Time Course of Gag Protein Assembly in HIV-1-Infected Cells:
A Study by Immunoelectron Microscopy

Milan V. Nermut,*,1 Wei-Hong Zhang,† Garry Francis,* Fedor Čiampor,‡ Yuko Morikawa,§ and Ian M. Jones¶

*National Institute for Biological Standards and Control, South Mimms, Hertfordshire, United Kingdom; †NERC, Institute of Virology, Oxford, United Kingdom; ‡Institute of Virology, SAS, Bratislava, Slovakia; §Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan; and ¶Animal and Microbial Sciences, University of Reading, Reading, United Kingdom

Recent biochemical studies have identified high molecular complexes of the HIV Gag precursor in the cytosol of infected cells. Using immunoelectron microscopy we studied the time course of the synthesis and assembly of a HIV Gag precursor protein (pr55gag) in *Sf* 9 cells infected with recombinant baculovirus expressing the HIV *gag* gene. We also immunolabeled for pr55gag human T4 cells acutely or chronically infected with HIV-1. In *Sf* 9 cells, the time course study showed that the first Gag protein appeared in the cytoplasm at 28–30 h p.i. and that budding started 6–8 h later. Colloidal gold particles, used to visualize the Gag protein, were first scattered randomly throughout the cytoplasm, but soon clusters representing 100 to 1000 copies of pr55gag were also observed. By contrast, in cells with budding or released virus-like particles the cytoplasm was virtually free of gold particles while the released virus-like particles were heavily labeled. Statistical analysis showed that between 80 and 90% of the gold particles in the cytoplasm were seen as singles, as doublets, or in small groups of up to five particles probably representing small oligomers. Clusters of gold particles were also observed in acutely infected lymphocytes as well as in multinuclear cells of chronically infected cultures of T4 cells. In a few cases small aggregates of gold particles were found in the nuclei of T4 lymphocytes. These observations suggest that the Gag polyprotein forms small oligomers in the cytoplasm of expressing cells but that assembly into multimeric complexes takes place predominantly at the plasma membrane. Large accumulations of Gag protein in the cytoplasm may represent misfolded molecules destined for degradation.   © 2002 Elsevier Science (USA)

INTRODUCTION

The assembly of retroviruses offers an attractive target for the development of anti-viral agents but requires detailed knowledge of the assembly pathway and of the intermediates involved. To date most information concerning retroviral assembly has come from biochemical and molecular biological studies (reviewed in Kräusslich, 1996; Weldon and Hunter, 1997; Jones and Morikawa, 1998; Freed, 1998; Garnier *et al.,* 1998) and has concentrated on identification of the interacting sequences. Little is known about the assembly pathway and the cellular sites at which it begins.

The major retroviral structural protein, pr55gag, which forms a submembrane shell in immature virus particles, is synthesized on free ribosomes (Eisenman and Vogt, 1978). Electron microscopy studies have provided evidence that this protein assembles into regular arrays at the inner surface of the plasma membrane before or concomitant with the process of budding (Nermut *et al.,* 1994, 1998). This is the first stage in the formation of an immature Gag shell. Incorporation of viral glycoproteins gp120/41 and the enzymatic proteins completes the assembly process.

Biochemical studies have shown the presence of multimeric assemblies in the cytoplasm of human immunodeficiency virus (HIV)-infected cells. Spearman *et al.* (1997) reported the presence of dimers, trimers, and multimers of HIV p17gag (MA) both in the cytosol and in the membrane fraction of infected cells. Lee and Yu (1998) described a "detergent-resistant" HIV gag protein complex in the supernatant of Nonidet-lysed cells as opposed to a "detergent-sensitive" Gag protein associated with cellular membranes. Lee *et al.* (1999) purified the detergent-resistant complex and showed that it could be disrupted by high salt treatment. The presence of HIV Gag oligomers in solution was reported by Scarlata *et al.* (1998). HIV MA trimers in solution were reported by Morikawa *et al.* (1998) and large Gag complexes (about 1000 kDa) were found in the cytosol of *Spodoptera frugiperda* (*Sf* 9) cells infected with a recombinant baculovirus expressing HIV pr55gag by Morikawa *et al.* (2000). A recent study by Wang *et al.* (2001) showed that deletions within the MA domain resulted in the formation of assembly intermediates in the cytoplasm and budding from most cellular membranes, including the plasma membrane. A similar conclusion was reached by Le

Blanc *et al.* (2002), who found that the wild-type (wt) Human T-cell leukemia virus (HTLV) Gag protein does not bind to cytoplasmic vesicles, but is free in the cytosol and is seen in the form of punctate fluorescence, which they concluded indicated that HTLV Gag protein assembly starts in the cytoplasm. The precise oligomeric state of the Gag polyprotein in the cytoplasm, the mechanism of transport of Gag molecules to the plasma membrane, and the site of the first steps in the assembly of immature Gag shells have not been resolved by the above studies. Interestingly, pulse/chase experiments (Tritel and Resh, 2000; M. Suomalainen, personal communication) have provided evidence for the fast transport of newly synthesized HIV Gag to the plasma membrane.

Previous observations of clusters of Gag protein in the cytoplasm of HIV-infected cells by immunoelectron microscopy (unpublished results) stimulated us to study the time course of Gag protein synthesis and assembly in HIV-infected cells with the aim of confirming or refuting the notion that Gag protein assembly starts in the cytoplasm of expressing cells. Unlike biochemical analyses, immunoelectron microscopy has the benefit of determining the topography and distribution of the protein in the cytoplasm at different stages of the infectious process. Initially, we took advantage of the high levels of HIV gag protein expressed by recombinant baculovirus-infected cells, as this allowed easy evaluation of the results of time course labeling. In addition, acutely or chronically HIV-infected T4 lymphocytes were also studied. The results provided evidence that only a limited degree of Gag oligomerization takes place in both cellular systems in the cytoplasm. It thus appears that multimeric prebudding complexes assemble predominantly at the plasma membrane.

RESULTS

The time course of Gag protein assembly in recombinant baculovirus-infected *Sf*9 cells

No immunolabeling for Gag antigen was observed in infected *Sf*9 cells prior to 24 h p.i., consistent with the known temporal pattern of polyhedrin promoter-driven transcripts (O'Reilly *et al.,* 1992). However, gold particles were observed after 28–30 h p.i., first close to the nuclear membrane and later scattered throughout the cytoplasm mainly in ribosome-rich areas (Fig. 1a). In a few cases, however, an accumulation of gold particles was also observed over dense areas free of ribosomes. The number of gold particles increased substantially between 34 and 36 h p.i. During this period a common feature was single gold particles, doublets, or only small groups (3–5 gold particles) in the cytoplasm of many cells. Groups of 10 to 70 gold particles were regularly observed (Figs. 1b and 1c), but very large accumulations (up to 200 gold particles) were rare (Fig. 2). In most cases gold aggregates were associated with a dark background, indicat-

ing the presence of proteinaceous material (Figs. 1b–1d). Most gold clusters were larger in diameter than average-size virus particles (about 130 nm).

We classified the counted gold particles in *Sf*9 cells into five categories: single particles, doublets, 3 to 5 particles, 6 to 10 particles, and over 10 particles. This evaluation was done in cells at 30, 32, 34, 36, and 44 h p.i. The great majority of gold particles was small groups of up to 5 gold particles (from 80 to 90%), while the number of colloidal gold clusters in the over-10 category was small and heterogeneous in size (from 4 to 15%). Clusters containing very large numbers of gold particles also biased the total number of gold particles in that category (Figs. 1b–1d and 2). Despite the high m.o.i. used during infection, synchronous expression of Gag protein was not achieved. Some cells produced virus-like particles (VLP) after 36 h p.i., while the majority of cells reached the state of VLP budding and release at about 40 to 44 h p.i. At this time point the cytoplasm was virtually free of gold particles and only a few gold particles were associated with the plasma membrane, while free VLP were heavily labeled (Fig. 1e).

Gold immunolabeling of Gag protein in T4 lymphocytes

As the levels of Gag antigen synthesis using the recombinant baculovirus system were high, it was possible that Gag protein accumulation within the cell could be simply the consequence of abnormally high protein concentrations. To verify that this was not the case, a study of Gag protein expression in acute and chronic infection of lymphocytes was undertaken. In keeping with the altered replication kinetics, the time scale of Gag expression was much longer than that in *Sf*9 cells, with the first budding observed after 2 or 3 days depending on the m.o.i. used. Similarly, the number of gold particles in the cytoplasm was substantially lower than that in the baculovirus system; however, distinct clusters of colloidal gold were observed both deep in the cytoplasm and close to the cell surface (Figs. 3a and 3b) together with a low number of single gold particles. The low level of labeling prevented a statistical evaluation of the gold particle distribution in acutely infected T4 cells. In a few cases small groups of gold particles were also observed in nuclei in both the C8166 and the CEM cells (Fig. 3c).

Chronically infected JM cells provided further evidence for the presence of Gag protein aggregates in the cytoplasm. In multinuclear syncytia small or large groups of gold particles either mixed with ribosomes or more frequently associated with small, occasionally large, dense areas of irregular shape were observed (Fig. 4a). The number of gold particles in such clusters varied from a few to about 20, representing up to 100 copies of the Gag precursor (see below). By comparison, the average number of gold particles per section of a released virus
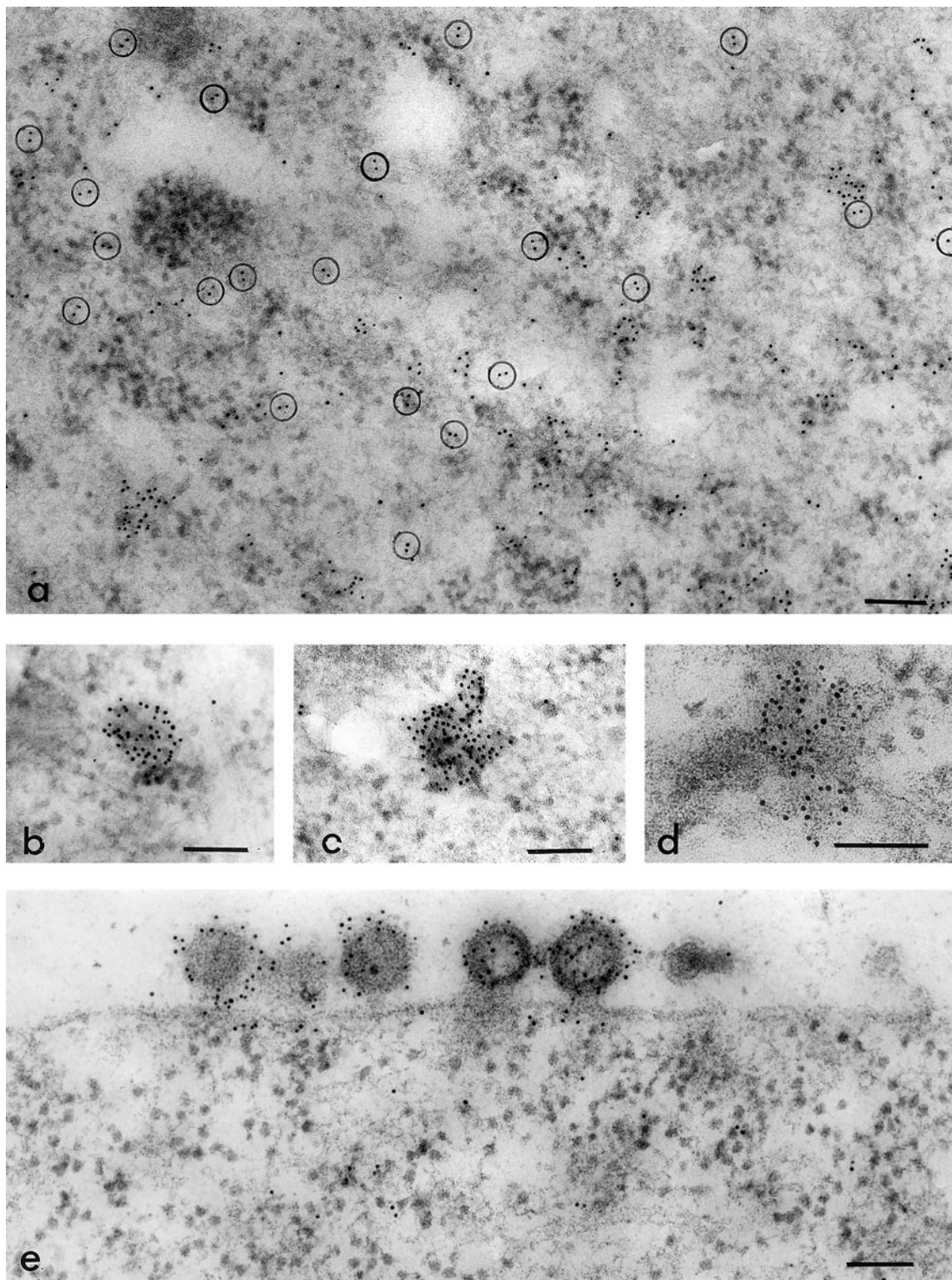
FIG. 1. *Sf* 9 cells after immunolabeling with monoclonal antibody for p55/24 and 5-nm colloidal gold. (a) Gold particles are scattered throughout the cytoplasm of *Sf* 9 cells 30 h after infection with recombinant baculovirus-expressing HIV p46gag. Some doublets of gold particles are circled. (b) Aggregates of colloidal gold particles in *Sf* 9 cells 36 h p.i. and (c) 32 h p.i. (d) Large group of colloidal gold particles associated with a homogenous high-electron-density area in *Sf* 9 cell 34 h p.i. (e) *Sf* 9 cell 44 h p.i. with released VLP. Note very low density of gold particles in the cytoplasm. Bars, 100 nm.

particle was 13.7 (SD 4.4, $N = 64$). Very large clusters of gold particles such as those observed in *Sf* 9 cells were not seen. The surface area covered by gold clusters in

the cytoplasm varied from 1000 to over 10,000 nm^2, while the area of virus particles was about 1000 nm^2. Single cells were usually free of gold particles. Some syncytia
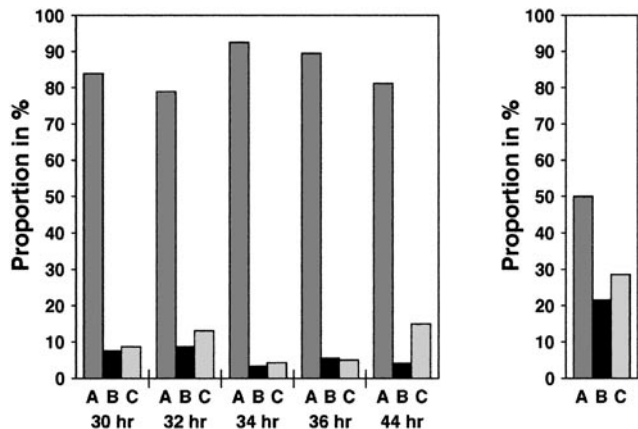
FIG. 2. (Left) Distribution of HIV gag molecules in *Sf* 9 cells at different times p.i. A, Groups of 1–5 gold particles. B, Groups of 6 to 10 gold particles. C, Groups of more than 10 gold particles. Total counts of gold particles in cells 30 h p.i. = 2360; 32 h p.i. = 1935; 34 h p.i. = 959; 36 h p.i. = 3570; 44 h p.i. = 1616. An efficiency of 20% was used to calculate the number of Gag copies. Category 1 to 5 gold particles may represent small Gag oligomers (dimers or trimers), category 6 to 10 gold particles may represent small assemblies of dimers or trimers, category over-10 gold particles may represent multimers or aggregates of Gag protein. (Right) Distribution of HIV Gag molecules in chronically infected JM cells. A, Groups of 1–5 gold particles. B, Groups of 6–10 gold particles. C, Groups of more than 10 gold particles. Gold particle counts in A = 3160, B = 1360, C = 1800.

contained only a few gold particles in the cytoplasm, but in such cases large numbers of free virus particles were observed close to the cell surface, often between the microvilli, and also budding into large syncytial vesicles filled with virus particles (Fig. 4b). Occasionally, such large vesicles contained clusters of gold particles of various shapes not typical of virus particles (Fig. 4c). The distribution of gold particles in multinuclear cells (Fig. 2) revealed differences when compared with the distribution of Gag molecules in *Sf* 9 cells. Specifically, the proportion of small groups of 1 to 5 gold particles was lower in chronically infected cells (50%) than in *Sf* 9 cells, reflecting the level of Gag expression achieved by each system. On the other hand, the number of gold particles in the category of over 10 was only 28.5%, and there were 21.5% gold particles in the category of 6–10. Some giant cells were in an early stage of virus production (see above), while others were surrounded by masses of free virus particles (VP) heavily labeled with antibody to HIV p24.

DISCUSSION

Investigation of retroviral Gag protein synthesis and assembly has, so far, largely concerned biochemical and molecular biology approaches. While this approach has provided much information concerning the fate of Gag protein *en masse,* the pathway of assembly at the individual cell level has been harder to define. We have used

immunoelectron microscopy to visualize the presence of Gag protein in the cytoplasm of expressing cells within a period from synthesis to release of immature virus-like particles or wt viruses. The aim of this study has been to contribute to the discussion of the initial site of Gag protein assembly (i.e., early multimerization): in the cytoplasm or exclusively at the plasma membrane. Answering these questions will improve our knowledge of the process of the assembly of HIV and may have parallels for other budding viruses.
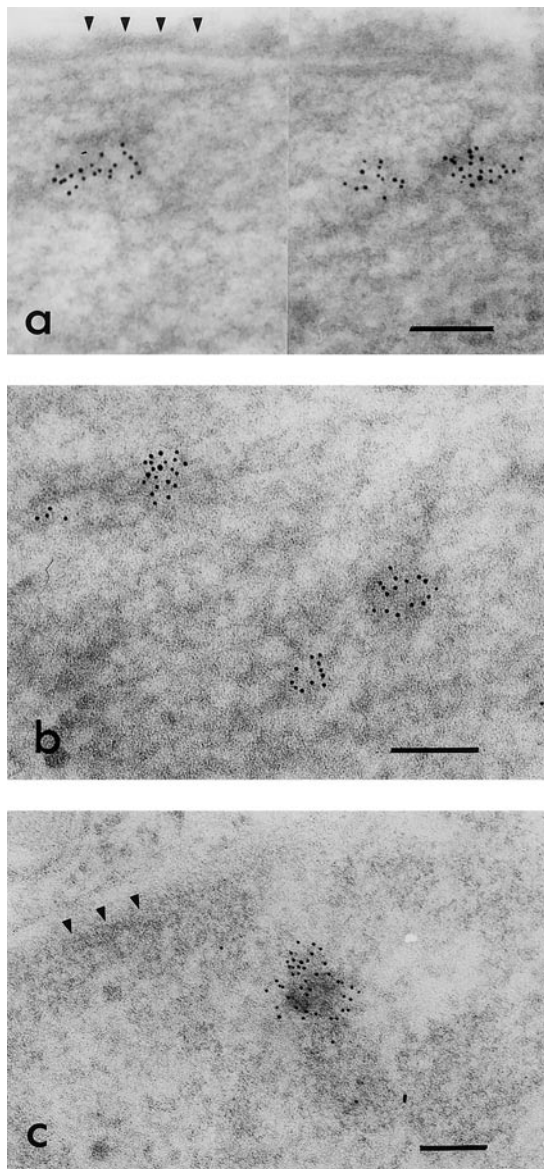

FIG. 3. Clusters of gold particles found below plasma membrane (a) or deep in cytoplasm (b) of acutely HIV-1-infected C8166 cells (48 h p.i.). Single gold particles were very rare in this cell. (c) A small group of gold particles in a nucleus of an acutely infected C8166 cell. Arrowheads point to plasma membrane in (a) and to nuclear membrane in (c). Bars, 100 nm.
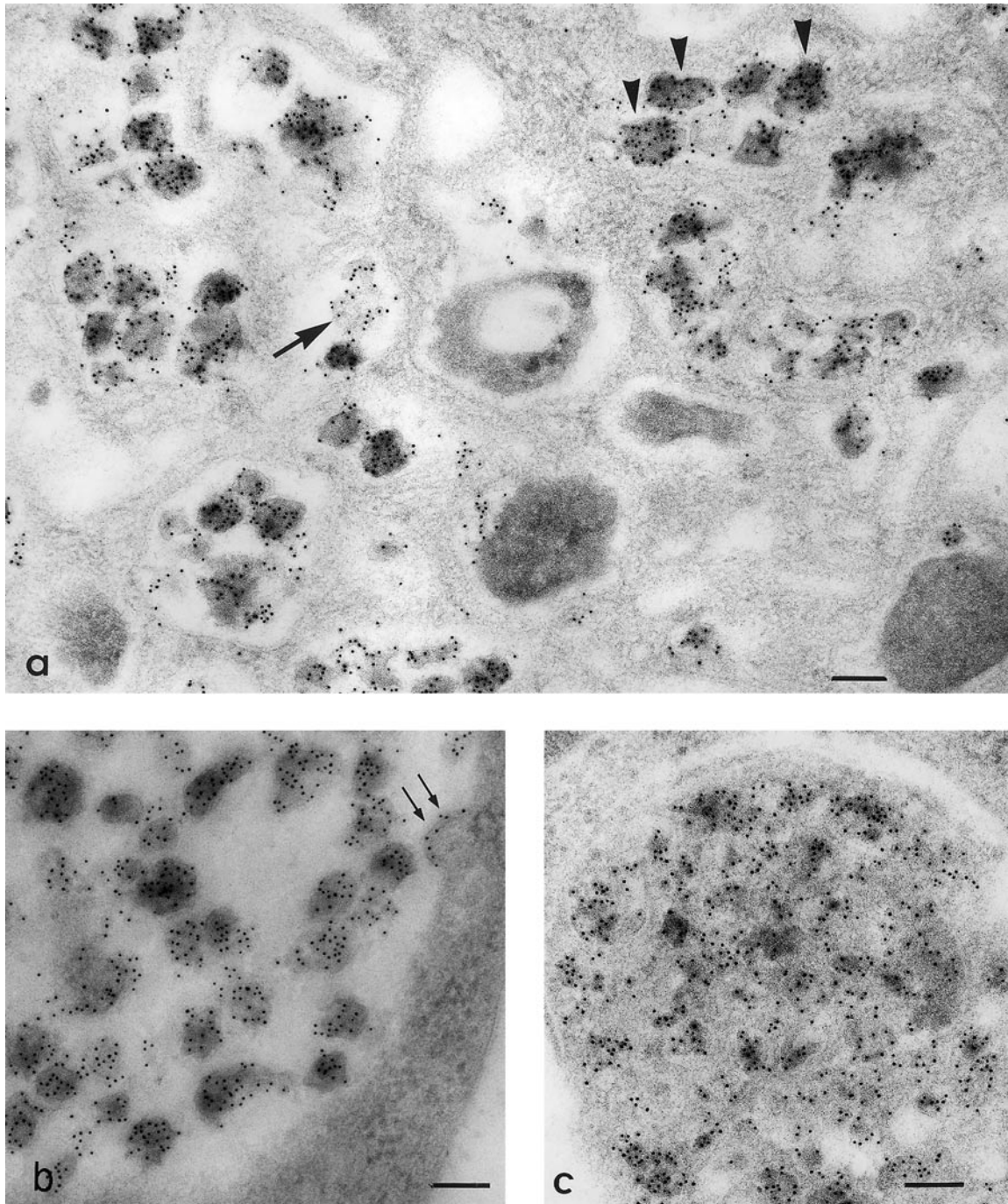
FIG. 4. (a) The cytoplasm of a multinuclear cell of chronically infected JM cells with groups of about 20 gold particles situated above dense cytoplasmic structures (arrowheads) or scattered in the cytoplasm (arrow). (b) Virus particles released into a large vacuole. Arrows point to a hemispherical structure, probably a budding virus particle. (c) Large vacuole filled with gold particles either single or in clusters of varying sizes and shapes. Bars, 100 nm.

Distribution of colloidal gold particles in the cytosol and calculation of the number of corresponding Gag molecules

Colloidal gold immunolabeling of thin sections of *Sf* 9 cells infected with a recombinant baculoviruses expressing Pr46gag provided convincing evidence for the pres-

ence of Gag molecules in the cytoplasm. These findings correlate well with biochemical studies reporting the presence of dimers, trimers, or small oligomers in solution (Spearman *et al.,* 1997; Scarlata *et al.,* 1998; Morikawa *et al.,* 1998) and large accumulations of Gag in the cytosol (Lee and Yu, 1998; Lee *et al.,* 1999; Morikawa *et al.,* 2000). However, the distribution of colloidal gold

particles (Fig. 2) showed clearly that the majority of Gag molecules (about 80 to 90%) was found in small groups of up to 10 gold particles. To convert these figures into numbers of Gag protein molecules requires the knowledge of the immunolabeling efficiency in our experimental conditions, the value of which depends on the quality of the antibodies used, the accessibility and preservation of the specific epitope, and the preparative techniques. Generally, the efficiency of gold immunolabeling for electron microscopy has been estimated at 1–10% (Griffiths, 1993), but this can be higher when labeling cell surface antigens (Nermut and Nicol, 1989; Erlandsen et al., 2001). Using the procedure described under Materials and Methods, we concluded that the efficiency in our experimental conditions was about 20%. This efficiency factor would indicate that most Gag molecules in the cytoplasm existed as relatively small oligomers of tens of Gag copies. Accordingly, gross multimerization of Gag into 2D sheets, which then induce the curvature typical of early budding, takes place predominantly at the plasma membrane. It is interesting to note that variation in labeling efficiency from 17 to 27% (the credible range of values that could be applied using known Gag and virus dimensions) would not substantially alter these conclusions. While the number of large clusters of gold particles was small when compared with the number of small groups, the overall number of Gag molecules in the aggregates was substantially larger (from a few dozens to hundreds of gold particles, see Figs. 1b–1d and 2). This discontinuity of size class, the sporadic occurrence, and the relative Gag content suggest that large Gag complexes are not part of the normal pathway of assembly and may represent misfolded Gag that fails to enter the assembly pathway and is destined for degradation. It has been reported that up to 80% of Gag may be degraded by proteasomes because of misfolding (Schubert et al., 2000; Tritel and Resh, 2000). Based on the numbers of large aggregates observed, our data suggest that at least 20% of Gag molecules might be destined for degradation in Sf9 cells, although the exact proportion would probably depend on the type and physiological state of the expressing cell.

Are cytoplasmic Gag oligomers the first stages of organized virus assembly?

Our study confirms the previous findings of Gag in the cytosol of HIV-infected cells (Lee and Yu, 1998; Lee et al., 1999; Morikawa et al., 2000). Extending the above reports we made an attempt to determine the size of the Gag oligomers by classifying gold particles observed in the cytoplasm into groups of up to 10 gold particles and above. Our data showed that a great majority of Gag molecules exist as small assemblies (Fig. 2). Since Gag is synthesized on free ribosomes (Eisenman and Vogt, 1978) and released into an aque-

ous environment, self-association into dimers, trimers, or oligomers will take place when a critical concentration of protein is reached, aided by hydrophobic residues on the protein surface (Matthews et al., 1994; Hill et al., 1996) and the formation of hydrogen bonds (see Turner and Summers, 1999, and refs. therein). Electrostatic interactions between charged groups on MA (Hill et al., 1996; Forster et al., 2000) may also enhance the probability of limited protein oligomerization in the cytosol. Trimers or multimers of MA have been observed in cell lysates of Gag-expressing cells (Scarlata et al., 1998; Morikawa et al., 1998), but no electron microscopy of the larger Gag protein complexes observed by Lee et al. (1999) has been reported to date. However, our examination by electron microscopy of the high-molecular-weight Gag aggregates reported by Morikawa et al. (2000) has failed to reveal any regular structural complexes (M.V. Nermut, unpublished data), consistent with their probable identification as misfolded and aggregated material.

Thus, while small Gag assemblies are formed in the cytosol, we conclude that the formation of ordered higher molecular weight complexes (2D sheets and spherical capsids) takes place only after association of Gag with the inner surface of the plasma membrane. This conclusion is supported by subcellular fractionation (Tritel and Resh, 2000) and, in particular, by confocal imaging, which has shown the presence of Gag in the plasma membranes (and in cellular vesicles) of infected cells (Hermida-Matsumoto and Resh, 2000). Our previous electron microscopy study revealed small patches, as well as occasionally large, triangular 2D sheets of Gag protein on the protoplasmic surface of the plasma membrane of Sf9 cells (Nermut et al., 1994, 1998). This does not rule out formation of spherical or part-spherical Gag shells in the cytoplasm under specific conditions. For example, high concentrations of HIV Gag in Sf9 cells producing a C-terminal truncation mutant of Gag showed preformed Gag shells free in the cytoplasm or budding into cytoplasmic vacuoles (see Fig. 4 in Hockley et al., 1994). Recently, Wang et al. (2001) reported that deletion of all but the first 15 MA residues resulted in the formation of intracellular virus particles and concluded that wild type Gag–Gag interactions occur initially in the cytoplasm to form "assembly intermediates" which subsequently interact with plasma membrane and cellular vesicles. This conclusion may be premature, as the >10 groups of gold particles observed in our study showed neither the typical structural features nor the right diameter to be qualified as virus particles. Also, the number of gold particles was, in most cases, larger than the average of 15 gold particles per immature or mature virus particle (see above).

Kinetics of the assembly process

Our observations suggested a 6- to 8-h period between initial Gag synthesis and the assembly of VLP. A similar delay (4–6 h after pulse) was observed in the experimental conditions used by Tritel and Resh (2000) and Morikawa *et al.* (2000). Slow transport of Gag is unlikely, since the results of pulse/chase experiments have revealed that a subpopulation of Gag protein reaches the plasma membrane within 5–10 min (Tritel and Resh, 2000; M. Suomalainen, personal communication).

Interestingly, budding from individual cells started approximately in synchrony in keeping with the accumulation of a critical minimum of small oligomers before arrangement into the periodic 2D assemblies destined to form spherical Gag shells (Nermut *et al.,* 1998). This notion is supported by the observation that the high number of gold particles observed in the cytoplasm for several hours reduces rather quickly approximately 1 h or more before the start of budding. The requirement to diffuse within lipid bilayers to lipid rafts, the presumed site of final assembly (Nguyen and Hildreth, 2000; Ono and Freed, 2001), and/or the incorporation of envelope glycoproteins might also provide a barrier to the budding and release process which is suddenly overcome when a critical concentration is reached. Lastly, the experimental system we have used to generate significant levels of Gag in order to follow its routing, recombinant baculoviruses, is a transient system in which a "wave" of Gag synthesis is followed by cell senescence. This may, itself, enforce a degree of synchrony.

Specific situation in multinuclear cells

The image in chronically infected mammalian cells differed from that seen in *Sf* 9 cells in respect to the lower density of gold particles in the cytoplasm and also to the size of the gold aggregates (Fig. 2). There were substantially less gold particles in the 1–10 class in chronically infected cells. On the other hand, the proportion of clusters containing more than 10 gold particles was higher than in *Sf* 9 cells. No data concerning the proportion of degraded Gag in chronically infected cells have been published, but assuming that the interpretation of the dark background to gold clusters as sites of degradation is correct, our observations would suggest that Gag digestion takes place both in the cytoplasm (Fig. 4a) and also in some large "syncytial vesicles" (Fig. 4c) possibly equivalent to secondary lysosomes or multivesicular bodies. Other large vesicles contained numerous virus particles (Fig. 4b). There is an uncertainty about the nature of round or oval large structures (occasionally labeled with anti-CA antibody) reminiscent of cellular vesicles (Fig. 4a). The medium used for low-temperature embedding does not visualize vesicular structures well enough to enable their identification, but as a whole our observations are in line with reports by Grief *et al.* (1991) and others (reviewed in Harris, 1993), which showed that the major producers of the virus were multinuclear cells, while single cells were mostly free of signs of assembly or budding. No immunolabeling for Gag was done in the above papers, limiting our ability to make direct comparisons.

In conclusion

Our observations have shown that (1) there is a long delay between the start of synthesis, budding, and release of immature virus particles (such delay might indicate a limited efficiency of the "synthesis engine" and also that the organization of Gag oligomers into periodic structures at the plasma membrane is a slow process); (2) in the "intermediate period" (from 30 to about 40 h p.i.) the cytoplasm is predominantly filled with small Gag assemblies, which suggests that the process of Gag assembly into 2D and 3D structures takes place at the plasma membrane, in agreement with previously published electron microscopy observations; and (3) the presence of large aggregates of gold particles in the cytoplasm during the intermediate period is consistent with findings that a large proportion of newly synthesized Gag is degraded.

MATERIALS AND METHODS

Spodoptera frugiperda cells were infected with recombinant baculoviruses expressing truncated HIV Gag precursor (p46gag, Jowett *et al.,* 1992) at an m.o.i. of about 5 and grown at 27°C in liquid TC 100 medium supplemented with 10% fetal bovine serum. Cells were harvested at 24, 28, 30, 32, 34, 36, 44, and 48 h postinfection and fixed in 3% paraformaldehyde for several hours. The cells were washed in phosphate-buffered saline (PBS) and processed for low-temperature embedding in Lowicryl HM20 using the routine "progressive lowering of temperature" protocol.

Human T4 (C8166) cells were infected with HIV-1 (RF) and processed after 2 days for low-temperature embedding after freeze-substitution (see below). In addition, CEM SS cells were also infected with HIV-1 (strain LAV) and immunolabeled 3, 6, 9, and 10 days later. In a study of chronic infection, JM cells (NIBSC Repository, ARP 008) were infected with HIV-1, strain GB8 (ARP 131.1), and processed as follows: Cells were fixed in 3% paraformaldehyde overnight, washed in PBS, and embedded in 1.5% agarose. The cell pellet solidified in agarose was cut into small pieces which were equilibrated in 2.3 M sucrose overnight. Small pieces of agarose-embedded cells were rapidly frozen in liquid ethane and freeze-substituted in absolute methanol with 0.5% uranyl acetate (Grief *et al.,* 1994). Thin sections were cut with Reichert–Jung Ultracut E microtome.

Gold immunolabeling

Thin sections were preconditioned in Tris-buffered saline (TBS), pH 8.2, containing 0.1% bovine serum albumin (BSA-C, Aurion, Netherlands) and 0.1% fish gelatine (Bio-Cell International, Cardiff, UK) followed by monoclonal antibody to p55/24 (ARP 313) diluted 300× with the above buffer (TBG). Control grids were incubated in TBS or in a nonspecific monoclonal antibody. After 60 min at room temperature, grids were washed 3× for 5 min each in TBG, followed by incubation for 45 min in 10× diluted gold anti-mouse antibody conjugated to 5-nm colloidal gold (Bio-Cell). After three washes in TBG, followed by TBS and distilled water, sections were stained in alcoholic uranyl acetate for 8 to 10 min. Carbon-coated sections were examined in a Philips CM12 electron microscope operated at 80 kV. The surface area of virus particles or colloidal gold clusters was measured using a Kontron Digiplan magnetic tablet.

For calculating gold immunolabeling efficiency in low-temperature-embedded sections, we counted colloidal gold particles associated with VLP produced in Sf 9 cells and also with virus particles observed in chronically infected lymphocytes. The obtained average figures were related to the number of Gag protein molecules supposedly exposed in cross-sectioned VLP or VP. In immature virus particles such as VLP, the Gag polyprotein forms a radial palisade of Gag molecules with the major CA domain well exposed to specific antibodies. We calculated the number of exposed CA domains in a spherical shell 120 nm in diameter by dividing its circumference by 5 nm, which is an average spacing between Gag molecules in frozen hydrated HIV VLP (5.5 nm by Fuller et al., 1997) and murine leukemia virus immature particles (4.5 nm by Yeager et al., 1998). The counts of gold particles from sections of VLP (Sf 9 cells) provided an average of 17.7 (STD 9.1, N 51) and 13.75 (STD 4.4, N 64) of wt HIV particles from chronically infected T4 cells. This difference is understandable, since the VLP Gag shell is of immature form, while, in HIV virions, the condensed core provides a smaller surface area for antibody binding. For practical reasons we amalgamated the above data, which resulted in a mean of 15.5 (SD 6.2, $N = 115$). The immunolabeling efficiency based on those figures was 24% for 100-nm-wide virus particles and 20% for 120-nm-wide particles. We used the more conservative 20% in our estimates of Gag molecules represented by a single gold particle.

ACKNOWLEDGMENTS

REFERENCES

Eisenman, R. N., and Vogt, V. M. (1978). The biosynthesis of oncovirus proteins. *Biochim. Biophys. Acta* **473,** 187–239.

Erlandsen, S. L., Greet Bittermann, A., White, J., Leith, A., and Marko, M. (2001). High resolution cryo-FESEM of individual cell adhesion molecules (CAMs) in the glycocalyx of human platelets: Detection of P-selectin (CD62P), GPI-IX complex (CD42A/CD42B alpha, B beta) and integrin GPIIbIIIa (CS41/CD61) by immunogold labeling and stereo imaging. *J. Histochem. Cytochem.* **49,** 809–819.

Freed, E. O. (1998). HIV-1 gag proteins: Diverse functions in the virus life cycle. *Virology* **251,** 1–15.

Forster, M., Mulloy, B., and Nermut, M. V. (2000). Molecular modelling study of HIV p17gag (MA) protein shell utilising data from electron microscopy and X-ray crystallography. *J. Mol. Biol.* **298,** 841–857.

Fuller, S. D., Wilk, T., Gowen, B. E., Krausslich, H.-G., and Vogt, V. M. (1997). Cryoelectron microscopy reveals ordered domains within the immature HIV-1 particle. *Curr. Biol.* **7,** 729–738.

Garnier, L., Bowzard, J. B., and Wills, J. W. (1998). Recent advances and remaining problems in HIV assembly. *AIDS* **12**(Suppl. A), S5–S16.

Grief, Ch., Farrar, G. H., Kent, K. A., and Berger, E. G. (1991). The assembly of HIV within the Golgi apparatus and Golgi-vesicles of JM cell syncytia. *AIDS* **5,** 1433–1439.

Grief, Ch., Nermut, M. V., and Hockley, D. (1994). A morphological and immunolabelling study of freeze-substituted human and simian immunodeficiency viruses. *Micron* **25,** 119–128.

Griffiths, G. (1993). "Fine Structure Immunocytochemistry." Springer-Verlag, Heidelberg.

Harris, J. R. (1993). The ultrastructure of multinucleate giant cells. *Micron* **24,** 173–231.

Hermida-Matsumoto, L., and Resh, M. D. (2000). Localization of human immunodeficiency virus type 1 Gag and Env at the plasma membrane by confocal imaging. *J. Virol.* **74,** 8670–8679.

Hill, C., Worthylake, D., Bancroft, D., Christensen, A., and Sundquist, W. (1996). Crystal structures of the trimeric human immunodeficiency virus type 1 matrix protein: Implications for membrane association and assembly. *Proc. Natl. Acad. Sci. USA* **93,** 3099–3104.

Hockley, D. J., Nermut, M. V., Grief, Ch., Jowett, J. M. B., and Jones, I. M. (1994). Comparative morphology of Gag protein structures produced by mutants of the gag gene of human immunodeficiency virus type 1. *J. Gen. Virol.* **75,** 2985–2997.

Jones, I. M., and Morikawa, Y. (1998). The molecular basis of HIV capsid assembly. *Rev. Med. Virology* **8,** 87–95.

Jowett, J. B. M., Hockley, D. J., Nermut, M. V., and Jones, I. M. (1992). Distinct signals in human immunodeficiency virus type 1 Pr55 necessary for RNA binding and particle formation. *J. Gen. Virol.* **73,** 3079–3086.

Kräusslich, H.-G. (1996). Morphogenesis and maturation of retroviruses. *Curr. Top. Microbiol. Immunol.* **214,** pp. 344, Springer, Berlin.

Le Blanc, I., Blot, V., Bouchaert, I., Salamero, J., Goud, B., Rosenberg, A. R., and Dokhelar, M.-Ch. (2002). Intracellular distribution of human T-cell leukemia virus type 1 Gag proteins is independent of interaction with intracellular membranes. *J. Virol.* **76,** 905–911.

Lee, Y., and Yu, X. (1998). Identification and characterization of virus assembly intermediate complexes in HIV-1-infected CD4$^+$ T cells. *Virology* **243,** 78–93.

Lee, Y. M., Liu, B., and Yu, X. F. (1999). Formation of virus assembly intermediate complexes in the cytoplasm by wild-type and assembly-defective mutant human immunodeficiency virus type 1 and their association with membranes. *J. Virol.* **73,** 5654–5662.

Matthews, S., Barlow, P., Boyde, J., Russell, R., *et al.* (1994). Structural similarity between the p17 matrix protein of HIV-1 and interferon-gamma. *Nature* **370,** 666–668.

Morikawa, Y., Zhang, W. H., Hockley, D. J., Nermut, M. V., and Jones, I. M. (1998). Detection of a trimeric human immunodeficiency virus type 1 Gag intermediate is dependent on sequences in the matrix protein, p17. *J. Virol.* **72,** 7659–7663.

Morikawa, Y., Hockley, D. J., Nermut, M. V., and Jones, I. M. (2000). Roles of matrix, p2 and N-terminal myristolyation in human immunodeficiency virus type 1 Gag assembly. *J. Virol.* **74,** 16–23.

Nermut, M. V., Hockley, D. J., Jowett, J. B. M., Jones, I. M., Garreau, M., and Thomas, D. (1994). Fullerene-like organization of HIV gag-protein shell in virus-like particles produced by recombinant baculovirus. *Virology* **198,** 288–296.

Nermut, M. V., Hockley, D. J., Bron, P., Thomas, D., Zhang, W. H., and Jones, I. M. (1998). Further evidence for hexagonal organization of HIV gag protein in prebudding assemblies and immature virus-like particles. *J. Struct. Biol.* **123,** 143–149.

Nermut, M. V., and Nicol, A. (1989). Colloidal gold immunoreplica method. *In* "Colloidal Gold" (M. A. Hayat, Ed.), Vol. 1, pp. 349–373. Academic Press, San Diego.

Nguyen, D. H., and Hildreth, E. K. (2000). Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. *J. Virol.* **74,** 3264–3272.

Ono, A., and Freed, E. O. (2001). Plasma membrane raft play a critical role in HIV-1 assembly and release. *Proc. Natl. Acad. Sci. USA* **98,** 13925–13930.

O'Reilly, D. R., Miller, L. K., and Luckow, V. A. (1992). "Baculovirus Expression Vectors: A Laboratory Manual," pp. 347. Freeman, New York.

Scarlata, S., Ehrlich, L. S., and Carter, C. A. (1998). Membrane-induced alterations in HIV-1 Gag and matrix protein–protein interactions. *J. Mol. Biol.* **277,** 161–169.

Schubert, U., Anton, L. C., Gibbs, J., Norbury, C. C., Yewdell, L. W., and Bennink, J. R. (2000). Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* **404,** 770–774.

Spearman, P., Horton, R., Ratner, L., and Kuli Zade, I. (1997). Membrane binding of human immunodeficiency virus type. *J. Virol.* **71,** 6582–6592.

Tritel, M., and Resh, M. D. (2000). Kinetic analysis of human immunodeficiency virus type 1 assembly reveals the presence of sequential intermediates. *J. Virol.* **74,** 5845–5855.

Turner, B. G., and Summers, M. F. (1999). Structural biology of HIV. *J. Mol. Biol.* **285,** 1–32.

Wang, J. J., Sandefur, S., Spearman, P., Chiou, C. T., Chiang, P. H., and Ratner, L. (2001). Tracking the assembly pathway of human immunodeficiency virus type 1 deletion mutants by immunogold labeling. *Appl. Immunohistochem. Mol. Morphol.* **9,** 371–379.

Weldon, R. A., Jr., and Hunter, E. (1997). Molecular requirements for retrovirus assembly. *In* "Structural Biology of Viruses" (W. Chiu, R. G. Burnett, and R. L. Garcea, Eds.), pp. 381–410. Oxford Univ. Press, New York.

Yeager, M., Wilson-Kubalek, E. M., Weiner, S. G., Brown, P. O., and Rein, A. (1998). Supramolecular organization of immature and mature murine leukemia virus revealed by electron cryo-microscopy: Implications for retroviral assembly mechanisms. *Proc. Natl. Acad. Sci. USA* **95,** 7299–7304.