Role of the \(gag\) and \(pol\) genes of human immunodeficiency virus in the morphogenesis and maturation of retrovirus-like particles expressed by recombinant vaccinia virus: an ultrastructural study

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An ultrastructural study was performed on rabbit epithelial RK-13 cells and CD4+ human T lymphocyte lines infected with various recombinant vaccinia viruses (RVVs) expressing genes of human immunodeficiency virus (HIV): the mature p17 or p24 gag domain alone, the entire or truncated \(gag\) gene, the reverse transcriptase domain, or the \(gag-pol\) genes with a frameshift mutation. Cells infected with RVVs that produced the gag polyprotein with a predicted Mr of more than 48K showed budding and release of HIV-like particles into the extracellular space. These particles were not observed in cells expressing a truncated \(gag\) gene (p17 and p24 regions). Mature HIV-like particles were observed extracellularly when the entire \(gag\) gene and the protease region of the \(pol\) gene were expressed. In contrast, in cells infected with RVVs that contained the \(gag-pol\) gene with a frameshift mutation, neither recognizable budding structures nor extracellular HIV-like particles could be detected. These results suggest that the \(gag\) gene, particularly its 3' terminus, is necessary for the assembly of HIV particles. In addition, the protease region of the \(pol\) gene seems to be required for morphological maturation of HIV particles, but complete proteolytic cleavage of the gag protein may prevent bud formation.

Introduction

Human immunodeficiency virus types 1 and 2 (HIV-1 and -2) (Coffin et al., 1986) have been classified as members of the lentivirus subfamily of the retroviruses. Morphologically, HIV-1 and -2 resemble visna virus (Gonda et al., 1985; Munn et al., 1985); mature virions of HIV have an electron-dense and bar-shaped core (Klatzmann et al., 1984; Munn et al., 1985; Palmer et al., 1985; Meyenhofer et al., 1987; Gelderblom et al., 1988, 1989; Hockley et al., 1988). Immature particles with a doughnut-shaped core have also been observed extracellularly in HIV-infected cell cultures (Munn et al., 1985; Palmer et al., 1985; Gelderblom et al., 1988, 1989; Hockley et al., 1988).

The genome of HIV consists of three major genes: \(gag\), \(pol\) and \(env\) (Muesing et al., 1985; Ratner et al., 1985; Wain-Hobson et al., 1985). The initial protein products of the \(gag\) and \(pol\) genes are a gag polyprotein precursor of about 55K (Pr55\(^{gp}\)) and a large gag–pol fusion protein translated from the \(gag\) and \(pol\) regions by ribosomal frameshifting (Jacks & Varmus, 1987; Jacks et al., 1988).

The frequency of frameshifting and the ratio of gag to gag–pol protein are relatively constant (Murphy et al., 1978; Jacks & Varmus, 1987; Jacks et al., 1988) and the maintenance of that ratio may be important for the production of infectious virus particles. A major function of the \(gag\) protein is assembly of the virus core. Expression of the murine leukaemia virus (MuLV) or the simian immunodeficiency virus \(gag\) gene alone is thought to be sufficient for assembly and release of virus particles (Yoshinaka & Luftig, 1977; Shields et al., 1978; Katoh et al., 1985; Delchambre et al., 1989). HIV gag proteins also assemble into virus-like particles when expressed in insect cells (Gheysen et al., 1989), COS 7 cells (Göttlinger et al., 1989), and CMT3 COS cells (Smith et al., 1990).

The \(pol\) region is required for gag function. The 5' region of the HIV \(pol\) gene encodes the viral protease responsible for cleavage of the gag and gag–pol precursors to their processed mature forms. Immature virus particles containing the uncleaved gag precursor have been observed in MuLV (Yoshinaka & Luftig, 1977) and Rous sarcoma virus (Voynow & Coffin, 1985).
with a mutation in the protease region. It has been assumed that formation of infectious retrovirus particles requires cleavage and processing of the gag-pol protein by viral protease (Shields et al., 1978; Katoh et al., 1985; Felsenstein & Goff, 1988).

Recombinant vaccinia virus (RVV) has been shown to express foreign genes authentically. An approach using RVVs expressing different regions of the gag and pol genes would help to clarify the relationship between the gag and pol functions in the formation of HIV particles.

Methods

Cells and viruses. A rabbit kidney epithelial cell line RK-13 and a thymidine kinase-negative (TK-) human cell line 143TK- were grown essentially as summarized by Sambrook and its recombinants were propagated in RK-13 cells.

DNA manipulations. Plasmid DNA manipulations were performed essentially as summarized by Sambrook et al. (1989) except that hybridization was done in a solution containing 6 x standard saline citrate, 0.5% SDS and 2% non-fat dry milk as described (Yasuda et al., 1990). DNA sequence analysis was done by the dideoxyribonucleotide-chain termination method described by Sanger et al. (1980). A plasmid encoding a proviral clone of HIV-1 (HTLV-IIIB strain), pBH10 (Ratner et al., 1985), was used as the source of HIV-1 viral DNA in all constructs. A 5 kb SstI-SalI restriction fragment (encoding the entire gag, pol and vif genes) was subcloned into M13mp18 phage and served for oligonucleotide-directed mutagenesis (Kunkel, 1985).

All restriction and modification enzymes and random-primer labelling and sequencing kits were purchased from Boehringer Mannheim. An in vitro mutagenesis kit was obtained from Bio-Rad.

In vitro mutagenesis and construction of insertion plasmids. Oligonucleotides complementary to the DNA sequences shown in Fig. 1(a) were synthesized with a DNA synthesizer (ABI type 381 A) and used as primers for in vitro mutagenesis. The primer Gag-N adds two cytidines upstream of the ATG codon of the gag gene (position 334; all nucleotide positions according to Ratner et al. (1985)), to create an Ncol site for excluding the HIV-1 packaging signal located upstream of the gag gene (Lever et al., 1989). The primer 24-N adds AGG upstream of position 730 to create a unique SfiI site. RT-N adds TAGGG upstream of position 2130 and changes cytidine at position 2132 to thymine, which also creates a site.

For the construction of p24 or RT open reading frames (ORFs), the ATG codon was produced in pAK10 by Ncol digestion and then polymerase I treatment. The resulting blunt end was ligated to that of the SstI site of the p24 or RT fragment. pAK10 also has stop codons in the polylinker and generates additional sequence coding for six amino acids (Pro Asp Leu Glu Lys Leu) or five amino acids (Ser Arg Ser Phe Lys) at the C terminus of the G5 and p48 ORFs, respectively. Frameshift (FS) mutations (designated as GFVS and GSFS; Fig. 2) were introduced into both the G5 and G5 fragments at the BglII site of the gag and pol overlap region. DNA was cleaved with BglII, filled with DNA polymerase I, and religated with T4 ligase. These treatments added four nucleotides at the BglII site and put the gag and pol genes in frame (Fig. 1c and 2).

The recombinant plasmids pAKGV, pAKGVFS, pAKG5FS, pAKP48, pAKP41, pAKP17, pAKP24 and pAKRT thus prepared were used for insertion of the HIV-I genes into the TK gene of vaccinia virus.

Construction of RVVs. RK-13 cells infected with vaccinia virus (m0 strain) were transfected with calcium phosphate-precipitated plasmid DNA of pAK10 recombinants as described (Yasuda et al., 1990). TK- viruses were isolated by plaque assay on 143TK- cells in the presence of 5-bromo-2'-deoxyuridine at a concentration of 25 μg/ml. TK- viruses were amplified through RK-13 cells and screened for recombinants of interest by dot hybridization with a 32P-labelled HIV-I DNA probe. RVVs, designated as m0GV, m0GVFS, m0G5, m0G5FS, m0P48, m0P41, m0P17, m0P24 and m0RT (Fig. 2) were isolated, plaque-purified and propagated. Genomic structure analyses of these RVVs by ethidium bromide staining and Southern blot hybridization confirmed that the HIV-I DNA was correctly integrated into the TK gene of the vaccinia virus genome, and that no rearrangement or deletion had occurred during their construction (data not shown).

Western immunoblot analysis of recombinant HIV-I proteins. RK-13 cells infected with RVVs were harvested 24 h post-infection (pi) and concentrated by low-speed centrifugation. Cell pellets were lysed in 1 x sample buffer (65 mM-Tris–HCl pH 6.8, 3% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue) by boiling for 10 min. Samples were applied to 12% SDS polyacrylamide gels and electrophoresed (Laemmli, 1970). Proteins were electrotransferred onto Immobilon P membranes (Millipore) for 1 h at 170 mA with a TEFCO electrophoresis unit. The membranes were incubated in blocking solution (2% non-fat dry milk in PBS), and were exposed to a mixture of monoclonal antibodies directed against either p17, p24 or reverse transcriptase (RT), followed by biotinylated anti-mouse IgG (Vector Laboratories) and peroxidase-conjugated avidin D (Bio-Rad). Protein bands were visualized with 4-chloro-1-naphthol (Wako) as the substrate. The RT activity in RVV-infected cells was confirmed according to the method of Hoffman et al. (1985).

Preparation of RVV-infected specimens for ultrastructural study. Monolayers of RK-13 cells were infected with RVV at an m.o.i. of 2 for 60 min at 37°C, washed with MEM-2% FCS, and then cultured in MEM-5% FCS for 12 to 72 h. Infected cells were harvested with scrapers and concentrated by low-speed centrifugation. CD4+ human T cells were concentrated to 5 x 10⁶ cells/ml in MEM-2% FCS and then diled to 5 x 10⁵ cells/ml with RPMI-1640-10% FCS. After centrifugation, the cell pellets were gently resuspended in growth medium and cultured for 12 to 72 h at 37°C. Infected T cells were recovered by gentle pipetting, and collected by centrifugation.

Electron microscopic examination. Pelleted cells were fixed with cold 2.5% glutaraldehyde in 0.067 M-cacodylate buffer (pH 7.2) for 2 h and post-fixed with 1% OsO₄ in phosphate buffer at 4°C for 1 h. After dehydration in a graded ethanol series, the specimens were embedded in Epon 812 (Poly/Bed). Ultrathin sections were cut, stained with
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(a) DNA sequences complementary to synthetic oligonucleotides used for mutagenesis. The initiation codon (O) and stop codons (Q) as well as restriction enzyme cleavage sites (underlined) produced by in vitro mutagenesis are shown. Deduced amino acid sequences at the N or C termini (Ratner et al., 1985) of HIV-1 proteins are also shown above the nucleotide sequences.

(b) Restriction fragments of HIV-1 proviral DNA inserted into the transfer plasmid vector pAK10. M13mp18 containing a SalI–SaiI fragment of pBH10 with the mutations indicated in (a) was digested with the restriction enzymes shown in parentheses. The size of the resultant fragments is expressed by the nucleotide position numbers (Ratner et al., 1985) of the 5' and 3' ends.

(c) Introduction of frameshifting mutation. The SalI–SaiI fragments mutated in vitro for construction of GV and G50RFs shown in (b) were recovered from M13mp18 recombinants and transferred into pUC18. The BglII site (V) of HIV-1 DNA in the pUC18 recombinants was cleaved, filled with four nucleotides (V) by DNA polymerase I, and religated. Amino acid sequences of the gag and pol frames are shown below the nucleotide sequences.

(d) Structure of the transfer plasmid vector pAK10 containing the early–late promoter of the vaccinia virus 7.5K polypeptide (P7.5) (Cochran et al., 1985; Mackett et al., 1985) and a 95 bp synthetic polylinker sequence which has the ATG codon (O) in the NcoI site and stop codons (Q) downstream. The P7.5 and polylinker regions are flanked by the vaccinia virus DNA fragments (TK1, TK2) containing the TK gene.

Fig. 1. (a) DNA sequences complementary to synthetic oligonucleotides used for mutagenesis. The initiation codon (O) and stop codons (Q) as well as restriction enzyme cleavage sites (underlined) produced by in vitro mutagenesis are shown. Deduced amino acid sequences at the N or C termini (Ratner et al., 1985) of HIV-1 proteins are also shown above the nucleotide sequences. (b) Restriction fragments of HIV-1 proviral DNA inserted into the transfer plasmid vector pAK10. M13mp18 containing a SalI–SaiI fragment of pBH10 with the mutations indicated in (a) was digested with the restriction enzymes shown in parentheses. The size of the resultant fragments is expressed by the nucleotide position numbers (Ratner et al., 1985) of the 5' and 3' ends. (c) Introduction of frameshifting mutation. The SalI–SaiI fragments mutated in vitro for construction of GV and G50RFs shown in (b) were recovered from M13mp18 recombinants and transferred into pUC18. The BglII site (V) of HIV-1 DNA in the pUC18 recombinants was cleaved, filled with four nucleotides (V) by DNA polymerase I, and religated. Amino acid sequences of the gag and pol frames are shown below the nucleotide sequences. (d) Structure of the transfer plasmid vector pAK10 containing the early–late promoter of the vaccinia virus 7.5K polypeptide (P7.5) (Cochran et al., 1985; Mackett et al., 1985) and a 95 bp synthetic polylinker sequence which has the ATG codon (O) in the NcoI site and stop codons (Q) downstream. The P7.5 and polylinker regions are flanked by the vaccinia virus DNA fragments (TK1, TK2) containing the TK gene.
Results

Expression of HIV-1 protein by RVVs

Expression of inserted gene products in RVV-infected cells was examined by Western blot and indirect immunofluorescence analyses using a mixture of monoclonal antibodies against p17, p24 and RT, or sera obtained from AIDS patients. Fig. 3 shows a Western immunoblot of gag or pol proteins produced by RK-13 cells infected with various RVVs. The Pr55<sup>ag</sup> precursor protein was mainly detected concomitantly with its proteolytic processing products p17, p24/25 and intermediate precursor p41 in lysates of m0GV- and m0G5-infected cells (Fig. 3, lanes 1 and 3). Several weak bands of intermediate Mr were also detected. In contrast, m0GVFS- and m0G5FS-infected cells (lanes 2 and 4) showed trace or no detectable amounts of Pr55<sup>ag</sup>, but produced predominantly p24/25 and p17 mature gag proteins and p41 intermediates. A band of 65K (RT) was also detected in m0GVFS-infected cells. Extracts of cells infected with m0P48, m0P41, m0P17 and m0P24 revealed major gag-related products of 48K (lane 5), 41K (lane 6), 17K (lane 7) and 24K (lane 8), respectively. A prominent single band of 65K was detected in m0RT-infected cells (lane 9). RT activity was expressed by the m0RT, m0GVFS and m0GV recombinants (data not shown). Similar results were obtained with CD4<sup>+</sup> T cell lines, although the expression of gag-pol proteins was not as high as in RK-13 cells.

Effect of host cells on RVV infection and HIV-like particle formation

RK-13 cells and CD4<sup>+</sup> human T lymphocyte lines infected with various RVVs were examined by electron microscopy during the course of infection. At all times, oval-shaped vaccinia virions about 250 nm in length were present in the cytoplasm of RVV-infected cells, sometimes in clusters. These RVVs were also released into the extracellular space (Fig. 4a, b). The number of cytoplasmic RVVs gradually increased between 12 and 72 h post-infection (p.i.). Infected cells also showed increasing degrees of cytopathic damage such as dilation of endoplasmic reticulum, swelling of mitochondria, formation of myelin figures and lysis.

When RK-13 cells were infected with m0GV, m0G5 or m0P48 recombinants, crescent- or ring-shaped budding structures were formed on the cell surface by 12 h p.i. Budding structures often appeared in cytoplasmic blebs and surface folds as well as at the tips of microvilli. In addition, HIV-like particles were detected extracellularly (Fig. 4a, b). The formation of HIV-like particles by m0GV, m0G5 or m0P48 infection was also seen in CD4<sup>+</sup> human T lymphocyte lines HUT-78 (Fig. 4c), H-9 and Molt-4.
Fig. 4. (a) RVVs and HIV-like particles generated by m0GV-infected RK-13 cells. Low magnification, bar represents 1000 nm. (b) Higher magnification reveals intracytoplasmic vaccinia virions, a crescent-shaped budding structure (arrow) and extracellular HIV-like particles (arrowheads), as well as extracellular vaccinia virions (double arrows); bar represents 200 nm. (c) An m0GV-infected HUT-78 lymphocyte. Extracellular HIV-like particles of pleomorphic shape and size are seen. The arrow indicates a virion containing an eccentric electron-dense body; bar represents 200 nm. (d) An m0G5-infected RK-13 cell. Many HIV-like particles are present in the extracellular space of an RK-13 cell. The arrow indicates a particle with an eccentric electron-dense body. Extracellular and intracytoplasmic vaccinia virions are seen; bar represents 200 nm. (e) Particles budding into an apparent cytoplasmic vacuole of an m0G5-infected RK-13 cell; bar represents 200 nm.
Formation of morphologically mature HIV-like particles

In m0GV- and m0G5-infected cells, many extracellular particles with a diameter varying from 70 nm to more than 160 nm had a bar- or cone-shaped core (Fig. 4a to d). In transverse sections the core had a circular appearance. Eccentric electron-dense bodies were also observed in some particles. These morphological characteristics are very similar to those of mature HIV particles (Munn et al., 1985; Palmer et al., 1985; Meyenhofer et al., 1987; Gelderblom et al., 1988, 1989; Hockley et al., 1988). Mature HIV-like particles connected to the cell surface were not identified by transmission electron microscopy. Spherical double-shelled particles with an electron-lucent centre were observed together with mature HIV-like particles in the extracellular space (Fig. 4d). In some instances, the budding and free particles were seen in what appeared to be cytoplasmic vacuoles (Fig. 4e). Surface projections (knobs) were not detected on the budding or released particles.

Immature HIV-like particles derived from the gag gene product

HIV-like particles were scattered outside cells infected with m0P48 that expressed a truncated 48K gag polyprotein (Fig. 3 and 5a, b). They were usually round, 90 to 120 nm in diameter, and had an electron-lucent centre. These particles closely resemble immature HIV particles (Munn et al., 1985; Palmer et al., 1985; Katsumoto et al., 1987; Hockley et al., 1988; Gelderblom et al., 1989). There was no evidence of surface knobs on the viral particles. Mature HIV-like particles were not identified in the extracellular space of m0P48-infected cells. On the other hand, no HIV-like particles were observed by infection with m0P41, which expressed another truncated 41K gag polyprotein (Fig. 3).

No HIV-like particle formation by RVVs expressing mature gag or pol products

In contrast to m0GV, m0G5 and m0P48 infections, neither budding structures nor extracellular HIV-like particles were identified in cells infected with either m0P24, m0P17 or m0RT; or with the wild-type m0 strain of vaccinia virus. Similarly, double (m0P17 + m0P24) or triple (m0P17 + m0P24 + m0RT) infections resulted in no detectable HIV-like particles or bud formation. Furthermore, in cells infected with m0GVFS or m0G5FS, which expressed cleaved mature p17 and p24 but scarce or no detectable gag precursor polyprotein (Fig. 3), neither budding structures nor free particles were identified by electron microscopy.

The morphological findings are summarized in Table 1.

Table 1. HIV-like particle formation and maturation

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<th>Budding and particle formation</th>
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Discussion

Recent studies focusing on the gag gene function of HIV, the causative agent of AIDS, have shown that one of the major roles of the HIV gag precursor polyprotein, Pr55gag, is particle formation, a final step in the viral life cycle (Gheysen et al., 1989; Göttlinger et al., 1989; Smith et al., 1990). Our morphological observations of cell lines infected with RVVs that express the gag and gag-pol genes of HIV-1 confirm this. The synthesis, transport and biological properties of the recombinant products of RVVs are very similar to those of native proteins (Morita et al., 1987; Watanabe et al., 1989; Yasuda et al., 1990). RVVs constructed here expressed the inserted HIV-1 gene segments and processed the gag-pol proteins as expected. The Pr55gag precursor and HIV-like particles were produced with both m0GV and m0G5, which carried the entire gag gene and the entire or truncated pol gene, but were scarcely detectable in infections with their respective FS mutants, m0GVFS and m0G5FS. An FS mutation was introduced at the BglII site just four codons downstream of the normal FS site of HIV-1 (Jacks et al., 1988) which causes a shift from the gag reading frame to the pol reading frame. This shift occurs only four codons after the initiation of the pol frame. Therefore, the primary products of FS mutants are expected to resemble closely the products generated by normal FS in HIV-infected cells, which are large gag-pol fusion polyproteins consisting of a truncated gag region (p48 minus four amino acids) and the pol proteins (Debouck et al., 1987; Gendelman et al., 1987; Jacks et al., 1988). Proteolytic cleavage of the fusion proteins generates gag products, a p41 intermediate and fully processed gag proteins. In accordance with this, Pr55gag is absent in lysates of cells infected with FS mutants. Furthermore, p41 and its cleaved products, p24/25 and p17, are major gag proteins in the infected cells.

The present study also provides valuable information on functional relationships that may be involved in budding and particle formation of HIV-1. m0P48, containing a gag gene truncated by deletion of the 3' terminus (p7 domain), also allowed assembly of immature HIV-like particles on the cell surface (Fig. 5a, b). This morphological evidence indicates that the entire gag gene is not a prerequisite for viral particle formation, and that the proline-rich p7 region is not required for the budding and particle formation of the virions. However, a small amount of a protein migrating around 55K is observed in m0P48-infected cell lysates. The protein is unlikely to be the full-length gag product, Pr55gag, because m0P48 does not contain the full-length gag gene as judged by hybridization with a probe encoding the region downstream of p48 (data not shown). Although we are not able to characterize the minor protein, it may be a product with a deduced Mr of about 53K which is generated by readthrough of the stop codon provided by the polylinker of pAK10.

In contrast to m0P48, m0P41, carrying another truncated gag gene that has a larger 3'-terminal deletion and encodes the p17 and p24 domains, was not able to produce virions. Recently, similar results have been reported by Gheysen et al. (1989) using a baculovirus-insect cell system, where deletion of the 3' terminus (p16) of the gag gene abolished spherical particle formation. The lack of particle production is apparently not dependent on the expression system utilized or host cell products, but rather is dependent on the progressive 3'-terminal deletion of the gag gene. Therefore, critical sequences responsible for virion formation are most likely to be located in the 3' terminus (p9) of the gag gene. The middle region of p9 has two blocks of an amino acid sequence motif with repeating Cys residues that is conserved in nucleic acid-binding proteins and in the Zn2+ finger proteins (Berg, 1986; Covey, 1986). We are presently evaluating a possible role of the p9 region in particle formation by constructing RVVs which contain truncated gag genes with progressive deletions in the p9 region.

Major components of HIV particles are the cleaved mature gag (p17 and p24), pol (RT) and env (gp120 and gp41) products. In our experiments, neither budding structures nor free particles were observed in cells infected with m0P17, m0P24 or m0RT, indicating that the mature gag products (p17 and p24) or pol products (RT) alone cannot assemble into viral particles. The gag gene-encoded precursor protein is processed into p17, p24 and p15 by the specific viral protease derived from pol gene products (Muesing et al., 1985; Ratner et al., 1985; Wain-Hobson et al., 1985; Mervis et al., 1988). In experiments involving double or triple infection, no viral particles were observed by electron microscopy, although these proteins are simultaneously produced in infected cells. Accordingly, the coexistence of the cleaved mature gag proteins and/or RT seems to be insufficient for particle formation. Furthermore, in FS mutant-infected cells, neither recognizable budding structures nor extracellular viral particles could be detected. These RVVs expressed mainly cleaved mature p17 and p24/25 (Fig. 3). However, a 25K protein instead of p24 was predominantly observed in cell lysates. This protein is an intermediate precursor from which p24 is generated by removal of 14 C-terminal amino acids, and is detected in HIV-infected cell lysates but scarcely detected in HIV particles purified from culture medium (Henderson et al., 1988; Mervis et al., 1988). These findings suggest that p25 is generated in the cytoplasm, and that effective cleavage for p24 production is achieved at the budding site at the cell membrane during
and/or after assembly. Lack of viral assembly with FS in the extracellular space of cells infected with the m0GV and m0G5 constructs which encode the protease gene (Fig. 2 and 4a to e). On the other hand, only immature HIV-like particles were detected by transmission electron microscopy when the p48 region, which does not contain the protease region, was expressed (Fig. 5a, b). Therefore, the protease gene appears to be involved in the morphological maturation of viral particle cores. In accordance with this, recent evidence shows that the introduction of mutations within the retrovirus protease gene blocks maturation of virions and infectivity (Katoh et al., 1985; Meyenhofer et al., 1985; Peng et al., 1989). Both m0GV and m0G5 contain the entire gag gene and the protease-encoding region of the pol gene, and express not only the Pr55\textsuperscript{gp} precursor and p41 intermediate but also measurable amounts of cleaved mature p17 and p24. Therefore, molecular maturation and processing of the gag and gag-pol large precursor polyproteins appears to occur either during or after, but not prior to, viral assembly.

References

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