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# Bovine Leukemia Virus Gag Particle Assembly in Insect Cells: Formation of Chimeric Particles by Domain-Switched Leukemia/Lentivirus Gag Polyprotein

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A key stage in the life cycle of C-type retroviruses is the assembly of Gag precursor protein at the plasma membrane of infected cells. Here we report the assembly of bovine leukemia virus (BLV) *gag* gene product into virus-like particles (VLPs) using the baculovirus expression system. Expression of BLV $Pr44^{Gag}$ resulted in the assembly and release of VLPs, thereby confirming the ability of retroviral Gag polyprotein to assemble and bud from insect cells. Efficient particle formation required a myristoylation signal at the N-terminus of BLV $Pr44^{Gag}$. Recombinant baculoviruses expressing matrix (MA) or capsid–nucleocapsid (CA–NC) proteins of BLV were generated but neither of these domains was capable of assembling into particulate structures. To assess the compatibility of Gag domains between leukemia and lentivirus groups three different recombinant chimeras each expressing MA of one virus (e.g., simian immunodeficiency or BLV) and CA–NC of another (e.g., BLV or human T-cell leukemia virus type-I) were constructed. Each of the chimeric proteins assembled efficiently and budded as VLPs, suggesting that the MA and CA domains of these two evolutionary divergent retrovirus groups can be functionally exchanged without perturbation of Gag VLP formation. The lenti-leukemia chimeric Gag approach has potential for studying protein–protein interactions in other retroviruses. 

## INTRODUCTION

Bovine leukemia virus (BLV) is a transactivating, oncogenic retrovirus that causes enzootic bovine leukosis worldwide in cattle. The disease is characterized by inapparent infection, persistent B-cell lymphocytosis, leukemia, and lymphosarcoma (Burny *et al.,* 1988), leading to a decrease in milk production. BLV is structurally and biologically similar to human T-cell leukemia viruses (HTLV-I and -II) and simian T-cell leukemia virus, and these viruses are, therefore, grouped together as the BLV–HTLV virus group within the *Retroviridae* family. BLV has become a model system for animal studies for HTLV-I and -II (Kettmann *et al.,* 1994; Sagata *et al.,* 1985). The BLV–HTLV group shares the properties of C-type assembly and the absence of an "intermediate" submembrane layer with lentiviruses such as human and simian immunodeficiency viruses (HIV and SIV, respectively) (Nermut and Hockley, 1996). All retroviruses including BLV and HTLV employ a single *gag* gene product, the Gag precursor polyprotein, as a principal building scaffold for virus assembly and budding (Wills and Craven, 1991). Retroviral Gag has all the necessary morphogenetic information to mediate intracellular transport, direct assembly, and catalyze the budding process (Freed, 1998).

The retrovirus particle assembly begins with association of Gag polyprotein with the host cell plasma membrane and immature particles bud from the plasma membrane. Viral environment (Env) protein also migrates to the same site and is incorporated into the budding particle. The events associated with the Gag-mediated budding appear to be similar among different retroviruses. Each step of virus assembly requires a great degree of precision and possibly involves highly specific macromolecular interactions including protein–protein and protein–RNA interactions (Freed, 1998; Gottlinger *et al.,* 1989; Hunter, 1994). Protein–protein interactions are required during the self-assembly process, which involves oligomerization of Gag polyprotein at the plasma membrane and envelopment during budding (Nermut *et al.,* 1998; Yu *et al.,* 1992).

BLV Gag is initially synthesized as a polyprotein precursor ($Pr44^{Gag}$) that is endoproteolytically cleaved into major structural subunits, matrix (MA, p15), capsid (CA, p24), and nucleocapsid (NC, p12), during or shortly after budding (Kettmann *et al.,* 1994). Of these proteins, MA plays a significant role in virus assembly and membrane targeting and has a multifunctional role in virus morphogenesis (Bryant and Ratner, 1990; Morikawa *et al.,* 1995).

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Both MA and CA contribute to the packing of Gag polyproteins during assembly (Dorfman *et al.,* 1994; Franke *et al.,* 1994; Hunter, 1994; Morikawa *et al.,* 1998; Zhang *et al.,* 1996). The linear order of MA, CA, and NC proteins within the Gag precursor is invariant and the assembly domains of different retroviruses may be functionally conserved.

Although different retroviral Gag proteins have similar functions, there is little sequence homology among the Gag proteins from evolutionarily divergent retroviruses. The three-dimensional structures of several retroviral MA proteins have been determined and provide an interesting insight into retrovirus assembly and MA organization (Christensen *et al.,* 1996; Conte *et al.,* 1997; Conte and Matthews, 1998; Hill *et al.,* 1996; Massiah *et al.,* 1996; Matthews *et al.,* 1996; Rao *et al.,* 1995). Recently, the crystal structure of SIV MA (Rao *et al.,* 1995) and NMR solution structure of BLV MA (Matthews *et al.,* 1996) have been resolved. BLV MA shows structural homology with the MAs of SIV and HIV-1. The X-ray crystallographic data revealed a trimeric nature of SIV and HIV-1 (Hill *et al.,* 1996; Rao *et al.,* 1995). Similar models have been predicted for BLV and subsequently for HTLV-II as well as for the D-type Mason–Pfizer monkey virus (M-PMV) MA proteins (Christensen *et al.,* 1996; Conte *et al.,* 1997; Matthews *et al.,* 1996). The high level of similarity among the MAs of these retorviruses suggests a common mode of transport and assembly of Gag polyprotein at the plasma membrane. The conservative nature of the MA structure and linear order of the Gag protein products raise the issue of whether MA and CA–NC proteins of different retroviruses are compatible.

Many heterologous expression systems have been used for analysis of the retrovirus particle assembly and release (Boulanger and Jones, 1996). Of all the systems, the baculovirus system appears to be the best for retroviral Gag expression and thus has been used extensively for studying the assembly and release of virus-like particles (VLPs) in bovine immunodeficiency virus (BIV), feline immunodeficiency virus (FIV), HIV-1, and SIV (Delchambre *et al.,* 1989; Cheysen *et al.,* 1989; Morikawa *et al.,* 1991; Rasmussen *et al.,* 1990). However, to date, no reports are available on the expression of either BLV Gag or VLPs made of heterologous components using a baculovirus expression system although recombinant vaccinia virus has been previously demonstrated to produce BLV Gag VLPs (Hertig *et al.,* 1994).

The present study was, therefore, undertaken to investigate the synthesis of BLV Gag particles in insect cells using a recombinant baculovirus system and to study the efficiency of BLV MA or CA–NC proteins to direct protein–protein interactions to assemble into particulate structures. The importance of the N-terminal myristoylation of BLV Gag was investigated by mutating the site of myristoylation (Gly2 → Ala) and expression of mutant Gag in insect cells. Subsequently the compatibility of MA and CA–NC domains of different viruses, BLV, HTLV-I, and SIV, was assessed by monitoring VLP formation by domain-switched chimeric Gag particles.

## RESULTS

### Assembly of BLV Gag and formation of virus-like particles in insect cells

The baculovirus expression system has been used extensively to analyze lentiviral Gag precursor assembly. However, it has yet to be demonstrated that the Gag polyprotein of a leukemia virus (BLV–HTLV) group has a similar inherent property of VLP assembly in insect cell cultures. To determine the protein–protein interactions required for BLV and HTLV Gag assembly, it was important to develop an assay system that will facilitate detection of particle formation. To establish such a system, a recombinant vector, pAcB*gag,* was therefore constructed (Fig. 1A) by cloning full-length BLV*gag* into pAcYM1 and recombinant baculovirus AcB*gag* obtained as described under Materials and Methods.

The expression of the BLV Gag polyprotein by recombinant baculovirus in infected insect cell lysate was monitored by Western blot analysis (Fig. 2A, lane 2). The expressed protein was approximately 44 kDa and it corresponded to the predicted size of the BLV precursor Gag ($Pr44^{Gag}$). The expressed protein reacted specifically with a polyvalent anti-BLV serum and had an electrophoretic mobility similar to that of the 44-kDa protein band of fetal lamb kidney cells infected with BLV (FLK/BLV cells) (Fig. 2A, lane 6). To determine whether this recombinant $Pr44^{Gag}$ could assemble and subsequently be released into the medium as VLPs, we used sucrose density gradient fractionation as a standard method of Gag VLP isolation. The gradient fractions were collected and examined for BLV $Pr44^{Gag}$ by Western blot (Fig. 3, lanes 2–11). Although some protein was pelleted as aggregate (Fig. 3, lane 11), the majority of the Gag protein segregated in fractions 5 to 8, corresponding to 35–45% sucrose (Fig. 3, lanes 6–9), equivalent to the positions of the HIV/SIV Gag particles (Gheysen *et al.,* 1989; Jowett *et al.,* 1992), indicating that the AcB*gag* expressed BLV $Pr44^{Gag}$ polyprotein assembled and was released as a particulate structure in the culture supernatant.

Electron microscopy was performed to monitor the morphology and the site of budding of VLPs. BLV Gag protein synthesized in insect cells assembled into VLPs and appeared to bud from the plasma membrane in a manner typical of C-type retroviruses (Fig. 4A). However, a number of VLPs formed tubular structures of different lengths and about 90 nm in diameter as opposed to 110 nm in diameter for the spherical forms (Fig. 4B). The immunogold labeling of the thin-sectioned infected insect cells confirmed these VLPs and the tubular structures to be of BLV origin (Figs. 4C and 4D).

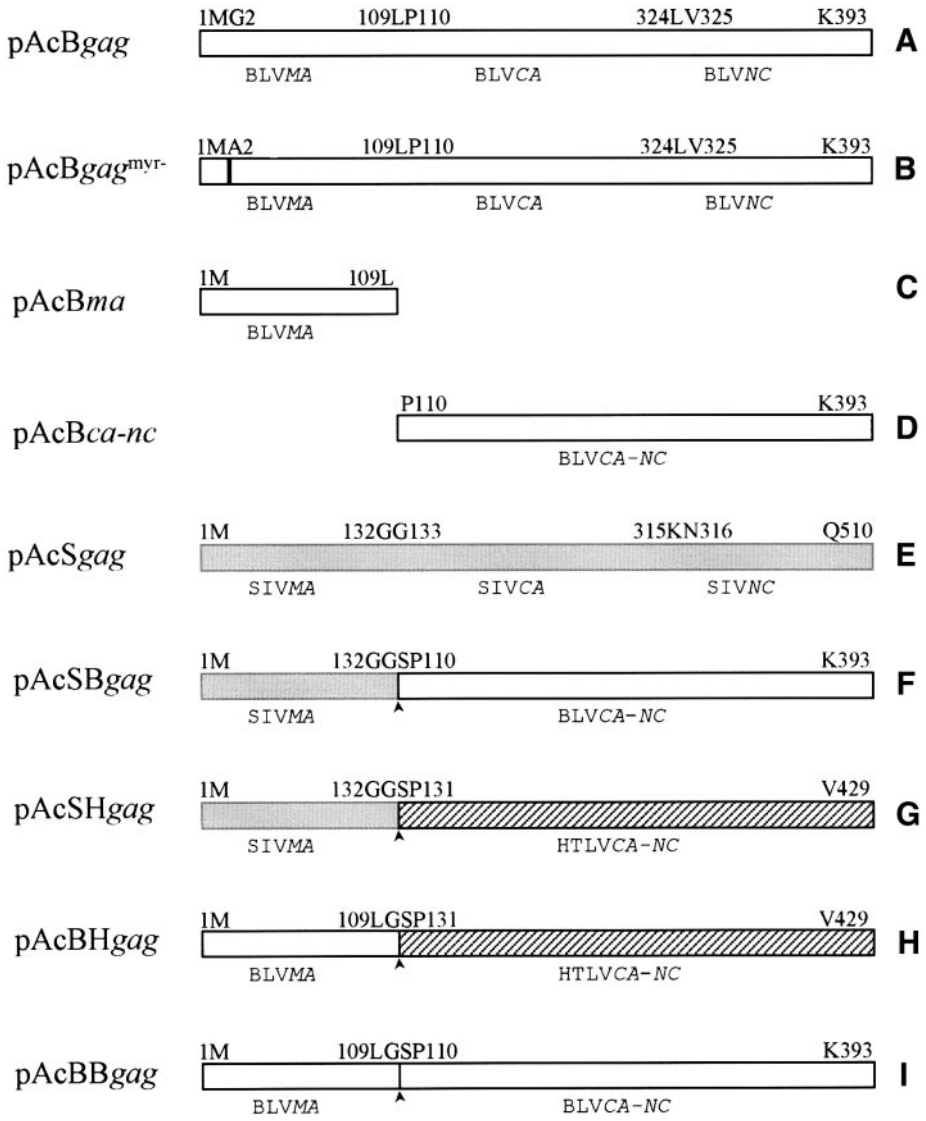


FIG. 1. Schematics of Gag polyprotein constructs used to generate recombinant transfer vectors. The recombinant transfer vector encoding the respective Gag protein is shown on the left. Source of Gag protein in each construct is indicated as (A) BLV (empty box), (E) SIV (shaded box), and (G and H) HTLV-I (hatched box). The major domains are numbered with their starting and ending residues in the respective Gag protein. Black bar (B) indicates the mutation of the second codon to code for Ala. Arrowheads (F, G, H, I) indicate the fusion protein junction.

## Myristoylation is essential for BLV Pr44$^{Gag}$ assembly and budding

The Gag protein of most retroviruses, including BLV, is myristoylated at the N-terminal glycine residue and plays an essential role in targeting the Gag precursor to the cell membrane and in the subsequent assembly and budding of extracellular particles (Towler *et al.,* 1988). To examine the role of myristoylation in the process of assembly and budding of BLV Gag, a mutant transfer vector, pAcB*gag*$^{myr-}$, was constructed altering GCA for GGA (Fig. 1B) by site-directed mutagenesis as described under Materials and Methods. This mutation exchanged Ala for the myristate acceptor Gly (Gly2 → Ala), thereby preventing myristoylation of the BLV Gag precursor polyprotein. The transfer vector pAcB*gag*$^{myr-}$ was used to cotransfect *Spodoptera frugiperda* (*Sf*) cells and recombinant baculovirus AcB*gag*$^{myr-}$ was obtained as described.

*Sf* cells infected with AcB*gag*$^{myr-}$ expressed a recombinant protein of 44 kDa that corresponded to wild-type BLV Pr44$^{Gag}$ and reacted specifically with polyvalent rabbit anti-BLV serum in Western blot (Fig. 2A, lane 4). The expression level of unmyristoylated Gag protein was similar to the wild-type BLV Gag. To investigate the particulate nature of the mutant BLV*gag* gene product, the culture supernatant was analyzed for the presence of 44-kDa antigen after concentration through a 20% sucrose cushion. No BLV protein could be detected in the culture supernatant (Fig. 2A, lane 5). To locate the BLV Gag$^{myr-}$ protein within AcG*gag*$^{myr-}$ infected cells, *Sf* cells

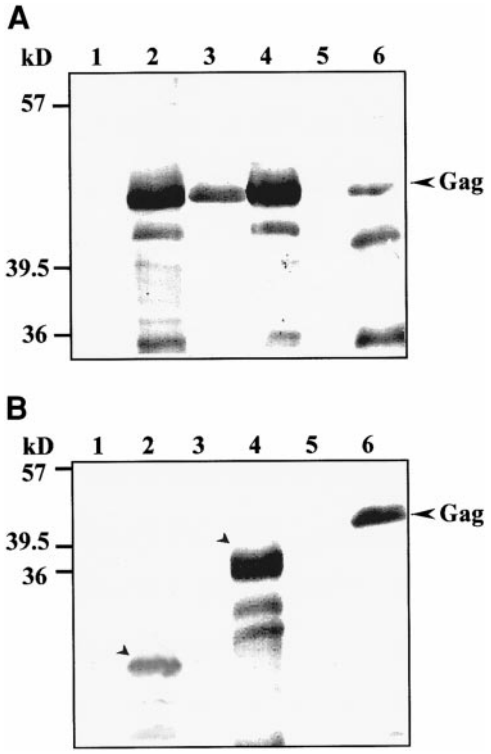


FIG. 2. Western analysis of BLV Gag expression and particle release in insect cells. Protein samples were resolved either on a (A) 10% or on a (B) 15% SDS–PAGE: (A) Mock-infected *Sf* cells (lane 1); BLV $Pr44^{Gag}$ in the cell lysate (lane 2, note some breakdown products that are common for retrovirus Gag expression in *Sf* insect cells) and supernatant (lane 3); Pr44 $Gag^{myr-}$ in the cell lysate (lanes 4) and supernatant (lane 5; note the absence of BLV $Gag^{myr-}$ in the supernatant); cell lysate from BLV infected FLK cells (lane 6). (B) Presence of BLV MA p15 (lane 2) and CA–NC p36 (lane 4) in the cell lysate indicated by arrowheads. Note that both proteins are absent in the supernatant (lanes 3 and 5). Lanes 1 and 6 are the mock-infected *Sf* cells and purified BLV $Pr44^{Gag}$, respectively. The position of molecular weight markers is indicated on the left.

were processed for thin-section electron microscopy. The unmyristoylated Gag protein was not found at the plasma membrane and no budding particles were visualized (results not shown). These results indicated that AcB*gag*$^{myr-}$ infected cells expressed a BLV protein similar in size to wild-type $Pr44^{Gag}$ that was unable to assemble as VLPs at the plasma membrane of insect cells.

## Neither BLV MA nor CA–NC alone is sufficient for assembly and release

The retroviral MA is important for the targeting and assembly of viral structural proteins at the plasma membrane of infected host cells. The SIV MA protein has been reported to be sufficient for the production of particulate structures when expressed by a vaccinia virus system (Gonzalez *et al.,* 1993). These observations have been recently confirmed (Giddings *et al.,* 1998). However, using the same expression system HIV-1 MA failed to assemble into a particulate structure in the absence of other viral molecules (Giddings *et al.,* 1998). No reports are available so far to demonstrate the capability of oncoviral (BLV/HTLV) MA alone to assemble into such structures. To assay whether individual domains of oncoviral Gag form particulate structures like SIV MA, we have dissected BLV Gag into two domains, MA and CA–NC. To investigate whether BLV MA or CA–NC in the absence of all other viral proteins forms particulate structures, we generated two recombinant baculoviruses, AcB*ma* and AcB*ca-nc,* that expressed only the MA (Fig. 1C) or CA–NC (Fig. 1D) protein of BLV.

*Sf* cells infected individually with AcB*ma* or AcB*ca–nc* expressed BLV MA protein of 15 kDa (p15, Fig. 2B, lane 2) or CA–NC protein of 36 kDa (p36, Fig. 2B, lane 4), respectively, that specifically reacted with polyclonal rabbit anti-BLV serum in Western blots. To monitor the release of particulate structures, the culture supernatant from infected *Sf* cells was concentrated through a 20% sucrose cushion and the pelleted material was analyzed for the presence of p15 or p36 antigen. By Western analysis using polyclonal rabbit anti-BLV serum we failed to detect any of the p15 (Fig. 2B, lane 3) or p36 (Fig. 2B, lane 5) antigen. These results indicated that BLV MA or CA–NC protein is not released into the culture medium as particulate structures.

Recombinant BLV MA or CA–NC protein was also examined within the infected *Sf* cells by thin-section electron microscopy. As expected, no VLP assembly or budding particles were detected at the plasma membrane of cells expressing BLV MA or CA–NC protein (results not shown). However, a few rings and small vesicles containing dense material were found in cytoplasmic vacuoles in *Sf* cells infected with AcB*ma.* Immunogold labeling of AcB*ca–nc* infected cells with a polyclonal rabbit anti-BLV antibody also demonstrated that the CA–NC protein was synthesized in sufficient amounts within the cytoplasm but failed to assemble and bud as particulate structures (Fig. 5A). In some cells long bundles of closely packed parallel structures (possibly cross sections of flat sheets) were observed, which also labeled with anti-BLV antibody (Figs. 5A and 5B), indicating that the recombinant products were concentrated as

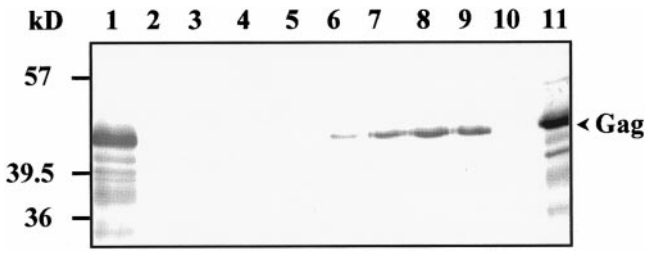


FIG. 3. Western analysis of sucrose density gradient fractions of BLV $Pr44^{Gag}$. The fractions were collected from top to bottom (fractions 1 to 10). *Sf* cell lysate expressing BLV $Pr44^{Gag}$, lane 1; top fraction, lane 2; and bottom fraction, lane 11. The position of molecular weight markers is indicated on the left.

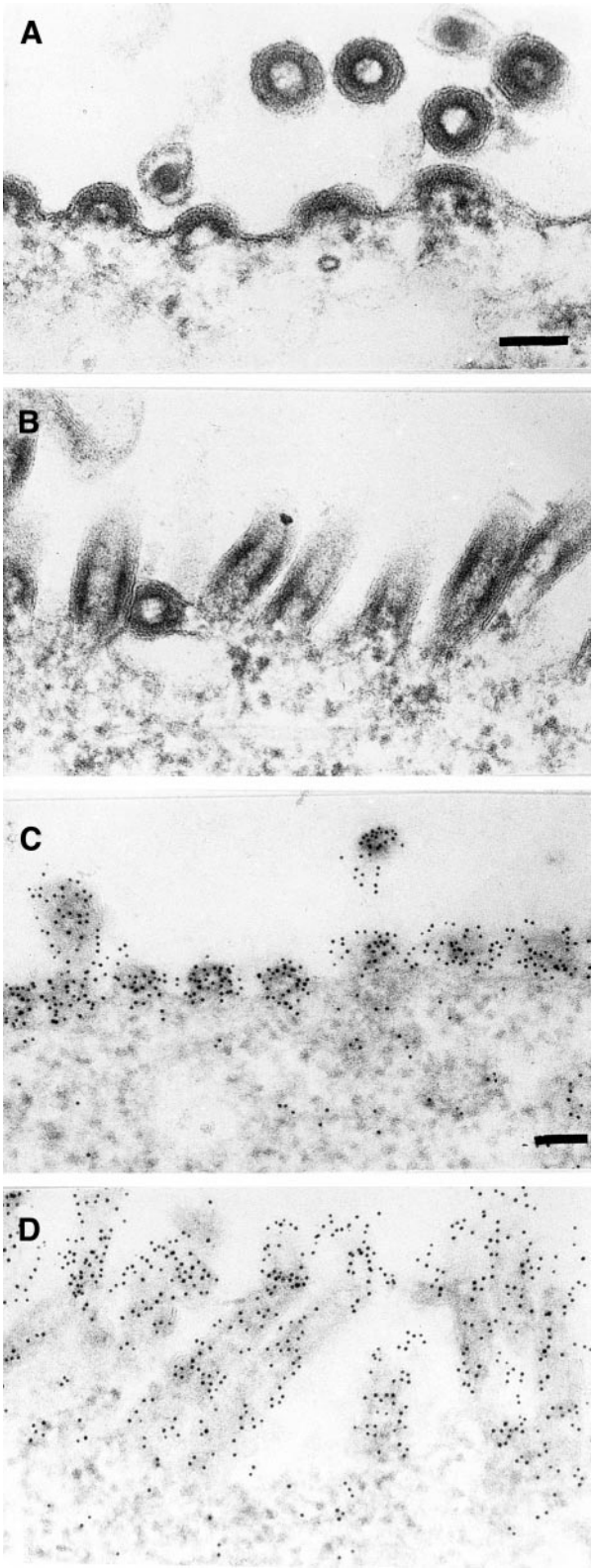


FIG. 4. Electron micrographs of infected *Sf* cells showing the expression, assembly, or budding of BLV Gag proteins. Thin section of budding and free VLPs of *Sf* cell expressing BLV $Pr44^{Gag}$ (A); same after immunogold labeling with polyclonal antibody to BLV (C); some cells also demonstrated assembly and budding of tubular structures at the plasma membrane (B); same after immunogold labeling (D). Bar represents 100 nm.

paracrystalline structures. These results indicated that although abundant protein was synthesized, BLV MA alone or MA-deficient BLV CA–NC was unable to assemble as particulate structures at the plasma membrane of infected insect cells.

### Chimeric Gag polyproteins consisting of SIV, BLV, and HTLV-I domains in insect cells assemble into VLPs

Recent X-ray crystallography and NMR structural analysis demonstrated that the MA proteins of SIV/HIV and BLV/HTLV share similar structures, although these proteins are quite distinct at the primary sequence level (Christensen *et al.,* 1996; Hill *et al.,* 1996; Matthews *et al.,* 1996; Rao *et al.,* 1995). To investigate whether the MA and CA–NC domains of two distinct members of the *Retroviridae* would complement and could be exchanged without perturbing Gag particle formation, three different chimeric constructs were designed. In two of these constructs the MA domain was derived from SIV, while in the third, MA was derived from BLV. However, for the first chimera, the CA–NC was derived from BLV, while the last two contained HTLV-I CA–NC. The cloning of two fragments (*MA* and *CA–NC*) in one vector would lead to insertion of six additional nucleotides (GGATCT) at the junction of *MA* + *CA–NC.* To ensure that the coding region of chimeric *gag* is not influenced by the cloning procedure, as a control, using the same two BLV*gag* gene fragments (*MA* and *CA–NC*), were constructed another vector, pAcBB*gag* (Fig. 1I), and obtained recombinant baculovirus, AcBB*gag.* Western analysis of infected cell lysate (Fig. 6A, lane 2) and sucrose density gradient purified Gag VLPs from culture supernatant (Fig. 6A, lane 3) indicated that the two additional amino acids at the junction of MA + CA–NC did not affect the ability of the Gag protein to form VLPs.

Further, as a positive control, the full-length SIV*gag* gene was also cloned in pAcYM1 constructing pAcS*gag* (Fig. 1E) and recombinant virus AcS*gag* was obtained. When a AcS*gag* infected *Sf* cell lysate was analyzed, as expected, a 57-kDa ($Pr57^{Gag}$) protein was clearly identified, which specifically reacted with polyclonal monkey anti-SIV serum in Western blot (Fig. 6B, lane 4). Similarly, analysis of sucrose density gradient purified SIV Gag VLPs demonstrated the presence of SIV $Pr57^{Gag}$ (Fig. 6B, lane 5). Thin sections of infected cells by electron microscopy confirmed the assembly and budding of VLPs at the plasma membrane similar to published reports (Delchambre *et al.,* 1989; Gonzalez *et al.,* 1993). Subsequently, recombinant viruses AcSB*gag,* AcSH*gag,* and AcBH*gag* expressing SIV MA + BLV CA–NC, SIV MA + HTLV-I CA–NC, and BLV MA + HTLV-I CA–NC, respectively, were derived and Gag polyprotein formation was analyzed as described.

*Sf* cells infected with recombinant chimeric virus AcSB*gag* expressed a 46-kDa protein that bound specifically

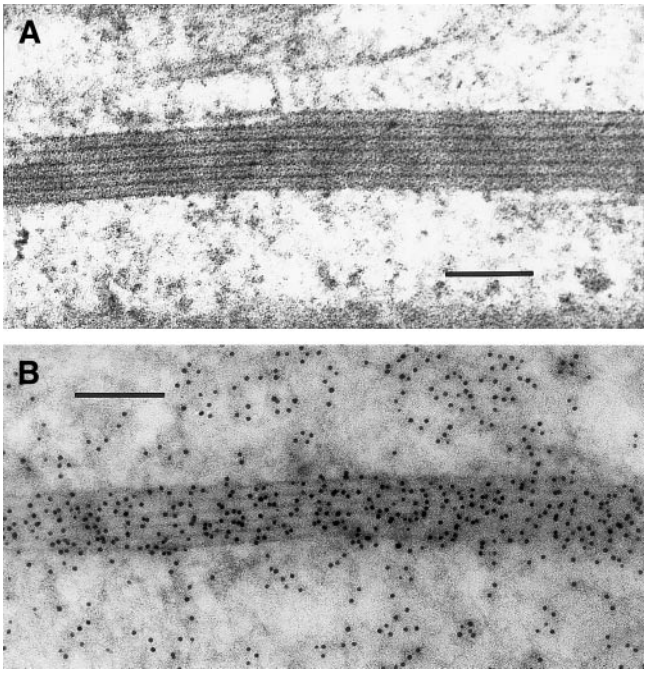


FIG. 5. (A) Closely packed parallel sheets in cells expressing CA–NC; (B) same after immunogold labeling with a polyclonal antibody to BLV. Bar represents 100 nm.

both to polyclonal rabbit anti-BLV serum (Fig. 6A, lane 6) and to monkey anti-SIV serum (Fig. 6B, lane 6) in Western blots, confirming its chimeric nature. The expressed protein had a faster electrophoretic mobility than SIV Gag (Fig. 6B, lane 4) but a slower electrophoretic mobility than BLV Gag (Fig. 6A, lane 2) did. Likewise, cells infected with AcSH*gag* expressed a 52-kDa protein that had the slowest mobility of all the chimeric polyproteins (Fig. 6B, lane 8). The AcBH*gag* infected *Sf* cells expressed a protein of 44 kDa that specifically reacted with polyclonal rabbit anti-BLV serum (Fig. 6A, lane 10) and had an electrophoretic mobility similar to that of BLV Gag (Fig. 6A, lane 2).

The clarified culture supernatant from each of the recombinant chimeric virus infected *Sf* cells was analyzed for the presence of the respective Gag antigen in sucrose density gradient fractions as described earlier. In each case the secreted Gag protein was detected in fractions 5 to 8 corresponding to 35–45% sucrose and some protein was detectable in the pellet as aggregates similar to BLV $Pr44^{Gag}$ (data not shown). Each of the chimeric proteins reacted specifically with their respective polyclonal antisera in Western analysis and exhibited a gel mobility corresponding to their predicted molecular weights (Figs. 6A and 6B, lanes 7, 9, and 11).

Further, thin sections of infected insect cells in each case also showed typical assembly and budding of VLPs at the plasma membrane. In the case of AcSB*gag* many VLPs were spherical in morphology and occasionally budded into cytoplasmic vacuoles (Fig. 7A). Interestingly, AcSH*gag* infected cells expressed VLPs that were apparently larger and some distinctly larger (140–170 nm) than average particles (Fig. 7B). The AcBH*gag* infected cells demonstrated budding of mainly spherical VLPs (Fig. 7C). In addition, some tubular structures were observed in each case. From these results it is clear that all three chimeric proteins assembled at the plasma membrane of infected cells and budded as VLPs similar to BLV $Pr44^{Gag}$.

## DISCUSSION

Expression of BLV $Pr44^{Gag}$ by recombinant baculovirus AcB*gag* resulted in the assembly, budding, and release of VLPs. This is consistent with similar studies on other lentiviruses and leukemia viruses (Delchambre *et al.,*

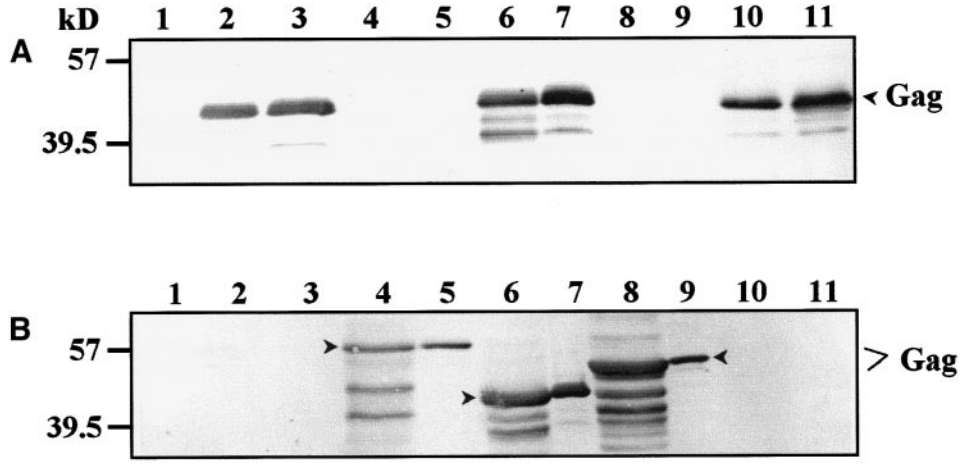


FIG. 6. Western analysis of Gag synthesis by homologous and chimeric constructs in cell lysates and supernatants. Mock-infected *Sf* cells (lane 1), religated BLV $Pr44^{Gag}$ (lanes 2 and 3), SIV $Pr57^{Gag}$ (lanes 4 and 5), SIV/BLV $Pr46^{Gag}$ (lanes 6 and 7), SIV/HTLV $Pr52^{Gag}$ (lanes 8 and 9), and BLV/HTLV $Pr44^{Gag}$ (lanes 10 and 11), respectively. The position of each Gag polyprotein is indicated by arrowheads. Immunoblots were developed with either the polyclonal rabbit anti-BLV (A) or the polyclonal monkey anti-SIV antibody (B). The position of molecular weight markers is indicated on the left.

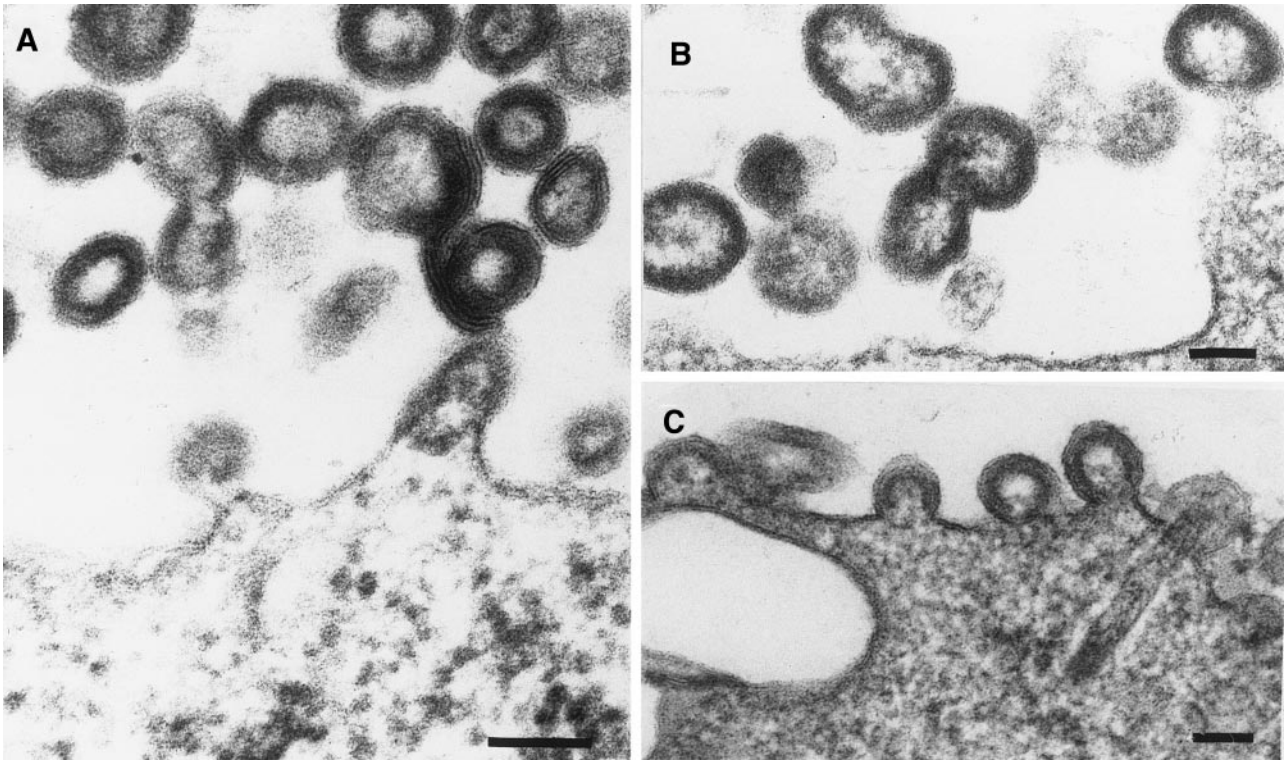


FIG. 7. Electron micrographs of thin sections of *Sf* cells showing the assembly and budding of Gag chimeras at the plasma membrane of cells infected with recombinant baculoviruses AcSB*gag* (A), AcSH*gag* (B), and AcBH*gag* (C). Bar represents 100 nm.

1989; Gheysen *et al.,* 1989; Hertig *et al.,* 1994; Morikawa *et al.,* 1991; Rasmussen *et al.,* 1990) using a variety of expression systems. Furthermore, these experiments demonstrated that retroviral Gag assembly could occur in the absence of viral enzymatic proteins, envelope glycoproteins, and genomic RNA.

Gag proteins of most mammalian retroviruses (e.g. HIV, Moloney murine leukemia virus (MuLV), M-PMV, and SIV) require N-terminal myristic acid to become associated with the plasma membrane prior to budding from the cell (Bryant and Ratner, 1990; Delchambre *et al.,* 1989; Gheysen *et al.,* 1989; Gottlinger *et al.,* 1989; Rein *et al.,* 1986; Rhee and Hunter, 1987). We have utilized the baculovirus system to analyze the role of myristoylation in assembly of BLV $Pr44^{Gag}$ by expressing the unmyristoylated Gag. This mutation abolished VLP assembly and budding from the plasma membrane but did not interfere with the polyprotein synthesis as an unmyristoylated Gag protein of 44 kDa could be detected in cell lysates of AcB$gag^{myr-}$ infected *Sf* cells. Similar results have been reported for HIV-1 and other retroviruses (Mergener *et al.,* 1992). Mutation of N-terminal Gly codons of MuLV*gag* and SIV*gag* gene products led to the accumulation of Gag proteins within the cytoplasm without release of particles in the supernatant of the infected cell culture (Delchambre *et al.,* 1989; Rein *et al.,* 1986). Thus myristoylation of BLV Gag polyprotein seems to be essential for the association of the precursor protein with the cell membrane, which in turn is critical for budding and release of Gag particles.

SIV MA alone has been reported to assemble into particulate structures (Giddings *et al.,* 1998; Gonzalez *et al.,* 1993). To study whether the BLV MA alone or CA–NC proteins have similar assembly characteristics, we expressed each of these proteins in insect cells and found that neither of these synthesized proteins was capable of assembling into a particulate structure. These results are consistent with the reported findings on HIV-1 MA (Giddings *et al.,* 1998). Although BLV MA and CA–NC proteins were expressed efficiently in infected *Sf* cells, no particle-like structure was visualized at the plasma membrane. Thus, BLV MA alone is insufficient to assemble into particulate structures. The failure of the BLV CA–NC domain to assemble and bud as a particulate structure suggests that the presence of the MA domain is essential for the *in vivo* assembly of BLV Gag at the plasma membrane. This result points to the leading role of retroviral MA in the plasma membrane assembly of the Gag precursor and formation of the spherical shell during budding. Retroviral MA has been shown to play a decisive role in transport, plasma membrane association, and formation of the spherical shell (Morikawa *et al.,* 1995; Nermut *et al.,* 1994).

To locate the functional equivalents of retroviral MA and CA–NC in evolutionary divergent retroviruses, we

made Gag chimeras. Both lenti-leukemia (SIV MA + BLV CA–NC and SIV MA + HTLV-1 CA–NC) and leukemia–leukemia (BLV MA + HTLV CA–NC) chimeras were capable of assembling VLPs at the plasma membrane of the infected cells. The particles produced by these chimeras were similar in morphology to wild-type Gag particles. These data are consistent with the earlier reports that Rous sarcoma virus MA + HIV-1 CA–NC chimeras were functional for assembly and budding and that their late assembly domains that function in a positional-independent manner can be exchanged (Bennett *et al.,* 1993; Parent *et al.,* 1995). However, retrovirus-like particles were not detected at the plasma membrane in human spuma retrovirus–HIV chimeras but could be detected within the cell in the cytoplasm only (Carriere *et al.,* 1995). The chimeric MuLV virions containing either the HIV-1 MA or the CA domain were inefficiently assembled into mature virus particles because of improper intracellular targeting and were partially defective in the late stage of replication (Deschamps *et al.,* 1981).

The successful generation of lenti-leukemia and leukemia–leukemia functional Gag chimeras suggests that these two evolutionary divergent retrovirus groups have a similar arrangement of assembly domains that serve identical functions and are interchangeable. Thus the replacement of leukemia viral MA with lentiviral MA as in SIV MA + BLV CA–NC and SIV MA + HTLV-I CA–NC or replacement of its CA–NC with other leukemia viral CA–NC as in BLV MA + HTLV-I CA–NC had no detrimental effects on the assembly capacity of the resulting Gag chimera. Further, the MA domain of one retrovirus could modulate the assembly of another as observed in all three chimeras even though their sequences within the Gag precursor are not conserved. These findings strongly suggest that the surface properties of the relevant domains are the dominant features in protein–protein interactions.

In conclusion the results in the present study indicate that BLV $Pr44^{Gag}$ assembles into VLPs just like other retroviral Gag. However, when we expressed the unmyristoylated form of BLV Gag, VLP formation was abrogated. BLV MA or CA–NC protein alone was unable to assemble as a particulate structure. All three lenti-leukemia or leukemia–leukemia chimeric Gag proteins efficiently assembled into VLPs, confirming that homologous MA–CA interaction is not essential for particle formation. These observations raise an important issue about how the different parts of Gag interact prior to or subsequent to proteolysis and particle maturation. Further these chimeras have the potential to be used to study *in vitro* protein–protein interactions and design inhibitors of retrovirus assembly.

## MATERIALS AND METHODS

### Cells and viruses

*Spodoptera frugiperda* (*Sf*9) cells were propagated in suspension or monolayer cultures at 28°C in TC-100 medium (Gibco BRL, UK) supplemented with 5% fetal bovine serum. The wild-type baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) and the recombinant BAcPAK-6 virus containing the *lacZ* (*β-galactocidase*) gene under the control of the AcNPV polyhedrin promoter were used to generate recombinant viruses (Kitts and Possee, 1993). Fetal lamb kidney cells infected with BLV were kindly supplied by L. Willems, Department of Biochemistry and Applied Biology, University of Brussels, Gembloux, Belgium.

### DNA manipulations

Plasmid DNA manipulations were undertaken essentially as described (Sambrook *et al.,* 1989). Polymerase chain reaction (PCR) was used for amplification of DNA fragments. PCR products after purification and restriction enzyme digestion were ligated into pAcYM1 (Matsuura *et al.,* 1987) and transformed into *Escherichia coli* XL-1 Blue competent cells. Plasmid minipreps were prepared from overnight grown cultures of a single bacterial transformed clone and digested with appropriate restriction enzymes to check the desired size and correct orientation of the insert. DNA fragments and plasmids after digestion were separated on agarose gel and purified using a Qiaex II gel extraction kit (Qiagen Inc.). All the DNA mutations and junction sites were confirmed by dideoxy nucleotide sequencing (Sanger *et al.,* 1977).

### Constructions of recombinant and chimeric transfer vectors

All recombinant and chimeric transfer vectors were constructed by PCR amplification of DNA fragments and subsequent cloning in baculovirus vector pAcYM1 downstream of the polyhedrin promoter. Oligonucleotide primers used for cloning DNA fragments were designed to include a *Bam*HI (GGATCC) or *Bgl*II (AGATCT) restriction site at their 5′ termini (Table 1). BLV, SIV, and HTLV-I full-length *gag* or their DNA fragments were amplified from the original clones, pBLVT-15 (Deschamps *et al.,* 1981), p239SpSp5′ (SIVmac239 isolate), and pUCES6CH (proviral HTLV-1 cDNA), respectively, to generate the recombinant transfer vectors described below.

*pAcBgag, pAcBgag$^{myr-}$, pAcBma, pAcBca–nc, and pAcSgag.* Recombinant transfer vector pAcB*gag* (Fig. 1A) containing the full-length (1179 bp) PCR amplified BLV-*gag* gene was constructed using forward and reverse primers BLVF1 and BLVR394, respectively (Table 1). pAcB$gag^{myr-}$ (Fig. 1B) was constructed similarly by cloning PCR amplified DNA such that the second DNA codon of BLV*gag* at the 5′ terminus was mutated (GGA → GCA)

TABLE 1

Oligonucleotide Primers Used in PCR

| Primer | Sequence[a] |
|---|---|
| BLVF1 | GCGC**AGATCT**AAAATGGGAAATTCCCCCTCCTATAACCCC |
| BLVF1$^{myr-}$ | GCGC**AGATCT**AAAATGGCAAATTCCCCCTCCTATA |
| BLVF110 | GCGA**AGATCT**CCAATTATATCTGAAGGAAAT |
| BLVF110S | GCGC**AGATCT**AAAATGCCAATTATATCTGAAGGA |
| BLVR109 | GCAC**GGATCC**CAAAATGGCGGGGGGGTCA |
| BLVR109*X* | GCAC**GGATCC**TTACAAAATGGCGGGGGGGTCA |
| BLVR394 | GCGC**GGATCC**TTAGTTTTTTGATTTGAGGGTTGG |
| HTLVF131 | GCGC**AGATCT**CCAGTCATGCATCCACAT |
| HTLVR429 | GCGCTTA**GGATCC**AACCTCCCCCCCTATGAA |
| SIVF1 | GAGC**AGATCT**AAAATGGGCGTGAGAAACTCC |
| SIVR132 | GAAT**GGATCC**TCCTCTGCCGCTAGATGG |
| SIVR511 | GCGC**AGATCT**CTACTGGTCTCCTCCAAA |

[a] Restriction enzyme sites are shown in boldface type and mutated sequences (substitution or addition) are underlined.

to code for Ala. Oligonucleotides BLVF1$^{myr-}$ (forward) and BLVR394 (reverse) were used for generating a 1179-bp BLV*gag*$^{myr-}$ DNA. pAcB*ma* (Fig. 1C) and pAcB*ca-nc* (Fig. 1D) were constructed by cloning 327- and 852-bp DNA fragments corresponding to MA and CA–NC region genes, respectively, of BLV*gag.* The forward and reverse primers used for pAcB*ma* were BLVF1 and BLVR109*X,* respectively, with a stop codon (ochre) in the reverse primer. For pAcB*ca-nc* the forward primer BLVF110S was designed to introduce a start codon at the 5′ terminus and BLVR394 was used as reverse primer. Recombinant transfer vector pAcS*gag* (Fig. 1E) containing the full-length (1530 bp) PCR amplified SIV*gag* gene was constructed similarly by using forward and reverse oligonucleotide primers SIVF1 and SIVR511, respectively (Table 1). The PCR generated DNA fragments after restriction enzyme digestion and purification in each case were cloned at a unique *Bam*HI restriction site of baculovirus vector pAcYM1.

*pAcSBgag, pAcSHgag, and pAcBHgag.* Chimeric transfer vectors were constructed in two steps. In the first step the *MA* fragment from SIV or BLV*gag* (containing a 396-bp fragment of SIV*MA* or a 327-bp fragment of BLV*MA*) was cloned individually into pAcYM1 to generate an intermediate transfer vector, pAcS*MA* or pAcB*MA,* respectively. The forward and reverse oligonucleotide primers used to generate PCR fragments were SIVF1 and SIVR132 or BLVF1 and BLVR109, respectively (Table 1). In the second step the *CA–NC* fragment from BLV or HTLV-I*gag* was cloned in each of the two intermediate vectors to generate three recombinant chimeric *gag* genes in pAcYM1. DNA fragments BLV *CA–NC* (852 bp) or HTLV-I *CA–NC* (897 bp) were synthesized using forward and reverse primers BLVF110 and BLVR394 or HTLVF131 and HTLVR429, respectively (Table 1). These *CA–NC* fragments were cloned into the *Bam*HI restriction site of pAcS*MA* and pAcB*MA* to construct (a) pAcSB*gag* (Fig. 1F), incorporating *MA* from SIV and *CA–NC* from BLV; (b) pAcSH*gag* (Fig. 1G), incorporating *MA* from SIV and *CA–NC* from HTLV-1; and (c) pAcBH*gag* (Fig. 1H), incorporating *MA* from BLV and *CA–NC* from HTLV-I.

*pAcBBgag.* A recombinant transfer vector consisting of the *MA* fragment and *CA–NC* fragment of BLV was similarly constructed by cloning the *CA–NC* fragment of BLV into the *Bam*HI restriction site of pAcB*MA* (Fig. 1I).

## Cotransfection and generation of recombinant viruses

The recombinant baculovirus transfer vectors were used for *in vivo* recombination together with *Bsu*36I-linearized BAcPAK-6 DNA. Cotransfection was performed by the lipofectin (Gibco BRL) method following the basic protocol (King and Possee, 1992). The recombinant baculovirus harvested after cotransfection was plaque purified and propagated in *Sf* cells (King and Possee, 1992). Viruses were used at a multiplicity of infection of 0.1–0.2 for virus stocks preparation and of 5–10 to detect expression of recombinant proteins.

## SDS–PAGE and Western blot analysis

*Sf* cells were harvested at 48 h postinfection (p.i.), washed with phosphate-buffered saline, and treated with lysis buffer (2.3% SDS, 10 mM Tris–HCl, pH 6.8, 5% 2-mercaptoethanol, 10% glycerol) as described elsewhere (Hughes *et al.,* 1993). The protein samples were electrophoresed on 10 or 15% PAGE containing 0.1% SDS. For Western blot analysis, proteins resolved by SDS–PAGE were electrophoretically transferred to an Immobilon-P membrane (Millipore International) by standard blotting procedure (Towbin *et al.,* 1979). The membrane after electroblotting was probed with appropriately diluted specific antiserum (primary antibody) in blocking buffer for 1 h. After three washes, the membrane was incubated with the secondary antibody conjugated with alkaline phosphatase and the bound antibody was detected by NBT-BCIP (Gibco BRL).

## Sucrose density gradient centrifugation

The released particles were isolated from infected cell supernatant by sucrose density gradient centrifugation using the method described previously (Hughes *et al.,* 1993). In brief, supernatant from an infected cell culture was harvested at 60 h p.i., and cells and cellular debris were removed by low-speed centrifugation. The clarified supernatant was then concentrated through 20% sucrose in 10 mM Tris–HCl and 1 mM EDTA, pH 8.0 (TE), by centrifugation at 20,000 rpm for 2 h. The pellet from high-speed centrifugation was resuspended in TE and centrifuged on a 20 to 60% continuous sucrose gradient at 26,000 rpm for 1 h. One-milliliter fractions were collected for analysis.

### Electron microscopy

Infected *Sf* cells were harvested after 48 h p.i. and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for conventional embedding in Araldite as described previously (Hockley *et al.,* 1988). For gold immunolabeling, cells were fixed in 3% paraformaldehyde and processed with the progressive lowering of temperature procedure followed by embedding in Lowicryl HM20 using a CS-Auto unit at −45°C (Reichert-Jung, Vienna). Lowicryl sections were gold-immunolabeled with polyclonal antibody against BLV Gag protein and poststained with alcoholic uranyl acetate for 5–8 min (Nermut and Nicol, 1989). Preimmune rabbit serum was used as a control.

### Antisera and enzyme conjugates

Rabbit anti-BLV serum was kindly supplied by D. Portetelle, Department of Microbiology, University of Brussels, Gembloux, Belgium. Monkey anti-SIV serum (ADP 416, antiserum to SIV*mac* 251) from E. J. Stott was obtained from the NIBSC Centralized Facility for AIDS Reagents supported by EU Programme EVA (Contract BMH4 97/2515) and the UK Medical Research Council. Goat anti-rabbit IgG (A-3687) and affinity-isolated rabbit anti-monkey IgG (A-1929) alkaline phosphatase conjugates (Sigma Inc., St. Louis, MO) were obtained commercially and used as second antibodies.

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