Linker Insertion Mutations in the Human Immunodeficiency Virus Type 1 *gag* Gene: Effects on Virion Particle Assembly, Release, and Infectivity

ALISE S. REICIN,[1] SANDRA PAIK,[2] ROBERT D. BERKOWITZ,[2] JEREMY LUBAN,[1]
ISRAEL LOWY,[1] AND STEPHEN P. GOFF[2,3*]

*Department of Medicine,[1] Department of Biochemistry and Biophysics,[2] and Howard Hughes Medical Institute,[3]
College of Physicians and Surgeons, Columbia University, New York, New York 10032*

The phenotypes of a series of mutant human immunodeficiency virus type 1 proviruses with linker insertion and deletion mutations within the *gag* coding region were characterized. These mutants were tested for their ability to make and release viral particles in COS7 cells and for their viability in vivo. Of the 12 mutant proviruses, 4 did not make extracellular virion particles when transfected into COS7 cells. All four of these mutants had mutations in the C-terminal domain of CA. These mutants appeared to have defects both in the ability to accumulate high-molecular-weight intracellular structures containing Gag and Pol products and in the ability to release virion particles. Seven of the mutant proviruses retained the ability to make, release, and process virion particles from COS7 cells. These particles contained the Env glycoprotein, viral genomic RNA, and the mature products of the Gag and Gag-Pol polyproteins, yet they were noninfectious or poorly infectious. The defect in these mutants appears to be in one of the early steps of the viral life cycle. Thus, multiple regions throughout Gag appear to be important in mediating the early steps of the viral life cycle.

The retroviral *gag* gene encodes the major structural proteins of the virion. Gag polyproteins can be assembled and released from cells as virion-like particles in the absence of other viral genes, suggesting that *gag* is the only viral gene essential for viral assembly (13, 18, 25, 31, 36, 38, 39). Human immunodeficiency virus type 1 (HIV-1) synthesizes a Gag precursor protein, Pr55^{gag}, and also a large Gag-Pol fusion protein, Pr160$^{gag-pol}$. During the process of virion maturation, Pr55^{gag} is cleaved by the viral proteinase to yield the individual proteins that make up the mature virion: MA (matrix; p17), CA (capsid; p24/p25), NC (nucleocapsid; p7), and the C-terminal peptide p6 (20).

MA, at the amino terminus of Pr55^{gag}, is intimately associated with the inner face of the viral membrane (12). MA is thought to play a central role in targeting the Gag precursor protein to the site of assembly on the plasma membrane (10, 41, 45, 52) and is essential for the stable association of the HIV-1 Env glycoprotein with the viral capsid (9, 45, 51). In addition, HIV-1 MA has recently been shown to be associated with the preintegrative complex and may be important in targeting this complex to the nucleus in quiescent cells (5, 6).

The CA protein makes up the major component of the mature virion electron-dense core. In murine leukemia virus, deletions and insertions in the CA domain of the Gag precursor protein interfered with viral assembly and release (14, 19, 26, 37). In Rous sarcoma virus (RSV), however, most of the CA coding region can be deleted without affecting viral assembly (46–48). Recent work with HIV suggests that portions of the N-terminal domain of CA may be deleted without affecting particle production (45), while mutations in the C terminus of CA block assembly of particles in baculovirus and vaccinia virus expression systems (7, 21, 24, 44). Within CA, there is a conserved motif designated the major homology region (MHR). This region has been shown to be important for par-

ticle assembly and viral infectivity in HIV-1 and Mason-Pfizer monkey virus (30, 42). Lastly, CA mutants which lack Pr160$^{gag-pol}$ in the virion have been reported, implying that CA may be important in the specific incorporation of Pr160$^{gag-pol}$ into virion particles. (40).

Between the C-terminal domain of CA and the N-terminal domain of NC is a short peptide termed p2. During the proteolytic processing of Pr55^{gag}, cleavage of the CA/p2 junction is slow, such that an intermediate form of CA, p25, can accumulate. The mature product of CA, p24, is generated from this intermediate by the removal of the 14 C-terminal amino acids. p25 is readily detected in the lysates of HIV-infected cells, but it is scarcely detected in HIV particles from culture medium (32). It has been suggested that effective cleavage of p25 to produce p24 is achieved at the budding site during or after assembly (22).

Nucleocapsid (p7) contains two zinc finger-like motifs, termed Cys-His boxes, and is responsible for the packaging of genomic RNA (2, 3, 8, 15). Portions of NC may also be important in the assembly and release of capsid particles, since mutations in the nucleocapsid which cause reductions in particle assembly have been described (8, 24). Finally, the C-terminal peptide sequence of HIV-1 contains the p6 protein, which appears to be important in mediating viral budding from an infected cell (16).

While it is clear that Gag proteins are essential for the formation and release of viral particles, these proteins have also been shown to play a role in the early steps of the viral life cycle (23, 42, 45, 50). It is likely that the structure of the Gag proteins is important for viral entry, efficient disassembly and release of a transcriptase-active core, and the import of the preintegrative complex into the nucleus of the cell.

We have previously reported the phenotype of a series of linker insertion mutations in the context of a Gag-protease polyprotein expressed in bacteria (29). In this bacterial system, protease was active, resulting in the cleavage of the Gag polyprotein into its mature products. Six linker insertion mu-

A

B

Mutant	Amino acid preceding insertion	Amino acid changes	
		From	To
H852	22	R - P	R-<u>LELE</u>-P
A906	40	E - L	E-<u>LELE</u>-L
A975	63	Q - L	Q-<u>LELE</u>-L
P1144	119	A - A	A-<u>ARAR</u>-A
Dr1337	183	D - <u>L</u> - N	D-<u>FSSSR</u>-N
A1411	208	E - A	E-<u>ARAR</u>-A
R1509	241	S - T	S-<u>LELE</u>-T
D1696	304	L - R	L-<u>TRARV</u>-R
Dr1787	333	I - <u>L</u> - K	I-<u>FSSSR</u>-K
A1906	374	A - T	A-<u>RARA</u>-T

C

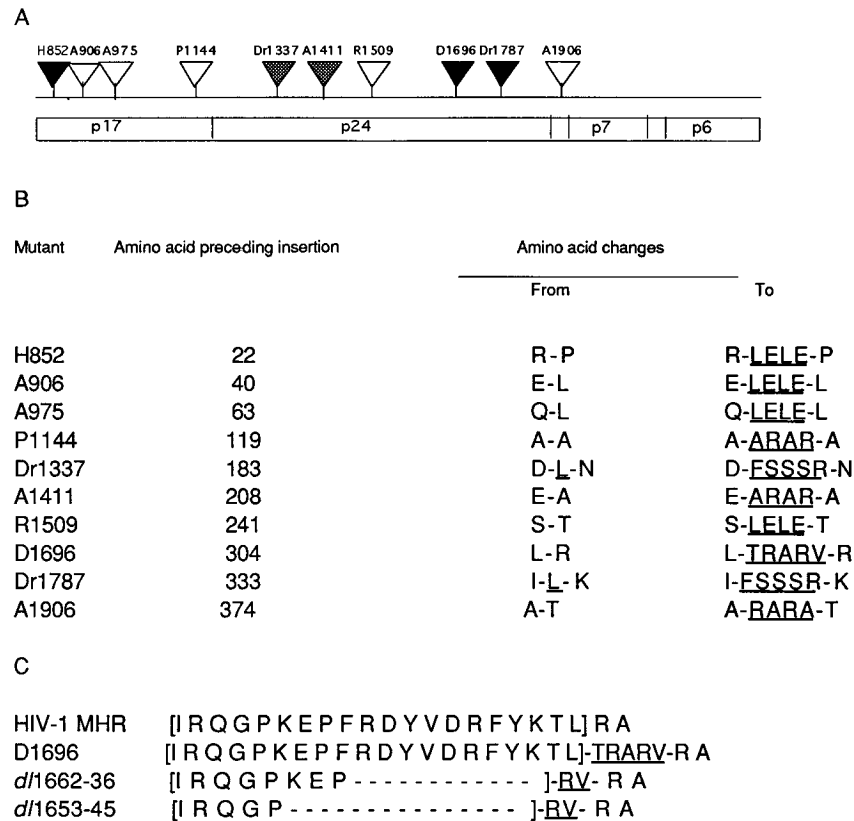|	
HIV-1 MHR	[I R Q G P K E P F R D Y V D R F Y K T L] R A
D1696	[I R Q G P K E P F R D Y V D R F Y K T L]-<u>TRARV</u>-R A
*dl*1662-36	[I R Q G P K E P - - - - - - - - - - - -]-<u>RV</u>- R A
*dl*1653-45	[I R Q G P - - - - - - - - - - - - - - -]-<u>RV</u>- R A

FIG. 1. (A) Insertion mutations in HIV-1 *gag*. The letter at the beginning of each mutant name indicates the restriction site used for linker insertion: A, *Alu*I; D, *Dde*I; Dr, *Dra*I; H, *Hae*I; P, *Pvu*II; R, *Rsa*I; the number indicates the nucleotide position of the restriction site with respect to the 5′ edge of the 5′ long terminal repeat of the HIV-1 (HXBc.2) provirus. White triangles indicate mutants that make wild-type-like particles; black triangles indicate mutants with defects in particle assembly and release; hatched triangles indicate mutants which release particles with an altered p24/p25 ratio. (B) Encoded protein sequences of wild-type and mutant Gag proteins. Amino acids lost or gained as a result of the mutation are underlined. (C) The 20-amino-acid sequence in the brackets represents the sequence of the MHR in HIV-1. Amino acids which are added as a result of the mutation are underlined. Amino acids which are lost as a result of the mutation are shown as dashed lines.

tations were found to disrupt the proteolytic processing of the Gag protein in this system, probably by preventing polyprotein dimerization. To further characterize the domains of the HIV-1 Gag precursor which are involved in particle assembly, particle budding, and the early steps of the viral life cycle, we have now placed 10 of these linker insertion mutations into wild-type proviral DNA. The mutant proviral DNAs were tested for their effects on infectivity, viral assembly, and the incorporation of the Env glycoprotein gp120, Pr160$^{gag-pol}$, and viral RNA. These studies indicate that the carboxy-terminal domain of CA is important in virion particle assembly and release, while multiple regions throughout Gag are important in mediating the early steps of the viral life cycle.

MATERIALS AND METHODS

Recombinant DNAs. The parent wild-type HIV-1 proviral DNA used in this study was plasmid R73/BH10 (generously provided by Mark Feinberg) (1). R73/BH10 contains the HXBc.2 provirus modified to be Vpu$^+$ and Nef$^+$ on a vector containing a simian virus 40 origin of replication. The construction of the bacterial expression plasmid pT7HG(pro+) and the linker insertions has been described previously (29). The 1.5-kb *Cla*I-*Bcl*I fragment containing each of these linker insertions was used to replace the corresponding wild-type fragment of the parent vector. To make deletions of the C-terminal region of the MHR of capsid, plasmid PT7HGD1696 (29), containing a *Xho*I linker insertion adjacent to the C terminus of the MHR, was linearized with *Xho*I. This linear fragment was then treated with BAL 31 exonuclease, blunted with the Klenow fragment of DNA polymerase, and digested with *Cla*I to yield fragments of approximately 660 to 680 bp. These fragments were then used to replace the corresponding 680-bp piece of PT7HGD1696. The plasmids from individual clones were se-

quenced to determine the sizes of the deletions. Two of these deletions were then placed into the wild-type parent R73/BH10 as described above for the linker insertion mutations.

Cell culture, transfections, and infections. COS7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Jurkat cells were maintained in RPMI 60 medium with 10% fetal calf serum. COS7 cells were transfected with 20 μg of DNA per 2×10^6 to 4×10^6 cells by the calcium phosphate precipitation technique (17). Sixty hours posttransfection, culture supernatants were harvested and filtered through a 0.45-μm-pore-size filter. The virion particles were concentrated by centrifugation through a 25% sucrose cushion in 10 mM Tris-HCl–10 mM NaCl–1 mM EDTA (TNE) at 4°C for 2 h at $100,000 \times g$. For infectivity assays, 1 μg of proviral DNA was used to transfect Jurkat cells by the DEAE-dextran method (34). To test for the spread of the virus throughout the cell culture, viral supernatants were harvested every 2 to 3 days and assayed for reverse transcriptase (RT) activity (34).

RT assays and Western blotting (immunoblotting). Virion particles released from COS7 transfected cells were collected from filtered supernatants by centrifugation through a 25% sucrose cushion in TNE at 4°C for 2 h at $100,000 \times g$. Pelleted virions were resuspended in 25 mM Tris-HCl (pH 7.5)–5 mM dithiothreitol–50 mM KCl–0.025% Triton X-100–50% glycerol. An aliquot was removed to be analyzed by the RT assay as previously described (34). To the remaining sample, additional dithiothreitol (final concentration, 100 mM), Tris-HCl (final concentration, 50 mM), and sodium dodecyl sulfate (SDS; final concentration, 2%) were added. The samples were then boiled for 5 minutes, separated by electrophoresis on an SDS–12% polyacrylamide gel, transferred to nitrocellulose, and probed with HIV-1-infected patient antiserum no. 256 (kindly provided by John Moore). Nitrocellulose-bound proteins were immunodetected by enhanced chemiluminescence as previously described (27). For detection of virion-associated gp120^{env}, filtered supernatants from transfected COS7 cells were pelleted over a 25 to 45% sucrose step gradient for 16 h at $100,000 \times g$. The 25%-45% interface was harvested, diluted fivefold with TNE, and repelleted for 2 h at $100,000 \times g$. The pellets were subjected to electrophoresis and Western
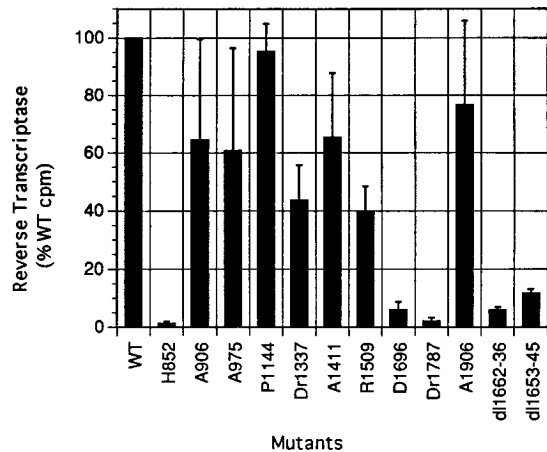
FIG. 2. Release of RT-containing viral particles by Gag mutant proviruses. Virion-associated RT activities of mutants are presented as percentages of wild-type (WT) activity.
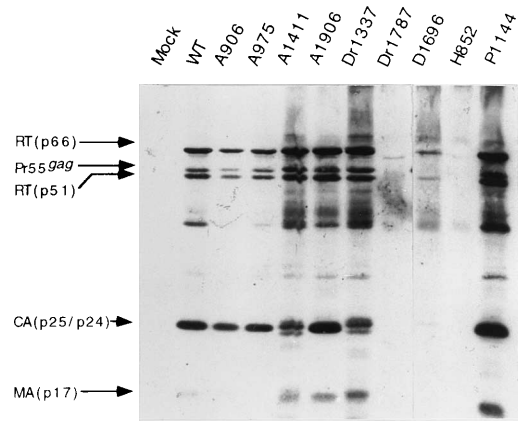

FIG. 3. Western blot of virion-associated viral proteins. Virion-associated proteins released by COS7-transfected cells were separated by electrophoresis on an SDS–12% polyacrylamide gel, transferred to nitrocellulose, and probed with HIV-1 antiserum. The positions of $p66^{RT}$, $p51^{RT}$, $Pr55^{gag}$ (the major Gag precursor), p25/p24^{capsid}, and p17^{matrix} are indicated. WT, wild type.

immunoblotting with sheep anti-gp120^{env} (AIDS Research and Reference Reagent Program Antiserum no. 288) as described above.

Pulse-chase experiments. COS7 cells were transfected with proviral DNA as described above. At 36 to 48 h posttransfection, cells were placed in methionine- and cysteine-free medium for 30 min and then subjected to a 30-min pulse-label with 500 μCi of [^{35}S]methionine and [^{35}S]cysteine. The cells were then washed with phosphate-buffered saline and either lysed (time zero) or incubated in fresh medium for 1, 2, or 4 h prior to cell lysis. Cells were lysed in 1 ml of cold lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 0.05 M Tris-HCl [pH 7.5]), the extract was centrifuged for 5 min at 10,000 × g to pellet nuclei, and SDS was added to each supernatant to a final concentration of 0.1% (4). Viral proteins were immunoprecipitated with 5 μl of polyclonal anti-p24 (American Biotechnology Laboratories) as previously described (4), separated by electrophoresis on an SDS–10% polyacrylamide gel, and subjected to autoradiography.

Analysis of intracytoplasmic high-molecular-weight aggregates of Gag proteins. Transfected COS7 cells were lysed and incubated for 1 h at room temperature in Triton lysis buffer containing 0.5% Triton X-100, 0.25 M sucrose, 1.0 mM EDTA, 0.14 M NaCl, 50 mm Tris-HCl (pH 7.5), 1 mM dithiothreitol, 100 Kallikrein inhibitor units of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride. The samples were centrifuged at 10,000 × g for 5 min, and the supernatant was removed (soluble fraction). This soluble fraction was then layered over a 25% sucrose cushion and centrifuged at 100,000 × g, and the pellet was recovered (pelleted fraction). The samples were then subjected to Western immunoblotting as described above. The antibodies used in this assay were mouse monoclonal anti-p24 and anti-p17 (Dupont Laboratories).

RNase protection assays. Cytoplasmic RNA from transfected COS7 cells was obtained 60 h posttransfection by lysis at 4°C in Nonidet P-40 buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 5 mM MgCl$_2$, 0.5% Nonidet P-40). Cell debris and nuclei were removed by centrifugation. The supernatant was adjusted to 0.2% SDS and 150 μg of proteinase K per ml, incubated at 37°C for 15 min, and extracted twice with phenol-chloroform and once with chloroform. RNA was collected by ethanol precipitation and suspended in water.

To prepare virion-associated RNA, virus from the culture medium of COS7-transfected cells was pelleted through sucrose as described above and disrupted in proteinase K buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM EDTA, 1% SDS, 100 μg of proteinase K per ml, 10 μg of yeast tRNA per ml) for 30 min at 37°C, and the nucleic acids were purified by phenol-chloroform and chloroform extractions. RNA was collected by ethanol precipitation and suspended in water.

An antisense RNA probe spanning the 5′ end of the HIV-1 genome was synthesized by T7 RNA polymerase as described previously (3), using plasmid pHRCS (28) linearized with NdeI as the template. The riboprobe (10^5 cpm [approximately 400 pg]) was mixed with either 5% of the cytoplasmic RNA preparation or 25% of the virion RNA preparation. After hybridization, the samples were subjected to RNase digestion using the Ambion RPA II RNase protection kit (Ambion Inc., Austin, Tex.). The protected fragments were subjected to denaturing polyacrylamide gel electrophoresis, and the gels were dried and subjected to autoradiography.

RESULTS

Structures of mutants. To define the roles of the various regions of Gag in viral assembly and infectivity, 10 linker in-

sertion mutations throughout the gag coding region were placed into the wild-type HIV-1 genome of plasmid R73/BH10. The location of each of the linker insertion mutants and the corresponding change in the amino acid sequence are shown in Fig. 1. H852, A906, A975, and P1147 contain linker insertion mutations in the matrix domain of the gag coding region; Dr1337, A1411, R1509, D1696, and Dr1787 contain linker insertion mutations in the capsid domain of the gag coding region. A1906 contains a linker insertion mutation in the p2 spacer peptide between capsid and nucleocapsid.

Virion assembly by mutants. To test for the ability of the mutants to assemble and release virions containing Pr160$^{gag-pol}$, COS7 cells were transfected with wild-type or mutant proviral DNA. Sixty hours posttransfection, culture supernatants were harvested and pelleted through a 25% sucrose cushion. The presence of virion particles in the pellet was determined by the RT assay (Fig. 2). Each value represents the mean determined from three different COS7 cell transfections. The majority of the mutants had only a two- to threefold decrease in pelletable RT activity compared with the wild type, suggesting that assembly and release were normal in these mutants. However, <5% of wild-type activity was observed in cells transfected with mutants H852, D1696, and Dr1787.

To determine whether the virions contained normally processed proteins, pelleted proteins were denatured, separated by electrophoresis on an SDS-polyacrylamide gel, and transferred to nitrocellulose. The mature cleavage products of Pr55^{gag} and Pr160$^{gag-pol}$ were detected on Western immunoblots, using antiserum from an HIV-1-infected patient. This antiserum recognizes the Gag polyprotein Pr55^{gag}, partially processed p41^{gag}, and the mature cleavage products of Gag, CA and MA (Fig. 3). The mature cleavage products of Pr160$^{gag-pol}$, p66^{RT} and p51^{RT}, are also recognized. Seven of the ten mutants had levels of all mature cleavage products which were within two- to threefold of the wild-type level (examples shown in Fig. 3). Mutants H852, D1696, and Dr1787, the three mutants with <5% of wild-type levels of pelletable RT activity, had virtually no detectable virion-associated proteins in the pelleted culture medium. Mutants A1411 and Dr1337 produce pelletable viral particles containing most of the mature cleavage products of Pr160$^{gag-pol}$ and Pr55^{gag}, but the linker insertions in these two mutants partially interfered with the cleavage of the p2 spacer peptide from p25 to produce p24, the
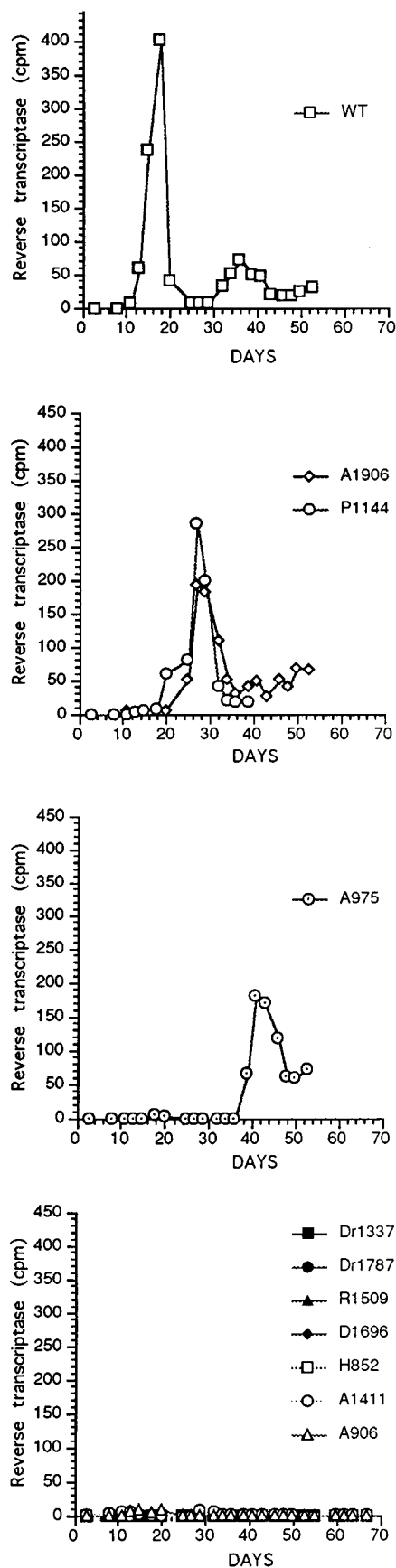
smaller form of CA. A1411 and Dr1337 had both p25 and p24 in the pelleted virions, whereas wild-type HIV-1 virions had no evidence of p25.

These two mutations and mutation R1509 affected the recognition of the capsid protein. A monoclonal antibody against p24 (Dupont) and serum from a different HIV-1-infected patient were unable to recognize the capsid proteins of these mutants (data not shown). Mutants A1411, Dr1337, and R1509 all have linker insertion mutations in the amino terminus of CA. Thus, they may define a region of the wild-type protein which is highly antigenic.

Infectivity of the virions. To determine whether the mutants produced infectious particles, Jurkat cells were transfected with wild-type and mutant proviral DNAs. Culture medium was harvested every 2 to 3 days and tested for virion-associated RT activity. These assays require viral spread to generate sufficient progeny virus to be detected by the RT assay. R73/BH10 induced the appearance of RT activity in culture supernatants by day 13 (Fig. 4). As expected, the three mutants (H852, Dr1787, and D1696) with severe defects in viral assembly failed to induce any detectable RT activity even after 45 days. The seven mutants which had minimal defects in particle formation and release in the COS7 viral assembly assay had significant defects in viral infectivity. A906, A1411, Dr1337, and R1509 did not induce RT activity even when the cultures were tested to day 50, demonstrating that these mutants are all noninfectious. The remaining three mutants, P1144, A1906, and A975, all showed markedly delayed growth kinetics in Jurkat cells. An increase in RT activity was detected after delays of 8, 12, and 26 days, respectively, relative to the appearance of wild-type RT activity (Fig. 4). Preliminary analysis of the virus recovered from these assays suggested that at least one of the mutants, A975, had reverted to generate infectious virus that replicated with wild-type kinetics (data not shown). In this revertant, analysis of the viral DNA showed retention of the original linker insertion, implying that a second-site suppressor mutation was responsible for the reversion. Further analysis of this revertant is ongoing.

Incorporation of gp120^{env} and viral genomic RNA into mutant viral particles. During assembly, wild-type virions incorporate viral Env glycoproteins and incorporate viral RNA. To further characterize the components of the particles released by COS7-transfected cells, the mutant virion particles were assayed for virion gp120^{env} incorporation and RNA packaging. COS7 cells were transfected with proviral DNA, and 60 h later supernatants were harvested and layered over a 25 to 45% sucrose step gradient. After centrifugation, the viral particles were harvested at the 25%-45% interface, repelleted over a 25% sucrose cushion, and subjected to Western immunoblotting with sheep anti-gp120^{env} antiserum (Fig. 5). All seven mutants, including those with linker insertion mutations in the matrix coding region, incorporated gp120^{env} into the mature virions. The gp120^{env} detected on the Western blot appears to represent protein that is specifically incorporated into the viral particles, since mutant H852, which does not release viral particles, had no evidence of gp120^{env} incorporation.

To determine whether genomic viral RNA was efficiently packaged by the Gag mutants, RNase protection assays of cytoplasmic extracts and pelleted supernatants of COS7-trans-

FIG. 4. Replication of the Gag linker insertion mutants in Jurkat cells. The cells were transfected with R73/BH10 (wild type [WT]) and mutant proviral DNAs. The infectivity was monitored as RT activity in the culture supernatant over time. Each RT value represents the amount of activity in 1.5 μl of culture supernatant.
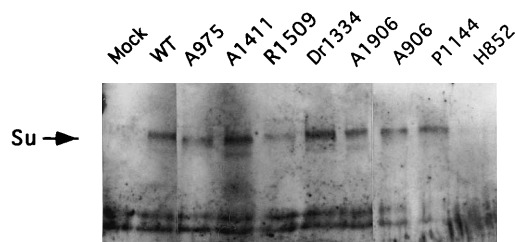
FIG. 5. Western blot of virion-associated Env glycoprotein gp120. Virion-associated proteins were separated on an SDS–7% polyacrylamide gel, transferred to nitrocellulose, and probed with sheep anti-gp120^{env} antiserum. WT, wild type; Su, gp120^{env}.

fected cells were performed. RNA was isolated 60 h posttransfection and was used to protect a radiolabeled antisense RNA probe spanning the U3/R junction. The protected fragments were separated on a denaturing polyacrylamide gel and subjected to autoradiography. All of the tested Gag mutants were able to efficiently package genomic RNA (Fig. 6). The ratio of virion-associated RNA to cytoplasmic viral RNA in the mutants was virtually identical to the wild-type ratio (data not shown). Thus, defects in gp120^{env} incorporation or RNA packaging did not contribute to the delayed growth kinetics of these mutant viruses. It appears that the defects in these mutants are in the early stages of the viral life cycle, including viral entry, uncoating, reverse transcription, or entry into the nucleus.

Pulse-chase studies of the assembly-defective mutants. To verify that the defect in viral assembly seen in mutants H852, D1696, and Dr1787 was not due to instability of the mutant Gag protein, pulse-chase experiments were performed. COS7 cells transfected with wild-type and mutant proviral DNA were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine for 30 min. The cells were then lysed (time zero) or incubated with fresh unlabeled medium prior to cell lysis. The presence of cellular Gag proteins was detected by immunoprecipitation of the clarified cellular extracts with polyclonal anti-p24 serum (Fig. 7). Mutants H852, D1696, and Dr1787 synthesized Pr55^{gag} and processed the protein to its cleaved products. However, in all three mutants, the CA was present only as p25; it was not cleaved to its mature form, p24. It has been suggested that the cleavage of p25 to p24 occurs at the budding site during and/or after assembly (22); similar suggestions have been made with respect to the RSV system (49). Therefore, the lack of normal virion assembly in these mutants may have interfered with p24 production, resulting in the accumulation of p25 in the cytoplasm of infected cells. The possibility that all three mutants had an altered p25 protein with an inaccessible p24/p25 proteolytic cleavage site cannot be ruled out but seems less likely.

The amount of Gag proteins detected during the chase periods was moderately reduced from the wild-type control in all three mutants, and thus these proteins may have slightly reduced stability. However, mutants D1696 and Dr1787 had at least wild-type levels of Pr55^{gag} at steady state, as seen by immunoblotting (Fig. 8). Some of the reduction of protein
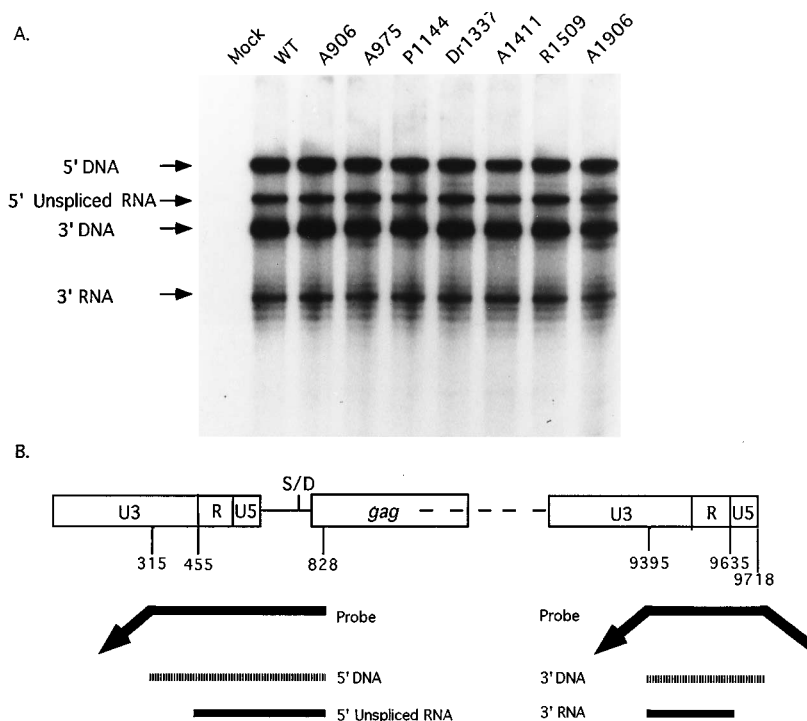

FIG. 6. (A) Packaging of RNA into virions from cells transfected with wild-type (WT) and mutant proviral DNA. RNA isolated from the pelleted supernatants of COS7-transfected cells was used to protect a ^{32}P-labeled antisense RNA probe spanning the 5′ major splice donor. The protected fragments were run on a denaturing polyacrylamide gel and subjected to autoradiography. Identities of the fragments are indicated at the right. The 5′ and 3′ DNA fragments are the result of plasmid DNA used in the COS7 transfection. (B) Regions of complementarity between the antisense riboprobe and viral RNA or proviral DNA. At the 5′ end, the riboprobe is complementary to nucleotides 315 to 828 but has a 3′ extension of sequence from the transcription vector SP72. At the 3′ end, the riboprobe is complementary to sequences 9395 to 9718. Fragments resulting from protection with viral RNA are shown by black bars. Fragments resulting from protection with proviral DNA are shown by hatched bars. S/D, splice donor.
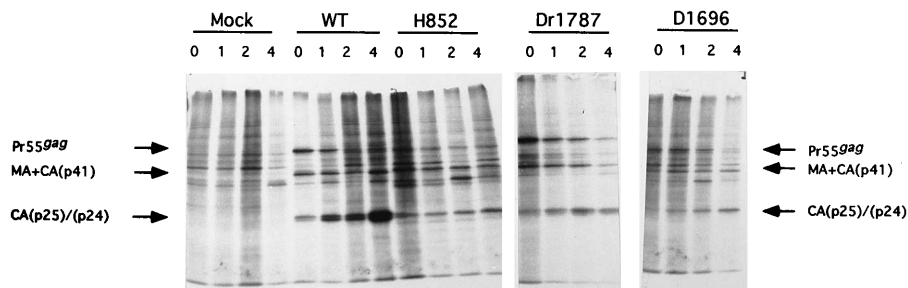
FIG. 7. Pulse-chase studies of intracellular viral proteins. COS7-transfected cells were pulse-labeled with 0.5 mCi of [^{35}S]methionine for 30 min, the label was removed, and the cultures were incubated with unlabeled methionine for 0, 1, 2, and 4 h. Cells were lysed, and cell-associated viral proteins were immunoprecipitated with rabbit anti-p24 antiserum. WT, wild type.

detected in the pulse-chase experiments for these two mutants may have reflected a problem with the antigenicity of the mutant protein for the p24 antibody used in the immunoprecipitation assay. The major defect in mutants D1696 and Dr1787 appears to be in the actual assembly and release of the virions, since their Gag proteins were stable enough to be present at wild-type levels with a steady-state analysis. However, steady-state levels of mutant H852 cellular Gag proteins, as measured by Western immunoblotting, were markedly reduced (data not shown). In the pulse-chase experiments, this mutant appeared to have only a moderate reduction in Gag protein stability, and thus it is likely that the protein was unstable only over a longer time scale. This instability would likely contribute to the defect in assembly seen with this mutant.

Detection of intracytoplasmic Gag protein aggregates. The analysis of the 10 linker insertions mutants showed that only two mutants, D1696 and Dr1787, were specifically defective in virion release. Mutant D1696 has a linker insertion mutation adjacent to the C-terminal end of the MHR. Since we were interested in assessing the importance of this region, we made two deletion mutants in this region (Fig. 1C). Both deletion mutants showed a significant reduction in the ability to release virion particles in COS7 cells. Mutant *dl*1662-36 was reduced 20-fold, while *dl*1653-45 was reduced 8-fold (Fig. 2).

To determine whether the Gag proteins of mutants D1696, Dr1787, *dl*1662-36, and *dl*1653-45 were deficient in the ability to form aggregates in the cytoplasm or to release viral particles, we examined lysates for the presence of pelletable Gag protein. In this assay, virus-expressing cells were lysed in 0.5% Triton X-100 lysis buffer, and the nuclei were removed; the resulting supernatants contained viral proteins which were both soluble and in aggregated forms. Crude intracytoplasmic aggregates of viral proteins were recovered after centrifugation through a 25% sucrose cushion. Western immunoblotting was then performed on portions of the supernatant and the pelleted proteins. Wild-type intracytoplasmic aggregates which contained uncleaved Pr55^{gag} and Pr160$^{gag-pol}$ (data not shown) were preferentially pelleted in this protocol, while the mature cleavage products of Pr55^{gag} and Pr160$^{gag-pol}$ remained in the soluble fraction (Fig. 8). These results were consistent with earlier work suggesting that immature capsids which contain unprocessed Pr55^{gag} and Pr160$^{gag-pol}$ are resistant to 0.5% Triton X-100, while mature viral capsids are disrupted under these detergent conditions (33, 35, 43). Although we cannot be sure that the pelleted proteins represent true virion capsids, the presence of both Gag and Pol proteins in the pellets and the preferential pelleting of unprocessed Gag and Pol lends some credence to the possibility that the pelleted proteins represent particle formation.

All four mutants had at least as much intracellular Gag proteins in the soluble fraction as the wild type did; in fact, mutant D1696 and the two MHR deletion mutants appear to have a two- to fourfold excess of unprocessed Pr55^{gag} relative to the wild type (Fig. 8). While this increase may reflect their accumulation in the cytoplasm due to the lack of particle release, it may also reflect a partial reduction in protease activation in those particles which are not released. All four mutants showed reduced levels of CA compared with the wild type, but this decrease is likely due to a change in antigenicity of the mature CA since the amount of MA is the same in all of the samples. Despite nearly equivalent or increased amounts of soluble intracellular proteins, all four of the mutants had a two- to fourfold reduction in pelletable intracytoplasmic aggregates. It is possible that even this small reduction in pelletable aggregates reflected a decreased stability of the aggregates in detergent. Since these mutants had an 8- to 100-fold reduction in the ability to release extracellular particles, it is likely that these mutants had defects both in the ability to make stable intracytoplasmic particles and in the ability to release particles. The nature of the aggregates could be probed by electron microscopy of transfected cells.

DISCUSSION

We have characterized the phenotypes of a panel of linker insertion mutations throughout the *gag* coding region. Surprisingly, 7 of 10 mutants were still able to assemble and release
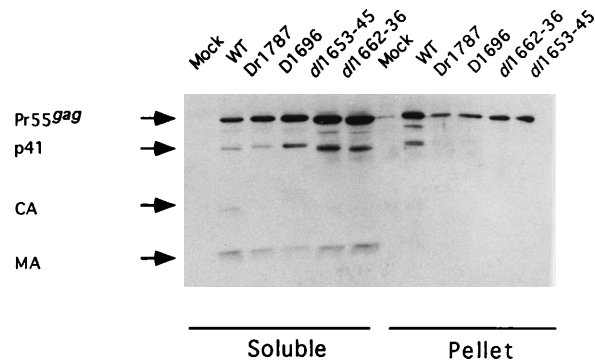

FIG. 8. Western blot of cell lysates and intracytoplasmic Gag aggregates. COS7 cells were transfected with wild-type (WT) and mutant proviral DNA. Sixty hours posttransfection, the cells were lysed in lysis buffer containing 0.5% Triton X-100. The cellular lysates were clarified and run on an SDS–polyacrylamide gel (soluble) or were pelleted over a 25% sucrose cushion prior to being run on an SDS-polyacrylamide gel (pellet). The proteins were transferred to nitrocellulose and probed with anti-p24 and anti-p17 antisera.

virion particles. Thus, it seems that the ability of the virus to form and release particles is relatively resistant to mutations in the *gag* gene. The infectivity of the viral particles, however, was extremely sensitive to mutagenesis.

Our results show that the C-terminal third of CA is important in vivo for viral assembly in mammalian cells; mutants D1696, Dr1787, *dl*1653-45, and *dl*1662-36, with linker insertion and deletion mutations in this region, were severely impaired in viral assembly. In both baculovirus and vaccinia virus expression systems, Gag proteins with mutations in the C-terminal region of capsid have been reported to be defective in viral particle assembly. (7, 21, 24). In addition, this same region appears to be critical for dimerization in the yeast two-hybrid system (11). The C terminus of capsid and the N terminus of nucleocapsid seem to contain regions critical for particle assembly in RSV (46–48). It is possible that the C-terminal third of capsid is a part of a similar region in HIV-1.

The assembly-defective CA mutants were able to make intracytoplasmic pelletable aggregates of Gag and Pol proteins and showed evidence of Gag and Pol proteolytic processing, implying that PR protein dimerization occurred. The amount of pelletable Gag aggregates after lysis in 0.5% Triton was decreased by only a small amount in comparison with the wild type. These defects were not enough to account for the larger reduction in extracellular particles, suggesting additional blocks. Similar analysis of intracellular aggregate formation by using a lower concentration of detergent, 0.1% Triton, showed that one of these mutants, *dl*1653-45, made wild-type levels of intracytoplasmic aggregates (data not shown). This finding implies that this mutant formed aggregates which were somewhat sensitive to 0.5% detergent. Electron microscopy should help elucidate the morphology of these aggregates and their location within the cell.

Within the C-terminal region of CA is an area, termed the MHR, which is highly conserved among retroviruses. The phenotype of HIV-1 MHR point mutants has recently been reported: many of the mutants were defective in assembly (30). Interestingly, the defects reported with some of the point mutations appear to be similar, though more severe, than those caused by our linker insertion and deletion mutations. In our studies, as more of the MHR was deleted, particle formation appeared to improve. It is possible that the presence of modified MHR sequences interferes more drastically with particle formation than does removal of all or part of the MHR. If true, this phenomenon may explain why the entire MHR can be deleted in RSV without impairing particle formation or release (48).

The three linker insertion mutants which had mutations within the N-terminal two-thirds of the capsid protein had minimal effects on viral particle formation and release. Consistent with this finding is a previously reported mutant with a 56-amino-acid deletion in this region which was found to have no effect on particle formation (45). Linker insertions into the same site as R1509 and A1411 have been reported in baculovirus to interfere with viral budding. Although the discrepancy between these results may be due to differences in the exact amino acids inserted in the mutants, it is also possible that the defect in budding was unique to the baculovirus system.

The remainder of our linker insertion mutants within both matrix and the p2 spacer peptide had only minimal effects on viral assembly. None of these mutants were found to have major defects in the incorporation of Env into the viral particles. In these experiments, it is clear that the Env protein that was detected in pelleted supernatants was virion associated, because the extra pelleting step over a 25 to 45% sucrose step gradient eliminated the detection of gp120^{env} that was not

specifically virion associated (data not shown). In previous reports (9, 52), small deletions throughout the matrix coding region were found to inhibit Env incorporation. Thus, it is likely that within matrix, some regions are critical for Env incorporation while other regions are not.

The results of our study, like those of previous studies, highlight the fact that multiple regions of the Gag protein appear to be important in the early stages of the viral life cycle. Seven of the linker insertion mutations had minimal defects in viral assembly, Env incorporation and RNA packaging, yet all had significant defects in viral infectivity. Thus, regions throughout matrix, capsid, and the p2 spacer peptide appear to be involved in early steps. It is possible that the defect in infectivity reflects the fact that although particles are formed, the multimerization of these mutant proteins could lead to the formation of particles with incorrect morphology, and early steps in the viral life cycle (i.e., viral entry and uncoating) may be strictly dependent on the correct morphology of the particles. One of the linker insertion mutations (R1509) has previously been shown to interfere with the ability of Gag to interact with the cellular protein cyclophilin (27). This mutant was able to assemble and release virion particles, but these particles were noninfectious, implying that the Gag-cyclophilin interaction may play a significant role in the early stages of the viral life cycle.

This laboratory previously reported the effect of the same linker insertion mutations on the activation of viral protease in the context of a Gag-PR expression plasmid in bacteria. As shown in Table 1, the bacterial system did not accurately indicate which regions were critical to viral assembly in the context of the full virus. Only one of the five mutants found to be impaired in protease activity in the bacterial system had a dramatic defect in particle assembly. Interestingly, two of these five mutants (A1411 and Dr1337) had a subtle defect in the ability to cleave p25^{capsid} to p24^{capsid} when tested in proviral constructs. This defect may reflect conformational changes in the mutant precursor protein which make the cleavage site

TABLE 1. Comparison of mutant phenotypes in *E. coli* and animal cells

Mutant	Location in Pr55^{gag}	Protease activity in Gag-PR expressed in *E. coli*[a]	Virion release from COS7 cells[b]	Proteolytic cleavage in whole virions
H852	Matrix	+	−	+/−[c]
A906	Matrix	+/−	+	+
A975	Matrix	+	+	+
P1144	Matrix	+	+	+
Dr1337	Capsid	+/−	+	+/−[d]
A1411	Capsid	+/−	+	+/−[d]
R1509	Capsid	+/−	+	+
D1696	Capsid (MHR)	+	−	+/−[c]
Dr1787	Capsid	+/−	−	+/−[c]
A1906	p2 spacer peptide	+	+	+

[a] +, wild-type protease activity; +/−, partial proteolytic activity.
[b] +, viral assembly and release of particles which was at least 30% of wild-type assembly and release; −, viral assembly and release which is less than 5% of wild-type assembly and release.
[c] Viral proteolytic cleavage of Gag intracellularly which is wild type except for the impaired cleavage of p25 to p24. Proteolytic cleavage of these mutants could not be assessed in mature virions, since these mutants interfered with the release of virions from COS7 cells.
[d] Proteolytic cleavage of Gag within mature virions which is wild type except for the impaired cleavage of p25 to p24.

partially inaccessible. Such changes may also have interfered with proteolytic processing in the bacterial assay system. The change in antigenicity seen with these two mutants may lend further evidence that such conformational changes may have occurred. These results emphasize the need to analyze mutants in vivo.

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