagent (Pierce).

Nuclear matrix samples from HEp2 were subjected to SDS-PAGE as previously described (12). Proteins were blotted to nitrocellulose and filters were blocked in 5% dry milk in PBS. Strips were incubated with 150 to 200 ug of pp65 per strip in 5% milk overnight at room temperature with gentle

rocking (2). The strip was washed in PBS three times, 10 min per wash, and, following the final wash, was incubated with the pp65-specific MAb 28-19 for 4 h at 37°C, then washed in PBS as described above. The strip was then incubated with a rabbit anti-mouse IgG secondary antibody for 1 h at 37°C. The filter was washed again, then incubated with ^{125}I-protein A for 30 min at 37°C. After washing, filters were dried and mounted, and bound antibody was detected by autoradiography. For Western blot analysis, nuclear matrix samples were separated electrophoretically then blotted. Strips were processed as previously described (12).

RESULTS

Tegument and Capsid Protein Components of HCMV Are Associated With the Nuclear Matrix

Phosphoprotein 65 (pp65) is one of the most abundant protein components of extracellular virions and dense bodies. Shortly after HCMV infection of human fibroblasts, pp65 can be detected in the nucleus of infected cells, suggesting that it is actively transported to this cellular compartment (26, 61). Immunofluorescence microscopy of AD169-infected HF cells as well as of Cos7 cells transfected with a plasmid containing the pp65 genomic sequence revealed

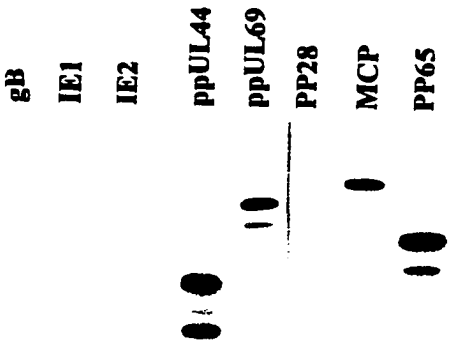
the accumulation of pp65 into discrete structures in the nucleus (Fig. 1A to D). Similarly, in studies utilizing a pp65-green fluorescent protein fusion, compartmentalization of the protein was observed as well as focal accumulation along the periphery of the nucleus (Fig. 1E to F). Together with previously reported findings which indicated that deletion mutants of pp65 lacking the carboxy-terminal nuclear targeting signals continued to accumulate in the nucleus (26, 61), these results suggested that pp65 might contain additional domains which could mediate nuclear retention by targeting the protein to a specific nuclear structure.

To characterize the interaction of pp65 with subnuclear structures, we investigated the binding of the protein to the nuclear matrix of HCMV-infected human fibroblasts. Extraction of isolated nuclei with 2 M NaCl resulted in an insoluble pellet containing pp65 as demonstrated in Western blots using the pp65-specific monoclonal antibody (MAb) 28-19 (Fig. 2A). Because pp65 was an abundant structural protein, we examined the possibility that other virion components were also present in the nuclear matrix. Western blot analysis of infected cells revealed that several virion structural proteins were associated with the nuclear matrix. Structural components including the MCP (pUL86) and the

FIG. 1. HCMV pp65 is a nuclear protein that is detected in subnuclear structures. HF cells were grown on coverslips and infected with AD169. Three to 5 days post-infection (100% cpe), cells were fixed and stained in immunofluroescence assays with the pp65-specific (A) mAb 65-8, (B) mAb 28-19, or (C) polyclonal guinea pig serum against bacterially-expressed pp65. Cos7 cells transfected with a vector expressing (D) pp65 or a (E, F) EGFP-pp65 fusion protein were fixed and stained with mAb 28-19 (D only). Magnification for panels A-C, 400X; for panels D-F, 1,000X.

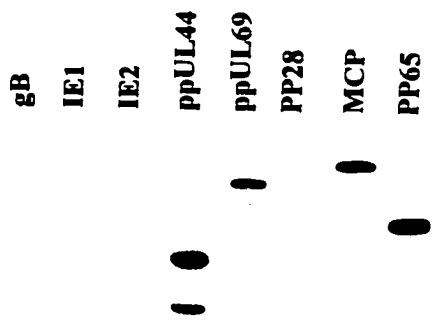
FIG. 2. HCMV-encoded structural and non-structural proteins are retained in the nuclear matrix of HCMV AD169-infected human fibroblasts. Nuclei from AD169-infected HF cells were isolated by treatment with non-ionic detergent then treated with DNase and high salt to remove soluble components from the nuclear matrix fraction. (A) Nuclear matrix-containing filters were probed with mAbs specific for gB, IE1, IE2, ppUL44, ppUL69, pp28, MCP, and pp65. (B) Filters containing proteins from nuclear matrix fractions washed with 1 M guanidine hydrochloride prior to electrophoretic separation were probed with the antibodies listed above.

A.



HIGH SALT EXTRACTION

B.



1M GUANIDINE HCl WASH

tegument protein ppUL69 were also retained in the nuclear matrix fraction. In addition, the non-structural viral polymerase accessory protein ppUL44 and previously described smaller molecular weight forms of this protein were associated with the nuclear matrix fraction of infected cells. The binding of these proteins was stable and resisted three washes with 1 M guanidine hydrochloride except for an observable decrease in the 50- to 52-kDa forms of pp65 (Fig. 2B). The interaction of these proteins was also specific as other virus-encoded proteins previously shown to localize in the nucleus of infected cells, including the 72-kDa IE-1 and 86-kDa IE-2, were not detected in the nuclear matrix (Fig. 2). In addition, two cytoplasmic virion proteins, gB (gpUL55) and pp28 (ppUL99), were not detected in the nuclear matrix of infected cells (Fig. 2).

The presence of structural components of the capsid and tegument on the nuclear matrix suggested that this nuclear structure was a potential assembly site for subviral structures. Furthermore, the strength of the association between viral and cellular proteins of the nuclear matrix as reflected by their binding following washes with 1 M guanidine hydrochloride suggested a direct interaction between individual virion proteins and components of the nuclear matrix.

Alternatively, individual virion structural proteins could be tethered to the matrix through interactions with other virus-encoded proteins. In order to gauge the relative strength of these protein-protein interactions, we measured the quantity of pp65 distributed between the different pools collected during the fractionation procedure (Fig. 3). As shown in Fig. 3B and Table 1, only a small amount of the total cellular pp65 can be detected in the soluble fraction isolated by treatment of cells with 0.2% NP-40. This fraction contained the cytoplasm and nuclear proteins released by this treatment as demonstrated by the presence of IE-1 in Fig. 3A. The nuclear and nuclear matrix fractions contained more pp65 per microgram of protein than the soluble fraction (Fig. 3B; Table 1). In addition, quantitation of signal intensity showed that the relative amount of pp65 was not greatly reduced by the fractionation procedure (Fig. 3B, lanes 2 to 4; Table 1). In fact, there was a relative enrichment of pp65 on the nuclear matrix (NM) and in the guanidine hydrochloride-washed (GW) pellet (Table 1). Of interest was the apparent loss of the 50- to 52-kDa forms of pp65 during the guanidine hydrochloride wash (Fig. 3; Table 1), suggesting that these products were not as strongly retained as the full-length protein. Consistent with our

FIG. 3. Quantitative Western blots of nuclear matrix preparations from AD169-infected HF cells. HCMV-infected cells were fractionated and protein content in each fraction was determined as described in Materials and Methods. Twenty-five micrograms of each of the fractions was loaded into the respective lanes (C, cytoplasm, soluble protein; N, detergent-treated nuclei; NM, nuclear matrix pellet; GW, guanidine hydrochloride washed NM) and transferred to nitrocellulose filters. Filters were reacted with mAb p63-27 against IE-1 (panel A) or mAb 28-19 against pp65 (panel B) and processed for autoradiography. Counts for each fraction were determined on a phosphorimager and were as follows: IE-1, 449874.4, 13951.6, 9332.5, and 9514.3, for C, N, NM and GW fractions, respectively; pp65 (68 kDa), 239720.7, 2048547.0, 1206468.7, and 1172115.9, for C, N, NM, and GW fractions, respectively; pp65 (50 kDa form), 33155.2, 1003560.2, 774609.0, and 188991.4, for C, N, NM, and GW fractions, respectively.

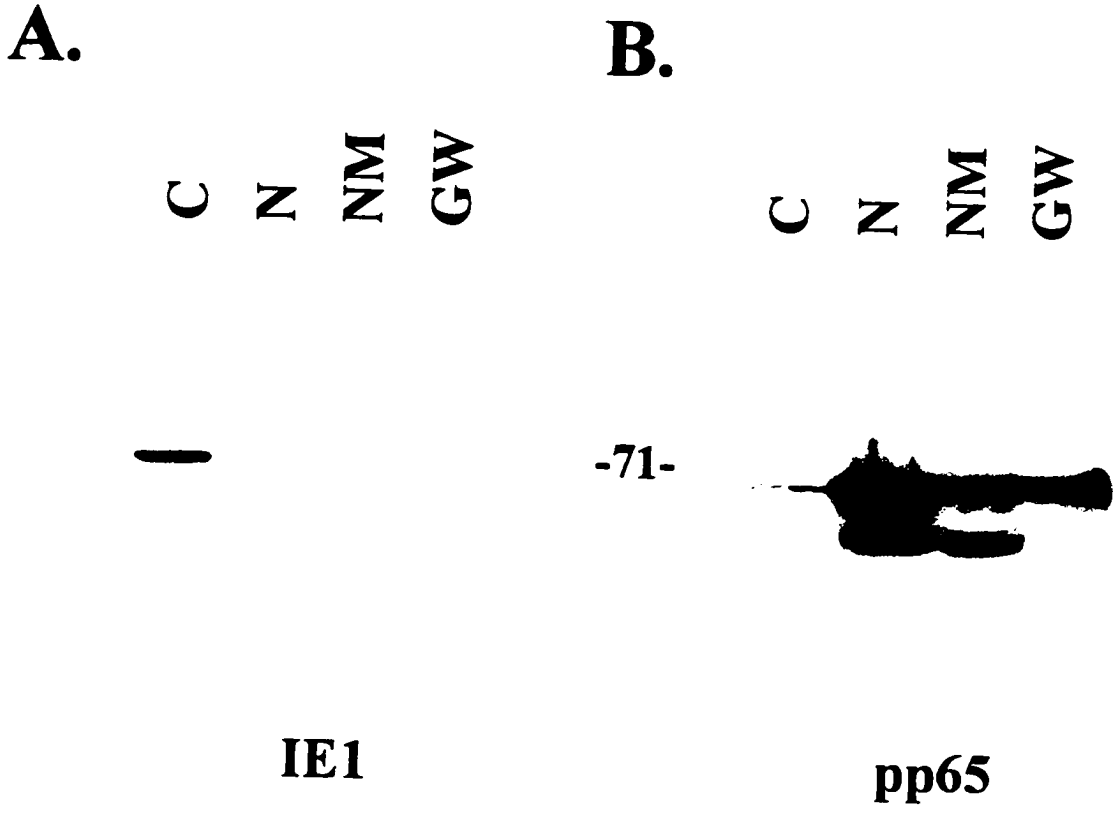


TABLE 1. Enrichment of pp65 on the nuclear matrix

Fraction[a]	% Total cellular protein[b]	% of protein in fraction[c]		
		IE-1	pp65 (68 kDa)	pp65 (50 kDa)
Cytoplasm/ soluble protein	86.3	93.2% (1.08)[d]	5.1% (0.06)	1.7% (0.02)
Detergent-treated nuclei	8.9	2.9% (0.33)	43.9% (4.93)	50.2% (5.64)
Nuclear matrix	3.0	1.9% (0.63)	25.9% (8.63)	38.7% (12.94)
Guanidine-HCl washed nuclear matrix	1.8	2.0% (1.11)	25.1% (13.94)	9.4% (5.22)

[a]HCMV-infected HF cells were fractionated into the indicated cellular fractions as described in Materials and Methods.

[b]The amount of total protein represented in each fraction was determined as described in Materials and Methods and the percentage of total cellular protein was calculated as follows: % = (total protein in fraction/total cellular protein) X 100.

[c]The amount of IE-1, pp65 (68 kDa), or pp65 (50 kDa) in each fraction was determined by phosphorimaging. The results are presented as the percentage of total IE-1, pp65 (68 kDa), or pp65 (50 kDa) in each fraction as calculated by the formula: % = (counts in fraction/total counts for protein) X 100.

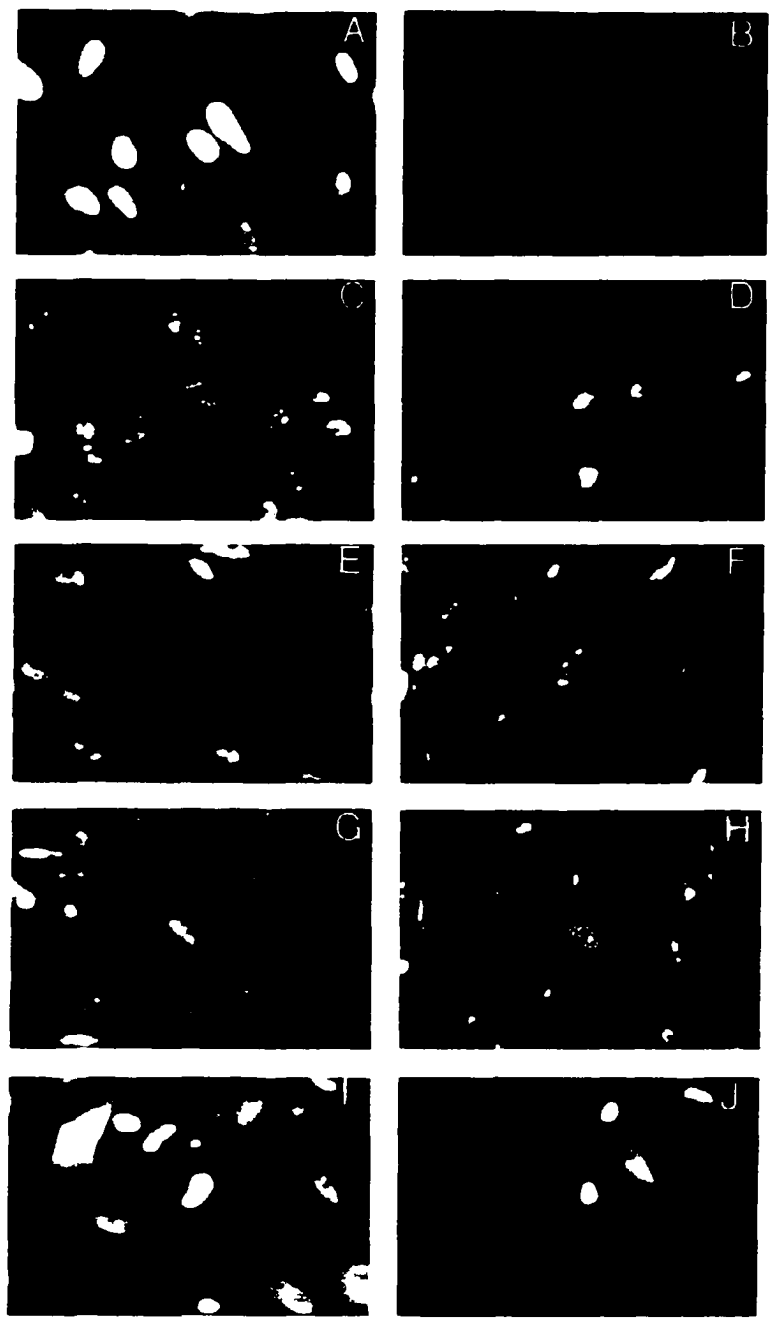
[d]The specific enrichment of each protein in a specific fraction was determined by the following formula: Enrichment = % of protein in a fraction[c]/% of total cellular protein in a fraction[b].

findings shown in Fig. 2, the IE-1 protein was not enriched in the nuclear matrix fraction and was contained in the soluble fraction (Fig. 3).

To further examine the compartmentalization of virion structural components on the nuclear matrix, we performed

immunofluorescence assays of in situ-extracted, HCMV-infected human fibroblasts. These assays were also used to estimate the quantity of protein removed during the extraction procedure. As shown in Fig. 4A, the IE-1 protein was readily detected in fixed cells but not in extracted cytoskeletal frameworks following treatment with high salt and DNase (Fig. 4B). The absence of IE-1 in in situ-extracted cells confirmed the results of the Western blot analysis which suggested that a significant amount of IE-1 was not retained on the nuclear matrix. In contrast, ppUL44, MCP, ppUL69, and pp65 were retained in the insoluble nuclear matrix fraction (Fig. 4D, F, H, and J, respectively). Furthermore, the pattern of immunofluorescence suggested that these proteins were localized to subnuclear structures and not evenly distributed throughout the nucleus. In addition, we also observed the NM retention of the minor capsid protein (pUL85) and the small capsid protein, p12 (pUL48/49)(data not shown). Note the lack of reactivity of primary and secondary antibodies with uninfected cells which were present in these preparations, demonstrating the specificity of these monoclonal antibodies.

FIG. 4. Immunofluorescence assays of in situ-extracted, AD169-infected HF cells. HF cells grown on glass coverslips were infected with HCMV AD169 five days prior to harvesting. Infected cells were untreated (A, C, E, G, I) or extracted with detergent, DNAse, and high salt (B, D, F, H, J) before fixation with 2.5% paraformaldehyde. Coverslips were then reacted with mAbs specific for IE-1 (A, B), ppUL44 (C, D), MCP (E, F), ppUL69 (G, H), or pp65 (I, J). Antibody binding was detected with FITC-conjugated goat anti-mouse IgG antibody and recorded by conventional fluorescence microscopy. Magnification for all frames was 400X.

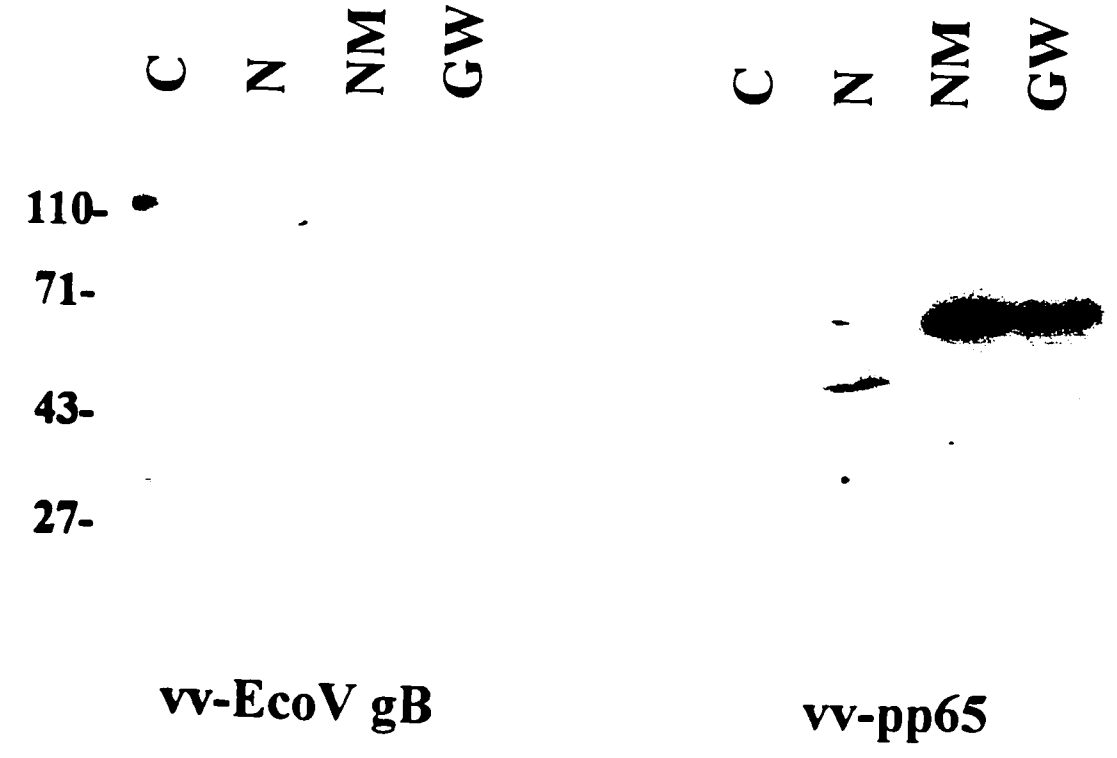


The Tegument Phosphoprotein pp65 Binds to the Nuclear Matrix

The interaction between pp65 and the nuclear matrix could be explained by either a direct binding of pp65 to a component of the nuclear matrix or an indirect binding through an association with another virus-encoded protein and/or DNase-resistant nucleic acid which was associated with a protein component of the nuclear matrix. We initially addressed this question by examining the association of pp65 with the nuclear matrix of cells infected with a recombinant vaccinia virus expressing pp65. Recombinant pp65 expressed in the absence of other HCMV-encoded proteins was enriched in the nuclear matrix and 1 M guanidine hydrochloride washed fractions (Fig. 5). As a control for the extraction procedure, similar experiments were performed on cells expressing a truncated form of HCMV gB. We could not detect enrichment of the 130-kDa precursor form of this gB or of its 30-kDa cleavage product in the nuclear matrix or guanidine hydrochloride-washed pellets. These results indicated that pp65 was retained in the nuclear matrix of cells in the absence of other viral proteins.

In order to directly investigate the specificity of the protein-protein interactions between pp65 and nuclear matrix components, far-Western blots were performed with nuclear

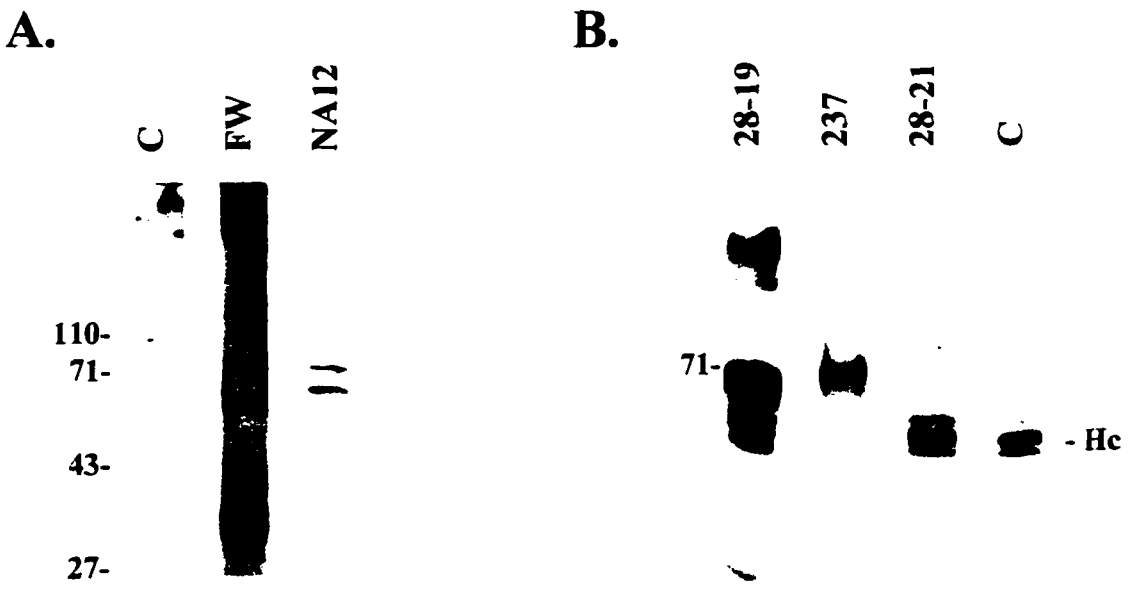
FIG. 5. pp65 expressed by a recombinant vaccinia virus, vv-pp65, is retained in the nuclear matrix of infected BSC-1 monkey cells. Cytoplasmic (C), nuclear (N), nuclear matrix (NM), and guanidine-washed nuclear matrix (GW) fractions were prepared from recombinant vaccinia virus, vv-Eco V gb (panel A) or vv-pp65 (panel B), infected BSC-1 cells as described in Material and Methods. The samples (10 ug per lane) were analyzed by Western blotting using a gB-specific or pp65-specific mAb and developed with ^{125}I-protein A.



matrix material derived from the human cell line, HEp2, as the substrate for binding. A pp65 fusion protein containing a His_6 tag at the amino-terminus was purified from *E. coli* and used to probe the nitrocellulose membrane containing electrophoretically separated nuclear matrix proteins. Binding of pp65 to the nuclear matrix proteins was then detected with the pp65-specific MAb 28-19. The results of this experiment indicated a direct interaction between pp65 and at least three cellular proteins which migrated between 50 and 70 kDa and two other proteins which migrated at approximately 30 kDa (Fig. 6A, lane FW). Interestingly, the 50- to 70-kDa bands were of the approximate molecular size of nuclear lamins which together represent major protein constituents of the nuclear matrix.

In earlier experiments we observed focal accumulations of pp65 along the periphery of the nucleus (Fig. 1E and F). Together these findings were consistent with the association of pp65 with proteins of the nuclear lamina, the protein network which provides the structural framework of the nuclear envelope (27, 47). Using a Mab against human lamin B1, the 68-kDa band detected in the far-Western blot was found to comigrate with the 68-kDa band of the lamin B1 Western blot (Fig. 6A, lane NA12). When viewed together,

FIG. 6. pp65 binds to a nuclear matrix protein which comigrates with lamin B1 in vitro. (A) Nuclear matrix material was isolated from the human carcinoma cell line, Hep2, as described in Materials and Methods. Proteins were electrophoretically separated then blotted onto nitrocellulose. Filters were then reacted with (C) pp65-specific mAb 28-19, (FW) purified pp65 followed by anti-pp65 mAb 28-19 or (NA12) anti-lamin B1 mAb NA12. Antibody binding was detected by addition of anti-mouse IgG antibody followed by ^{125}I-protein A and autoradiography. Migration of molecular weight markers is shown in left margin. (B) Coprecipitation of pp65 and lamins from soluble nuclear matrix extracts. Soluble nuclear matrix extracts prepared from AD169-infected HF were immunoprecipitated with mAbs against pp65. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose for Western blotting with antibodies specific for pp65 (28-19), lamins (237), and a control mAb specific for ppUL44 (28-21). Antibody binding was detected by addition of rabbit anti-mouse IgG antibody (lanes 28-19 and 28-21 only) followed by ^{125}I-protein A and autoradiography. Immunoglobulin heavy chains were detected with a rabbit anti-mouse immunoglobulin antibody followed by addition of ^{125}I-protein A and autoradiography (lane C). Migration of molecular weight marker and immunoglobulin heavy chains is shown in margins.



these results suggested a direct interaction between pp65 and proteins of the nuclear matrix, possibly components of the nuclear lamina.

pp65 Interacts with Lamins in the Nuclear Matrix Fraction

Direct investigations of protein-protein interactions with nuclear lamins have been complicated by the insolubility of lamins. We approached the study of pp65 interactions with these proteins by preparing soluble nuclear matrix proteins through step-dialysis of nuclear matrix extracts from AD169-infected cells. The soluble nuclear matrix proteins were immunoprecipitated with a mixture of pp65-specific MAbs, and the precipitated proteins were separated by SDS-PAGE then transferred to nitrocellulose membranes. The membranes were probed with an anti-pp65 MAb 28-19, a polyvalent rabbit antiserum specific for lamins (237) (29), and a control MAb, 28-21, which is specific for ppUL44. The pp65-specific MAb detected pp65 and several forms of this protein (Fig. 6B). The lamin-specific antiserum 237 detected lamins in the immunoprecipitated complex (Fig. 6B). The immunoreactive protein(s) migrated at approximately 68 kDa, consistent with the migration of lamins. Antiserum 237 failed to react with recombinant derived pp65 produced in

bacteria, indicating that the reactivity for lamins was specific (data not shown). In contrast, MAb 28-21 reacted with three proteins which ranged in size between 50 and 45 kDa (Fig. 6B). Although we cannot rule out the possibility that at least one of these bands may represent a ppUL44-related protein, we observed the same three bands in the membrane developed with the pp65-specific MAb, suggesting that these proteins represented reactivity of the anti-mouse IgG second antibody with the different forms of the immunoglobulin heavy chain present in the original immunoprecipitate (Fig. 6B). The identity of these bands was confirmed by probing a filter with rabbit anti-mouse IgG antibody (lane C) which produced the same pattern of reactivity as the control anti-ppUL44 antibody. Together with our findings from the far-Western analysis, these findings indicated that pp65 interacted directly with lamins.

To further examine the interactions between pp65 and lamins, in situ-extracted AD169-infected HF cells were reacted with an anti-pp65 MAb and a rabbit polyclonal serum against lamins a and c (17). We observed localization of pp65 along the periphery of the nucleus of infected cells (Fig. 7A). Similarly, the distribution of lamins a and c along the nuclear periphery was consistent with previous

FIG. 7. Colocalization of pp65 and lamins. Nuclear matrix extracted (panels A-C) or unextracted (D-F) HCMV-infected cells were stained with a murine mAb against pp65 (green, A, D) and a rabbit antiserum against lamins a/c (red, B) or against lamin b (red, E). Colocalization (blue) of pp65 and lamins is shown in panels C and F. Magnification for all panels, 1,000X.

studies (Fig. 7B) (17, 29). As shown in Fig. 7C, we noted colocalization of pp65 and lamins a and c. We also examined HCMV-infected HF cells late in infection when extensive cytopathic effects were present. In some cells, we observed extranuclear, vacuole-like structures containing both pp65 and lamin b (Fig. 7D to F). These results were consistent with the biochemical data suggesting a direct interaction between pp65 and proteins of the nuclear lamina.

DISCUSSION

In this report we have described the subcellular distribution of several structural and non-structural proteins of human cytomegalovirus. Specifically, we have investigated the intranuclear localization of protein components of the virion tegument and capsid. Because these proteins were colocalized in a specific nuclear structure, the nuclear matrix, we have proposed that the nuclear matrix is a potential site of particle morphogenesis in the HCMV-infected cell.

Intranuclear compartmentalization of proteins from several DNA viruses has been well documented. Studies of adenovirus-infected cells have described the spatial separation of viral transcription and replication sites (51). Virus-

encoded proteins involved in replication of the adenovirus genome have been localized to discrete nuclear structures which are distinct from sites of transcription. de Bruyn Kops and Knipe as well as other groups (41, 42, 44) described the spatial organization of viral replication structures in HSV-infected cells and suggested that the arrangement of these structures was defined by preexisting nuclear architecture (18, 44). Similarly, intranuclear HCMV replication compartments were recently characterized by Sarisky and Hayward, who characterized the viral proteins associated with the formation of these sites and for oriLyt-dependent DNA replication (59). A more complete description of intranuclear compartmentalization of HSV proteins was recently reported by Ward et al., who described HSV viral structures within the nucleus which they termed assemblons (64). These nuclear subcompartments were reported to segregate proteins into groups associated with specific functions, including replication of viral DNA and assembly of subviral particles. The mechanisms driving accumulation of proteins into these structures are not clear, but they are likely to involve interaction of viral proteins with the architectural framework of the nucleus, thereby providing spatial organization to an already temporally-regulated replicative process.

Previous studies of several DNA viruses have also shown that virion structural proteins localized to the nuclear matrix (5, 7, 37, 66). Recent studies have documented the presence of newly synthesized adenovirus virions on the core filaments of the nuclear matrix, suggesting that this nuclear structure may be a site of adenovirus assembly (66). In this same study, Zhonghe et al. have suggested that newly formed adenovirus particles track along 10-nm core filaments of the nuclear matrix, providing some evidence that this filamentous network may also provide a function critical to nuclear egress of progeny virions. Herpes simplex virus proteins have been reported to associate with the nuclear matrix (5, 7). In these studies, proteins comigrating with capsid and DNA-binding proteins of HSV were found in the nuclear matrix fraction (7). In addition, HSV capsids were observed in a filamentous network within the nucleus (5). These findings, together with studies which have shown that the nuclear matrix is an important site of transcription and replication, were consistent with a model in which this nuclear compartment could serve as the site of assembly for viruses which encapsidate nucleic acid in the nucleus (7, 37, 66). Our findings were also in agreement with these previous findings in adenovirus- and HSV-infected cells and

suggested that HCMV may also assemble subviral particles in association with the nuclear matrix of infected cells. However, finding virion structural proteins associated with the nuclear matrix does not indicate that in each case there is a direct interaction between individual viral proteins and proteins of the nuclear matrix. In some cases the association could have resulted from virion protein interactions with a limited number of virus-encoded proteins bound to the nuclear matrix; however, the maintenance of this association following washes in 1 M guanidine hydrochloride suggested a very stable interaction.

We focused the majority our studies on the tegument protein pp65 because of its abundance in extracellular particles as well as its nuclear expression shortly after infection of permissive fibroblasts (36, 61). Previous studies have shown that, almost immediately after infection, pp65 is transported to the nucleus of fibroblasts where it accumulates until late in infection (61, data not shown). Targeting of the protein has been attributed to a bipartite nuclear targeting signal at the extreme carboxy terminus of the molecule (61) and, more recently, to a second signal proximal to this conventional nuclear localization signal (NLS) (26). However, in both of these reports mutated forms

of pp65 which lacked these signals could still be localized to the nucleus when expressed in recombinant systems, suggesting that there were other domains mediating nuclear localization of pp65. Such domains could mediate nuclear retention in addition to nuclear localization associated with previously described NLS (26, 61). As shown in Fig. 1, pp65 was observed in subnuclear structures and also in patches along the periphery of the nucleus. Additional domains within pp65 could therefore mediate retention on a particular nuclear structure such as the nuclear lamina. Such protein interactions could explain the findings of earlier studies which documented the nuclear accumulation of mutant forms of pp65 which lacked NLS but were of such a size as to allow passive diffusion into and out of the nucleus (20, 26, 61).

The nuclear matrix of HCMV-infected fibroblasts was isolated according to the method of Mirkovitch which consisted of high salt extraction of DNase-treated nuclei which were initially isolated by non-ionic detergent treatment of viable cells (45). The resulting pellet of nuclear material represented insoluble nuclear proteins and was essentially devoid of DNA. Western blot analysis of nuclear matrix material demonstrated the retention of several HCMV

structural proteins which have previously been characterized as nuclear proteins (Fig. 2A). In addition, the polymerase accessory protein ppUL44 and the associated products of this ORF were also present in the nuclear matrix pellet. In contrast, the nuclear 72-kDa IE-1 and 86-kDa IE-2 non-structural proteins were not detected in this assay, suggesting that the association of HCMV proteins with the nuclear matrix was specific. Furthermore, we failed to detect two abundant tegument proteins, pp28 (ppUL99) and pp150 (ppUL32), as well as glycoprotein B (gpUL55) in the nuclear matrix, providing additional evidence for the specificity of the protein-nuclear matrix interactions we have described (data not shown). These results were confirmed by immunofluorescence of in situ-extracted HCMV-infected cells (Fig. 4). Together these data suggested that several proteins which were incorporated into nuclear subviral particles were sequestered on the nuclear matrix.

Enrichment of pp65 on the nuclear matrix was demonstrated by quantitative Western blot (Fig. 3; Table 1). The results from this experiment suggest that pp65 is strongly associated with the nuclear matrix. Moreover, the localization of pp65 to the nuclear matrix in the absence of other HCMV proteins indicated a direct interaction with components

of the nuclear matrix. Although initial studies also documented the association of pp65 with the nuclear matrix of monkey cells (Fig. 5) and insect cells (data not shown), we obtained additional evidence of the direct interaction of pp65 with the nuclear matrix by performing far-Western blots with recombinant-derived pp65 and the nuclear matrix of HEp2 cells (Fig. 6A). The results of this experiment indicated that pp65 associated with a limited number of protein constituents of the nuclear matrix. The binding of pp65 to a restricted set of nuclear matrix proteins underscored the specificity of the protein/protein interactions and suggested the possibility of a sequence-specific targeting signal for the localization of pp65 to the nuclear matrix. Although the identity of the nuclear matrix proteins detected by far-Western blot has not been conclusively established, the higher-molecular-weight bands were of a similar molecular size as lamins a, b, and c which together represent major protein components of the nuclear matrix (27, 47). Western blot analysis of the nuclear matrix protein-containing filter showed that one of the proteins detected by far-Western blotting comigrated with lamin B1 (Fig. 6A). Additional biochemical evidence for the interaction between pp65 and lamins was provided by the coprecipitation of pp65 and

lamins from a soluble extract of nuclear matrix proteins from HCMV-infected fibroblasts (Fig. 6B). The polymerase accessory protein ppUL44 was not coprecipitated with pp65 and lamins, suggesting that this was a specific interaction (Fig. 6B). Thus, the binding of pp65 to a major constituent of the nuclear matrix and colocalization of pp65 and lamins (Fig. 7) was consistent with the hypothesis that at least one step in nuclear tegumentation of the HCMV capsid might localize to this subnuclear compartment. Finally, we have consistently observed cytoplasmic vacuole-like structures containing pp65 and nuclear lamins in cells transfected with pp65 expression plasmids which are similar to those illustrated in Fig. 7D and E. This observation and the accumulation of pp65 on the nuclear membrane suggest a possible role of pp65 in focal modifications of the nuclear envelope.

In summary, we have provided biochemical and imaging data of the association of nuclear tegument and capsid proteins of HCMV with the nuclear matrix. Together these findings argued for the nuclear matrix being a potential site for assembly of subviral particles of HCMV and suggested that protein/protein interactions between virus-encoded proteins and this nuclear structure might provide spatial

coordination for the highly regulated and temporally coordinated replication of this virus.

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HUMAN CYTOMEGALOVIRUS PHOSPHOPROTEIN 65 (UL83) CONTAINS CELL-TYPE SPECIFIC NUCLEAR LOCALIZATION SIGNALS

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ABSTRACT

To further understand the mechanisms of human cytomegalovirus (HCMV) tegument assembly, we investigated the cellular trafficking of pp65. pp65 is the only HCMV tegument protein which is detected in the nucleus and in the cytoplasm late in infection. Analysis of the predicted pp65 protein sequence revealed a putative nuclear targeting signal (NTS) at the extreme carboxy terminus of the protein. Previous studies have demonstrated that this putative signal could function to direct pp65 or a beta-galactosidase fusion protein to the nucleus but that there were additional signals in pp65 also mediating nuclear localization (12, 26). pp65 deletion mutants lacking the carboxy terminal NTS continued to accumulate in the nucleus, suggesting the presence of second, novel NLS, or possibly a nuclear retention domain mediating the localization of mutant forms of pp65 which were able to enter the nucleus by passive diffusion. Our analysis of full-length or truncated forms of pp65 revealed that the nuclear localization of pp65 can be attributed to additional signals in the pp65 sequence. The presence of a second, inefficient nuclear targeting signal between amino acids 415 and 437 of pp65 was confirmed by experiments utilizing a green fluorescent protein-pp65 fusion protein and

truncated mutants of this chimera. Our strategy with these constructs was to uncouple active transport from diffusion by increasing the molecular size of pp65 and its truncated mutants in order to ablate passive transport. By using the green fluorescent protein reporter system, we have demonstrated that there was a second NTS that mediated nuclear accumulation of truncated pp65 mutants that lacked the bipartite NTS at the carboxy terminus as was proposed by Gallina et al. (12). This second NTS was active in rapidly dividing Cos7 cells but was ineffective in growth-arrested human fibroblast (HF) cells. Our studies in HF cells revealed the presence of a hydrophobic segment in pp65 which contributed to nuclear localization of truncated forms of this protein in these cells. Taken together, our observations bring into question whether the newly described NTS plays a significant role in the nuclear targeting of pp65 during a normal infection since HCMV demonstrates a particular tropism for non-dividing cells in vitro.

INTRODUCTION

Human cytomegalovirus is the largest member of the human herpesvirus family. Its linear 250-kb pair genome encodes for over 200 proteins of which an estimated 50 are

virion structural proteins (19). The function of many of these proteins in the replicative cycle of HCMV are unknown, but tentative assignments of function have been made based on homologous proteins of herpes simplex virus-1 (HSV-1) (19). In many cases positional homologs for HCMV proteins are not present in HSV. Prominent among unique HCMV proteins is the product of the UL83 open reading frame (ORF) (19). This 65 kilodalton (kDa) phosphoprotein is an abundant component of the virion tegument region and a major constituent of the non-infectious dense body particle (16). The function of this protein is unknown, but in vitro it appears to be dispensable for virus replication (27). In contrast to this finding, this protein is highly conserved in all clinical isolates of HCMV and also represents the dominant target of host derived cellular immune responses (1, 17, 18). Thus, it appears that the pp65 protein serves an essential function in the replcative cycle of HCMV in vivo and that understanding its function could further advance our understanding of the biology of HCMV.

Shortly after HCMV infection of permissive human fibroblasts, pp65 can be detected in the nuclei of the infected cells (26). In addition, although this gene has been described temporally as an early gene, its synthesis begins

very shortly after infection of permissive cells and its expression increases throughout the replicative cycle (13). The protein has been shown to be phosphorylated at least at one consensus casein kinase II phosphorylation site and does not contain any additional post-translational modifications (20). Pulse-chase analysis of the synthesis of pp65 has revealed that at least two smaller, phosphorylated forms of the protein with estimated molecular weights of 52- and 50-kDa are generated during its synthesis (4). Interestingly these smaller forms of pp65 can be found within the nuclear fraction of infected cells but are not incorporated into the virion or dense bodies (4). Recent findings from our laboratory have also demonstrated that these proteins may interact differently with the nuclear matrix of infected cells as compared to the full length protein (24). Their functional role in the replicative cycle of HCMV is unknown.

A unique characteristic of pp65 is its intracellular localization within the infected cell. Early in the infectious cycle, pp65 remains localized to the nucleus; however, late in the infection the protein can be detected in both the nucleus and cytoplasm of infected cells (10, 25, 26). Recently several studies have examined structural features of this protein which direct it to the nucleus of infected

cells: An initial study by Schmolke et al. suggested that pp65 contained a unique bipartite nuclear targeting signal (NTS) in the extreme carboxyl terminus which could direct heterologous proteins such as beta-galactosidase to the nucleus of Cos7 cells (26). In this same study, these authors proposed the presence of additional NTS because pp65 deletion mutants in which the bipartite signal had been removed continued to localize in the nucleus (26). In a related study, Gallina et al. reported that a second bipartite nuclear targeting signal was located approximately 150 (aa) upstream of the carboxy-terminal signal (12). The exact location of this sequence was not defined and the size of the reporter constructs which were generated were such that passive diffusion into the nucleus could not be excluded as an explanation for nuclear localization of the reporter fusion protein (12). Furthermore, in both studies the function of the proposed NTS was not assayed in the context of pp65, raising the possibility that these signals may function differently in HCMV pp65 than in the reporter constructs. Lastly, these studies were also carried out almost exclusively in rapidly dividing, transformed cells and not in primary, non-transformed human fibroblasts which are quiescent, terminally-differentiated cells. Thus, the findings

of these studies may not extrapolate directly to the transport of pp65 in terminally differentiated cells which are permissive for HCMV replication.

In this study we have investigated the sequences within pp65 which facilitate nuclear localization of this protein. We utilized a deletional mutagenesis strategy to generate the constructs used throughout the study. We believe this is the most relevant approach for the identification of nuclear localization signals in this protein because it requires these signals to function in the context of the HCMV protein and not in an unrelated protein. Furthermore, we generated amino terminal fusion proteins with the reporter protein green fluorescent protein (GFP) to examine the activity of nuclear localization signals in truncated forms of pp65, eliminating the potentially confounding mechanism of passive diffusion of proteins which are less than 50-kDa in size (6). These GFP fusion proteins allowed us to distinguish between the contributions of active and passive transport processes to the localization of truncated forms of pp65. Our results demonstrated the presence of a nuclear localization signal between aa 415-438 when truncated forms of pp65 were transiently assayed in rapidly dividing Cos7 cells. Truncated forms of pp65 which did not contain the aa

sequence *RKRK* located between aa 434-438 abrogated nuclear localization. Interestingly, this sequence failed to facilitate nuclear localization of pp65 when truncated mutants were expressed in terminally differentiated primary human fibroblasts, suggesting that transport of pp65 into the nucleus was cell-type dependent. Finally, a nuclear localization signal for transport of pp65 into the nucleus of human fibroblasts was mapped between aa 481-508.

MATERIALS AND METHODS

Cells, Viruses, and Antibodies

Primary human foreskin fibroblasts (HF) were maintained at 37°C in Medium 199 supplemented with 5% newborn calf serum and antibiotics. Cos7 cells were maintained in 5% CO_2 at 37°C in DMEM containing 10% fetal calf serum and antibiotics. For transfection assays, cells were grown on 12-mm glass coverslips at 37°C in 5% CO_2.

The recombinant vaccinia virus expressing T7 RNA polymerase was propagated in HF cells (11). The reactivity of pp65-specific murine monoclonal antibody 28-19 (mAb) has been described previously (4). The murine monoclonal antibody specific for the green fluorescent protein was purchased from Clontech (Palo Alto, Calif.).

Generation of pp65 Mutants

The pp65 genomic sequence (a gift from Dr. Bodo Plachter, University of Mainz, Mainz, Germany) was cloned into the *BamH1* site of a modified pcDNA3 plasmid (Invitrogen, San Diego, Calif.) containing an *Nhe1* linker inserted into the *Xba1* site. This linker encodes translational stops in all three reading frames and construction of the plasmid destroyed the *Xba1* site in the plasmid. Deletions were made from the 3' end into the pp65 coding sequence following an exonuclease III digestion protocol. Briefly, the plasmid pcDNA3/*Nhe1*-pp65 was digested with *Xho1* and *Xba1* which recognize sites in the plasmid and insert, respectively. The *Xba1* site is located 3' to the pp65 stop codon, and digestion with the enzyme generated a 5' overhang 3' to the insert. This site served as the target for exonuclease III digestion which proceeded into the gene. Mung bean nuclease followed by the Klenow fragment of DNA polymerase I were used to generate blunt ended products which were then ligated with T4 DNA ligase. This procedure yielded a mixture of plasmids with a wide range of deleted sequences. These products were subjected to restriction enzyme analysis and

sequencing (Sequenase kit, Version 2.0, Amersham, Arlington Heights, Ill.).

The enhanced green fluorescent protein-pp65 fusion was constructed in the plasmid pEGFP C1 (Clontech, Palo Alto, Calif.). The pp65 sequence was ligated in-frame into the plasmid at the *BamH1* site. Exonuclease digestion of this plasmid was performed as described above with some modifications. The plasmid was digested with *Xba1*, then this site was protected from exonuclease digestion by filling-in the overhang with 5'-phosphorothioate dNTPS (Promega, Madison, Wis.) using the Klenow fragment of DNA polymerase 1. This DNA was then digested with *Not1* which cuts within the pp65 gene at nucleotide 1359. Exonuclease digestion and characterization of the products were described above.

Transfections and Immunofluorescence Assays

Cos7 cells were seeded on glass coverslips and transfected the following day using a calcium phosphate kit (5'-3', Boulder, Colo.). For vaccinia virus-T7 (vv-T7) based transfections, HF or Cos7 cells were grown to confluency on glass coverslips then infected with the recombinant virus

expressing T7 RNA polymerase 2 h prior to transfection via lipofection (23).

Cos7 cells were harvested 36-48 h post-transfection while the vv-T7 infected cells were harvested approximately 24 h after infection. Cells were fixed either in 2% para-formaldehyde or acetone. Immunostaining of these cells was performed as previously described using mAb 28-19 and a FITC-conjugated anti-mouse IgG second antibody (24). For each mutant, 50-100 positive cells were counted per experiment and scored for protein expression as follows: n > c, n = c, or n < c. The percent positive cells was determined and an average was calculated in each of these categories. For graphing purposes, the contribution made by cells exhibiting whole cell fluorescence (i.e., n = c) was not included; the percentage of nuclear (n) or cytoplasmic (c) cells for each construct assayed was determined using the following formulas: $n = \%(n > c)/[\%(n > c)+\%(n < c)]$; $c = \%(n < c)/[\%(n > c) + \%(n < c)]$.

Western Blot Analysis

Cos7 cells grown in 35-mm dishes were transfected with the plasmids as described above. Cells were harvested 36-48

h post-transfection and protein expression was analyzed by Western blotting as previously described (3). Briefly, cell pellets were solubilized in SDS-containing sample buffer and samples were subjected to SDS-PAGE. Electrophoretically separated proteins were transferred to nitrocellulose and filters were blocked then reacted with either mAb 28-19 specific for pp65 or antibody against GFP. Blots were washed then incubated with rabbit anti-mouse IgG antibody. Filters were again washed and reacted with ^{125}I-protein A. Antibody binding was visualized by autoradiography.

RESULTS

Generation of pp65 Mutants

We constructed an expression plasmid containing the genomic sequence of pp65 (UL83) in the eukaryotic expression plasmid, pcDNA3 (Invitrogen, San Diego, Calif.). We modified this plasmid to include a translational stop in all three reading frames downstream of the pp65 sequence. To identify sequences which directed pp65 to the nucleus, nuclear localization signals (NLS), we carried out exonuclease III digestion of the wild type sequence and assayed the intracellular location of the mutant proteins following transient expression in Cos7 cells. A wide range of mutant pp65

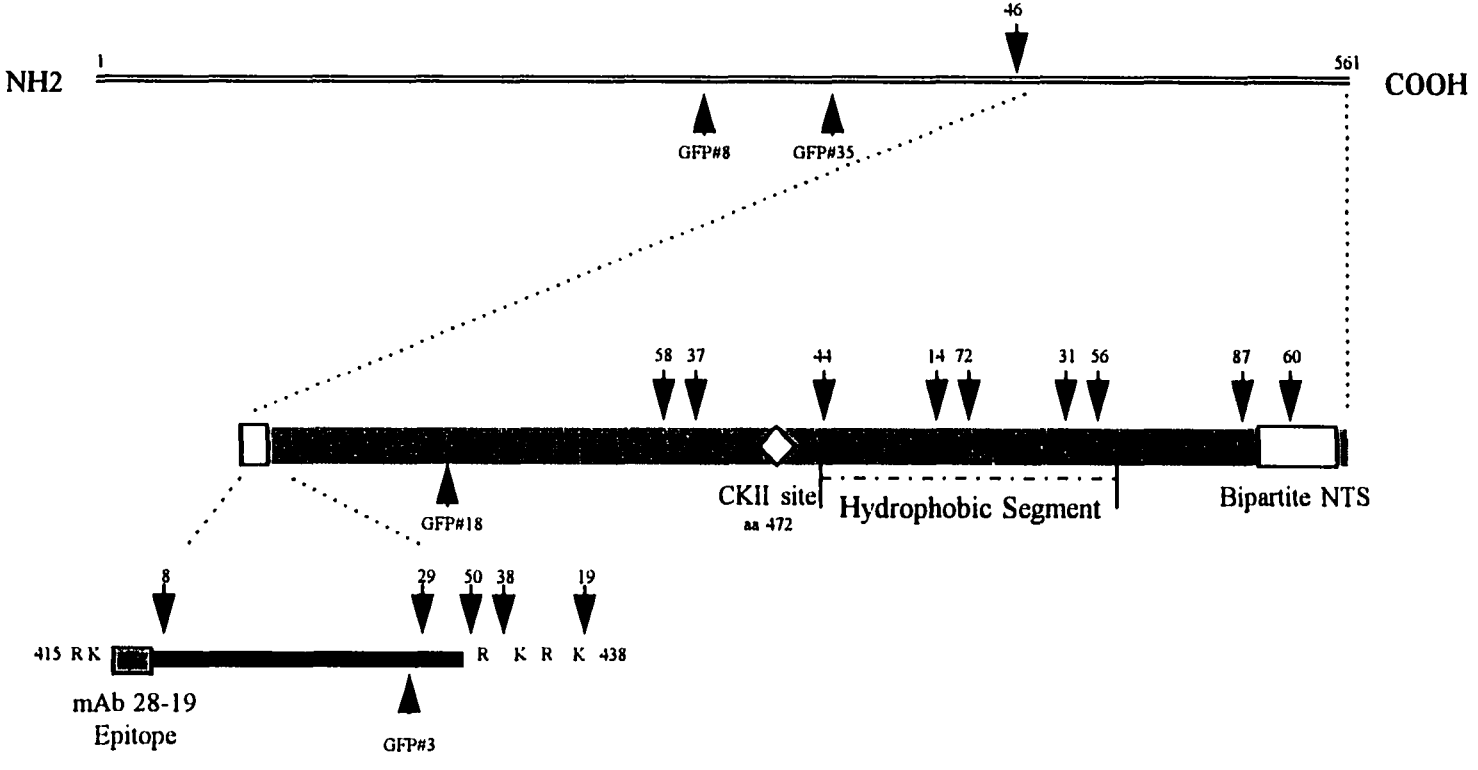
polypeptides were generated ranging in size from 550 aa to less than 200 aa (Table 1; Fig. 1). Protein expression in Cos7 cells was monitored 36-48 h post-transfection by

TABLE 1. Reactivity of pp65 deletion mutants with mouse mAb

Mutant	AA size	Reactivity with mAb 28-19
46	410	-
8	425	+
29	432	+
50	434	+
38	435	+
68	437	+
19	438	+
35	438	+
58	461	+
37	463	+
44	481	+
14	490	+
72	496	+
31	508	+
56	511	+
87	524	+
60	550	+
GFP# 8	285	-
GFP#35	334	-
GFP# 3	430	+
GFP#18	453	+

immunofluorescence assays using several different mAbs specific for pp65 (4). Analysis of the truncated products revealed that there was a limit of reactivity exhibited by the group of mAbs which were used in this study. Transiently expressed proteins which terminated before aa 422 were not

FIG. 1. Schematic of pp65 protein domains. Locations of putative nuclear targeting signals are shown in open white boxes. The casein kinase II site is denoted as an open diamond at aa 472 and the epitope for mAb 28-19 is shown at aa 417 as a shaded box. The termination sites for pp65 mutants are shown above the line with downward pointing arrows. The termination sites for GFP-pp65 mutants are shown below the line with upward pointing arrows. The location of the hydrophobic region of pp65 is also shown.



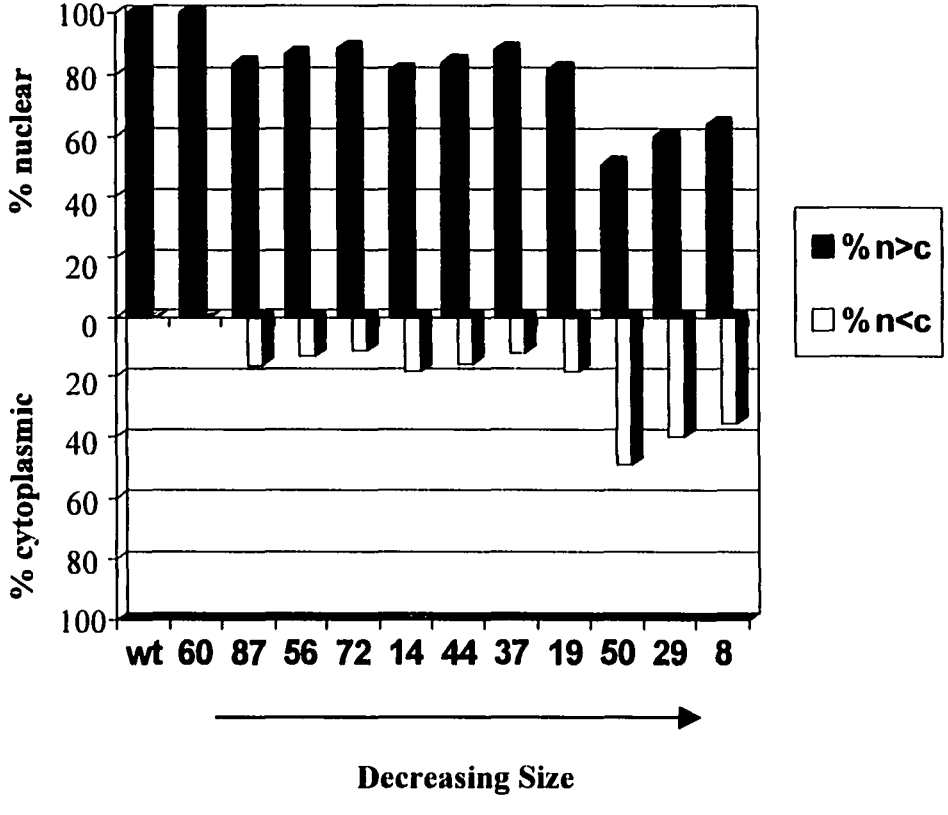
reactive with mAb 28-19 which was used for analysis in the remainder of this study.

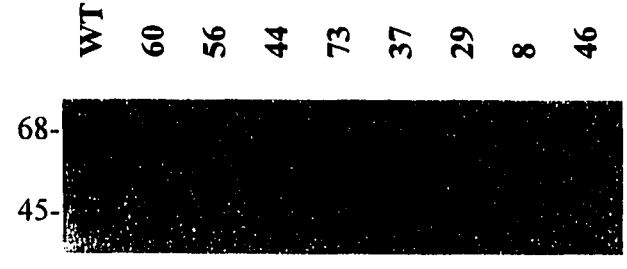
A diagram of pp65 with the previously proposed nuclear targeting signals (NTS) and additional structural features of this molecule is provided in Fig. 1. The location of the mutants we constructed for this study are shown as arrows above the sequence. Four green fluorescent protein (GFP) fusion proteins containing pp65 sequences are designated as GFP (Fig. 1) and their location is indicated by arrows below the sequence. The bipartite NTS described by Schmolke et al. is shown at the extreme carboxyl terminus (26). Gallina et al. have recently proposed that a second NTS is located between aa 415-438 (Fig. 1) (12).

Nuclear Localization of pp65 Mutants

As shown in Fig. 2, all truncated forms of pp65 analyzed were detected in the nucleus of the transfected cell including mutants 8 and 29 which did not contain either the carboxy-terminal NTS or the putative bipartite NTS located between aa 415-438 (12, 26). The nuclear localization phenotype of pp65 mutant 19 (aa 1-438) and 50 (aa 1-434) were appreciably different in that mutant 19 was detected predominantly in the nucleus of transfected cells whereas there

FIG. 2. Expression of pp65 deletion mutants in Cos7 cells. (A) Cellular distribution of pp65 deletion mutants. Cos7 cells were stained for immunofluorescence assays with mAB 28-19. Percentages of cells expressing truncated pp65 proteins in the nucleus or cytoplasm were calculated as described in Materials and Methods. (B) Western blot of truncated pp65 proteins probed with mAb 28-19.



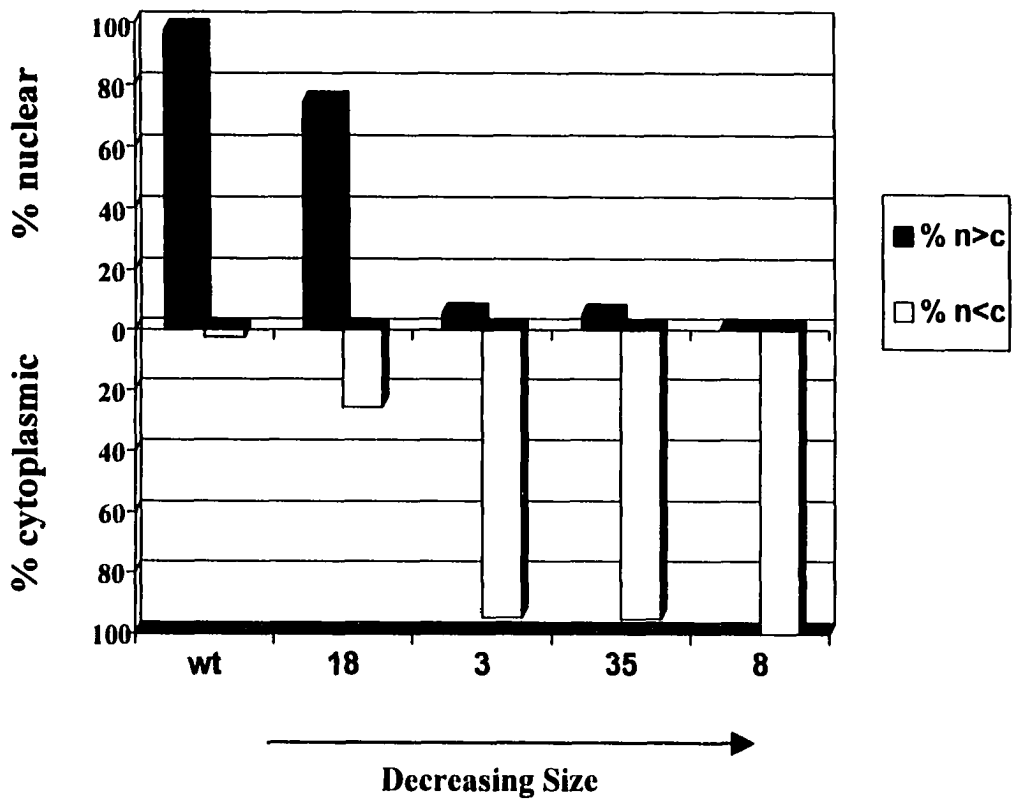


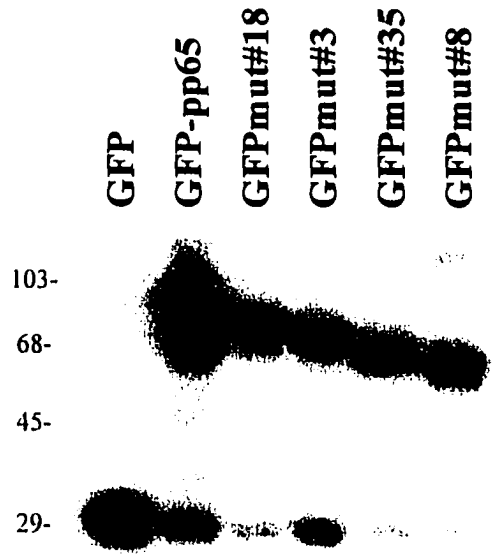
was a nearly equal distribution of cells with predominantly nuclear or cytoplasmic expression of mutant 50 (Fig. 2A). Interestingly, these mutants differed by 4 aa, specifically the sequence *RKRK*, which was contained within the second, proposed NTS of pp65 (Fig. 1) (12, 26). We could demonstrate expression of these mutant forms of pp65 in immunoblot assays, although the level of expression of the smaller mutants 29 and 8 was significantly decreased compared to the other mutants (Fig. 2B). We have no explanation for the decreased levels of protein expression by these mutants, but it was not assay dependent as we also noted decreased fluorescence intensity in cells expressing the smaller mutants as compared to the full-length, wild type pp65 (data not shown). Taken together, these findings were consistent with previous results and indicated that pp65 can be directed to the nucleus Cos7 cells in the absence of the previously described bipartite NTS signal at the carboxyl terminus. Furthermore, the apparent phenotypic difference between mutant 19 and 50 suggested that the aa sequence *RKRK* present between aa 435-438 could function as a NLS in the context of a pp65 molecule lacking the bipartite NTS at the carboxyl terminus.

Several of the mutant proteins analyzed were approximately 50 kDa in size, raising the possibility that nuclear accumulation of these proteins could be explained by passive diffusion and not by a specific targeting sequence (6). If this explanation accounted for the nuclear accumulation of the smaller mutants of pp65 such as mutants 50 and 29 of estimated mass of approximately 45 kDa, then it should be possible to block diffusion of these forms of pp65 by constructing a fusion protein with a reporter protein which would, in turn, increase their size. To test this hypothesis, we constructed a pp65-green fluorescent protein fusion using the vector pEGFP-C1 which would increase the mass of each mutant by approximately 27,000 Da (Clontech, Palo Alto, Calif.). The pp65 gene was cloned in-frame at the *BamHI* site downstream of the EGFP coding sequence and the plasmid was subsequently subjected to exonuclease digestion as described above. The cellular distribution of the full-length and truncated fusion proteins was then assayed in transiently transfected Cos7 cells. Mutant GFP 18, which has an estimated mass of 75 kDa, yet lacks the carboxyl terminal NTS, was detected almost exclusively in the nucleus, indicating that a second, active transport signal was present (Figs. 3 and 4A). Surprisingly, truncated proteins from GFP

FIG. 3. Cellular distribution of green fluorescent protein-pp65 deletion mutants in Cos7 cells. (A) Wild type pp65-GFP; (B) pp65-GFP mutant #18; (C) pp65-GFP mutant #3.

FIG. 4. Expression of pp65-GFP deletion mutants in Cos7 cells. (A) Cellular distribution of pp65-GFP deletion mutants. Transfected Cos7 cells were fixed 36 h post-transfection. Percentages of cells expressing truncated pp65-GFP proteins in the nucleus or cytoplasm were calculated as described in Materials and Methods. (B) Western blot of truncated pp65-GFP proteins probed with mAb specific for GFP.





mutants 3, 35, and 8 which did not contain the *RKRK* sequence at aa 435-438 were excluded from the nucleus (Figs. 3 and 4A). GFP expressed alone did not localize exclusively within the nucleus but was distributed throughout the nucleus and cytoplasm of infected cells (data not shown).

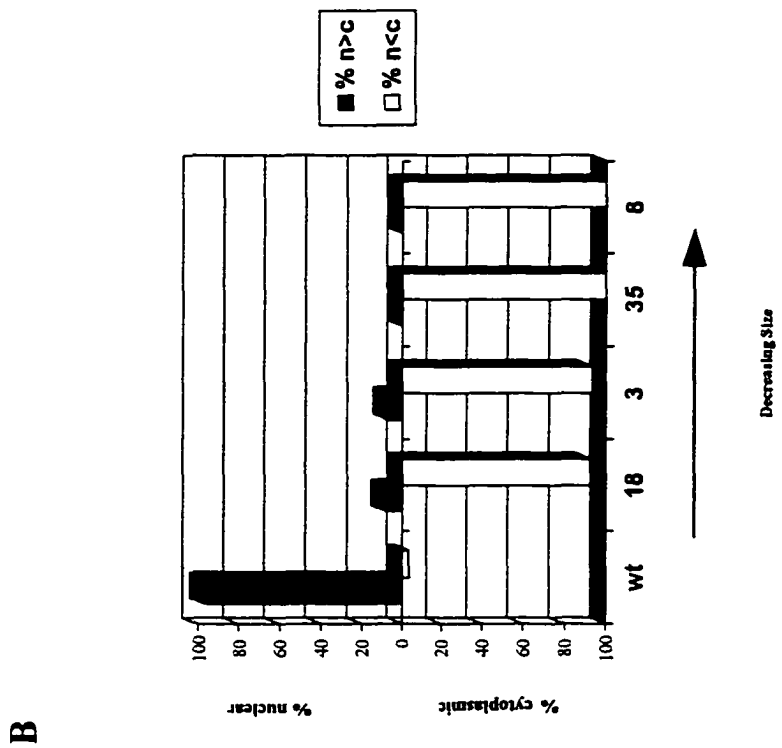
Immunoblot analysis of the GFP-pp65 fusion proteins revealed that proteins of the predicted sizes were produced as well as a small amount of an amino terminal cleavage product containing the GFP epitope (Fig. 4B). These findings and the previous results of Schmolke et al. suggested that pp65 contained two NLS which facilitated nuclear accumulation of the protein following transient expression assays in Cos7 cells (26). Deletion of both signals resulted in conversion of the nuclear phenotype of this protein into a cytoplasmic protein. These results were also consistent with the presence of a functional NLS in pp65 sequence between aa 415-438, a finding consistent with the NTS proposed by Gallina et al. (12).

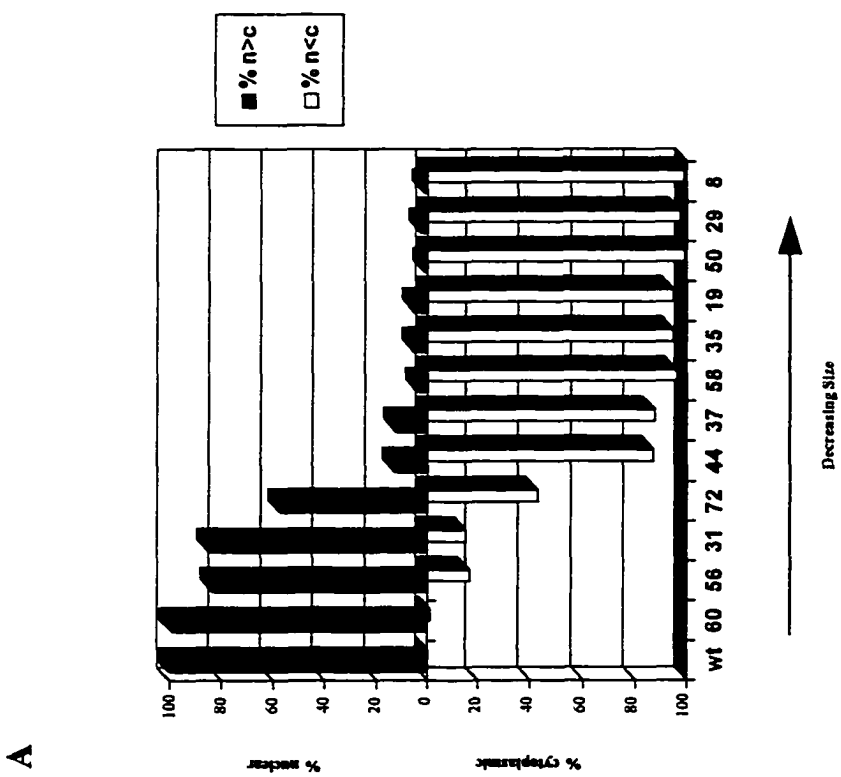
Nuclear Localization of pp65 Differs in Resting Cells

To investigate the importance of the cell type to the intracellular localization of pp65, we examined the trafficking of these proteins in primary HF cells. Examination

of the phenotypic expression of the mutant pp65 proteins in this cell type was felt to be relevant to understanding the function of nuclear localization signals in pp65 because primary HF cells represent the prototype of a terminally differentiated, non-dividing cell which is permissive for HCMV replication. The nuclear architecture and transport mechanisms in rapidly dividing, transformed cells such as Cos7 cells could differ significantly from human primary fibroblasts (14, 21). To achieve detectable expression of these proteins we utilized T7 RNA polymerase expressed from a recombinant vaccinia virus. Protein expression was monitored 12-18 h post-transfection using mAb 28-19 as described above. Unexpectedly, the nuclear localization phenotype of the mutated proteins was markedly different in this cell system. In contrast to the observations in Cos7 cells, mutants which contained only the second putative NTS were not detected in the nucleus of the transfected fibroblasts (Fig. 5A). A very obvious difference in the phenotype of the pp65 mutant 44 could be seen. This mutant protein exhibited a nearly exclusive nuclear localization in Cos7 cells, yet it was detected only in the cytoplasm in over 85% of HF cells (Figs. 2A and 5A). Similar results were obtained with mutant GFP 18 such that this protein was predominantly

FIG. 5. Expression of pp65 and pp65-GFP deletion mutants in human fibroblasts driven by vv-T7. (A) Cellular distribution of pp65 deletion mutants. HF cells were stained for immunofluorescence with mAb 28-19. Percentages of cells expressing truncated pp65 proteins in the nucleus or cytoplasm were calculated as described in Materials and Methods. (B) Cellular distribution of pp65-GFP deletion mutants. HF cells were fixed 36 h post-transfection. Percentages of cells expressing truncated pp65-GFP mutants were calculated as described in Materials and Methods.





nuclear in Cos7 cells yet was distributed in the cytoplasm of HF cells (Figs. 4A and 5B). The most likely explanations for these observations was that the second putative nuclear localization signal which was located between aa 415-438 of the pp65 sequence was either inefficient or non-functional in primary fibroblasts, perhaps because of additional structural requirements for nuclear accumulation in non-dividing cells. Alternatively, it was possible that vaccinia virus infection of the cells non-specifically altered the intracellular trafficking of the proteins. Therefore, we repeated the same assays in Cos7 cells which were infected with vv-T7 to drive expression of the pp65-containing plasmids and found no qualitative differences between mutant protein localization in Cos7 cells transfected with the expression plasmids in the presence or absence of the recombinant vaccinia virus (data not shown). Taken together, our data were most consistent with a model of nuclear transport proposed by Feldherr and Akin which suggested that proliferating cells demonstrate more rapid rates of nuclear transport compared to quiescent cells (7-9). The second NLS could be an inefficient signal as has been suggested by Schmolke et al., and therefore, accumulation of pp65 mutants would only be detected in rapidly dividing cell types such

as Cos7 cells. In quiescent human fibroblast cells, the kinetics of transport of these proteins into the nucleus could be so slow that the nuclear accumulation of transported proteins was not observed.

DISCUSSION

The nuclear localization of pp65 has been documented in several previous studies. Weiner et al. used an immunofluorescence assay to demonstrate the nuclear compartmentalization of this protein over a decade ago (28). Since this early study, several other investigators have also noted the nuclear localization of this abundant virion protein during HCMV infection of permissive fibroblasts (4). Studies in other cell types such as the U373 astrocytoma cell line and primary macrophages have also shown that pp65 accumulated in the nucleus during productive infection (5, 10). Recently, two laboratories have reported studies which investigated the location of aa sequences in this protein which directed the protein to the nucleus (12, 26). Initial studies by Schmolke et al. characterized a bipartite NTS at the carboxyl terminus of the protein (aa 537-561) which was similar to NTS signals described for other nuclear proteins, such as the *Xenopus* protein nucleoplasmin (15, 22, 26). This signal

functioned as an NTS when fused with a heterologous protein, beta-galactosidase; however, its function as an NTS required both stretches of basic amino acids, P*KRRRHR* and P*KKHR* located between aa 537-561 (26). This was somewhat surprising as the sequence between aa 537-543 (P*KRRRHR*) closely resembled the NTS of SV40 large T antigen, a signal sufficient to facilitate nuclear import of large proteins (15, 27). Two copies of this sequence expressed in tandem but separated by a 5-aa spacer resulted in nuclear localization of a heterologous protein (26). Thus, it appears that the NTS at the carboxyl terminus of pp65 represented a variation of the class of NTS previously defined in other systems, a finding consistent with the continually expanding list of NTS (2, 15, 26). In this same report, these investigators suggested that additional signals were present based on the nuclear localization of mutant forms of pp65 which lacked the bipartite NTS at the carboxyl terminus of the protein (26). A more recent report also described the presence of a putative second NTS between aa 348 and 465 of the protein (12). This sequence has not been definitively identified but is thought to include a short stretch of amino acids (*RKRK*) between positions 435-438 (12). Gallina et al. mutagenized this sequence in a truncated form of pp65 by replacing each amino

acid in this sequence with alanine (12). The resulting mutant protein localized primarily in the cytoplasm of infected cells; however, the protein was still detected in the nucleus (12). From these data the authors concluded that, in the absence of the bipartite NTS located between aa 537-561, this short stretch of amino acids could act as an NTS (12).

Although our findings were generally in agreement with both earlier reports, several of our results suggested different interpretations. The bipartite NTS between aa 537-561 was apparently not required for nuclear localization of pp65 in Cos7 cells as evidenced by the phenotype of mutant 60 (aa 1-550), which suggested that in this mutant there was either a second signal responsible for the nuclear localization of the protein or that the P*KRRRHR* SV40-like NTS located between aa 537-543 was sufficient for nuclear targeting (Fig. 2A). Similarly, mutant 87 (aa 1-524) was also found almost exclusively within the nucleus of transfected Cos7 cells, suggesting that, in addition to the elements of the bipartite signal, a second signal must be present in the remaining pp65 sequence. These results were similar to that noted by both earlier reports, suggesting that the primary sequence of pp65 specifies additional nuclear localization

signals other than the bipartite NTS described by Schmolke et al. (26). Whether these sequences function independently or together with the bipartite NTS located between aa 537-561 remains to be determined.

An alternative explanation for the nuclear accumulation of the mutant forms of pp65 could be diffusion of smaller forms of this protein into the nucleus. Previous studies have suggested that proteins of molecular weight below 50 kDa can enter the nucleus by passive diffusion, although the rate of diffusion varies between cell type and specific proteins (7-9). We addressed this possibility by constructing GFP-pp65 fusion proteins. GFP-pp65 mutant 18 exhibited nuclear comparmentalization, yet it contained only aa 1-453 of the pp65 sequence (Figs. 3 and 4). The addition of the 27 kDa from the GFP resulted in a fusion protein of estimated mass of 75 kDa, a protein larger than wild type pp65 and one which would be expected to be too large to passively diffuse into the nucleus. In contrast, GFP-pp65 fusion mutant 3 (aa 1-430) was found only in the cytoplasm of transfected cells (Figs. 3 and 4A). This finding indicated that a second nuclear localization signal was present between aa 430-453 of the pp65 sequence. Together with the finding that pp65 deletion mutant 19 (aa 1-438) exhibited a predominantly

nuclear phenotype in Cos7 cells whereas mutant 50 (aa 1-434) was detected mostly in the cytoplasm suggested that the proposed nuclear localization signal between aa 415-438 could be demonstrated experimentally. Furthermore, a likely candidate for this nuclear localization sequence was the short stretch of basic aa *RKRK* located between aa 435-438. Schmolke et al. have proposed that this stretch of amino acids together with an *RK* sequence located at position 415-416 could act as a bipartite signal nuclear localization signal (26). Gallina et al. fused an approximately 117 aa stretch (aa 348-465) of pp65 which contained the proposed NTS between aa 415-438 to the reporter protein chloramphenicol acetyltransferase (CAT) and demonstrated nuclear localization (12). Although consistent with the proposed function of this predicted NTS, the size of this fusion protein was approximately 40 kDa, raising the possibility that nuclear localization resulted from diffusion into the nucleus (6). The results of this study combined with our findings clearly indicated that the region of pp65 between aa 415-438 contained sequences which could mediate nuclear localization, yet their contribution to the overall nuclear transport of full-length pp65 was unclear at this time.

An important finding of our study was that, at least in the context of pp65, only mutants containing the aa 1-508 could localize within the nucleus of primary HF cells, a cell type permissive for HCMV replication (Fig. 5A). In agreement with this finding was the cytoplasmic distribution of GFP-pp65 fusion protein mutant 18 (aa 1-453)(Fig. 5B). Thus, in marked contrast to the behavior of this mutant form of pp65 in Cos7 cells, the second NLS between aa 430-453 failed to facilitate nuclear localization in fibroblast cells. Moreover, the localization of mutant 56 (aa 1-511) suggested that, in addition to the bipartite signal between aa 537-560, additional NLS could promote nuclear accumulation of pp65 in primary fibroblasts. From our limited data in fibroblasts, we would suggest that an NLS is located between aa 481-508 (mutant 44 and 31, Fig. 5A). Although examination of the aa sequence of this region of pp65 does not reveal a described NTS, the switch from a nuclear to a cytoplasmic phenotype suggested that such a signal was present. The examination of a different cell type, especially a primary, non-dividing cell, demonstrated the cell-specific nature of NTS and suggested that our findings and those of others in non-permissive, proliferating cells may not be

entirely relevant to the intracellular transport and function of this HCMV protein.

Recent studies have suggested that there are differences in the rates of nuclear transport of macromolecules between proliferating and quiescent cells. Elegant studies by Feldherr and Akin in proliferating and growth-arrested BALB/c 3T3 cells indicated that there were differences in the transport of large nuclear-targeted gold particles between these cells such that nuclear uptake of the particles was significantly less in the quiescent cells (8, 9). This decrease was correlated with a decrease in the functional size of the nuclear pore (NP) transport channel in these cells from ~230 A in dividing cells to ~130 A in growth-arrested cultures; therefore, the NPs were hypothesized to be less permeable (8, 9). The differences in signal-mediated, active transport in these cells were dependent on the size of the targeted molecule as there was no difference in the nuclear uptake of relatively smaller gold particles in proliferating or growth-arrested cells (8, 9). Separate studies in HeLa cells suggested that there were also cell-cycle dependent changes in NP permeability (7). From these and other reports, it is clear that there are many factors that contribute to the nuclear accumulation of proteins,

including the size of the target protein, the number of signals in the protein and their efficiency, accessibility to these signals by cellular factors, and the metabolic state of the cell in which the protein is expressed.

In summary we have provided evidence that the abundant tegument protein of HCMV, pp65, contained several signals which could facilitate nuclear localization. At least two of these sequences resemble previously described bipartite signals which contain short stretches of basic aa. When examined in primary fibroblasts, we could demonstrate that a stretch of aa between position 481-508 could also mediate localization in the nucleus; however, mutants lacking this region of the molecule but containing the putative bipartite signal between aa 415-438 remained within the cytoplasm of transfected cells. This result provided evidence for cellular specificity in the nuclear localization of proteins and pointed to the importance of selecting a representative cell type for transport studies of viral proteins.

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GENERAL DISCUSSION

Studies of herpesvirus morphogenesis have outlined both the similarities and differences between different members of this virus family (20, 27, 28, 30, 31, 51, 56, 62, 64, 75, 85). While the mechanisms of replication of the genome and assembly of the virion appear to be very similar among the alpha, beta, and gamma herpesviruses, closer inspection of these processes has indicated that the divergence between these viruses goes beyond differences in host specificity and kinetics of viral infection.

The herpesvirus virion consists of a large, double-stranded DNA genome contained within a capsid of icosahedral symmetry (63, 75). The capsid shell is surrounded by an amorphous layer of tegument proteins which is also in contact with the lipid bilayer containing many virion-encoded glycoproteins (63, 75). In contrast to the replicative process of HSV-1, replication of HCMV does not yield a high titer of extracellular virus or lead to rapid lysis of the infected cell (51). In addition, the distribution of several conserved virion structural proteins in HCMV-infected

cells differs from that in HSV-infected cells (42, 51, 64, 75).

The assembly of herpesvirus particles has been studied using several approaches. Perhaps the most informative of these strategies is the creation of null or temperature-sensitive mutants for the study of the roles of specific proteins in morphogenesis. These genetic approaches have been very successful for the study of herpes simplex viruses, but the generation of recombinant HCMV and gamma herpesviruses has proved difficult because few cell types are permissive to infection and because of prolonged replication and low viral yield (51, 64). To date, only one HCMV mutant with a deletion in a virion structural protein has been constructed (67). This recombinant virus, AdRV, which does not express pp65 has been reported to produce comparable titers of progeny virus to the parental strain Ad169 (67); however, the phenotype of this virus resembles that of clinical isolates. AdRV also fails to produce dense bodies; therefore, this virus could be used to study mechanisms of HCMV particle morphogenesis.

An alternative method to studying herpesvirus assembly relies on the generation of virus-specific immunologic reagents for use in immunoelectron microscopy. By utilizing

antibodies to specific proteins, the locations of capsid, tegument, and envelope assembly have been investigated. However, electron microscopy provides only static images of particles and assembly intermediates with no information about other characteristics such as infectivity or structural complexity.

Our approach to understanding the mechanisms of virion morphogenesis was to examine the cellular trafficking of several different structural proteins in recombinant systems and HCMV-infected cells. By using a combination of imaging and biochemical techniques, we attempted to define not only the cellular compartment where each protein is acquired by the virion but also the biochemical characteristics of the proteins. Combining this information has allowed us to propose a general assembly pathway which could then be dissected in greater detail in future studies.

The first step to understanding HCMV assembly was to define the composition of the virion. Our analysis of purified virions suggested that these particles contained several abundant tegument proteins including pp65, ppUL69, pp28, and pp150. We focused on the proteins of the tegument because understanding the assembly of this region of the virion will help to define the cellular site of envelopment.

As an example, a previous report localized at least one of these proteins, pp28, to a non-nuclear compartment of the infected cell (42). This observation, therefore, suggested that envelopment occurred at a non-nuclear site as has been suggested for VZV (27, 31, 40, 80, 85). Our strategy was to learn about envelopment and egress by using the proteins of the tegument as probes for nuclear and cytoplasmic stages of particle morphogenesis.

We initially concentrated our studies on pp65 because, among the HCMV tegument proteins described, it is the only protein detected in the nucleus early in infection and in the cytoplasm late in infection (26, 66); therefore, pp65 could potentially serve as a marker for nuclear egress and cytoplasmic envelopment of virions.

The signals mediating nuclear targeting of pp65 have been the subject of two previous reports (26, 66). In these studies, a conventional nuclear targeting signal (NTS) at the extreme carboxy terminus of pp65 was identified (66). The results from both of these investigations suggested that other signals that could mediate nuclear localization were present in the pp65 sequence, as neither group could generate a mutation in pp65 that resulted in an exclusively cytoplasmic distribution. Our data confirmed the presence of a

second NTS as suggested by Gallina et al.(26); however, our results from experiments using a GFP-pp65 fusion protein indicated that this signal was only functional in dividing cells. Our investigations also suggested that pp65 contained a sequence which could mediate nuclear retention of this protein. We are presently examining the ability of this 20 aa, hydrophobic sequence to promote nuclear retention of a heterologous protein. A hydrophobic retention signal was recently described in the nuclear protein hnRNPC (53). This segment was shown to override nuclear export of a heterologous protein presumably by mediating protein-protein or possibly protein-lipid interaction within the nucleus (53). It is possible that the pp65 hydrophobic segment modulates interactions between pp65 and proteins of the nuclear matrix.

The role of nuclear pp65 in the replication of HCMV in infected cells has been of particular interest to several groups. Our studies revealed that pp65 associates with the nuclear matrix of HCMV-infected cells. This interaction was not dependent on the presence of other HCMV proteins as pp65 expressed in recombinant systems was also retained on the nuclear matrix. An unexpected finding was the observation

that the 50-52 kDa forms of pp65 were not as tightly associated with the nuclear matrix as was the full-length protein. These results prompted us to investigate the nature of the smaller products which are detected in HCMV-infected, recombinant vaccinia virus- and recombinant baculovirus-infected cells. Like the full-length pp65, the 50-52 kDa forms of pp65 are phosphorylated, but these products are not detected in extracellular virions (11). Our preliminary data suggest that these proteins are proteolytic products resulting from an amino-terminal and possibly a carboxyl-terminal cleavage of pp65. Our evidence for a cleavage site at the amino-terminus comes from studies of the GFP-pp65 fusion protein. In transient transfection assays, this protein is cleaved such that the GFP fragment is released from the pp65 portion of the molecule. Evidence for a cleavage site near the carboxyl-terminus comes from two sources. We have generated site-directed mutants of pp65 in which the consensus casein kinase II (CK II) phosphorylation site was mutated to an alanine or an aspartate. GFP fusion proteins of these pp65 mutants formed the same subnuclear structures as the wild type pp65; however, the Western blot analysis indicated that the alanine mutant did not produce the 65 kDa, the 50-52 kDa, or GFP cleavage products. Secondly, as

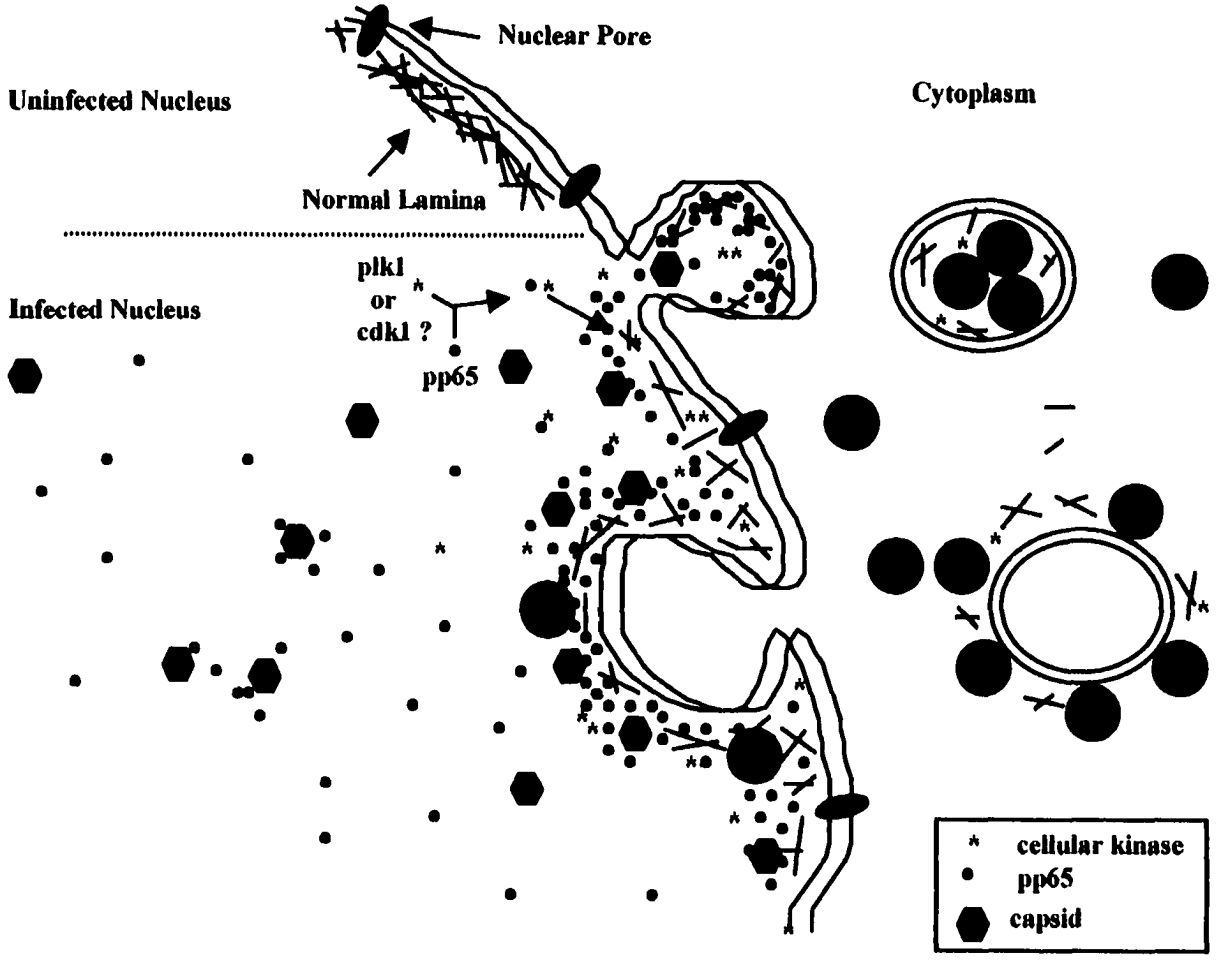
mentioned in the previous section, the 50-52 kDa forms of pp65 are not tightly retained on the nuclear matrix. The CK II site is directly upstream of the hydrophobic sequence implicated as a potential NRS (53). In addition, cleavage of pp65 at a site adjacent to the CK II site would yield fragments approximately 50-52 kDa in size. We are currently pursuing these lines of investigation in order to determine the significance of these sequences in pp65 localization and function.

Our studies into the nuclear matrix association of pp65 suggested that this protein interacted directly with proteins of the nuclear lamina. pp65 exhibits focal accumulation on the nuclear membrane in HCMV-infected cells and in recombinant systems. In addition, transient transfection of pp65 in Cos7 cells results in nuclear accumulation of pp65 as well as a distribution in cytoplasmic packets. These cytoplasmic packets exhibit positive immunostaining with antibodies against lamin B, suggesting that these vesicles may be derived from the nucleus (14). We have also observed the formation of nuclear buds in cells expressing the GFP-pp65 fusion protein. These observations suggested to us that the vacuolar-type of cytoplasmic staining observed late in HCMV infection might be the result of nuclear

fragmentation. We have demonstrated that a subpopulation of the cytoplasmic vacuoles observed in HCMV-infected fibroblasts also contain lamin B and lamina-associated polypeptide 2 (LAP2) (24). We are currently investigating whether the localization of these nuclear proteins in cytoplasmic vacoules results from the disruption of the structural integrity of the nucleus or from aberrant trafficking of these proteins due to infection. It is tempting to speculate that HCMV-infection and perhaps pp65 itself causes focal modifications of the nuclear lamina which could result in the release of unenveloped, immature subviral particles from the nucleus into the cytoplasm. Such a mechanism of nuclear egress would be consistent with information derived from EM micrographs of HCMV-infected cells showing unenveloped, tegumented particles associated with vacuoles in the cytoplasm. Support for this model is the finding that lamins a, b, and c are modified during HCMV-infection of human fibroblasts (60). The exact nature of these modifications is not clear, but perhaps these are changes in the phosphorylation state of the lamins. The dynamic disassembly and repolymerization of the lamina at mitosis is regulated by phosphorylation, so it follows that focal changes in the lamina caused by the accumulation of HCMV proteins could result in local

disruption of the network and lead to membrane blebbing (Fig. 1). The association of pp65 with the lamina suggests that pp65 might play a role in modifying the nuclear membrane. It has been observed that pp65 has an associated protein kinase activity. Work from our laboratory and others has indicated that pp65 does not have intrinsic kinase activity but instead binds to a cellular kinase which phosphorylates serines and threonines and is similar in enzymatic properties to CK II. A recent report has suggested that pp65 interacts with polo-like kinase, Plk1, in a two-hybrid system (68, 77). This kinase regulates assembly of the mitotic spindle and is also thought to regulate cdk1 kinase activity (43). Cdk1 is the mitotic kinase that controls entry into M phase and mediates breakdown of the nuclear lamina. It is also possible that pp65 might bind to cdk1 itself. Although HCMV-infected fibroblasts have been reported to be blocked in either G1 or G2/M of the cell cycle, upregulation of cyclin B and cdk1 kinase activity has also been reported (7, 22, 37). Cyclin B is the regulatory subunit of cdk1. We are currently investigating the trafficking of cyclin B in HCMV-infected fibroblasts in order to determine if the cdk1 complex plays some role in the

FIG. 1. Putative function of pp65 in nuclear egress of HCMV. In this model, pp65 localizes to the nucleus until late in infection when it exhibits focal accumulation along the inner nuclear membrane. Through its interaction with cellular kinases, perhaps plk1 or cdk1, pp65 targets these enzymes to the nuclear envelope ant the underlying lamina. These kinases could then modify the proteins of the lamina directly or indirectly causing focal disruptions of the network. This instability in nuclear architecture could result in alterations of the membrane, either invaginations or blebbing. Tegumented nucleocapsids are released from the nucleus within nuclear membrane vesicles or remain passively associated with the exterior or these compartments. Alternatively, immature virions could exit the nucleus by passage through breaks in the nuclear envelope.



putative disruption of nuclear morphology and subsequent egress of HCMV.

Our studies have also addressed the interactions of other structural and non-structural proteins with the nuclear matrix in infected cells. Our findings suggest that the capsid and nuclear tegument protein are associated with the NM; however, we have no information on whether these interactions are direct or whether pp65 might tether these proteins to this nuclear structure. We have also localized replication proteins to the NM which could indicate that replication is spatially coordinated with assembly in the nucleus. Our hypothesis is that the nuclear matrix serves as a staging site for the assembly of virions, and we are currently investigating methods which will permit us to visualize these processes in the nucleus of infected cells. By using resinless embedding procedures for electron microscopy (55), we anticipate that it will be possible to prepare nuclear matrix samples and examine replication, encapsidation, and tegumentation within HCMV-infected fibroblasts in combination with immunologic techniques.

We have also addressed envelopment of the virion from the point of view of tegument assembly in the cytoplasm. Our studies have indicated that pp150 and pp28 are localized

to the cytoplasm of HCMV-infected and transfected cells. We have focused on pp28 because the HSV-1 homolog of this protein UL11 is a myristylated, nuclear protein which has been suggested to play a role in envelopment of HSV-1 virions at the nuclear membrane (1). A temperature-sensitive mutant of HSV-1 UL11 is defective in envelopment at the nonpermissive temperature and virions accumulate at the inner nuclear membrane which contains unprocessed virion glycoproteins. Our hypothesis is that pp28 plays a similar role during HCMV infection, except that in this case tegument assembly and envelopment are coupled in the cytoplasm. Our findings have indicated that pp28 is myristylated in HCMV-infected cells and in recombinant systems. In addition, a mutation which removed the myristylation site in pp28 also changed the cellular distribution of pp28 (Fig. 2). Wild type pp28 is observed in cytoplasmic vacuoles in transfected cells but the non-myristylated mutant distributes in a diffuse pattern within the nucleus and cytoplasm. We are presently investigating the association of pp28 with envelope glycoproteins also present in the cytoplasmic vacuoles as well as examining these vacuoles for cellular markers in order to determine how particles are assembled in the cytoplasm. Studies from other laboratories have suggested that the envelope

FIG. 2. Cellular distribution of HCMV tegument proteins. Cos7 cells grown on glass coverslips were transfected with plasmids encoding HCMV pp28, a non-myristylated form of pp28, pp150, and gB. Cells were harvested 36 h post-transfection and fixed in 2% paraformaldehyde in PBS. After permeabilization and blocking, cells were stained with a murine monoclonal antibody specific for pp28 mAb 41-18 (A, C), a purified rabbit antiserum specific for pp150 (E), and a human antibody specific for gB (B, D, F). Cells were washed several times with PBS prior to addition of Texas Red-conjugated anti-mouse (A, C) or anti-rabbit (E) IgG and FITC-conjugated anti-human IgG antibodies. Magnification 400X.

glycoprotein gB recycles from the plasma membrane in endocytic vesicles (23). Perhaps the role of pp28 is to interact with gB via the cytoplasmic tail and to promote envelopment of partially tegumented, cytoplasmic virions at these vesicles. This process would increase the efficiency of envelopment and insure infectivity of progeny virion.

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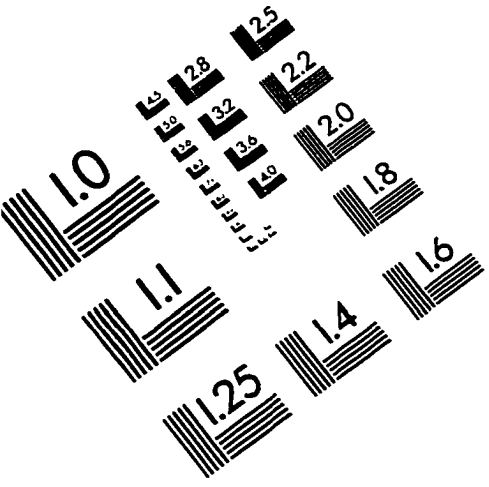

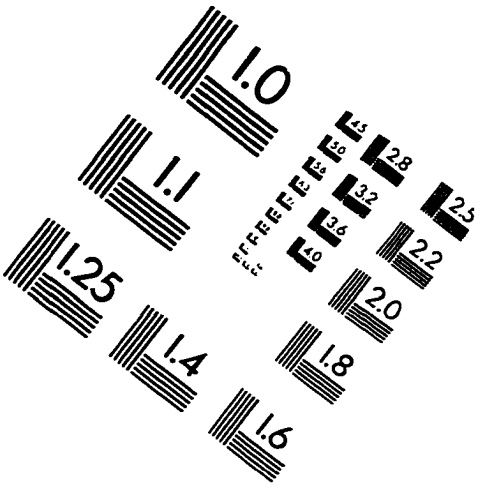

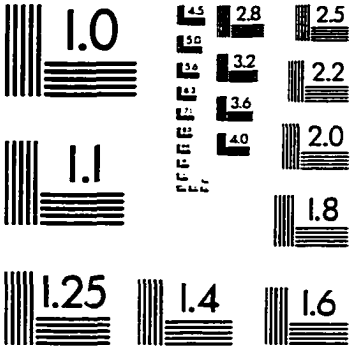

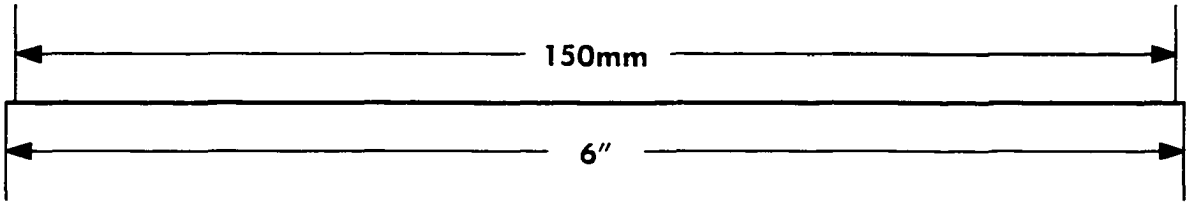

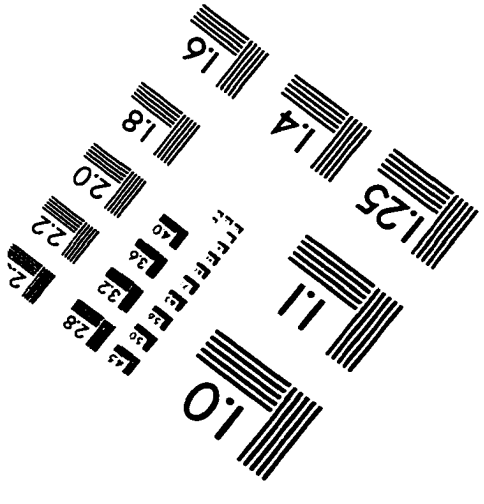

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