Mutations altering the Moloney murine leukemia virus p12 Gag protein affect virion production and early events of the virus life cycle

Bing Yuan, Xingqiang Li¹ and Stephen P.Goff^{1,2}

Integrated Program in Cellular, Molecular and Biophysical Studies and
¹Howard Hughes Medical Institute and Department of Biochemistry
and Molecular Biophysics, College of Physicians and Surgeons,
Columbia University, New York, NY 10032, USA

²Corresponding author
e-mail: goff@cuccfa.ccc.columbia.edu

The p12 Gag protein of Moloney murine leukemia virus is a small polypeptide of unknown function, containing two proline-rich motifs. To determine its role in replication, we introduced a series of deletion and alanine-scanning substitution mutations throughout the p12 coding region of a proviral DNA, and characterized the phenotypes of the resulting mutant viruses. Complete deletion of p12 and mutations affecting the PPPY motif caused substantial reduction in the yield of virions and a modest reduction in Gag processing. Proteolytic cleavage of the R-peptide from the cytoplasmic tail of the envelope protein TM was abolished in these mutants, suggesting that the PPPY motif is crucial for the viral protease to access the TM tail. The resulting virions were non-infectious, and unable to initiate DNA synthesis in infected cells. Mutants with alterations in both the N- and C-terminal portions of p12 exhibited a distinct phenotype. The production of virions and processing of Gag, Pol and Env precursors were normal. The viruses were able to direct synthesis of linear viral DNA, but there was almost no detectable circular DNAs or LTR–LTR junction. These data suggest that p12 plays a critical role in the early events of the virus life cycle.

Keywords: DNA circles/early events/TM processing/
virion production

Introduction

The retroviral Gag protein plays important roles at several stages in the viral life cycle (Wills and Craven, 1991; Freed, 1998). Late in infection, the Gag precursor protein acts to mediate the assembly and release of virion particles. During and soon after assembly, the Gag precursor is cleaved by the viral protease into a number of separate proteins found in the mature virion (Vogt *et al.*, 1975; Arcement *et al.*, 1976, 1977). These separated Gag proteins then play roles in the early stages of infection, and may facilitate virus entry, uncoating and possibly nuclear entry of the viral DNA. In the case of the Moloney murine leukemia virus (M-MuLV), these proteins are termed MA (for matrix or membrane-associated), p12 (an unnamed protein), CA (for capsid) and NC (for nucleocapsid) (Leis *et al.*, 1988). The functions of many of these proteins

have been determined by analysis of mutations, and the names that have been assigned to these proteins correspond to their localization and function (Wills and Craven, 1991).

Among the MuLV *gag* gene products, only the p12 protein remains without any clear function, and therefore is as yet unnamed. p12 is only 84 amino acid residues in length. The protein is very proline rich, and contains two polyproline motifs: one of sequence PPPY and one PPPS. A small fraction of the p12 molecules are phosphorylated, and some are modified by the covalent addition of ubiquitin (Sen *et al.*, 1976, 1977; Sen and Todaro, 1977; Ott *et al.*, 1998). The localization of the protein in the virion is unknown. Deletions affecting the N-terminal portion of p12 have been generated; the mutant genomes directed the assembly of normal levels of virions, but infection by these mutant virions was blocked at a very early stage before the synthesis of viral DNA (Crawford and Goff, 1984). These results suggest that p12 may play a role early in the life cycle.

Mutational studies of the Gag proteins of Rous sarcoma virus (RSV), Mason–Pfizer monkey virus (M–PMV) and human immunodeficiency virus 1 (HIV-1) have resulted in the identification of a proline-rich domain, which in each case is required for late stages of viral assembly and release (Wills *et al.*, 1994; Huang *et al.*, 1995; Parent *et al.*, 1995; Xiang *et al.*, 1996; Yasuda and Hunter, 1998). This ‘L-domain’, often containing the amino acid sequence PPPY, is located in different positions in the Gag proteins of various retroviruses: in the small p2b protein of RSV; in the p24 domain of M–PMV; in the C-terminal p9 protein of equine infectious anemia virus (EIAV); and in the p6 protein of HIV-1 (Wills *et al.*, 1994; Huang *et al.*, 1995; Puffer *et al.*, 1997; Yasuda and Hunter, 1998). The domain from one virus can often function in place of the corresponding sequence of another virus, suggesting that the sequences employ similar mechanisms and can act autonomously in many contexts (Parent *et al.*, 1995; Xiang *et al.*, 1996). The domain may serve as the docking site for particular host proteins, as the sequence is a nearly perfect match to the consensus binding site of the WW domain, a widely used interaction domain in many cellular proteins (Garnier *et al.*, 1996; Sudol, 1998). Mutations affecting the PPPY motif cause an arrest of virion budding at late stages, just before release from the cell, and a significant reduction in the yield of virus (Wills *et al.*, 1994; Xiang *et al.*, 1996; Yasuda and Hunter, 1998). In the case of M-MuLV, the closest match to these sequences lies in p12.

To identify the functions of the MuLV p12 protein, we have generated a series of deletion and substitution mutations throughout the p12 coding region of Gag. Some of these mutations are targeted to the PPPY motif of p12, and the others extend over the rest of the coding sequences. The results suggest that the PPPY motif may be the

Fig. 1. Structure of p12 mutants. (A) The M-MuLV Gag protein is represented by rectangular boxes. The location and size of p12 is indicated, and the full amino acid sequence of p12 is shown below. The blocks of residues changed to alanines in each substitution mutant and the names of the mutation are indicated. The PPPY and PPPS motifs are circled. (B) The structure of PPPY and PPPS mutants. Both deletion and alanine substitution mutations were introduced into the PPPY and PPPS motifs. Deletions are indicated (Δ) and alanine substitutions are shown as AAAAA aligned with wild-type sequence. Two of the mutants, DPYS and APYS, contain double mutations that affect both PPPY and PPPS motifs.

functional counterpart of the L domain of other retroviruses. This motif facilitates virus production, and is also essential for normal Gag processing, and surprisingly, envelope transmembrane (TM) processing. In addition, mutations elsewhere in p12 block early steps in infection, and some can strikingly and specifically block the formation of circular viral DNAs in the nucleus. These results suggest that p12 plays a crucial role in the early stages of infection, perhaps affecting trafficking or processing of the viral DNA.

Results

Construction of mutants

To determine the function of the p12 protein in the M-MuLV life cycle, a series of mutant proviral DNAs were generated. Mutant Del-p12 was created by removing the entire p12 coding region precisely at the cleavage sites between MA/p12 and between p12/CA. A series of mutants were made by introducing both deletion and alanine (Ala) substitutions in the PPPY and PPPS motifs (Figure 1B). Another series was made by replacing blocks of 4–6 amino acids throughout the rest of p12 by the same number of Ala residues (Figure 1A). Since the processing of the Gag precursor is critical for producing infectious virus (Crawford and Goff, 1984), the protease cleavage sites at the junction regions between MA/p12 and between p12/CA were not mutated.

Viability of p12 mutants

The effects of mutations on M-MuLV replication and infectivity were first analyzed by a virus spreading assay. All 18 mutant proviral DNAs were introduced into NIH 3T3 cells using the DEAE–dextran method and viral spread was monitored by assaying reverse transcriptase (RT) activity released into the culture medium. The wild-type viral DNA initiated a spreading infection and produced high levels of RT within 5 days. Twelve of the mutants failed to display any RT activity even 24 days after transfection (Figure 2A). These mutants included the Del-p12 mutant, the PPPY motif mutants (DPY and APY), the PPPY/PPPS double mutants (DPYS and APYS) and both N- (PM5, PM6, PM7 and PM8) and C-terminal (PM13, PM14 and PM15) mutants. These results suggest that both the N- and C-terminal regions of p12 must contain sequences that are important for virus replication.

Fig. 2. RT assay of p12 mutants. (A) Replication competence of p12 mutants by virus spreading assay. Proviral DNAs containing various mutations in the p12 region were introduced into NIH 3T3 cells by the DEAE–dextran method, and culture supernatants were harvested every 2 days up to 24 days after transfection. RT assays of the harvests at day 15 are shown as a measure of viral spread. (B) Viral assembly and particle release assayed after transient transfection of 293T cells. The mutations were subcloned into a plasmid vector containing the SV40 origin of replication, and 293T cells were transfected by the calcium phosphate method. Supernatants were collected 72 h after transfection and analyzed for RT activity. (C) Viral infection ability by virus spreading assay. Culture medium from (B) was collected 72 h after transfection and equal volumes were used to infect naïve NIH 3T3 cells. Culture supernatants were harvested every 2 days up to 15 days after infection. RT assays of harvests at day 9 are shown to indicate viral spreading ability. Mock, mock transfection and infection; WT, wild type; Del-p12, full-length p12 deletion mutant.

The six remaining mutants replicated with kinetics similar to that of the wild-type virus. All of these viable mutants (PM9, DPS, APS, PM10, PM11 and PM12) had alterations located in the central region of p12, including the PPPS motif, indicating that this region is not crucial for viral replication (Figures 1A and 2A). Of this set, only mutant PM12 showed a slight decrease in the rate of virus spread. The mutation in PM12 is located at the C-terminal border of this middle region, suggesting that these sequences may encroach on an important domain.

Virion production

To test directly whether the replication-defective mutants were affected in virion production, the mutant proviruses were moved into a plasmid containing an SV40 origin of replication, allowing high-level expression in cells expressing T antigen. 293T cells were then transfected

with the proviral DNAs by the calcium phosphate method, and supernatants were collected 72 h after transfection. The levels of virions produced were assessed by RT assay. All the mutants with alterations in the PPPY motif showed a significant defect in virion assembly or release. The Del-p12 mutant, the PPPY mutants and the PPPY/PPPS double mutants all produced 5- to 10-fold less virus than the wild-type virus (Figure 2B). These results suggest that p12, and especially the PPPY motif of p12, play a crucial role in virion production during the late stages of the life cycle. All the other mutants, including both the viable and non-viable mutants, produced wild-type levels of virions, suggesting no significant block to virion assembly or release (Figure 2B).

Early stages of viral infection

To examine whether the bulk of the non-viable mutants were blocked in the early phase of the life cycle, mutant viruses were collected from transfected 293T cells and used to infect fresh NIH 3T3 cells. The virus yields in the culture supernatants were monitored by RT assay for 2 weeks. The wild-type virus preparations infected the cultures efficiently and induced the formation of progeny virus as judged by the appearance of RT activity within 2–3 days. In contrast, mutants with alterations in the N- and C-terminal regions of p12 (PM5, PM6, PM7, PM8 and PM13, PM14, PM15) produced no RT activity even after 2 weeks (Figure 2C). Thus, although these mutants could assemble wild-type levels of virions in a transient transfection, these virions were not infectious. The mutants that produced only reduced yields of virus (the Del-p12 mutant and the PPPY mutants) were also unable to infect the cultures (Figure 2C). These data are consistent with the virus spreading assay (Figure 2A). Mutants with alterations only in the central region of p12 were fully viable, and could infect NIH 3T3 cells and initiate the production of virus. The time of appearance of RT activity for these mutants was similar to that for the wild-type virus.

Taken together, these data suggest that a single small sequence—the PPPY motif—is important in late steps of assembly; that the bulk of the p12 protein, including both the N- and C-terminus, is not important for assembly but is needed for early events; and that the central region is dispensable.

Analysis of virion proteins produced by p12 mutants

To characterize the virion particles produced by the mutants, the viral proteins and genomic RNA incorporated into the virions were examined directly. Seventy-two hours after transfection of 293T cells, the culture supernatants were collected and the virus particles were purified by ultracentrifugation through a sucrose cushion. The virions were lysed, the virion proteins were subjected to SDS-PAGE and a series of antibodies were used to detect viral proteins on Western blots.

The gag gene products

Because p12 is a component of the *gag* gene, mutations in p12 could affect the function and processing of the Gag precursor protein, as well as the levels of any of the cleavage products. To test these possibilities, antibodies

Fig. 3. Western blot analysis of *gag* gene products in the virions of p12 mutants. Viral particles collected from the culture supernatant of transfected 293T cells were lysed, and the virion proteins were separated by electrophoresis, blotted and probed with (A) anti-CA, (B) anti-p12 or (C) anti-MA polyclonal antiserum. The anti-p12 and anti-MA polyclonal antisera also show contaminating reactivity to CA. The positions of Pr65^{gag} precursor protein, CA, p12 and MA are indicated. WT, wild type; Del-p12, full-length p12 deletion mutant.

Fig. 4. Detection of RT protein in the virions of p12 mutants. Virion lysates were prepared as Figure 3, blotted and probed with anti-RT antiserum. The position of the RT protein is indicated by an arrow. WT, wild type; Del-p12, full-length p12 deletion mutant.

against CA, MA and p12 were used in Western blots to detect the levels of these proteins in the secreted virions. The wild-type virions contained high levels of each of the mature CA, MA and p12 proteins, and a small amount of unprocessed Pr65^{gag}. The majority of the mutants produced virions with nearly normal levels of CA, MA and p12 (Figure 3A–C). In many of these mutants, the position of migration of the p12 was slightly affected by the alteration in sequence. Those mutants that produced lower yields of virus based on RT levels—the Del-p12 mutant and the PPPY mutants (DPYS, DPY, APYS and APY)—showed obvious defects in virion production and Gag processing. The PPPY mutants produced 4- to 6-fold less of the CA and p12 proteins, and very low or no detectable MA protein in their virions. These same mutants showed an increased level of unprocessed Pr65^{gag} precursor protein compared with the control virus. Thus, there was a general inhibition in Gag processing and a specific failure to produce or accumulate the mature MA protein. The Del-p12 mutant showed a more severe defect, with even less *gag* gene products recovered in the virions.

RT protein

To assess the level of RT protein in virus particles, virion proteins were blotted and probed with anti-RT serum

Fig. 5. Western blot analysis of *env* gene products in the virions of p12 mutants. Virion lysates were prepared as Figure 3. (A) Anti-SU (gp70 Env) polyclonal antiserum was used to detect SU envelope protein. (B) Anti-TM monoclonal antibody was used to detect p15E and p12E TM proteins. The positions of gp70 Env, p15E TM and p12E TM proteins are marked as shown. WT, wild type; Del-p12, full-length p12 deletion mutant.

(Figure 4). The virions produced by the Del-p12 and PPPY mutants showed a ~5- to 10-fold decrease in the levels of RT protein, consistent with the reduced level of RT activity and the reduced levels of Gag proteins produced by these mutants. The virions of the remaining mutants contained nearly normal amounts of RT protein. Thus, there were no specific effects on the formation or incorporation of RT.

Envelope proteins

The envelope protein of M-MuLV is synthesized as a glycosylated precursor protein that is processed by cellular proteases into two subunits, SU and TM. To measure the levels of SU (gp70) protein present in virus, the virion proteins were blotted as before and probed with a polyclonal antibody. The Del-p12 and PPPY mutants produced significantly lower levels of SU protein compared with the wild-type virions (Figure 5A). Thus, per virion particle, the levels of envelope were close to normal. The virions of the remaining p12 mutants showed levels of SU similar to that of the wild-type virions. These findings are consistent with our other results that fewer virions were released by PPPY mutants and more severe decreases were seen with the Del-p12 mutant.

During virus maturation, the TM protein (p15E) is cleaved by the viral protease to yield a mature TM protein (p12E) and a 16-amino acid R peptide (Karshin *et al.*, 1977). This proteolytic cleavage of TM protein in the cytoplasmic tail is critical for virus infectivity (Ragheb and Anderson, 1994; Rein *et al.*, 1994). To test for the production of TM, virion proteins were separated by gel electrophoresis, blotted to nitrocellulose, and probed with a monoclonal antibody specific for the TM protein (Figure 5B). Strikingly, only the unprocessed p15E form was detected in the PPPY mutants; p12E TM protein could not be detected in these virions. Thus, the mutations in the PPPY motif completely abolished the p15E TM protein cleavage. The Del-p12 mutant also contained only p15E and no p12E; for this mutant there was significantly less total TM protein. PM8, an infection-defective mutant, showed a partial defect in TM cleavage, approximately a 1:1 ratio of p15E and p12E TM proteins. This mutant, with substitutions adjacent to the PPPY motif, also consistently showed subtle defects in p12, MA and RT protein levels in the virions (Figures 3B, 3C and 4), suggesting that the

Fig. 6. Detection of viral protein expression in transfected 293T cells. Seventy-two hours after transfection by mutant or wild-type proviruses, the transfected 293T cells were lysed and subjected to SDS-PAGE. Anti-CA polyclonal serum was used to detect viral CA and Pr65^{gag} protein level in the cell. CLN4 virus lysate was used as control to indicate the position of CA and Gag protein.

amino acids adjacent to PPPY motif may also play a role in viral assembly. The other mutant viruses contained normal levels of TM protein, and the bulk of the TM protein was cleaved to the mature p12E, suggesting that their TM proteins were processed normally. These results indicate that mutations in the PPPY motif not only impaired viral particle release but also affected the cleavage of the Gag precursor and the TM cytoplasmic tail. The failure of these viruses to cleave TM would account for the inability of the virions to enter the cell and carry out the early steps of the life cycle.

To rule out the possibility that the mutant viral proteins were poorly expressed by the transfected 293T cells, the intracellular levels of the proteins were measured. Cell lysates were prepared 72 h after transfection with wild type and five representative mutants (APY for PPPY mutants, PM6 for the N-terminal region, PM9 for the middle region, PM13 for the C-terminal region, and Del-p12) and the viral proteins were analyzed by Western blot. All the mutants, including APY and Del-p12, induced the expression of similar levels of Gag and CA proteins in the transfected cells, suggesting no significant defect in synthesis or stability of the mutant proteins (Figure 6). Therefore, the lower levels of virion release by the PPPY and Del-p12 mutants must be caused by defects in viral assembly or budding.

Encapsidation of genomic RNA into p12 mutant virions

To determine the effect of p12 mutations on viral RNA encapsidation, an RNase protection assay was performed to analyze viral genomic RNA in the mutant virions and transfected cells. Virions were collected from the supernatant of 293T cells transfected with the viral DNAs, and RNAs were extracted from both virions and transfected cells. An antisense radiolabeled RNA probe spanning the viral transcription initiation site and the major splice donor site was hybridized to the extracted RNAs, digested by single-strand-specific RNases, and analyzed by gel electrophoresis (Figure 7A). Virions produced by the wild-type virus contained high levels of the unspliced genomic RNA, and no significant levels of the spliced Env mRNA. The APY and Del-p12 mutants showed a 5- to 10-fold

Fig. 7. RNase protection assay of viral RNAs produced by p12 mutants. 293T cells were transfected with the p12 mutant or wild-type proviruses indicated. Seventy-two hours post-transfection, RNA from both transfected cells and virions were isolated and annealed with a radiolabeled antisense RNA probe. The RNAs were digested with single-strand-specific RNases and the protected RNA fragments were analyzed by denaturing PAGE and autoradiography. (A) Schematic diagram of the probe used in the RNase protection assay and the sizes of different protected fragments in the assay. The transcription initiation site and the splice donor site are indicated by an arrow and 'SD', respectively. (B) RNase protection assay results. For each of the indicated constructs, both cellular and virion-associated RNA levels were analyzed. F, fragment protected by the full-length unspliced viral genomic RNA (335 nt). S, fragment protected by the spliced viral RNA (205 nt). The anti-CA immunoblot assay was used to indicate the viral protein level in the corresponding cell or virion lysates.

reduction in the levels of genomic RNA recovered in purified virions, consistent with the reduced levels of virions released by these mutants. Thus, per virion particle produced, the mutants packaged normal levels of viral RNA. Wild-type levels of genomic RNA were detected in the virions produced by mutants PM6, PM9 and PM13. All of the mutants produced normal levels of intracellular RNAs, including both the unspliced and spliced RNAs (Figure 7B). Taken together with the previous Western blot results of viral protein incorporation, these results suggest that there was no significant defect in the incorporation of RNA.

To monitor the ability of the packaged RNA genome to serve as a template for reverse transcription, we performed endogenous RT reactions on preparations of selected mutant virions (PM6 and PM13). The virions were incubated with triphosphates, and the DNA was analyzed by gel electrophoresis followed by autoradiography. These assays showed that normal levels of minus-strand strong stop viral DNAs could be synthesized, indicating that the virion-associated RT enzyme and RNA were correctly assembled into a conformation capable of making viral DNA (data not shown).

Fig. 8. Southern blot detection of linear and circular viral DNA synthesis following infection of NIH 3T3 cells by p12 mutant viruses. NIH 3T3 cells were infected by the supernatants of transfected 293T cells as described in Figure 2B and the Hirt method was used to harvest low-molecular-weight DNA 24 h after infection. (A) Southern blotting was performed by using a radiolabeled viral DNA probe. The positions and sizes of viral linear and circular DNAs are indicated. A specific probe for mitochondrial DNA was also used to normalize the amount of DNA. (B) PhosphorImager quantitation of relative linear viral DNA products after normalizing for loading and background. The amount of linear DNA produced by wild-type virus (WT) is set as 100. (C) PhosphorImager quantitation of relative circular viral DNA products after normalizing for loading and background. WT is also set as 100.

Analysis of viral DNA synthesis in infected cells

Shortly after M-MuLV infection, RT synthesizes double-stranded viral DNA from the viral genomic RNA. This linear viral DNA then enters into the nucleus in normally dividing cells and is integrated into the host chromosomal DNA. Circular forms of the viral DNA containing one or two copies of the LTR can also be detected in the nucleus of the cell. Although these circles do not integrate into the host genome, they serve as a hallmark of nuclear entry by the viral DNA (Ellis and Bernstein, 1989; Lobel *et al.*, 1989).

To determine the stage at which the replication-defective mutants were blocked during the early phase of the life cycle, we tested whether the mutants could direct the synthesis of various viral DNA forms *in vivo*. NIH 3T3 cells were acutely infected with the mutant viruses harvested from transfected 293T cells, using equal amounts of virus as judged by the RT activity in the preparations. The viral DNA was then isolated 24 h after infection using the Hirt method and analyzed by Southern blotting with a viral probe (Figure 8A). The same Southern

blot membrane was also stripped and hybridized with a mitochondrial DNA probe as a loading control. The relative amounts of linear and circular viral DNA in cells infected by the different mutants were calculated after normalization to the control DNA. In these experiments, the wild-type virus directs the synthesis of high levels of linear DNA and the two circular DNAs (Figure 8).

The different mutants displayed a number of distinct phenotypes.

Linear DNA but no circular DNAs

Five of the mutants showed a remarkable result: two mutants with substitutions in the N-terminal region (PM5 and PM7) and all three mutants with substitutions in the C-terminal region (PM13, PM14 and PM15) showed nearly normal levels of linear viral DNA but no detectable circular DNAs in the Hirt extracts (Figure 8). Thus, these mutant virions were able to enter the cell and perform reverse transcription to generate linear DNA, but were apparently blocked before the formation of circles. Although there was some variation in the amount of linear DNA synthesized by these mutants in different experiments (20–100% of wild type), we did not detect circular viral DNA in any of the experiments. This result suggests that p12 must play a role in the intracellular transport of the DNA, or in the release of the viral DNA termini normally associated with formation of the circular forms. The PM12 mutant also showed a very mild version of this phenotype, producing about half the normal amount of circular DNA (Figure 8).

Less linear DNA, no circular DNAs

Mutants PM6 and PM8 showed a second phenotype: infected cells produced only low levels of linear DNA, ~10-fold less than the wild-type control. As in the first group, no circles could be detected. These results suggest that these two mutants are partially blocked during the very early stages of viral infection, before or during reverse transcription. Both of these mutants are located in the N-terminal region of p12.

Normal DNA synthesis

All of the mutants that were replication-competent and capable of mediating a spreading infection showed normal levels of linear and circular forms. These mutants included the PPPS mutants and the remaining substitution mutants affecting the central portion of p12. These mutants show no aberrant DNA synthesis.

Block to both linear and circular DNA synthesis

The Del-p12 and the PPPY mutants produced almost no viral DNA in the infected cells, indicating that the low levels of virions produced by these mutants were almost completely uninfected.

These analyses of DNA species were repeated several times in NIH 3T3 cells with similar outcomes. Experiments in Rat2 cells infected by these mutants also revealed similar results (data not shown), indicating that these phenotypes are not restricted to mouse cells.

Detection of circular viral DNA by PCR

To confirm further that circular viral DNAs were not made in cells infected by the replication-defective p12 mutants,

Fig. 9. Analysis of viral circular DNA in the infected NIH 3T3 cells by p12 mutant viruses. The Hirt DNAs prepared as described in Figure 8 were used as template and a circular DNA-specific PCR product flanking the LTR–LTR junction region of two-LTR circles was amplified. The migration positions of the PCR product are indicated with an arrow.

we used a more sensitive PCR method to amplify the LTR junction region of the two LTR DNA circles from extracts. Oligonucleotides annealing in the R and U3 regions of the LTR were used to prime PCR reactions with DNA isolated from acutely infected cells, and the products were analyzed by gel electrophoresis. The LTR–LTR junction was readily detected in cells infected by wild-type virus and by the viable mutants with mutations in the middle region of p12 (Figure 9). However, PCR products diagnostic of circular DNA were not detected in the replication-defective mutants, including those mutants that produce normal levels of the linear DNA (Figure 9). These results are fully consistent with those obtained by Southern blotting, and suggest that these mutants are strongly impaired in production of circular DNA. This is a novel phenotype for Gag mutants, and it suggests a major role for p12 in the early events of the viral life cycle. Mutant PM12 displayed low levels of the circular DNA, corresponding to the partial defect in its replication.

Discussion

The analyses of the p12 mutants presented above suggest that the functions of the MuLV p12 Gag protein are diverse and more complex than previously anticipated (Figure 10). First, the mutants suggest that p12 plays a role in viral assembly or release; mutants with alterations in the PPPY motif showed a significant defect in virion production. The yields of virus particles were reduced by a factor of 5–10, consistent with the notion that the PPPY motif is an L-domain, similar to those found in other retroviruses. Surprisingly, these PPPY mutants also reduced Gag protein processing and completely abolished TM protein maturation. Secondly, two mutants (PM6 and PM8) located in the N-terminal region of p12 showed a dramatic reduction in overall viral DNA synthesis, suggesting a role for p12 early in virus entry, either before or during reverse transcription. Thirdly, five of the mutants, including two with alterations in the N-terminal (PM5, PM7) and all three with alterations in the C-terminal (PM13, PM14, PM15) portions of p12, made normal levels of linear viral DNA but did not make detectable circles. Finally, the central portion was highly tolerant of mutations, consistent with earlier tests of linker insertion mutations (Lobel and Goff, 1984). It is remarkable that such a small protein can be involved in so many distinct steps in the life cycle.

Fig. 10. Functional domains of p12 protein. (A) Schematic diagram of the location of different functional domains within p12. (B) Properties of different p12 mutants. The mutants can be classified into four functional groups: (i) PPPY mutants, which affect viral assembly, particle release and protein maturation. (ii) PM6 and PM8, which are located at the N-terminus of p12 and affect early events in virus entry. (iii) PM5, PM7, PM13, PM14 and PM15 mutants, which can make linear viral DNA but not circular DNA. The major blocking step for these mutants must be after viral DNA synthesis. (iv) PM9, PPPS, PM10, PM11 and PM12 mutants, which are located in the middle region of p12 and have no effects on viral replication.

Function of PPPY motif in M-MuLV p12

All of the mutants with alterations in the PPPY motif showed a significant decrease in the yield of virions, though not an absolute block to virion production. The nearby PPPS motif was apparently unimportant for any aspect of virus replication. These results suggest that the PPPY motif is an important functional domain for virus assembly and release, and are consistent with the notion that it may serve as the L-domain for M-MuLV. The mutations showing this effect were quite narrowly restricted to the PPPY motif itself; of all the other mutants, only mutant PM8 which affected residues upstream of the PPPY motif showed even a partial effect on assembly. Previous studies have shown that the Gag precursor proteins of many diverse retroviruses contain a PPPY motif between the MA and CA domains with similar function. John Wills and his colleagues first found that the PPPY motif within the RSV p2b protein plays a key role in virus budding (Wills *et al.*, 1994). Mutants lacking the motif were arrested at late stages of virion budding,

with a stalk holding the virion to the cell surface. Similarly, the PPPY motif in M-PMV was demonstrated to be critical for viral particle release (Yasuda and Hunter, 1998). In lentiviruses, a small protein (p6 for HIV-1 and p9 for EIAV) located at the C-terminus of the Gag precursor protein functions as the equivalent L-domain. A PTAPP motif is conserved among L-domains of many lentiviruses; EIAV is an exception, containing a YXXX motif (Huang *et al.*, 1995; Puffer *et al.*, 1997). Interestingly, the PPPY motif of RSV Gag is position independent and functionally interchangeable with the HIV-1 p6 and EIAV p9 proteins (Parent *et al.*, 1995; Xiang *et al.*, 1996). These data suggest that different retroviruses may have similar mechanisms for viral particle release, although regions with diverse amino acid sequences seem to be able to mediate these processes.

One possible mechanism for the function of the L-domain is through interactions with host factors involved in assembly. The PPPY motif has been demonstrated to be a binding site for the WW domain, a sequence motif

containing 38–40 amino acids with two highly conserved tryptophan residues (W) spaced widely apart (Garnier *et al.*, 1996). The WW motif was first found in a signaling molecule called Yap which binds with the Yes protein tyrosine kinase (Sudol *et al.*, 1995a,b). Recent studies found that this motif mediates protein–protein interaction and appears in many signaling and cytoskeletal proteins (Sudol, 1996). The consensus sequence of the binding motif for WW domains is PPxY, similar to the PPPY motif in the late assembly domains of many retroviruses (Sudol, 1998). Thus, it is possible that in the late stages of virion assembly, a cellular protein containing a WW domain might interact with the PPPY motif of the L-domain and facilitate the budding process. The sequences immediately flanking the PPxY motif may also be important for the binding of different WW domains. A recent study of the mitotic prolyl isomerase, Pin1, and the ubiquitin ligase, Nedd4, found that the WW domains may not only bind to the PPxY motif, but can also function as phosphoserine- or phosphothreonine-binding modules (Lu *et al.*, 1999). Interestingly, the p12 protein is known to be phosphorylated on serine and threonine residues at a low level (Sen *et al.*, 1976, 1977; Sen and Todaro, 1977), and thus it is possible that the binding of a host protein WW domain could be regulated by phosphorylation. However, since the HIV-1 and EIAV L-domains have different core sequences, there may be different cellular proteins that provide this function (Puffer *et al.*, 1998). Further study is needed to identify the actual host protein that may interact with p12 to promote virion production.

The proteolytic processing of Gag and TM proteins was severely affected by mutations in the PPPY motif (Figures 3 and 5B). How the mutants may affect protease activity during virion maturation is not clear. Analysis of RSV mutants with alterations in p2, particularly the polypyrroline stretch, led to the suggestion that p2 might play a role in controlling protease activation directly (Bowles *et al.*, 1994). However, the primary effect of the PPPY mutations might be on assembly; in both RSV and M-PMV, mutations in the L-domain could still block virion budding even when the viral protease was inactivated (Wills *et al.*, 1994; Yasuda and Hunter, 1998). Protease activation is likely to be coupled to assembly, and effects on assembly might well result in defects in protease activation. Since the M-MuLV PPPY motif is located in the middle of a domain involved in Gag–Gag multimerization (Alin and Goff, 1996b; Li *et al.*, 1997), this motif may be important in bringing Gag and Gag–Pol proteins together in the correct conformation to serve as substrate for protein processing. It is possible that the mutant virions may have major defects in overall structure, morphology and maturation. The absence of MA seen in the mutant virions may reflect selective reduction in cleavage at the MA–p12 junction, enhanced lability of any mature MA to abnormal internal proteolysis or significant loss from the virions after maturation. Although our studies suggest that the density and sedimentation properties of the mutant virions are not grossly different from controls, electron microscopy of the budding structure or the mature virions may help identify more subtle aberrations.

The complete block to p15E TM protein maturation caused by the PPPY mutations is an unexpected and novel

phenotype. Previous work has suggested that in other retroviruses a different domain of Gag, MA, may be involved in binding to TM, and in the processing of TM. Specific mutations in HIV-1 MA can block the incorporation of wild-type HIV-1 envelope; deletions in the TM cytoplasmic tail can relieve this block and permit normal Env incorporation (Freed and Martin, 1995, 1996). HIV-1 virions pseudotyped with M-MuLV envelope protein can efficiently cleave the M-MuLV TM protein cytoplasmic tail by HIV protease, but certain mutations within the HIV MA coding region can inhibit this protein processing and impair the viral infectivity (Kiernan and Freed, 1998). Mutations affecting the M-PMV MA protein can severely block TM protein maturation (Brody *et al.*, 1992). In the murine viruses, it is possible that p12, either with or without MA, is involved in contacts to TM, and may control TM processing. p12 might directly hold the TM tail in a conformation that can be recognized by the viral protease, or it may organize the capsid structure to permit protease to have access to the TM tail. The functions of p12 could also be carried out by indirect mechanisms; the PPPY motif may bind with host proteins that can bring TM protein and protease together. Further study is needed to reveal the actual mechanism. However the effect is achieved, the failure of the p12 mutants to accomplish the cleavage of p15E TM to p12E should completely block the infectivity of the virus (Ragheb and Anderson, 1994; Rein *et al.*, 1994), and can fully account for the replication-defect phenotype of the mutant virions.

Function of p12 protein in early events of the virus life cycle

The majority of the mutations scattered throughout p12 caused a strong block in the early stages of infection. Some of the mutants near the N-terminus of p12 were blocked very early, and synthesized only very low levels of viral DNA. These mutants are similar to earlier deletion mutants in the N-terminal portion of p12 (Crawford and Goff, 1984). Mutations affecting several viral proteins, including MA, CA and RT, can prevent virus infection at this stage (Schwartzberg *et al.*, 1984; Yu *et al.*, 1992; Craven *et al.*, 1995; Alin and Goff, 1996a; Casella *et al.*, 1997; Kiernan *et al.*, 1998). It is likely that during infection, p12, MA and CA may together take part in an uncoating process to change the conformation of the intracellular virus and allow reverse transcription to commence.

Five mutants with substitutions affecting both N- and C-terminal portions of p12 could mediate normal virion assembly and maturation, and could synthesize linear viral DNA after acute infection of NIH 3T3 cells, but could not produce detectable circular viral DNAs. These are the first retroviral mutants identified to be blocked at this stage. Circular DNAs are not thought to serve as substrates for formation of the integrated provirus, but rather are side products generated as an alternative to integration. The formation of the two-LTR circular DNAs is thought to be mediated by host DNA ligases, and is considered to be a hallmark of entry into the nucleus. The failure of the p12 mutants to generate these DNAs could have several explanations. One possibility is that p12 may play an important role in the nuclear entry of the viral DNA. The process of nuclear entry is a poorly understood step in

the retrovirus life cycle and is likely to be a coordinated process regulated by many viral and cellular proteins. In HIV-1 and other lentiviruses, the viral DNA can enter the nucleus even in non-dividing cells, probably through an active import process (Goldfarb, 1995; Stevenson, 1996). Various viral proteins—MA, Vpr and IN—have been suggested to mediate this nuclear import (Farnet and Haseltine, 1991; Bukrinsky *et al.*, 1993; Heinzinger *et al.*, 1994; Gallay *et al.*, 1995; Popov *et al.*, 1998). However, in the simple mammalian viruses, the viral DNA cannot enter the nucleus of nondividing cells, and can only be detected in the nucleus after cells pass through mitosis (Roe *et al.*, 1993). It is possible that p12 is somehow involved in the incorporation or retention of the viral DNA into the nucleus during this process. The cells infected in our experiments are dividing rapidly, and it is not obvious how the p12 mutants would be prevented from having access to the nucleus.

An alternative possibility is that the termini of the viral DNA could be trapped or blocked in the mutant preintegration complexes, and not accessible to the ligases that would form circles. According to this model, the normal role of p12 would be to uncoat or release the viral DNA in a form that could be available for integration into the host genome. A variation on this theme is that p12 might be responsible for holding the DNA termini in a correct conformation for integration; this conformation might include the juxtapositioning of the two termini, which could promote both integration and end ligation. Recent work suggests that the termini of the viral DNA in the preintegration complex are normally held by a large protein complex (Wei *et al.*, 1997, 1998; Chen *et al.*, 1999). DNA footprints suggest that hundreds of base pairs near the termini are protected in the complex. However, the proteins involved are not known, and CA is the only Gag protein shown to be present in the preintegration complex (Bowerman *et al.*, 1989). Examination of the state of the DNA in the cells abortively infected by the p12 mutants might help reveal whether the complex is affected.

The folded structure of p12 is unknown. It is noteworthy that the mutations with a given phenotype tended to cluster in the primary sequence of p12, suggesting some domain structures. The PPPY motif was quite distinct from the rest of the molecule in function, and was sharply bounded by regions with other functions. The N- and C-terminus contained two stretches with a similar phenotype, suggesting that they might fold together; they were separated by a large region that was highly tolerant of mutations, perhaps looped out in the structure. The location of p12 in the virion is also unclear. The mutational study described here suggests that p12 may be in contact with TM in the virion, and thus near the envelope. Finally, the fate of p12 after virion entry is unclear. The results obtained here suggest that p12 is likely to be retained in the preintegration complex, and may play an important role in determining its conformation. We hope that further analysis of the state of the viral DNA in mutant virus-infected cells will help reveal that role.

Materials and methods

Cells and viruses

NIH 3T3 fibroblasts, 293T cells and Rat2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%

calf serum. Wild-type M-MuLV was harvested from either the 293T cells after transfection with the plasmid pNCS or the NIH 3T3 clone 4 (CLN4) cells, a cell line constitutively producing wild-type viruses (Schwartzberg *et al.*, 1983).

Plasmids and mutants construction

pNCA contains an infectious copy of M-MuLV proviral DNA (Colicelli and Goff, 1988); pNCS is a version of pNCA that carries a simian virus 40 (SV40) replication origin to allow high-level expression in 293T cells.

Mutations were generated by PCR using a subclone of the Gag region. The native *pfu* DNA polymerase (Stratagene) was used to limit possible random mutagenesis. Primers to amplify the *Bsr*GI-*Xho*I fragment were as follows: BSRGI (forward), 5'-CCTTTGTACACCTAAGCC-3'; DP12 (reverse), 5'-GGCGAAGCTTCTCGAGGGGAAAAGCG-3'.

The oligonucleotides used to introduce mutations of the PPPY and PPS motifs of p12 were as follows. Deletion mutants: PD1 (DPYS), 5'-CCTACTTACAGAAAGGGACCCAAGAGACAGGGACGG-3'; PD2 (DPY), 5'-CCTACTTACAGAAAGGGACCCAAGACC-3'; PD3 (DPS), 5'-AGGGACCCAAGAGACAGGGACGG-3'. Alanine substitution mutants: PM1 (APYS), 5'-CAGAAGACGCCGCGCTGCTAGG-GACCCAAGAGCAGCCGCTGCCGACAGGG-3'; PM2 (APY), 5'-CTA-CTTACAGAAGACGCGAGCTGCCGCAAGGGACCCAAGACC-3'; PM3 (APS), 5'-CTTATAGGGACCCAAGAGCAGCTGCCGCAAGACAGGG-ACGGAATG-3'.

The alanine scanning method was used to make mutations throughout the p12 coding regions. The primers were: PM5, 5'-CTCCTTCTC-TAGGCGCCGCGAGCTGCAGCAGCTGTTCTTTCTGACAGTGGG-3'; PM6, 5'-CGCCAAACCTAAACCTCAAGCAGCTGCAGCTGCAGG-GGGGCGCTCATC-3'; PM7, 5'-CTCAAGTTCTTTCTGACAGT-GCAGCTGCAGCTGCCGACCTACTTACAGAAGACC-3'; PM8, 5'-G-GGGGCGCTCATCGCAGCTGCAGCTGCCGACCTCCCGCCTT-ATAGGG-3'; PM9, 5'-GAAGACCCCCCGCTTATGCTGCAGCTG-CACACCCCTTCCGACAG-3'; PM10, 5'-CAAGACCACCCCTT-TCGCGAGCTGCAGCTGCAGCTGGAGAAGCGACCCCTG-3'; PM11, 5'-GACAGGGACGGAATGGTGCAGCTGCCGACCTGCCGGAG-AGGCACCG-3'; PM12, 5'-GAGAAGCGACCCCTGCCGCGAGCTG-CAGCTGCACCCCTCCCAATGGCATC-3'; PM13, 5'-GGGAGA-GGCACCGGACGCGAGCTGCAGCTGCATCTCGCCTACGTGG-3'; PM14, 5'-CCCTCCCAATGGCAGCAGCTGCAGCTGCAAGACG-GGAGCCCCCTG-3'; PM15, 5'-CATCTCGCCTACGTGGGGCAGCT-GCAGCTGCAGTGGCCGACTCCACTAC-3'.

The presence of all mutations was confirmed by DNA sequencing.

Mammalian cell transfection and viral infection

To examine virus viability, NIH 3T3 cells were transfected using the DEAE-dextran method. Assays for viral spreading were as previously described (Goff *et al.*, 1981; Gao *et al.*, 1997). To produce large amounts of virus and test the effects of mutations on viral assembly, 293T cells were transiently transfected by proviral DNAs containing the SV40 origin of replication using the calcium phosphate method. The culture medium was changed at 48 h post-transfection, and the virus was harvested 72 h post-transfection and used for further analysis. Infections of NIH 3T3 cells and Rat2 cells were carried out in the presence of polybrene (8 µg/ml) for 2 h.

Virus purification and analysis of viral proteins

Seventy-two hours after transfection of 293T cells with wild-type or mutant proviral DNAs, 7 ml of culture supernatant was collected from each plate (100 × 15 mm) of transfected cells. Cell debris was removed by filtering the medium through a 0.45 µm filter and HEPES buffer was added to a final concentration of 20 mM. Virions were first purified on a 25/45% sucrose-TNE step gradient for 2 h at 25 000 r.p.m. and 4°C in a Beckman SW41 rotor, suspended in 4 ml of TNE (50 mM Tris pH 7.5, 100 mM NaCl and 1 mM EDTA), and pelleted through a 25% sucrose cushion by centrifugation in the same rotor for 2 h at 25 000 r.p.m. The collected virions were suspended in TNE buffer and stored at -80°C.

For immunodetection of virion proteins, pelleted virus particles were lysed and subjected to SDS-PAGE. The proteins were transferred to nitrocellulose filters (Schleicher & Schuell) and probed by various antibodies. The membrane was stained by the ECL kit (Amersham Corporation) and exposed to X-ray film for detection. The filter could be stripped and re-probed with other antibodies. The antisera used in the Western blot assays included goat anti-CA serum (NCI serum #79S-804), goat anti-p12 serum (NCI serum #79S-559), goat anti-MA serum (NCI serum #76S-155), goat anti-gp70 serum (NCI serum #81S-127) and rabbit anti-RT serum (Blain and Goff, 1993). The monoclonal anti-

serum against TM envelope protein was collected from a rat hybridoma line (42-114) (Granowitz *et al.*, 1996).

Analysis of viral RNA incorporation in virions

An RNase protection assay was used to analyze levels of viral RNA incorporation in the virion. An antisense, ³²P-labeled RNA probe was prepared by the MAXIScript™ *In vitro* Transcription Kits (Ambion) using the plasmid pMoR (Berkowitz *et al.*, 1995). RNAs were extracted from both virions and cell lysates by the RNazol™ B method (Tel-Test, Inc.) and were then hybridized and digested using the RPA II™ Ribonuclease Protection Assay Kit (Ambion). An endogenous reverse transcription assay was used to detect minus-strand strong stop DNA synthesis as described previously (Telesnitsky *et al.*, 1995).

Analysis of viral DNA synthesized in vivo

Preintegrative viral DNAs were isolated from cells 24 h postinfection (Hirt, 1967) and analyzed by Southern blotting. PCR was used to detect circular viral DNA containing two LTRs. Primers to amplify the LTR–LTR junction were MR5784, 5′-AGTCTCCGATTGACTGAG-3′ and MR4091, 5′-CTCTTTTATTGAGCTCGGG-3′ (Smith *et al.*, 1997); PCR conditions were 94°C for 1 min, 52°C for 1 min, 72°C for 2 min, repeated for 35 cycles.

Acknowledgements

We thank Guangxia Gao, Eran Bacharach and Ari Fassati for helpful discussions. We also thank Gilda Tachedjian and Sharon Boast for early readings of the draft. This work was supported by Public Health Service Grant CA 30488 from the National Cancer Institute. X.L. is an Associate and S.P.G. is an Investigator of the Howard Hughes Medical Institute.

References

- Alin, K. and Goff, S.P. (1996a) Amino acid substitutions in the CA protein of Moloney murine leukemia virus that block early events in infection. *Virology*, **222**, 339–351.
- Alin, K. and Goff, S.P. (1996b) Mutational analysis of interactions between the Gag precursor proteins of murine leukemia viruses. *Virology*, **216**, 418–424.
- Arcement, L., Karshin, W., Naso, R., Jamjoom, G. and Arlinghaus, R. (1976) Biosynthesis of Rauscher leukemia viral proteins: presence of p30 and envelope p15 sequences in precursor polypeptides. *Virology*, **69**, 763–774.
- Arcement, L.J., Karshin, W.I., Naso, R.B. and Arlinghaus, R.B. (1977) ‘Gag’ polypeptide precursors of Rauscher murine leukemia virus. *Virology*, **81**, 284–297.
- Berkowitz, R.D., Ohagen, A., Hoglund, S. and Goff, S.P. (1995) Retroviral nucleocapsid domains mediate the specific recognition of genomic viral RNAs by chimeric Gag polypeptides during RNA packaging *in vivo*. *J. Virol.*, **69**, 6445–6456.
- Blain, S.W. and Goff, S.P. (1993) Nuclease activities of Moloney murine leukemia virus reverse transcriptase. Mutants with altered substrate specificities. *J. Biol. Chem.*, **268**, 23585–23592.
- Bowerman, B., Brown, P.O., Bishop, J.M. and Varmus, H.E. (1989) A nucleoprotein complex mediates the integration of retroviral DNA. *Genes Dev.*, **3**, 469–478.
- Bowles, N., Donnet, D., Mulhauser, F. and Spahr, P.-F. (1994) Site directed mutagenesis of the p2 region of the Rous sarcoma virus gag gene: effects on Gag polypeptide processing. *Virology*, **203**, 20–28.
- Brody, B.A., Rhee, S.S., Sommerfelt, M.A. and Hunter, E. (1992) A viral protease-mediated cleavage of the transmembrane glycoprotein of Mason–Pfizer monkey virus can be suppressed by mutations within the matrix protein. *Proc. Natl Acad. Sci. USA*, **89**, 3443–3447.
- Bukrinsky, M.I., Sharova, N., McDonald, T.L., Pushkarskaya, T., Tarpley, W.G. and Stevenson, M. (1993) Association of integrase, matrix and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. *Proc. Natl Acad. Sci. USA*, **90**, 6125–6129.
- Casella, C.R., Raffini, L.J. and Panganiban, A.T. (1997) Pleiotropic mutations in the HIV-1 matrix protein that affect diverse steps in replication. *Virology*, **228**, 294–306.
- Chen, H., Wei, S.Q. and Engelman, A. (1999) Multiple integrase functions are required to form the native structure of the human immunodeficiency virus type I intasome. *J. Biol. Chem.*, **274**, 17358–17364.
- Colicelli, J. and Goff, S.P. (1988) Sequence and spacing requirements of a retrovirus integration site. *J. Mol. Biol.*, **199**, 47–59.
- Craven, R.C., Leure-duPree, A.E., Weldon, R.A., Jr and Wills, J.W. (1995) Genetic analysis of the major homology region of the Rous sarcoma virus Gag protein. *J. Virol.*, **69**, 4213–4227.
- Crawford, S. and Goff, S.P. (1984) Mutations in gag proteins p12 and p15 of Moloney murine leukemia virus block early stages of infection. *J. Virol.*, **49**, 909–917.
- Ellis, J. and Bernstein, A. (1989) Retrovirus vectors containing an internal attachment site: evidence that circles are not intermediates to murine retrovirus integration. *J. Virol.*, **63**, 2844–2846.
- Farnet, C.M. and Haseltine, W.A. (1991) Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex. *J. Virol.*, **65**, 1910–1915.
- Freed, E.O. (1998) HIV-1 gag proteins: diverse functions in the virus life cycle. *Virology*, **251**, 1–15.
- Freed, E.O. and Martin, M.A. (1995) The role of human immunodeficiency virus type 1 envelope glycoproteins in virus infection. *J. Biol. Chem.*, **270**, 23883–23886.
- Freed, E.O. and Martin, M.A. (1996) Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions. *J. Virol.*, **70**, 341–351.
- Gallay, P., Swingle, S., Song, J., Bushman, F. and Trono, D. (1995) HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell*, **83**, 569–576.
- Gao, G., Orlova, M., Georgiadis, M.M., Hendrickson, W.A. and Goff, S.P. (1997) Conferring RNA polymerase activity to a DNA polymerase: a single residue in reverse transcriptase controls substrate selection. *Proc. Natl Acad. Sci. USA*, **94**, 407–411.
- Garnier, L., Wills, J.W., Verderame, M.F. and Sudol, M. (1996) WW domains and retrovirus budding. *Nature*, **381**, 744–745.
- Goff, S., Traktman, P. and Baltimore, D. (1981) Isolation and properties of Moloney murine leukemia virus mutants: use of a rapid assay for release of virion reverse transcriptase. *J. Virol.*, **38**, 239–248.
- Goldfarb, D.S. (1995) HIV-1 virology. Simply Marvelous nuclear transport. *Curr. Biol.*, **5**, 570–573.
- Granowitz, C., Berkowitz, R.D. and Goff, S.P. (1996) Mutations affecting the cytoplasmic domain of the Moloney murine leukemia virus envelope protein: rapid reversion during replication. *Virus Res.*, **41**, 25–42.
- Heinzinger, N.K. *et al.* (1994) The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc. Natl Acad. Sci. USA*, **91**, 7311–7315.
- Hirt, B. (1967) Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.*, **26**, 365–369.
- Huang, M., Orenstein, J.M., Martin, M.A. and Freed, E.O. (1995) p6Gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. *J. Virol.*, **69**, 6810–6818.
- Karshin, W.L., Arcement, L.J., Naso, R.B. and Arlinghaus, R.B. (1977) Common precursor for Rauscher leukemia virus gp69/71, p15 (E) and p12 (E). *J. Virol.*, **23**, 787–798.
- Kiernan, R.E. and Freed, E.O. (1998) Cleavage of the murine leukemia virus transmembrane env protein by human immunodeficiency virus type 1 protease: transdominant inhibition by matrix mutations. *J. Virol.*, **72**, 9621–9627.
- Kiernan, R.E., Ono, A., Englund, G. and Freed, E.O. (1998) Role of matrix in an early postentry step in the human immunodeficiency virus type 1 life cycle. *J. Virol.*, **72**, 4116–4126.
- Leis, J. *et al.* (1988) Standardized and simplified nomenclature for proteins common to all retroviruses. *J. Virol.*, **62**, 1808–1809.
- Li, X., Yuan, B. and Goff, S.P. (1997) Genetic analysis of interactions between Gag proteins of Rous sarcoma virus. *J. Virol.*, **71**, 5624–5630.
- Lobel, L.I. and Goff, S.P. (1984) Construction of mutants of Moloney murine leukemia virus by suppressor-linker insertional mutagenesis: positions of viable insertion mutations. *Proc. Natl Acad. Sci. USA*, **81**, 4149–4153.
- Lobel, L.I., Murphy, J.E. and Goff, S.P. (1989) The palindromic LTR–LTR junction of Moloney murine leukemia virus is not an efficient substrate for proviral integration. *J. Virol.*, **63**, 2629–2637.
- Lu, P.J., Zhou, X.Z., Shen, M. and Lu, K.P. (1999) Function of WW domains as phosphoserine- or phosphothreonine-binding modules. *Science*, **283**, 1325–1328.
- Ott, D.E. *et al.* (1998) Ubiquitin is covalently attached to the p6Gag proteins of human immunodeficiency virus type 1 and simian

- immunodeficiency virus and to the p12Gag protein of Moloney murine leukemia virus. *J. Virol.*, **72**, 2962–2968.
- Parent, L.J. *et al.* (1995) Positionally independent and exchangeable late budding functions of the Rous sarcoma virus and human immunodeficiency virus gag proteins. *J. Virol.*, **69**, 5455–5460.
- Popov, S. *et al.* (1998) Viral protein R regulates nuclear import of the HIV-1 pre-integration complex. *EMBO J.*, **17**, 909–917.
- Puffer, B.A., Parent, L.J., Wills, J.W. and Montelaro, R.C. (1997) Equine infectious anemia virus utilizes a YXXL motif within the late assembly domain of the Gag p9 protein. *J. Virol.*, **71**, 6541–6546.
- Puffer, B.A., Watkins, S.C. and Montelaro, R.C. (1998) Equine infectious anemia virus Gag polyprotein late domain specifically recruits cellular AP-2 adapter protein complexes during virion assembly. *J. Virol.*, **72**, 10218–10221.
- Ragheb, J.A. and Anderson, W.F. (1994) pH-independent murine leukemia virus ecotropic envelope-mediated cell fusion: implications for the role of the R peptide and p12E TM in viral entry. *J. Virol.*, **68**, 3220–3231.
- Rein, A., Mirro, J., Haynes, J.G., Ernst, S.M. and Nagashima, K. (1994) Function of the cytoplasmic domain of a retroviral transmembrane protein: p15E-p2E cleavage activates the membrane fusion capability of the murine leukemia virus Env protein. *J. Virol.*, **68**, 1773–1781.
- Roe, T., Reynolds, T.C., Yu, G. and Brown, P.O. (1993) Integration of murine leukemia virus DNA depends on mitosis. *EMBO J.*, **12**, 2099–2108.
- Schwartzberg, P., Colicelli, J. and Goff, S.P. (1983) Deletion mutants of Moloney murine leukemia virus which lack glycosylated gag protein are replication competent. *J. Virol.*, **46**, 538–546.
- Schwartzberg, P., Colicelli, J. and Goff, S.P. (1984) Construction and analysis of deletion mutations in the pol gene of Moloney murine leukemia virus: a new viral function required for productive infection. *Cell*, **37**, 1043–1052.
- Sen, A. and Todaro, G.J. (1977) The genome-associated, specific RNA binding proteins of avian and mammalian type C viruses. *Cell*, **10**, 91–99.
- Sen, A., Sherr, C.J. and Todaro, G.J. (1976) Specific binding of the type C viral core protein p12 with purified viral RNA. *Cell*, **7**, 21–32.
- Sen, A., Sherr, C.J. and Todaro, G.J. (1977) Phosphorylation of murine type C viral p12 proteins regulates their extent of binding to the homologous viral RNA. *Cell*, **10**, 489–496.
- Smith, C.M., Potts, W.B.III, Smith, J.S. and Roth, M.J. (1997) RNase H cleavage of tRNA^{Pro} mediated by M-MuLV and HIV-1 reverse transcriptases. *Virology*, **229**, 437–446.
- Stevenson, M. (1996) Portals of entry: uncovering HIV nuclear transport pathways. *Trends Cell Biol.*, **6**, 9–15.
- Sudol, M. (1996) The WW module competes with the SH3 domain? *Trends Biochem. Sci.*, **21**, 161–163.
- Sudol, M. (1998) From Src Homology domains to other signaling modules: proposal of the 'protein recognition code'. *Oncogene*, **17**, 1469–1474.
- Sudol, M., Bork, P., Einbond, A., Kastury, K., Druck, T., Negrini, M., Huebner, K. and Lehman, D. (1995a) Characterization of the mammalian YAP (Yes-associated protein) gene and its role in defining a novel protein module, the WW domain. *J. Biol. Chem.*, **270**, 14733–14741.
- Sudol, M., Chen, H.I., Bougeret, C., Einbond, A. and Bork, P. (1995b) Characterization of a novel protein-binding module—the WW domain. *FEBS Lett.*, **369**, 67–71.
- Telesnitsky, A., Blain, S. and Goff, S.P. (1995) Assays for retroviral reverse transcriptase. *Methods Enzymol.*, **262**, 347–362.
- Vogt, V.M., Eisenman, R. and Diggleman, H. (1975) Generation of avian myeloblastosis virus structural proteins by proteolytic cleavage of a precursor polypeptide. *J. Mol. Biol.*, **96**, 471–493.
- Wei, S.Q., Mizuuchi, K. and Craigie, R. (1997) A large nucleoprotein assembly at the ends of the viral DNA mediates retroviral DNA integration. *EMBO J.*, **16**, 7511–7520.
- Wei, S.Q., Mizuuchi, K. and Craigie, R. (1998) Footprints on the viral DNA ends in moloney murine leukemia virus preintegration complexes reflect a specific association with integrase. *Proc. Natl Acad. Sci. USA*, **95**, 10535–10540.
- Wills, J.W. and Craven, R.C. (1991) Form, function and use of retroviral gag proteins. *AIDS*, **5**, 639–654.
- Wills, J.W., Cameron, C.E., Wilson, C.B., Xiang, Y., Bennett, R.P. and Leis, J. (1994) An assembly domain of the Rous Sarcoma Virus Gag protein required late in budding. *J. Virol.*, **68**, 6605–6618.
- Xiang, Y., Cameron, C.E., Wills, J.W. and Leis, J. (1996) Fine mapping and characterization of the Rous sarcoma virus Pr76gag late assembly domain. *J. Virol.*, **70**, 5695–5700.
- Yasuda, J. and Hunter, E. (1998) A proline-rich motif (PPPY) in the Gag polyprotein of Mason–Pfizer monkey virus plays a maturation-independent role in virion release. *J. Virol.*, **72**, 4095–4103.
- Yu, X., Yu, Q.-C., Lee, T.-H. and Essex, M. (1992) The C terminus of human immunodeficiency virus type 1 matrix protein is involved in early steps of the virus life cycle. *J. Virol.*, **66**, 5667–5670.

Received June 22, 1999; revised and accepted July 16, 1999