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A block in virus-like particle maturation following assembly of murine leukaemia virus in insect cells

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Abstract

Expression of the murine leukaemia virus (MLV) major Gag antigen $p65^{Gag}$ using the baculovirus expression system leads to efficient assembly and release of virus-like particles (VLP) representative of immature MLV. Expression of $p180^{Gag\text{-}Pol}$, facilitated normally in mammalian cells by readthrough of the $p65^{Gag}$ termination codon, also occurs efficiently in insect cells to provide a source of the MLV protease and a pattern of $p65^{Gag}$ processing similar to that observed in mammalian cells. VLP release from $p180^{Gag\text{-}Pol}$-expressing cells however remains essentially immature with disproportionate levels of the uncleaved $p65^{Gag}$ precursor when compared to the intracellular Gag profile. Changing the $p65^{Gag}$ termination codon altered the level of $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ within expressing cells but did not alter the pattern of released VLP, which remained immature. Coexpression of $p65^{Gag}$ with a fixed readthrough $p180^{Gag\text{-}Pol}$ also led to only immature VLP release despite high intracellular protease levels. Our data suggest a mechanism that preferentially selects uncleaved $p65^{Gag}$ for the assembly of MLV in this heterologous expression system and implies that, in addition to their relative levels, active sorting of the correct $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ ratios may occur in producer cells.

Introduction

Murine leukaemia virus (MLV) is one of the most widely used vectors for gene delivery. Its productivity is limited however with routine titres of 10^{6-7} PFU/ml compared to 10^{12-13} PFU/ml for adenovirus-based systems (reviewed by Miller, 1997). Higher titres of MLV have been obtained when expression of the structural genes *gag-pol* and *env* were placed under the control of the CMV promoter (Soneoka et al., 1995), suggesting that high-level heterologous expression of the individual components of the virus might provide the basis for a system of high-titre retrovirus vector production. Expression of the major retrovirus capsid gene, *gag*, in a number of protein expression systems such as bacteria (Joshi and Vogt, 2000; Luschnig et al., 1995; Morikawa et al., 2001; Rumlova-Klikova et al., 2000), yeast (Sakuragi et al., 2002), insect cells (Gheysen et al., 1989; Johnson et al., 2001; Jones and Morikawa, 1998; Kakker et al., 1999; Luo et al., 1990; Morikawa et al., 1991; Nermut et al., 1994; Sommerfelt et al., 1993; Takahashi et al., 1999; Yamshcikov et al., 1995), and mammalian cells (Haynes et al., 1991; Jenkins et al., 1991; Karacostas et al., 1989; Krausslich et al., 1993; Shioda and Shibuta, 1990) has led to the synthesis of virus-like particles (VLPs) that mimic the immature form of the authentic virus. Particularly widespread use has been made of the expression of Gag in insect cells using recombinant baculoviruses, with VLP assembly being reported for HIV-1 and -2 (Gheysen et al., 1989; Luo et al., 1990), SIV (Yamshcikov et al., 1995), BIV (Rasmussen et al., 1990), FIV (Morikawa et al., 1991), BLV (Kakker et al., 1999), HTLV-II (Takahashi et al., 1999), RSV (Johnson et al., 2001), and M-PMV (Sommerfelt et al., 1993). In contrast coexpression of Gag with retroviral protease, either

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as part of the complete *gag-pol* gene or as Gag-Pro, fails to generate VLPs (Gheysen et al., 1989; Hughes et al., 1993; Johnson et al., 2001; Morikawa et al., 1991; Wagner et al., 1992), a finding suggested to be the result of premature activation of the retroviral protease in insect cells and consequential degradation of the Gag precursor prior to VLP assembly (Gheysen et al., 1989; Hughes et al., 1993; Morikawa et al., 1991; Wagner et al., 1992). Alteration of protease activity by mutagenesis or through the use of antiretroviral drugs alters the relative ratio of Gag and Gag-Pol in producer cells but fails to rescue VLP assembly unless the protease is essentially inactivated (Adamson et al., 2003), arguing that the coincorporation of Gag and Gag-Pol may be more active than previously thought. For all the retroviruses for which insect cell expression of gag-pol has been investigated, the viral protease is produced as part of the Gag-Pol fusion protein by frameshifting at the end of the gag gene. In MLV, however, $p180^{Gag\text{-}Pol}$ expression is achieved through an in-frame readthrough suppression of the $p65^{Gag}$ UAG termination codon by host cell $tRNA^{Gln}$ (Yoshinaka et al., 1985). Thus, in contrast to the situation where relative Gag and Gag-Pol levels are set by the rate of frameshifting, a relative level suitable for the assembly of MLV VLP with a mature phenotype could follow expression of the MLV structural genes in insect cells. Such a system could provide a method for the large-scale production of mature particles for structural study as well as higher titres of gene delivery vectors. Here we describe the expression of MLV $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ in insect cells using recombinant baculoviruses. We show that, in common with many retroviral precedents, MLV $p65^{Gag}$ assembles into immature VLPs but that, unlike in previous studies, incorporation of the Pol domain does not result in the cessation of VLP assembly but in the retention of synthesis of VLPs with an immature morphology. Purposeful alteration of the $p65^{Gag}$:$p180^{Gag\text{-}Pol}$ ratio through mutation of the readthrough site failed to recover mature particle assembly, whereas permanent readthrough resulted in complete loss of VLP assembly. Our data suggest that, irrespective of the molecular mechanism by which the ratio is achieved, additional factors may modulate the correct ratios of $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ required for mature particle formation.

Results

MLV $p65^{Gag}$ assembles into immature virus-like particles in insect cells

The normal pathway for the assembly of mature infectious MLV involves proteolytic maturation of the $p65^{Gag}$ precursor by the MLV encoded protease (Crawford and Goff, 1984; Katoh et al., 1985; Yoshinaka et al., 1985). The protease cleaves $p65^{Gag}$ into four major proteins; the matrix protein ($p15^{Gag}$), the late domain ($p12^{Gag}$), the capsid protein ($p30^{Gag}$), and the nucleocapsid protein ($p10^{Gag}$). Maturation results in a structural rearrangement producing virions with a characteristic mature morphology (Yeager et al., 1998; Yoshinka and Luftig, 1977). To assess if insect cell expression of MLV $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ could mimic this, the *gag* gene was excised from plasmid p6565 (Yap, 2000) and cloned into the baculovirus transfer vector pBAC4x-1 (Cambridge Bioscience), placing *gag* under the control of the baculovirus p10 promoter. A $p180^{Gag\text{-}Pol}$ transfer vector was similarly constructed except that the *gag-pol* gene was amplified by PCR from plasmid p*gag-pol*gpt (Markowitz et al., 1988) and placed under control of the pBac4x-1 polyhedrin promoter. Recombinant baculoviruses $AcMLV_{Gag}$ and $AcMLV_{Gag\text{-}Pol}$ were constructed by standard procedures using BacPAK6 viral DNA (Clontech). Following growth of virus stocks and high multiplicity infection (m.o.i. = 5), expression of $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ products were assessed at 2 days postinfection (pi) using 12% SDS–PAGE and Western blotting using anti-$p30^{Gag}$ antibody (Quality Biotech Inc.). Cells infected with $AcMLV_{Gag}$ showed abundant synthesis of uncleaved MLV $p65^{Gag}$ when blotted with anti-$p30^{Gag}$ that comigrated with $p65^{Gag}$ expressed in mammalian 293T cells (Fig. 1A, tracks 2 and 4). Some general proteolytic degradation of $p65^{Gag}$ was apparent but no distinct $p30^{Gag}$ band was present in

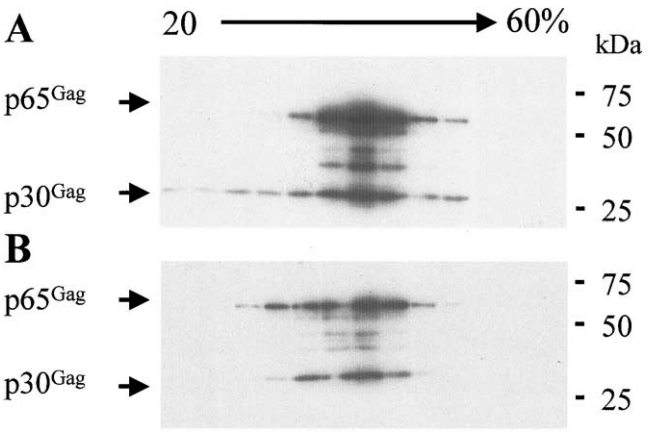

Fig. 1. Western blot analysis of MLV $p65^{Gag}$ proteins expressed in Sf9 and 293T cells. Cell lysates and supernatants were harvested and adjusted to an equivalent total protein concentration. Protein loading of cellular extracts was 20 μg/lane for mammalian cell samples and 2 μg/lane for insect cell samples. Supernatants were prepared by low-speed clarification followed by pelleting (14K rpm, 30′, 4°C) to eliminate secreted Gag monomers. Western blotting was done with a goat anti-$p30^{Gag}$ serum to detect MLV proteins and a monoclonal antibody to gp67, the major baculovirus glycoprotein, as marker for baculovirus infection. Primary antibodies were incubated with appropriate conjugates and the blots were finally developed using BM chemiluminescence (Roche). The samples analysed were as follows: 1, nontransfected 293T cells (control); 2, 293T cells transfected with pHIT60 (Soneoka et al., 1995) expressing $p180^{Gag\text{-}Pol}$; 3, Sf9 cells infected with BacPAK6 (control); 4, $AcMLV_{Gag}$; 5, $AcMLV_{Gag\text{-}Pol}$. Infected cell lysates (A and C) or supernatants (B) were collected 48 h postinfection and resolved by 12% SDS–PAGE before Western blotting using anti-$p30^{Gag}$ antibody (A and B) or anti-gp67 (C). The position of uncleaved $p65^{Gag}$, cleaved $p30^{Gag}$, and baculovirus gp67 is indicated.

keeping with a construct lacking the Pol domain. By contrast, cells infected with $AcMLV_{Gag\text{-}Pol}$ showed extensive proteolytic processing of $p65^{Gag}$ with cleaved $p30^{Gag}$ being the predominant Gag protein detected and only low levels of the uncleaved $p65^{Gag}$ when compared to $AcMLV_{Gag}$-infected cells (Fig. 1A, compare tracks 4 vs 5). The observed proteolytic processing of $p65^{Gag}$ following expression of the MLV $p180^{Gag\text{-}Pol}$ fusion protein indicated readthrough of the MLV $p65^{Gag}$ stop codon by insect cell tRNAs. The overall expression of $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ was estimated to be ~10-fold higher than that in mammalian cells on a per expressing cell basis, confirming the potential of heterologous expression for the production of high levels of viral particles. When supernatants from infected cells were probed with anti-$p30^{Gag}$, $AcMLV_{Gag}$-infected cells showed a strong band at $p65^{Gag}$, reflecting the intracellular expression profile of Gag and suggesting efficient release of uncleaved $p65^{Gag}$ (Fig. 1B, tracks 4 and 5). Supernatants from $AcMLV_{Gag\text{-}Pol}$-infected cells also showed predominantly $p65^{Gag}$, in contrast to the intracellular Gag profile, although some $p30^{Gag}$ was also visible by Western blot (Fig. 1B, track 5). A pattern of fully cleaved $p30^{Gag}$, with no $p65^{Gag}$ in the supernatant, was shown by MLV harvested from the supernatant of transfected 293T cells (Fig. 1B, track 2). Similar results were obtained when a $p15^{Gag}$ antibody was used for blotting (data not shown). Thus, despite suppression of the *gag* termination codon in insect cells to provide a mix of Gag and Gag-Pol products, predominantly uncleaved $p65^{Gag}$ entered the VLP assembly pathway.

To assess the nature of the MLV antigen released from infected Sf9 cells, the sedimentation profiles of antigen on sucrose velocity gradients, diagnostic of VLP assembly, were analysed. Supernatants from $AcMLV_{Gag}$-infected cells showed a major band at the position of $p65^{Gag}$ (Fig. 2A) that peaked in the 45% sucrose fraction as reported for other C-type retrovirus particles (Jowett et al., 1992). VLPs present in the supernatant from $AcMLV_{Gag\text{-}Pol}$-infected cells also peaked in the middle of the gradient and were similarly associated with $p65^{Gag}$, although some $p30^{Gag}$ cobanded and were also present at lighter densities (Fig. 2B). MLV particles released from transfected 293T cells banded at a similar position in the gradients but were associated only with fully cleaved $p30^{Gag}$ antigen (Fig. 2C). These data suggested that MLV virus-like particles are efficiently budded from insect cells following high-level expression of MLV $p65^{Gag}$ or $p180^{Gag\text{-}Pol}$. The Gag profile by blot however suggested that maturation of VLPs does not occur, or occurs with very low efficiency, despite demonstrably high MLV protease activity in the producer cells.

To confirm that MLV Gag-derived antigen released into the supernatant of $AcMLV_{Gag\text{-}Pol}$-infected *Sf*9 cells was in the form of nonmaturing VLPs, infected cells were fixed, thin-sectioned, and examined by transmission electron microscopy (TEM). Typical fields showed profuse budding of MLV VLPs with an immature morphology at the infected cell surface (Fig. 3A). Occasionally, a field of VLPs showed

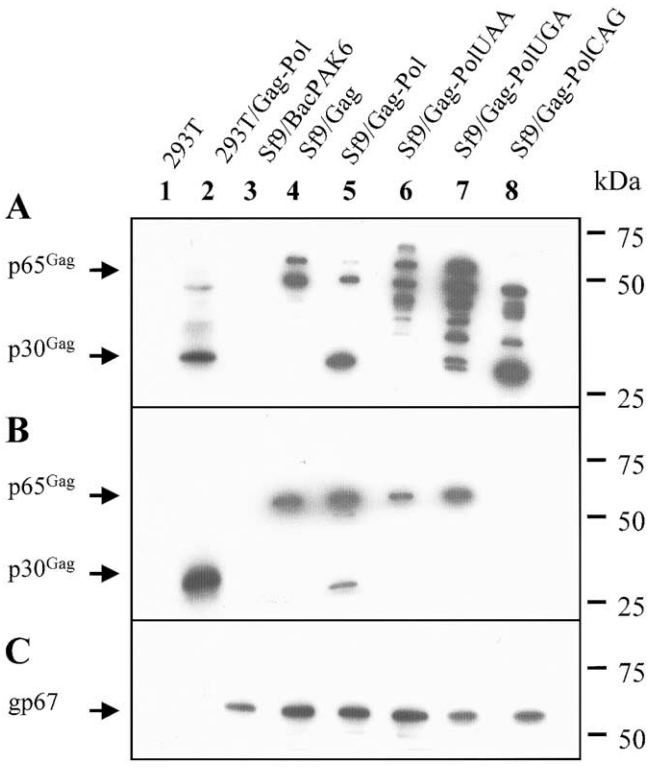

Fig. 2. Sucrose gradient analysis of VLPs assembled in Sf9 and 293T cells. VLPs present in the supernatant were sedimented through a 20–60% step sucrose gradient made up in PBS. Gradients were fractionated from the top and VLPs produced from Sf9 cells infected with $AcMLV_{Gag}$ (A) and $AcMLV_{Gag\text{-}Pol}$ (B) or from 293T cells transfected with pHIT60 (C) were detected by 12% SDS–PAGE and Western blotting using goat anti-$p30^{Gag}$ antibody followed by an anti-goat peroxidase conjugate and chemiluminescence.

a single particle with a morphology that would be consistent with a mature phenotype (Fig. 3B) but the overall level was extremely low (<0.1%), did not reflect the levels of $p30^{Gag}$ observed in the banded VLP preparations used for adsorption to the grids, and failed to be confirmed by reverse transcriptase assay or blot by anti-RT antibody, all of which which showed barely detectable activity when compared with mammalian cell-derived controls (data not shown).

Variable $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ expression levels fails to rescue mature VLP

Failure to assemble maturation competent VLPs, despite evidence for activation of the MLV protease in insect cells expressing MLV $p180^{Gag\text{-}Pol}$, could have been the result of an inappropriate balance of $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ in the expressing cells. To address this, the wild-type UAG codon at the end of the *gag* gene was mutated to each alternate stop codon, UAA and UGA. Previous studies showed that these alternate termination codons allowed equivalent rates of suppression to the original UAG termination codon, at least in mammalian cells and cell lysates (Feng et al., 1989). In addition, UAG was changed to CAG (encoding glutamine)

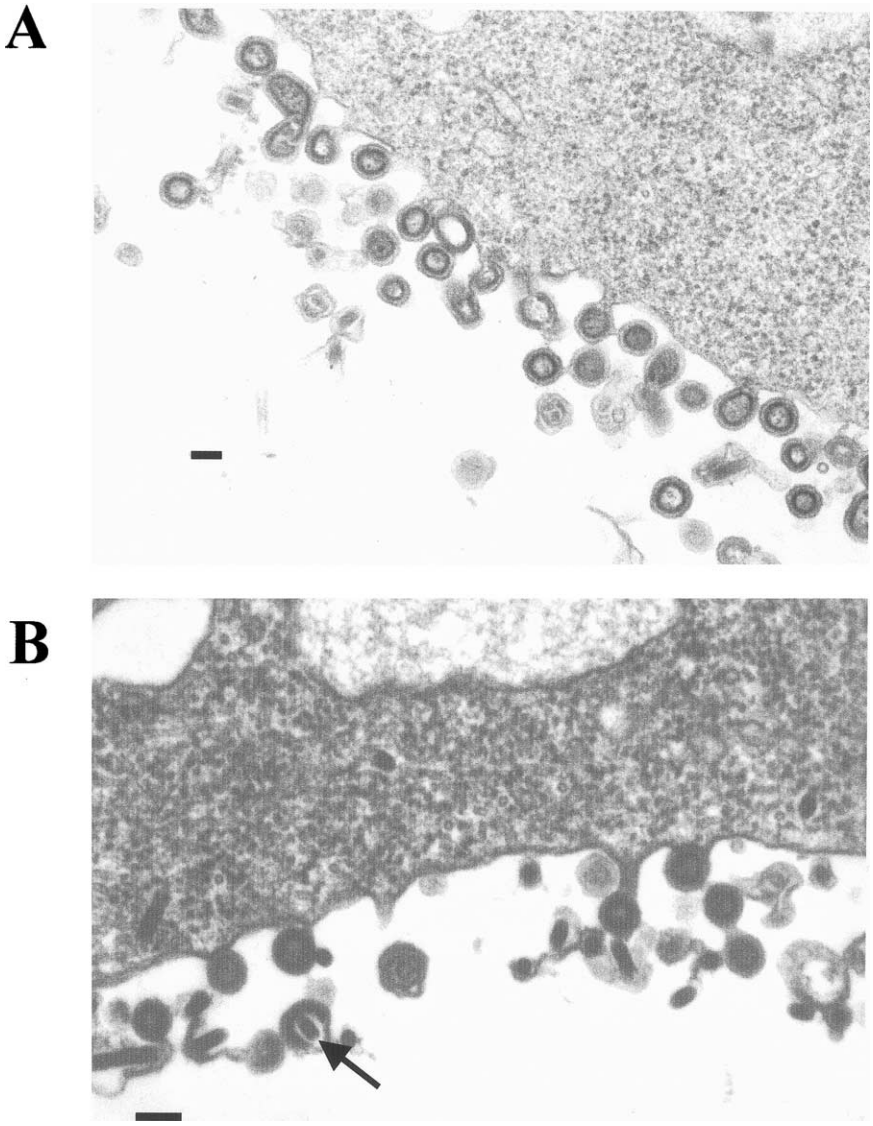

Fig. 3. Electron microscopy of assembled MLV VLPs. Representative thin-section electron micrographs of MLV VLPs were assembled in Sf9 cells infected with $AcMLV_{Gag\text{-}Pol}$. Samples were prepared as described by Jowett et al. (1992) and stained as described by Hockley et al. (1988) before being examined using a Philips CM12 electron microscope operating at 80 KV. The majority of VLPs examined had a morphology typical of immature viruses (A), but very occasionally a VLP with a morphology resembling that of a mature virion was observed (B, indicated). Rod-shaped baculoviruses are also present sectioned at various angles in both panels. Bar is 100 nm.

to produce constitutive expression of the Gag-Pol fusion protein. Following mutant construction and verification, recombinant baculoviruses $AcMLV_{Gag\text{-}Pol}$-UAA, $AcMLV_{Gag\text{-}Pol}$-UGA, and $AcMLV_{Gag\text{-}Pol}$-CAG were generated and their expression profile was assessed by SDS–PAGE and Western blotting using the anti-$p30^{Gag}$ antibody as before. A number of cleavage intermediates, but less fully cleaved $p30^{Gag}$ protein, was observed in cell extracts prepared from cells infected with $AcMLV_{Gag\text{-}Pol}$-UAA, suggesting that UAA is more poorly suppressed than the parental sequence by insect cell tRNAs. Similarly, change of UAG → UGA resulted in intermediate $p65^{Gag}$ and $p30^{Gag}$ levels compared to those associated with expression of parental $p180^{Gag\text{-}Pol}$ (Fig 4, compare track 7 with track 5), while change of UAG → CAG resulted in the production of $p180^{Gag\text{-}Pol}$ fusion protein independent of termination codon readthrough and showed very little $p65^{Gag}$ but prominent partial cleavage products and substantial levels of $p30^{Gag}$ consistent with high-level protease production and essentially complete $p65^{Gag}$ degradation (Fig 4A, track 8). To assess if altered $p65^{Gag}$ levels in the expressing cells influenced the Gag profile of the VLPs formed, supernatants from infected cells were concentrated and analysed as before. Gag-derived proteins released into the supernatant from $AcMLV_{Gag\text{-}Pol}$-UAA-infected cells consisted of $p65^{Gag}$ with no $p30^{Gag}$ (Fig 4B, track 6). Supernatants from $AcMLV_{Gag\text{-}Pol}$-UGA-infected cells similarly showed only $p65^{Gag}$ (Fig. 4B, track 7), while cells infected with $AcMLV_{Gag\text{-}Pol}$-CAG failed to

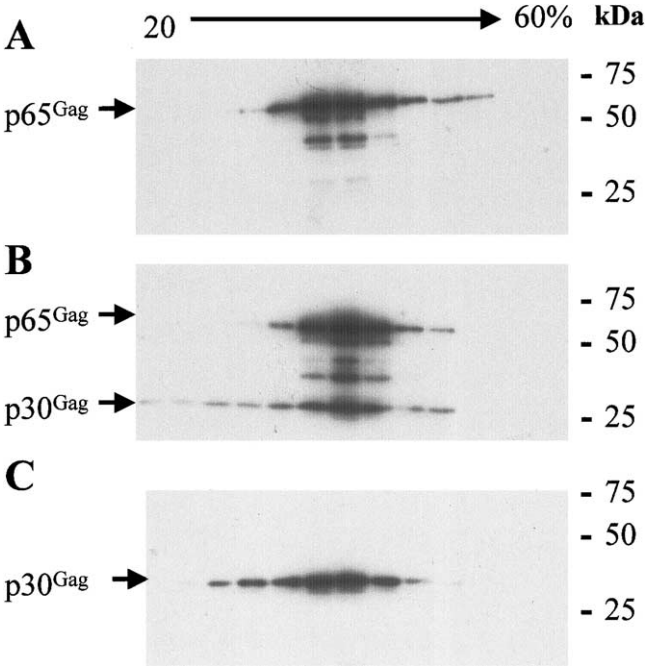

Fig. 4. Western blot analysis of $p65^{Gag}$ proteins expressed by $p65^{Gag}$ termination codon mutants in Sf9 cells. Cell lysates and supernatants were prepared as described for Fig. 1. The samples analysed were as follows: 1, nontransfected 293T cells (control); 2, 293T cells transfected with pHIT60 expressing $p180^{Gag\text{-}Pol}$; 3, Sf9 cells infected with BacPAK6; 4, $AcMLV_{Gag}$; 5, $AcMLV_{Gag\text{-}Pol}$; 6, $AcMLV_{Gag\text{-}Pol}$UAA; 7, $AcMLV_{Gag\text{-}Pol}$UGA; 8, $AcMLV_{Gag\text{-}Pol}$CAG. Infected cell lysates (A and C) or supernatants (B) were collected 48 h postinfection and resolved by 12% SDS–PAGE and Western blot using anti-$p30^{Gag}$ antibody (A and B) or anti-gp67 (C). The blot is a composite and the positions of uncleaved $p65^{Gag}$, cleaved $p30^{Gag}$, and the baculovirus expressed gp67 are indicated.

show any Gag-derived antigen in the supernatant (Fig. 4B, track 8), as expected of a construction that does not express any full-length $p65^{Gag}$ (Felsenstein and Goff, 1988). TEM analysis of pelleted VLP material failed to show any particles with an apparent mature morphology as was the case with VLP produced by $AcMLV_{Gag\text{-}Pol}$ and no supernatant had appreciable RT activity (data not shown). None of the stop codon mutations altered the level of $p30^{Gag}$ in the supernatant to the levels associated with the $p30^{Gag}$ present in the supernatant of transfected mammalian cells expressing MLV $p180^{Gag\text{-}Pol}$ (Fig. 4B, track 2). Thus, although modulation of the readthrough event through alteration of the stop codon clearly changed the relative levels of $p65^{Gag}$ and $p30^{Gag}$ in expressing cells, those profiles were not mirrored in the VLP population.

Coexpression of $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ fails to rescue mature VLP formation

Singular expression of the *gag-pol* gene in insect cells resulted in expression of $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ at a relative level dependent on the readthrough rate. To assess if the failure to incorporate the correct levels of $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ to allow both particle assembly and maturation was related to expression of the required proteins from a single transcriptional unit, cells were coinfected with $AcMLV_{Gag}$ and $AcMLV_{Gag\text{-}Pol}$-CAG to express uncleaved $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ from different mRNA transcripts. Coinfection by baculoviruses at high multiplicity is an efficient method for coexpression of two antigens in the same cell (Belyaev et al., 1995). Following coinfection, supernatants were harvested and the VLPs present were analysed by sedimentation through velocity gradients followed by SDS–PAGE and Western blotting. MLV Gag antigen was present in fractions of approximately 45% sucrose as before (Fig. 5A) but, despite coexpression of the readthrough $p180^{Gag\text{-}Pol}$ domain, the predominant band was at $p65^{Gag}$ with a relatively low level of $p30^{Gag}$ when compared to the cellular profile and no evidence of enhanced maturation beyond that was seen in cells expressing $AcMLV_{Gag\text{-}Pol}$ (Fig. 5B). As with the alternate stop codon mutants, no MLV particles with a mature morphology were apparent following EM examination (data not shown). Coexpression of $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ from different transcripts there-

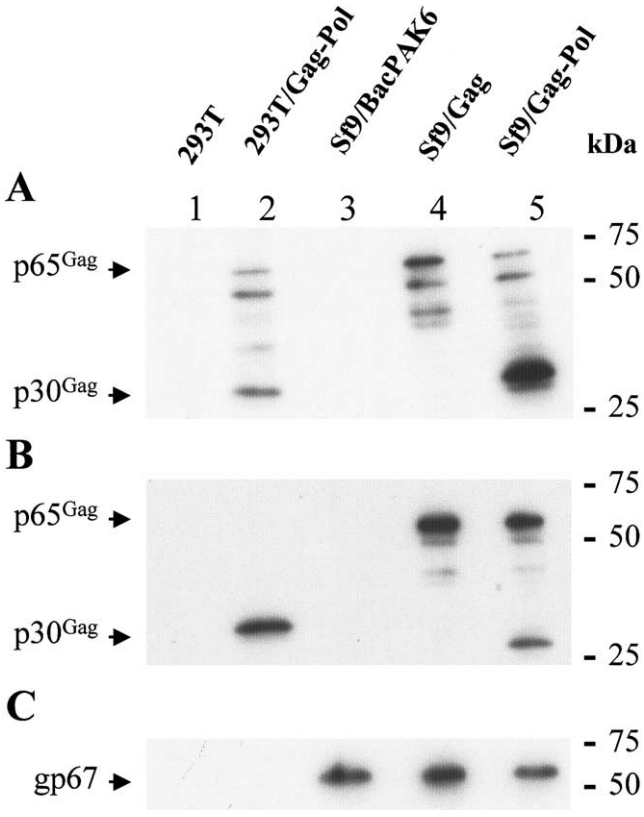

Fig. 5. Sucrose gradient analysis of VLPs assembled after Sf9 cell coinfection with $AcMLV_{Gag}$ and $AcMLV_{Gag\text{-}Pol}$CAG. VLPs present in the supernatant of Sf9 cells infected with $AcMLV_{Gag}$ or $AcMLV_{Gag}$ + $AcMLV_{Gag\text{-}Pol}$CAG were sedimented through a 20–60% step sucrose gradient and analysed as described for Fig. 2. (A) Gradient profile for $AcMLV_{Gag\text{-}Pol}$ only (as in fig. 2B); (B) gradient profile of coinfection of $AcMLV_{Gag}$ and $AcMLV_{Gag\text{-}Pol}$CAG. VLP were detected by 12% SDS–PAGE and Western blotting using anti-$p30^{Gag}$ antibody following gradient fractionation from the top.

fore makes no difference in the selection of uncleaved $p65^{Gag}$ during particle assembly and the failure to form particles with a mature morphology.

Discussion

The Gag precursor protein is the main structural unit of retroviral assembly and its ability to assemble into VLPs in heterologous expression systems has previously been demonstrated for numerous retroviruses with the exception of HTLV-1 (Bouamr et al., 2000) and RSV (Johnson et al., 2001). Assembly of immature VLPs following expression of MLV $p65^{Gag}$ was expected therefore, despite not being formally demonstrated previously. The expression of MLV $p180^{Gag\text{-}Pol}$ resulted in abundant levels of the cleaved $p30^{Gag}$ and indicated that suppression of the $p65^{Gag}$ termination codon occurred efficiently in insect cells. In studies on retroviruses whose relative Gag and Gag-Pol levels are set by frameshifting, activation of the protease in heterologous expression systems leads to cessation of particle assembly. Surprisingly however, in the case of MLV, expression of $p180^{Gag\text{-}Pol}$ resulted in the continued assembly of VLPs, which contained high levels of uncleaved $p65^{Gag}$ and exhibited an immature morphology by TEM. The failure to form mature particles could be due to one of two factors: firstly a level of $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ incompatible with a VLP structure that is enabled for further $p65^{Gag}$ processing, or alternately, the lack of a host specific factor or environment necessary for the correct packing of $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$. The former possibility seems unlikely as we observed efficient translational readthrough in insect cells and showed that mutation of the *gag* stop codon significantly altered intracellular Gag ratios yet did not concomitantly alter the profile of budded VLPs. In addition, coexpression of similar levels of $p65^{Gag}$ with a $p180^{Gag\text{-}Pol}$ fusion protein mutated to provide permanent readthrough, effectively boosting the intracellular level of protease, did not alter the form of budded VLP. Some cleaved $p30^{Gag}$ antigen was found associated with VLPs following coincorporation of $p180^{Gag\text{-}Pol}$ into VLPs but the lack of overt VLP maturation was consistent with that being nonspecifically associated with VLPs in, for example, membrane fragments released from lysed cells or by passive capture of $p30^{Gag}$ by the budding VLP. Retrovirus protease expression in insect cells has been variously observed to be uncontrolled, cytotoxic, and generally incompatible with VLP formation (Gheysen et al., 1989; Johnson et al., 2001). This was not the case for MLV where, in the case of coinfection of $AcMLV_{Gag}$ and $AcMLV_{Gag\text{-}Pol}$-CAG, high levels of protease were present but VLP synthesis was maintained and no particle maturation occurred. An alternate explanation is that particle assembly may involve an active sorting process requiring more than the presence of a suitable $p65^{Gag}$: $p180^{Gag\text{-}Pol}$ ratio and that key factors, either required for initiating maturation directly or required for loading assembling particles with the correct ratios of $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$, are poorly represented in, or absent from, insect cells. A similar conclusion was reached in a recent study which examined the rescue of mature HIV-1 VLPs from insect cells following modulation of protease activity (Adamson et al., 2003).

The existence of *cis*-acting "late" assembly signals has been known for retroviruses since 1991 (Gottlinger et al., 1991) and recent data show them to interact with host cell factors such as Nedd 4 and TSG101 (Demirov et al., 2002a; Garrus et al., 2001; Kilonyogo et al., 2001; Martin-Serrano et al., 2001; Myers and Allen, 2002; Puffer et al., 1998; VerPlank et al., 2001), whose function appears, principally, to direct virus particle trafficking and budding (reviewed by Freed, 2002). Interestingly, however, mutations in the late domain of HIV-1 Gag have been associated with defective Gag processing (Demirov et al., 2002b; Gottlinger et al., 1991; Huang et al., 1995) and the loading of assembling viruses with the normal amounts of the *pol*-encoded enzymes (Demirov et al., 2002b; Dettenhofer and Yu, 1999; Yu et al., 1998), providing a link between late domain function and the ability of the particle to mature. Moreover, the requirement for late factors can be bypassed if protease is missing or inactive (Huang et al., 1995; Schubert et al., 2000), although the reason for this remains unclear. Our data show that MLV budding, which also uses a late domain (Yuan et al., 2000), was efficient in heterologous cell types irrespective of the presence of active protease but that onward maturation did not occur. This might suggest that factors involved in late virus assembly have an active role in sorting Gag into the assembling virion in addition to allowing the assembled virus to bud efficiently from the plasma membrane. Our data on MLV assembly in insect cells, in which Gag readthrough rather than frameshifting achieves the relative balance of $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$, is consistent with the lack of maturation obtained in systems that have attempted heterologous expression of mature HIV (Adamson et al., 2003; Gheysen et al., 1989) and suggests a common defect that is not related to the molecular mechanism of Gag translational control. If the action of viral late factors, or the microenvironment they enable, can be understood in detail, there seems no reason retroviral vectors could not be assembled in heterologous cells with concomitant improvements in titre and safety.

Materials and methods

Cell culture

Insect serum-free adapted *Spodoptra frugiperda* (Sf9) cells were propagated at 28°C as described by O'Reilly et al. (1992) except that Sf-900 II media (Gibco-BRL Life Technologies) were used throughout. Mammalian 293T cells (cell bank, Oxford University) were propagated at

37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% v/v heat-inactivated foetal calf serum (Gibco-BRL Life Technologies) and 1% v/v 200 mM L-glutamine (Gibco-BRL Life Technologies).

Construction of recombinant transfer vector

Recombinant transfer vectors expressing MLV $p65^{Gag}$ and $p180^{Gag\text{-}Pol}$ were constructed. The *gag* gene was excised from plasmid p6565 (Yap, 2000) using *Bam*HI and *Eco*RI restriction sites. Restriction sites *Bgl*II and *Eco*RI were then used to clone the *gag* gene into the transfer vector pBAC4x-1 (Cambridge Bioscience), placing *gag* under the control of a p10 promoter. The Gag-Pol transfer vector was constructed by amplifying *gag-pol* by PCR from plasmid *pgag-pol*gpt (Markowitz et al., 1988). Gag-Pol was inserted into the *Bam*HI and *Not*I restriction sites of transfer vector pBAc4x-1, placing the gene under the control of the polyhedrin promoter.

Mutations to alter the $p180^{Gag\text{-}Pol}$ frameshift site by changing the UAG termination codon to alternative termination codons UAA and UGA or the glutamine codon CAG were introduced using the Quick Change Site-Directed Mutagenesis Kit (Stratagene). To carry out the mutagenesis procedure, an ~4-Kb 5′ fragment containing the Gag termination codon was subcloned into the *Xho*I restriction site of pBSK (Stratagene). The UAA mutation was introduced using the following primer pair: 5′-CTCCTGACCCTAGATGACTTAAGGAGGTCAGGGTCAGGAGCC-3′; 3′-GAGGACTGGGATCTACTGATTCCTCCAGTCCCAGTCCTCGG-5′. The UGA mutation was introduced using the following primer pair; 5′-CCTCCTGACCCTAGATGACTGAGGAGGTCAGGGTCAGGAGCC-3′; 3′-GGAGGACTGGGATCTACTGACTCCTCCAGTCCCAGTCCTCGG-5′. The CAG mutation was introduced using the following primer pair: 5′-CCCTCCTGACCCTAGATGACCAGGGAGGTCAGGGTCAGGAG-3′; 3′-GGGAGGACTGGGATCTACTGGTCCCTCCAGTCCCAGTCCTC-5′. Clones containing the correct mutations were identified by sequencing and cloned into the pBAC4x-1 backbone containing the 3′ fragment of the *gag-pol* gene.

Production of recombinant baculoviruses

All recombinant baculoviruses were derived from *Autographa californica* nuclear polyhedrosis virus (AcNPV). The baculoviruses were generated by cotransfecting Sf9 cells with recombinant transfer vector (0.5 μg) and linear BacPAK6 viral DNA (1 μL) (Clontech). Cell supernatants, harvested after 5 days, were plaque assayed and plaques were picked and grown for virus stocks as described by King and Possee (1992). Recombinant baculoviruses were confirmed by the immunodetection of Gag at 2 days pi and high-titre stocks were generated.

Expression and preparation of MLV proteins

MLV proteins were expressed in insect cells by infecting monolayers of 70% confluent Sf9 cells with recombinant baculovirus according to the methods described by King and Possee (1992). Infections were carried out at an m.o.i. of 5. MLV proteins were transiently expressed in mammalian 293T cells seeded at 70% confluence, using the profectin calcium phosphate transfection system (Promega). The cells were transfected with plasmid pHIT60 encoding the MLV *gag-pol* genes (Soneoka et al., 1995). Sf9 and 293T cells were harvested 48 h postinfection or transfection and lysed in cell lysis buffer (1× PBS, 1% v/v NP-40) for 20 min at 4°C followed by clarification at 14K rpm for 10 min at 4°C. Protein concentrations were determined using a Bio-Rad protein assay kit. Cell supernatant was clarified (4 K rpm, 4°C, 5′ followed by 10 K rpm, 4°C, 10′) and secreted protein was pelleted (14K rpm, 4°C, 30′). The resulting pellet was washed once in PBS and resuspended in 10 μl of H_2O.

SDS–PAGE and Western blotting

Protein samples were prepared in Laemmli sample buffer (Laemmli, 1970) and separated by 12% SDS–PAGE. For Western blots, proteins were transferred to PVDF membrane (Immobolin-P, Millipore) and blocked for 1 h at room temperature in PBS containing 0.1% Tween 20 (PBS-T) and 5% w/v milk powder. Incubation with anti-$p30^{Gag}$ antibody (1:5000, Quality Biotech Inc.) was conducted for 1 h at room temperature followed by washing several times with PBS-T. Membranes were incubated a further hour with peroxidase-conjugated rabbit anti-goat antibody (Sigma) and bound antibodies were detected by chemiluminescence (Roche). Relative band intensities of scanned images were estimated using Phoretix software (Phoretic International).

Isolation and purification of virus-like particles

VLPs present in the supernatant were collected 48 h postinfection or transfection by centrifugation at 24K rpm for 1 h at 4°C, in a SW28 rotor (Beckman). The pellet was resuspended in PBS and loaded onto a 20–60% w/v step sucrose gradient and centrifuged at 30K rpm, 4°C for 1.5 h in a SW41 rotor (Beckman). Fractions were collected from the top and a 10-μL aliquot of each fraction was analysed by SDS–PAGE and Western blot.

Reverse transcriptase assay

Particles in the supernatants were concentrated by sedimentation as described and reverse transcriptase (RT) activity was assessed in triplicate using the Quant-T-RT kit (Amersham Pharmacia), which utilizes the scintillation proximity assay (SPA) principle to measure reverse transcriptase activity by incorporation of [^{3}H]TTP by reverse

transcriptase into a DNA/RNA primer/template bound to SPA beads. The kit was used according to the manufacturers instructions except a modified assay buffer (50 mM Tris–HCl, pH 8.0, 20mM DTT, 0.6mM $MnCl_2$, 60 mM NaCl, 0.05% v/v NP-40) was used. Purified MLV reverse transcriptase (Gibco BRL Life Technologies), which gave an activity linearly to dose, was used as a positive control.

Electron microscopy

The procedure followed was that described by Jowett et al. (1992). Briefly infected or transfected cells were dislodged by gentle tapping and collected by low-speed centrifugation at 1K rpm for 5 min at 21°C. The cells were resuspended in 0.1 M sodium ca*cody*late buffer pH 7.4 containing 1% v/v gluteraldehyde (25% w/v EM grade). Following fixation, the cells were embedded in Araldite, sectioned, and poststained with uranyl acetate as described by Hockley et al. (1988). Specimens were examined using a Philips CM12 electron microscope operating at 80 kV.

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