GENETIC ANALYSIS OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) GAG PROTEINS

by

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Abstract

The core of HIV is composed mainly of the proteins Gag-Pol and Gag, which initially is synthesized as a 55 kilodalton precursor protein that ultimately becomes cleaved into the mature Gag protein matrix (MA), capsid (CA), nucleocapsid (NC), and p6. The processes of core assembly and the potential functions of HIV *gag* involved in other phases of the HIV life cycle are unclear. This thesis focuses on studies of HIV assembly and investigate the influence of *gag* mutations on HIV infectivity and processing. Infectivity of HIV *gag* mutants were screened by the transduction of a drug-resistance marker. Protein analyses showed that most HIV *gag* mutants could assemble and release virus particles, except a myristylation-minus (Myr^-) mutant and a mutant with a linker insertion at the central part of the matrix domain (AccI). Immunofluorescence studies revealed that the AccI mutant protein was trapped in a perinuclear area, implying that intracellular membranes may be involved in Gag transport. Although most HIV *gag* mutants could process virus particles, had wild-type (wt) particle densities, and possessed significant RT (reverse transcriptase) activities, they were noninfectious or poorly infectious, suggesting that HIV *gag* is functionally involved in postassembly, postprocessing stages of virus infectivity. Surprisingly, mutants with either a deletion of a 56 codons within the capsid domain or a deletion of about 80% of the matrix domain (dl.MA) still could assemble, process virions, had wt particle densities and wt RT activities. Reduced dl.MA virion-associated HIV Env indicated that the matrix domain (MA) interact

with HIV Env. A 10% wt infectivity demonstrated by the dl.MA mutant suggested that an intact HIV MA domain is not essential for reverse transcription, nuclear localization, or proviral integration. Finally, to define potential regions within the Gag protein domain for Gag-Pol incorporation into virions, a series of HIV Gag-β-gal fusion constructs were generated and assayed for assembly of β-gal activity into HIV particles. Results from these studies showed that HIV Gag-β-gal fusion proteins lacking the C-terminal two-thirds of the capsid domain (CA) were impaired in assembly into virions, indicating that the CA is the major determinant for the HIV Gag-β-gal incorporation into virus particles. Thus the HIV CA domain could be a potential antiviral target for interfering virus assembly.

Introduction

I. Discovery and Nomenclature

AIDS (acquired immunodeficiency syndrome), an unusual disease syndrome, was first reported in 1981 (24). This syndrome, which occurred initially among the homosexual men and intravenous drug users, was suspected of being caused by a transmissible agent (40). Subsequent increased incidence of AIDS among persons with hemophilia and blood transfusion recipients supported the transmission agent hypothesis (39, 40). In May 1983, Luc Montagnier and his colleagues first described a single virus isolate which was designated lymphoadenopathy-associated virus (LAV) and eventually proved to be the etiological agent of the AIDS (6). About half year later, Robert Gallo and his colleagues described their isolate from AIDS patients and provided strong evidence linked to the disease (58, 59, 151,155). The Gallo group chose the name of human T-lymphotropic virus type III (HTLV-III) for this virus because they found it similar to HTLV-I and II in preference for infecting T cells. Following the Gallo group, another group led by Jay Levy also identified an isolate from AIDS patients and called it the AIDS-associated retrovirus (ARV) (98). Subsequent sequence comparison showed that the three different designated isolates were variants of the same virus.

Besides LAV, HTLV-III, and ARV, immunodeficiency-associated virus (IDAV) and compound names (HTLV-III/LAV and LAV/HTLV-III) were also used in publication by 1987. It become an important issue to adopt an internationally acceptable name for this virus

group, because of widespread interest in its origin and AIDS and because of multiple names have been used. Dispute over who should get priority for discovering the AIDS virus extended to the viral nomenclature. In early 1986, International Committee on the Taxonomy of Viruses (ICTV) recommended Human immunodeficiency virus (HIV) as a replacement for the name of AIDS virus (115). The new name, HIV, without regard to priority of discovery, has become widely accepted.

Although the first AIDS case was described in the United States, retrospective seroepidemiologic studies suggest that this disease may have emerged from Central Africa (15, 141). Three years after the discovery of HIV-1, one other causative agent of AIDS, HIV-2 (LAV-2) was isolated from Western African patients with a clinical syndrome indistinguishable from HIV-induced AIDS (33). HIV-2, less virulent than HIV-1, is more closely related to simian immunodeficiency virus (SIV, also called simian T-cell lymphotropic virus type III), the causative agent of AIDS-like disease in captive macaques (45, 57). HIV-1 isolates vary in their replicative capacity (5), host cell tropism (27,87), and cytopathogenicity (25, 28, 180). Studies have suggested that the HIV envelope region contains the major determinants of these biological features (25, 37, 79, 159).

II. Classification

Retroviruses generally are divided into three groups: 1) the oncoviruses; 2) the spuma viruses or foamy viruses; 3) the lentiviruses or slow viruses (178). This classification is based on

pathogenic rather than on genomic relatedness. For instance, Rous sarcoma virus (RSV) and Moloney murine leukemia virus (M-MuLV), members of oncoviruses, can cause tumors in chickens and mice respectively (178). Retroviruses of mice are further designated amphotropic, ecotropic, and xenotropic by the species distribution of their receptors (178). Amphotropic virus can infect both mouse and non-mouse cells. Ecotropic virus can only infect mouse cells. Xenotropic viruses use receptors found on cells of most species except mouse. Spumaviruses, the least well characterized retrovirus family, can cause persistent infection but is not associated with tumors or any known disease (180). *In vitro*, a variety of mammalian cells can be transformed by spumaviruses and lead to formation of giant multinucleated cells with numerous vacuoles, giving the induced syncytia a foamy appearance.

HIV, a RNA retrovirus, shared many features with other members of the non-oncogenic and cytopathic lentivirus subfamily. Electron microscopic study of HIV particles revealed a cone-shaped central structure (63, 64), similar to the visna and equine infectious anemia virus, members of the lentivirus family (142). In addition, biological and genomic characteristics of HIV are more closely related to lentivirus than to other mammalian retroviruses (30, 142). Thus, HIV has been classified as a member of lentivirus family. Lentiviruses have been isolated from several other animal species including cat (131), sheep (43), and monkey (45). These viruses, including HIV, cause a slowly progressive, often fatal disease in their hosts.

III. Pathogenesis

AIDS is characterized by a progressive and irreversible immune defect that renders the body highly susceptible to opportunistic infections and neoplasms. HIV-induced profound immunosuppression is thought primarily due to the tropism of HIV for the $CD4^+$ T lymphocyte and its cytopathic effect, resulting in a selective depletion of the $CD4^+$ helper/inducer lymphocytes (51, 88).

In immune-competent individuals, the $CD4^+$ T helper/inducer cells give signals to suppressor T cells, natural killer (NK) cells, immunoglobin-producing B cells, and nonlymphoid cells as well (51). Certainly, depletion or functional impairment of $CD4^+$ T cells will result in a profound and global immunologic abnormalities. Molecular bases of HIV-induced cytopathic effects on host cells are not yet understood completely. Based on *in vitro* studies, a variety of mechanisms or factors that may contribute to the quantitative deficiency of $CD4^+$ T cells have been proposed (51). One important mechanism is the interaction between surface CD4 present on uninfected cells and HIV envelope proteins of infected cells, resulting in formation of multinucleated giant cells (syncytia) and cell death (102, 103, 163, 192). Cytolysis can be induced by a process independent of syncytium formation (171). For instance, large amounts of accumulated unintegrated viral DNA (158) and high levels of viral RNA and protein synthesis (164) have been found in HIV cytocidal infection. Autoimmune phenomena also may play a role in the immunopathogenesis associated with HIV infection (175). For instance, infected $CD4^+$ T cells, which express HIV envelope proteins on their surface are recognized as non-self, resulting in

immune clearance (51). Similarly, binding of free HIV envelope proteins (gp120) to the uninfected T cells might lead to elimination of such cells by antibody-dependent cellular cytoxicity (108).

In addition to the quantitative depletion of $CD4^+$ T cells, a number of HIV-induced functional defects of $CD4^+$ T cells have been observed (34, 71, 92). For instance, functions of uninfected $CD4^+$ T cells could be inhibited by free HIV envelope proteins (51). One possibility is that binding of free envelope proteins to the $CD4^+$ molecules of T cells could interfere the interactions between the T cell receptor (TCR) and the class II MHC (major histocompatibility complex) molecules of the antigen-presenting cells (114). Functional defects of T cells may also occurs by noncytopathic infection by HIV, presumably due to decreased expression of CD4 molecules on cell surface (51). Programmed cell death (apoptosis) of T cells from HIV-infected individuals have been recently proposed to be a potential mechanism that contribute to the depletion of T cells and HIV-induced immunopathogenesis (4, 122, 179).

Although the percentage of HIV-infected cells that express viral RNAs is very low (75), recent studies have confirmed that lymphoid system of HIV-infected individuals contains a large number of latently infected $CD4^+$ lymphocytes and macrophages during the clinically latent stage (49, 127). Active and progressive HIV infection in lymphoid tissues may contribute to much of immune depletion of AIDS despite the fact that minimal viral activity is detected during the period of clinical latency (75, 140, 161).

IV. HIV Life Cycle

A. Viral entry

The replication cycle of HIV is initiated by binding of its envelope protein complex (gp120/gp41 or SU/TM) to the human cellular $CD4^+$ receptor, found mainly on T helper lymphocytes, monocytes and macrophages (41, 80). Several lines of evidence suggest that entry appears to be the restrictive step in virus replication. It has been demonstrated that the host range of HIV can be expanded to CD4-negative cells by forming pseudotyped virus particles (107, 166) or transfection of HIV DNA into human or animal fibroblasts (99), leading to production of infectious virions. However, additional host factors may be required in the process of entry since murine cells and some human cells expressing $CD4^+$ permit binding but not infection (29, 32).

How HIV gains entry into target cells is not entirely clear. The entry route could be either via CD4 receptor-mediated endocytosis (130) or via direct fusion between the viral envelope and cell membrane (111, 169). Accumulated evidence that non-CD4 bearing cells could be infected by HIV (12, 26, 101, 177) indicates that the CD4 receptor is not the only binding site for viral entry (31). In agreement with this idea, infection of HIV can be enhanced by antibody via Fc receptor-mediated entry (176). Furthermore, *in vitro* demonstrations of HIV transmission by direct contact of HIV-infected cells with CD4-negative epithelial cells (137) indicate that HIV can spread by some novel ways (150). This evidence supports the notion that HIV entry does not absolutely require binding to the CD4 receptors (31).

B. Viral latency and activation

After adsorption and penetration into target cells, viral genomic RNA is transcribed into double-stranded DNA by reverse transcriptase which is carried within the virus particles. Viral linear double-stranded DNA migrates into the nucleus and then integrates into the host cell genome mediated by the viral integrase. It should be noted that the viral RNA or/and DNA transcript complex still appears to be associated with Gag proteins during the processes of reverse transcription, nuclear localization, and integration (13). However, the extent to which Gag proteins are involved in the early phases of viral replication cycle remains unclear.

The integrated HIV provirus can remain indefinitely silent as in a quiescent state until it is activated (62). Despite extensive studies, molecular mechanisms of the latent phase are poorly understood. In general, the latency stage could be defined as any block to the complete expression of HIV proteins. It could be "absolute latency" with integrated provirus but no RNA expression or "chronically persistent infection" with low level RNA expression but no detectable structural proteins (9, 62). Failure of intracelluar HIV to replicate may be due either to a defect in the integrated provirus, an absence of some cellular activators, or to inhibition of expression by viral repressor factors. Hypermethylation of integrated proviruses has been proposed as a factor to cause latency as restriction of HIV expression could be overcome by treatment with a DNA methylation antagonist (8).

HIV latency has been observed in both chronically infected T cells in culture and HIV-infected T cells isolated from AIDS patients

(62). These infected T cells expressed large amounts of multiply spliced viral mRNA, but little of the unspliced HIV genomic RNA that served as a mRNA for translation of structural proteins (62, 139); an expression pattern similar to the early stage of HIV-1 replication before Rev activity becomes detectable (86). Subsequent stimulation of these cells can lead to cytoplasmic accumulation of the singly spliced and unspliced HIV-1 mRNAs that encode the structural proteins (139, 197), suggesting that post-integration viral latency may be due in part to low levels of Rev proteins (139). Consistent with this hypothesis, the requirement of a critical amount of Rev for HIV activity has been suggested by *in vitro* demonstration of Rev multimerization on the Rev responsive element RRE (112). Latently HIV-infected quiescent cells may not only serve as an inducible HIV-1 reservoir but can escape the immune surveillance, making it extremely difficult to eliminate HIV from the infected host (19).

Initial studies found that HIV proviral genomes are unable to integrate into the resting T cell genome and remain as extrachromosomal forms (172), presumably some required cellular factors are present only in dividing cells. These unintegrated HIV proviral genomes could be induced to integrate and initiate virus production by activation of the infected cells (172). However, recent studies have shown that HIV can infect nondividing monocyte-macrophages (51) and cells arrested by irradiation (100), indicating that, in contrast to other retroviruses, the completion of early events (reverse transcription, nuclear import, and integration) of the HIV life cycle does not require a dividing host cell (18, 100).

Expression of the latent provirus in infected cells can be induced by a variety of factors. Antigens (51), mitogens (51), cytokines (55, 56, 149), UV light (168), and some other gene products from cotransfections or coinfections of heterogeneous virus (7, 42, 50, 65, 125, 152) could promote HIV expression. However, mechanisms of activation are unclear. Binding of activated host cellular transcriptional factors to the HIV promoter may activate the expression of latent HIV provirus.

Once activated, proviral DNA is transcribed into genomic and messenger RNAs. In a single cycle of infection or transfection, the HIV gene expression pattern features an early phase with predominant cytoplasmic multiply spliced HIV mRNA molecules (86). These early HIV mRNAs encode regulatory proteins including Tat (previously *tat-3*) and Rev (*art, trs*), which are absolutely essential for viral replication (44, 54, 181). Tat either alone or associated with cellular protein(s) binds to the trans-acting response element (TAR) in RNA and increases transcriptional initiation and/or elongation (53, 61, 93, 147). The shift from expression of viral regulatory proteins to expression of viral structural proteins is mediated by Rev. Rev, a nuclear protein, activates nuclear export of unspliced and singly spliced viral RNAs via an interaction with a Rev-responsive element (RRE) within the env coding sequence (46, 113, 123, 148). Downregulation of multiply spliced viral mRNAs and accumulation of genomic and subgenomic (singly spliced) viral RNAs within cytoplasm results in dominant expression of structural proteins Gag, Gag-Pol, and Env. Viral structural proteins are transported to the plasma membrane where self-assembly of core (Gag) protein

proceeds. While most viral genomic RNA serves as mRNA for translation of structural proteins, a portion of viral genomic RNA is encapsidated into virus particles. Viral envelope proteins are incorporated into particles when assembling virions bud out from the cellular surface. During or after budding, immature virions are processed by the viral protease into mature virions with acquisition of infectivity (90, 132).

V. HIV Genomic Composition

The genomes of all replication-competent retrovirus consist of three major genes; *gag*, *pol*, and *env*. The HIV genome is unique in that it contains several additional open reading frames (Figure 1). To date, at least six accessory genes have been identified in the HIV-1 genome. Functions in regulation of viral replication cycle have been ascribed to some of these accessory genes; however, the exact mechanisms are still unclear. The transactivators Tat and Rev have been discussed (see above). Nef (3' *orf*) has been thought to be a negative modulator of viral gene expression. For instance, expression of Nef proteins will inhibit LTR-directed CAT expression(1, 193), and HIV clones containing mutated Nef produce virus to higher titers than native clones with intact Nef (106). However, the role of Nef in negative regulation remains controversial because no effects of the Nef proteins on viral replication or expression were observed by other groups (73). Studies have shown that Nef, a myristylated protein (193) causes downregulation and loss of surface CD4 molecules (2).

Vpr, present in virus particles (35), may enhance the rate of infection and cytopathicity, presumably by transactivation of gene expression from the HIV-1 LTR (38a). Like Nef and Vpr, the *vif* (*sor*) and *vpu* gene products are dispensable for HIV-1 replication in culture. Vif (virion infectivity factor) may increase the virus infectivity by facilitating transmission of cell-free virus in culture (38a). The gene *vpu* is not found in all HIV-1 isolates and is not virion-associated. Vpu has been shown to be an integral membrane protein (173) and could promote the release of assembling virus particles (36, 89, 174). In addition, Vpu can induce the retention and rapid degradation of CD4 in endoplasmic reticulum (189). Vpu and Vif are not present in HIV-2, but an accessory gene *vpx* is found exclusively in HIV-2 and SIV genome. Similar to Vpr, Vpx also is virus-associated (84). The function of this gene is unknown although it has been proposed to play a role in cell-specific tropism for HIV-2 (72).

The *gag* (group of specific antigen) gene appears to play a central role in virus assembly. Without other viral components, the *gag* gene products still can assemble into viruslike particles (66, 160), and mutations in *gag* can block particle assembly (67, 83, 104, 156), suggesting that *gag* is necessary and sufficient for particle assembly (66, 160). The HIV Gag protein is synthesized initially as a precursor, Pr55. It is cotranslationally modified by removal of the N-terminal methionine and attachment of a myristic acid to the second glycine residue (154, 182, 187). Myristylation is required for stable association with membranes, and assembly of Pr55 (16, 70). Studies of other retroviruses have suggested that myristylation is necessary

but not in itself sufficient to target the Gag precursors to the plasma membrane (144, 145). Gag precursors Pr55 assemble into immature particles at the cytoplasmic surface of the plasma membrane. Subsequent processing of Pr55 by the viral protease yields p17 (matrix antigen, MA), p24 (major capsid, CA), p2, P7 (nucleocapsid, NC), p1, and P6 (77, 95, 121, 143, 185). An incomplete cleavage product, p41 composed of p17 (MA) and p24 (CA), can be found in the host cells and virus-containing media. Both HIV MA and CA have been shown to be phosphorylated proteins (20, 183); however, the significance of phosphorylation is unknown. Functions of p2 and p1 proteins are currently unclear.

The C-terminal Gag cleavage product, p6, has been demonstrated to play a role in the budding process as mutants lacking p6 can still assemble virus particles but the budding mutant virions are unable to release from the cell membrane (66, 69). The HIV nucleocapsid domain (p7) contains two Cys-His motifs similar to the "zinc-finger" of DNA binding proteins (11, 165). Mutations of the Cys-His motifs do not prevent virus assembly but inhibit encapsidation of viral genomic RNAs into virions (3, 68). Although there is no direct evidence, it is postulated that NC may function in selective encapsidation of viral RNA by interaction with the packaging signal (Psi) located between the major splice donor (MD) site and the initiation codon of *gag* (96). Alternatively, or in addition, it may stabilize the genomic RNA dimer and promote viral genomic RNA incorporation into particles (68). The CA protein, which is derived from the central part of Gag precursor, forms the major core structure surrounding the ribonucleoprotein (RNP) complex. The

matrix protein retains myristylated and may associate with the membrane (16).

The HIV *pol* gene encodes the viral protease, reverse transcriptase, RNAse H, and integrase, which are essential for virus replication. They are translated from the genomic RNA as part of a Gag-Pol fusion protein by -1 ribosomal frameshifting at a frequency of about 5-10% (81). This infrequent translation event leads to overproduction of *gag* structural proteins compared with the *pol* replicative enzymes. The ratio of the intracellular Gag versus Gag-Pol polyprotein level is critical for virus assembly. In Moloney murine leukemia virus (M-MuLV), Gag-Pol fusion proteins without Gag proteins can not assemble into virus particles, presumably due to steric hindrance by the larger Gag-Pol polyproteins (52). In HIV, it has been shown that expression of only Gag-Pol polyproteins leads to efficient intracellular proteolytic processing of structural proteins but failure of virus assembly (128). Thus, a critical amount of Gag proteins may serve as a buffer to prevent the premature activation of viral protease. Normally, the embedded protease is not activated until virus assembly occurs. Interactions among Gag-Pol molecules may create a dimer formation of the protease domain, which is required for activation of the viral protease (119, 124). The activated protease first cleaves itself out and then processes the Gag and Gag-Pol proteins. Processing of Gag proteins is not necessary for RNA packaging and particle assembly, but is essential for viral infectivity (70, 90, 132).

The HIV envelope glycoproteins are translated from the subgenomic mRNA as precursors gp160. Translated envelope protein

precursors start folding and oligomerization at the RER (188). They are cleaved by a cellular protease at the Golgi into an outer surface domain gp120 (SU), and the transmembrane protein gp41 (TM). Cleavage of the precursor gp160 is also required for HIV infectivity (116). This complex is transported to the plasma membrane via the secretory pathway (188).

VI. Viral Structure

A mature retrovirus particle has a core surrounded by the viral membrane, which is composed predominantly of a lipid bilayer and the viral envelope glycoproteins. The HIV envelope protein complex (gp120/gp41) derives from proteolytic cleavage of the precursor gp160. The HIV gp120 is anchored to the membrane by noncovalent associations with the transmembrane protein gp41(TM) and is easily shed from virus or cell membrane (118, 153). Binding of HIV to target cells is served by gp120, which has a high binding affinity for the CD4 receptor (94) and is the primary determinant for cellular tropism (14, 79). The transmembrane protein gp41 appears to play a major role in the fusion process (60, 91). TM is divided into the extracellular domain, transmembrane domain, and the cytoplasmic domain (184). Genetic studies have shown that two separate regions within extracellular domain of HIV TM are responsible for its noncovalent association with gp120 (23). Structure-function analysis also has defined a region within gp120 which is involved in stable association with virus particles (76, 190). Additionally, it has been demonstrated that mutations in the V3 domain of gp120 do not affect mutant envelope protein incorporation but reduce virus

infectivity and fusion activity (126). These observations suggest that perturbation of either SU or TM could lead to instability of their noncovalent association and loss of infectivity (10).

The cytoplasmic domain of gp41 appears to be critical for HIV infectivity (91). In RSV, truncation of the cytoplasmic domain does not affect the envelope proteins synthesis, transport, incorporation, and virus infectivity (135). However, mutants with variant truncations of HIV gp41 cytoplasmic domain will reduce virus infectivity and/or incorporation of envelope proteins into virus particles (196). This indicates that cytoplasmic domain of HIV TM proteins may be involved in incorporation of HIV Env into virions (196).

Several lines of evidence have suggested that MA is the likely candidate for interaction with Env in virions. MA lies immediately beneath the envelope and forms the viral matrix (64). It has been demonstrated that the matrix protein can be crosslinked to the viral lipid membrane in MLV and avian sarcoma viruses (133). In RSV, MA can be crosslinked to the transmembrane protein (134). Because of its intimate association with inner surface of plasma membrane, MA has been thought not only play a role in directing the Gag precursors to the plasma membrane for particle assembly but may be involved in incorporation of envelope proteins into virus particles. Recent studies have suggested that HIV MA appears to be involved in incorporation of HIV Env into particles (195). Possible additional functions of MA including RNA packaging (120, 167) and nuclear localization (17, 157) have also been proposed.

The capsid protein (CA) forms the core of the virion and may play a major role in particle assembly because it is the largest of the Gag cleavage product and has a strong tendency to aggregate (21, 48). In the case of M-MuLV, mutations within CA often block virus assembly (67, 104, 156). Within the capsid is the nucleocapsid protein (NC) which is associated with two copies of viral genomic RNA and together form a RNP (ribonucleoprotein) complex. Of note is that the RNP contains cellular tRNAs which serve as primers for initiating the reverse transcription by binding to the primer binding site (PBS) of genomic RNA. How the primer tRNA is selected and packaged into virus particles is unknown. Studies have shown that the PBS of the HIV genome is not involved in packaging of cellular tRNAs into virions (82). In murine leukemia virus (97) and avian sarcoma virus(136), packaging of host cell tRNA does not require the genomic RNA but may involve the Gag or Gag-Pol proteins. The *pol* gene products reverse transcriptase (RT) and integrase (IN) are also within the capsid.

VII. Assembly of HIV

Expression of HIV *gag* gene alone is sufficient to assemble and release virionlike particles (66, 85, 160). This indicates HIV Gag precursor contains the transport signals and the functional domains needed for self-assembly. In addition, incorporation of other viral components including viral RNA and envelope proteins depend on their interactions with Gag precursor. How the Gag precursors and other viral components assemble into virus particles is still unclear. In the late stage of HIV replication cycle, myristylated Gag and Gag-

Pol precursors travel to the plasma membrane where particle assembly proceeds. Myristylation is necessary for particle formation and it may serve to target Gag and Gag-Pol precursors to the plasma membrane (16). Studies of other retroviruses suggested that besides myristate moiety there may be other Gag domains required for Gag transport because myristylation is not sufficient for plasma membrane targeting (146). Mechanisms of Gag transport to the plasma membrane are poorly understood. In MLV, some studies suggested that the Gag precursor Pr65 is transported via interactions with elements of cytoskeletal system (47); data obtained from our lab suggested that Gag is associated with vesicles and transported to the plasma membrane (74).

Assembly of HIV is similar to type C retroviruses (63) in that both assemble at the plasma membrane. Beyond that, little is known about the mechanism of HIV assembly. Based on previous studies, we described a model of HIV assembly: viral structural proteins Gag, Gag-Pol, and envelope proteins are transported to the plasma membranes; Gag precursors Pr55 assemble into viral cores; Gag-Pol polyproteins are incorporated into the assembling particles by interactions of their *gag* determinants with Gag proteins; viral genomic RNAs are encapsidated simultaneously via interaction with the Gag proteins; virions bud out from the cell surface with incorporation of envelope proteins. During or after budding, Gag precursors and Gag-Pol polyproteins are processed by the viral protease into mature products.

VIII. Thesis Rationale

Gag proteins are not only required for core assembly but also are involved in other phases of virus life cycle. To investigate the processes of virus assembly and the potential functions of HIV Gag proteins in virus replication, we analyzed HIV Gag proteins by genetic approaches. We established a system by introducing a series of HIV *gag* mutations into a HIV replication-defective provirus (HIVgpt) which carried a drug-resistance gene in the *env* coding region. Cotransfection of COS7 cells with HIVgpt plus an amphotropic MLV envelope protein expression plasmid (A-MLV-env) resulted in production of infectious virions which could transfer the drug-resistance gene to the recipient cells. All HIV *gag* mutants were screened for particle assembly, and processing of Gag proteins by protein analyses. Infectivity of each mutant was determined by the ratios of its titer (number of drug-resistant colonies) versus wt titer in parallel experiments. Pseudotyping of HIV *gag* mutants with A-MLV-env provided higher titers in comparison to pesudotyping with HIV env and permitted us to examine the effects of *gag* mutations on postentry stages of virus infectivity. Because of pseudotyping with an amphotropic MLV envelope protein, perturbations of HIV Env functions by *gag* mutations could not be tested in this system.

In addition, we constructed HIV Gag-β-galactosidase fusion protein expression plasmids by fusion of the *E. coli.* β-gal gene to various portions of HIV *gag* gene. Assembly of each fusion construct into virus particles was determined by the ratios of β-gal activity release in the presence or absence of wt HIV Gag proteins. Although the HIV Gag-β-gal fusion protein was not identical to the natural HIV Gag-Pol protein, this system provided a convenient and sensitive

assays to define which region within Gag protein was important for fusion protein incorporation into virus particles.

Different HIV isolates differ in the cell tropism and replication capacity. In our system, only one HIV-1 strain was tested, selected in tissue culture, and HIV proteins were expressed in COS7 cells. We do not know whether our results apply to different HIV strains or different cell systems. For instance, the HIV-1 strain (HXB2) we used doesn't contain the *vpu* that can enhance virus release and *in vivo*, the rate of virus assembly and the efficiency of Gag protein processing may differ from studies in tissue culture. However, for initial studies, we believe use of the HIVgpt system is warranted.

We found that most HIV-1 *gag* mutants generated by linker-insertion or deletion still could assemble and process Gag proteins, and had significant RT activities, although they were noninfectious or poorly infectious (manuscript #1). These results suggested that HIV *gag* is functionally involved in the post-assembly and post-processing stages of virus infectivity. The findings of assembly-competent *gag* mutants with either a 56 amino acid deletion within CA (dl.NsiPst) or a deletion of most of MA (dl.MA) indicated that HIV *gag* is not completely sensitive to mutation. However, a mutant with a linker-insertion within middle portion of MA appeared trapped around perinuclear area, suggesting that HIV MA may play a role in Gag transport.

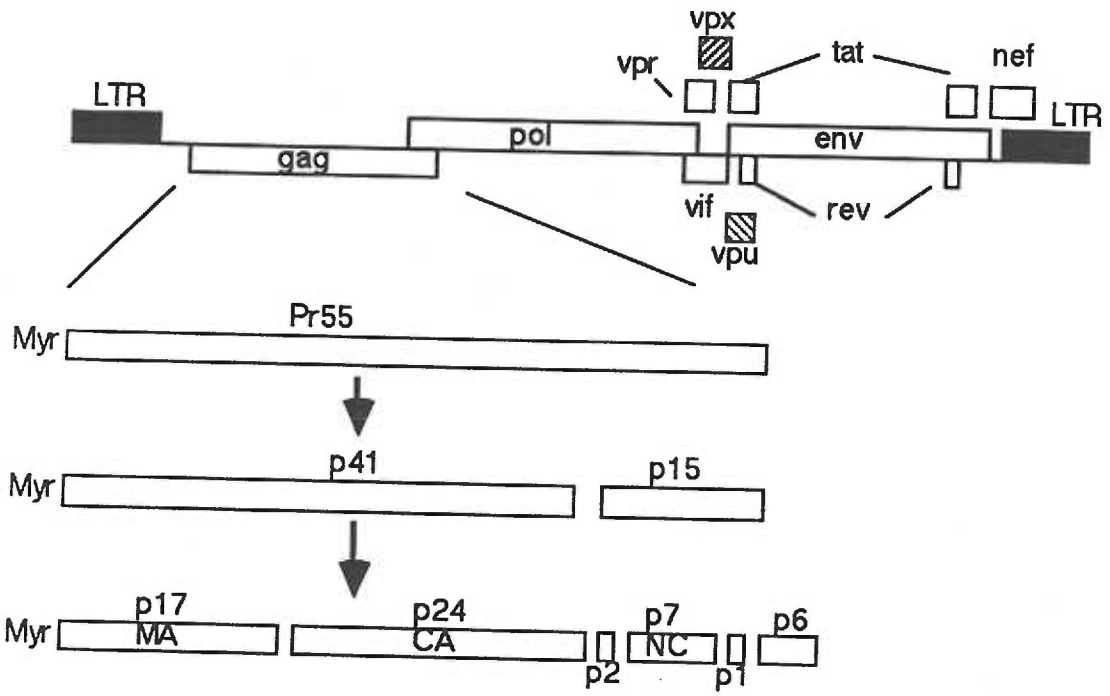
Several studies have suggested that MA also is involved in viral RNA packaging, reverse transcription, nuclear localization, or integration. However, data obtained from analyses of our HIV dl.MA mutant suggested that HIV MA is involved in HIV Env virion

incorporation or stability but is not absolutely required for viral replication (see manuscript #2).

Although the Gag-Pol fusion protein is thought to be incorporated into virions via its interaction with the Gag precursor, the mechanism remains unclear. We constructed plasmids expressing HIV Gag-β-galactosidase fusion proteins, which were versions of the natural HIV Gag-Pol fusion protein. Incorporations of a variety of mutant fusion proteins into HIV particles were analyzed by β-gal activity assay. We found that the HIV CA domain appeared to be the major determinant for Gag-Pol incorporation into particles. NC had a minor effect on the fusion protein incorporation and MA appeared not directly to be involved in incorporation of Gag-Pol into particles. Although myristylation is not required for Gag-Pol assembly into particles (129, 162), the amounts of virion-associated nonmyristylated HIV Gag-β-gal fusion proteins were much reduced in our experiments (manuscript #3).

Figure 1. HIV provirus and processing of the Gag precursor.
Shown are the HIV proviral forms, including *gag*, *pol*, *env*, and accessory genes. *Vpu* is present in HIV-1 but not in HIV-2, and *vpx* is found in HIV-2 but not in HIV-1. The Gag-Pol fusion protein is translated by ribosomal frameshifting, and the Gag-Pol and Gag precursors are cleaved by the viral protease (see the text).

Figure 1.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NC
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Myr
p2
p1

Manuscript #1

ASSEMBLY, PROCESSING AND INFECTIVITY OF HUMAN IMMUNODEFICIENCY VIRUS (HIV-1) GAG MUTANTS

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ABSTRACT

We studied the effects of *gag* mutations on human immunodeficiency virus (HIV-1) assembly, processing and infectivity using a replication-defective HIV expression system. HIV mutants were screened for infectivity by transduction of a selectable marker, and for assembly by monitoring particle release from transfected cells. Gag protein processing and reverse transcriptase activities of mutant particles also were assayed. Surprisingly, most Gag protein mutants were assembled and processed. The two exceptions to this rule were a myristylation minus mutant, and one *gag* matrix domain mutant which expressed proteins that were trapped intracellularly. Interestingly, a mutant with a 56 amino acid deletion within the HIV *gag* capsid domain still could assemble and process virus particles, exhibited a wild-type retrovirus particle density, and had wild-type reverse transcriptase activity. Indeed, although most HIV-1 *gag* mutants were non-infectious or poorly infectious, they produced apparently normal particles which possessed significant reverse transcriptase activities. These results strongly support the notion that the HIV-1 Gag proteins are functionally involved in post-assembly, post-processing stages of virus infectivity.

INTRODUCTION

Assembly of HIV virus particles occurs at the plasma membrane of infected cells (41) and results in the incorporation of several viral components. They are the viral RNA genome, core (Gag) structural proteins, envelope (Env) glycoproteins, and virion-associated enzymes encoded by the viral *pol* gene including protease, reverse transcriptase, RNase H, and integrase (6, 36). In addition to the characteristic retroviral *gag-pol-env* genome (47), HIV-1 encodes several novel proteins. One small accessory protein, Vpu, is involved in assembly process by assisting virus particle budding, although Vpu itself is not packaged into virions (7, 19, 43, 44). However, in the absence of other viral products, Gag polyproteins still can assemble as a virion-like particle, suggesting that *gag* is the only viral gene required for virus assembly (10, 39). The HIV Gag polyprotein is synthesized as a precursor Pr55, which is modified cotranslationally by methionine cleavage and the attachment of a myristic acid to the N-terminal glycine (45, 48). Myristylation is necessary for membrane association and virion formation (5, 13, 34). During or after budding, Pr55 is cleaved by the viral protease into p17 (matrix; MA), p24 (capsid; CA), p7 (nucleocapsid; NC), and p6 (17, 25, 29, 32). p17 is myristylated and membrane-associated; p24 is the major capsid structure; p7 containing two zinc finger motifs is a nucleic acid-binding protein (1, 2, 11, 40); and the p6 domain, located at the carboxyl end of Pr55, may play a functional role in the process of virus budding (10, 12).

Despite intensive efforts, the mechanism of HIV virus assembly is still unclear. One model, based on previous studies, is that myristylated Gag precursors are targeted to the plasma membrane where they self-assemble into particles. Env proteins might be incorporated by binding to Gag proteins at the plasma membrane; Gag-Pol fusion proteins would be incorporated into virions by virtue of their N-terminal *gag* determinants; and viral RNA might be encapsidated by interaction with Gag proteins at the RNA packaging signal (Psi) located around the initiation sequence of Gag (16, 27). While Gag proteins play a central role in the process of retroviral assembly, they also have been implicated in other functions. Studies have shown mutations of murine leukemia virus (MuLV) *gag* can block early stages of infection (8), and HIV *gag* mutants can interfere replication of wild-type (wt) virus (46). This evidence suggests that Gag proteins also may affect the processes of reverse transcription, nuclear transportation, or integration (4, 37, 38).

To investigate the mechanism of HIV assembly, and the potential functions of Gag proteins in other phases of the virus replication cycle, we have adapted a genetic approach. HIV *gag* mutations were created by deletion or linker insertion, and subcloned into a replication-defective HIV proviral genome (HIVgpt), which carries the drug-resistant *gpt* gene (30) in the *env* region (33). Cotransfections of wild-type or mutant HIVgpt constructs with an envelope expression plasmid permitted us to analyze how each *gag* mutation affected virus assembly and infectivity. Twelve HIV mutants were screened for infectivity, assembly, processing and reverse transcriptase (RT) activity. Surprisingly, results showed that

most mutants were assembled and released as particles. However, most mutants either were noninfectious or poorly infectious, although they had significant RT activities. Interestingly, a mutant with a 56 amino acid deletion in the capsid domain still was assembled and processed. Indeed, the only mutants incapable of particle assembly were a myristylation mutant and a linker insertion mutant in the central portion of the matrix domain. Immunofluorescence studies showed that Gag proteins of this insertion mutant localized to the perinuclear area, indicating that the matrix domain may be involved in transport or assembly of Pr55^{gag}. Overall, our results strongly support the notion that HIV Gag proteins are functionally involved in post-assembly and post-processing stages of viral infectivity.

MATERIALS AND METHODS

Cell culture, transfections, infections, and infectivity assays. HeLa and COS7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Gibco). Confluent COS7 cells were split 1:10 onto 10-cm dishes 24 hours before transfections. Fifteen micrograms plasmid DNAs of *gag* mutants or wt HIVgpt were transfected onto COS7 cells by calcium phosphate precipitation (14). At 48 to 72 hours after transfection, supernatants of COS7 cells were collected and filtered through a 0.45 µm-pore-size filter and frozen at -80°C. For infections, 15 µg plasmid DNAs of each mutant were cotransfected with 10 µg plasmid DNA of SV-A-MLV-env into COS7 cells. Two days later, cell-free supernatants of COS7 cells were used to infect HeLa cells which had been split and grown to 10% confluent at the time of infection. Adsorption of virus was allowed to proceed at 37°C in the presence of 4 µg/ml polybrene. Three days after infection, cells were trypsinized and split 1:8 onto 10 cm dishes containing selection medium. The selection medium was made of DMEM supplemented with 10% heat-inactivated fetal calf serum, penicillin plus streptomycin (Gibco), 50 µg/ml xanthine, 3 µg/ml hypoxanthine, 4 µg/ml thymidine, 10 µg/ml glycine, 150 µg/ml glutamine plus 25 µg/ml mycophenolic acid (Gibco). Cells were refed every 3-4 days with selection medium until colonies of drug-resistant cells formed. The number of colonies was converted into titer (infectious

units/ml). Infectivity of each mutant was determined by the ratio of their titers versus the titer of HIVgpt in parallel experiments.

Recombinant plasmids. The parent DNA in this study is pHXB2 (36). Plasmids HIVgpt and SV-A-MLV-env were generously provided by D. Littman (33). Construction and sequencing followed the protocols described in Maniatis et al. (28). The methods for engineering linker insertion mutants were described previously (15). Sequences in mutated regions are shown in Figure 1.

Protein analysis. Supernatants of transfected COS7 cells were collected and filtered through a 0.45 µm-pore-size filter. The filtered supernatants were centrifuged through 2 ml of 20% sucrose in TSE (10 mM Tris hydrochloride, 100 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride) at 4°C for 45 minutes at 274,000 X *g* (SW41 rotor at 40,000 rpm). Pellets were suspended in 100 µl IPB (20 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, 1% Triton X-100, 0.02% sodium azide) plus 0.1 mM PMSF (phenylmethylsulfonyl fluoride). Cells were washed twice with 10 ml ice cold phosphate-buffered saline (PBS) and collected in 1 ml of PBS for 10 cm plates. Cells then were pelleted and resuspended in 1 ml IPB plus 0.1 mM PMSF followed by microcentrifugation to remove debris. Lysate was homogenized and saved. Supernatant and cell samples were prepared for loading by adding an equal volume of 2X sample buffer (12.5 mM Tris hydrochloride [pH 6.8], 2% SDS, 20% glycerol, 0.25% bromphenol blue) and 5% β-mercaptoethanol and boiling for 4 to 5

min. Samples were subjected to SDS-polyacrylamide gel electrophoresis (21) and electroblotted onto a nitrocellulose filter. Procedures for immunodetection of nitrocellulose-bound proteins were described previously (18). Mouse anti-p24 monoclonal antibody (Epitope Inc., Beaverton, Oregon) was diluted at 1:20,000 as primary antibody. The secondary antibody was a goat anti-mouse IgG-alkaline phosphatase conjugate. HIV Gag proteins immunodetected on filters were quantitated by a scanning densitometry using a Bio-Rad model 620 video densitometer on reflectance mode. For immunofluorescence, protocols were as described elsewhere (18), using anti-p24 antibody at a 1:1,000 dilution as primary antibody and rhodomine-conjugated rabbit anti-mouse antibody at 1:100 dilution as secondary antibody.

Reverse transcriptase assay. Supernatants of transfected COS7 cells were collected and pelleted as described above. Pellets were suspended in 1X PBS followed by incubation for 2 hr at 37°C with 2X cocktail mix (100 mM Tris buffer pH 8.3, 40 mM dithiothreitol, 1.2 mM $MnCl_2$, 120 mM NaCl, 0.1% Nonidet P-40, 20 mM dTTP,10 µg/ml oligo$(dT)_{12-18}$, 20 µg/ml polyA and 20 microCi of Amersham [α-^{32}P] dTTP). Samples were precipitated with 10% TCA (trichloroacetic acid) after the 2 hr incubation. The precipitates then were washed three times with 10% TCA followed by liquid scintillation counting.

Sucrose density gradient fractionation. Linear sucrose gradients (20-50%) in TSE were poured into SW50.1 polyallomer tubes and allowed to sit at 4°C for 1 to 2 hrs. Cell-free supernatants from transfected COS7 cells were spun through a 20% sucrose cushion

at 4°C for 45 min at 274,000 X *g*. Pellets were resuspended in 1X PBS and carefully layered on top of the gradient. The gradients were centrifuged in an SW50.1 rotor overnight at 50,000 rpm (300,000 X *g*) at 4°C. Fractions of 0.4 ml were collected from top to bottom. Each fraction was measured for the density and analyzed for the Gag proteins by immunoblot.

RESULTS

Infectivity of HIV *gag* mutants

In order to define the functional domains of the *gag* gene in the processes of HIV-1 virus assembly and infectivity, a series of *gag* mutations was generated and introduced into a replication-defective HIV vector, HIVgpt. As described previously (33), HIVgpt contains a deletion of the HIV-1 *env* coding region and an insertion of a fragment from pSV2gpt (30). This insertion fragment contains the simian virus 40 origin of replication and early promoter driving expression of the selectable bacterial *gpt* gene. The two regulatory protein coding regions for *tat* and *rev* that are essential for virus replication are intact, and transient expression of HIVgpt in COS7 cells results in assembly and release of noninfectious HIV virus particles. When HIVgpt is cotransfected into COS7 cells with a retrovirus envelope protein expression plasmid, virus particles are produced which can transduce the *gpt* gene in a single round of infection (33).

As described in the Materials and Methods (see Figure 1), we constructed HIV mutations by deletion or linker insertion in the *gag* or *pol* genes of HXB2 (36). In addition, we were kindly provided with three previously created mutants (1, 5). All twelve mutations were introduced into the HIVgpt backbone, yielding the constructs illustrated in Figure 1. As shown, the Psi$^-$ construct contains a packaging signal deletion (nt 748 to 786; 1) which inhibits viral RNA

packaging (1). The Myr$^-$ construct possesses a second codon glycine to alanine mutation which reduces membrane association and blocks infectivity (5). The ClaI, AccI, HindIII and PvuII mutations were linker insertions into the HIV-1 matrix coding region at nucleotides 831, 960, 1085 and 1147, respectively (Figure 1B). Within the capsid domain, we generated a 56 amino acid deletion (dl.NsiPst) and a two codon linker insertion at nucleotide 1508 (SpeI). Further towards the Gag protein carboxy terminus are ApaI, a linker insertion in NC, and A14-15, a mutant which contains four individual mutations in the HIV-1 *gag* Cys-His motif, blocking RNA encapsidation (1). The final two mutations were BglII, located near the HIV-1 *gag-pol* frameshift region, and a linker insertion at the nt 2429 BclI site, in the protease (PR) coding region.

For infectivity analysis, each mutant was cotransfected into COS7 cells with the murine leukemia virus amphotropic *env* expression plasmid SV-A-MLV-env (33). At 48 or 72 h after transfection, virus-containing supernatants were used to infect recipient HeLa cells: infections and selections for drug-resistant colonies were performed as described in the Materials and Methods. In this regard, it should be noted that because cotransfections were not with a HIV *env* expression plasmid, potential *gag* mutations affecting HIV *env-gag* interactions could not be detected. Nevertheless, this system permitted analysis of *gag* mutants which perturbed other phases of the virus life cycle. Using our protocol, infectivity of each mutant was determined by the ratios of their titers versus the titers of wt HIVgpt in parallel experiments. Depending on the date of transfection, the wt HIVgpt construct

yielded titers which varied from 263 to 2080 colony forming units (cfu) for virus collections at 48 h, and from 3428 to 4916 for virus collected at 72 h. However, most of our *gag* mutant viruses were 20-fold less infectious than comparable wt stocks (Table 1). As shown in Table 1, several mutants (Myr^-, AccI, NsiPst, SpeI, A14-15, PR^-) appeared noninfectious. Some mutants such as Psi^-, HindIII, ApaI, and BglII retained low levels of infectivity. The PvuII mutant, within the *gag* MA domain, had levels of infectivity approximately 10% that of wt. Even higher levels of infectivity, averaging over 50% of wt levels, were demonstrated by the ClaI mutant, also within the MA domain.

Based on previous work, some of the results of our infection assays were expected. For instance, the Myr^- mutation, which has been reported to block virus assembly (5, 34), was noninfectious. Similarly, RNA encapsidation mutants mapping to the packaging signal region (Psi^-) and the nucleocapsid RNA binding motif also were poorly infectious. The other NC domain mutant, ApaI, which contained a six amino acid insertion between the two HIV Cys-His motifs, also had a low titer, as did our insertion within the protease region (PR^-). However, infectious virus particles were produced on cotransfection of PR^- with the noninfectious dl.NsiPst mutant, suggesting that NsiPst *gag-pol* proteins could provide a functional protease when incorporated into protease-minus particles. Also infectious were viruses produced by constructs with mutations just after the matrix myristylation signal (ClaI) and just before the MA-CA cleavage site (PvuII). These results suggested that, unlike other

gag protein regions, the ClaI mutation (and to lesser extent the PvuII mutation) defines an area where variation is tolerated.

Expression and assembly of HIV *gag* mutants

To assay Gag protein expression and to examine the effects of mutations on virus assembly, immunoblotting was performed to detect virus-associated Gag antigen in the media versus inside the cells. At 48 hrs after transfection of COS7 cells, cell-free supernatants and cell samples were prepared and subjected to SDS-PAGE followed by electroblotting onto a nitrocellulose membranes as described in the Materials and Methods. HIV Gag proteins then were immunodetected with anti-p24*gag* monoclonal antibody. As illustrated for several mutants in Figure 2, the mature Gag product p24*gag*, and incompletely processed proteins Pr55*gag* and p41*gag* were detected in media supernatants (lanes A to G). In cell samples, precursor Pr55*gag* and p41*gag* bands were visible (lanes I to O), while p24*gag* was variably detected (lanes I and O). In the case of dl.NsiPst (Figure 2, lanes F and N) a band at 49 kDa, and bands at 34 to 36 and 16 to 20 kDa were observed, consistent with a deletion of 56 amino acids. As shown, some mutant proteins were released efficiently from cells, whereas others were present in media supernatants at noticeably reduced levels (see AccI, Figure 2, lanes D, L).

Because these experiments involved collection of media supernatant virus proteins which were pelleted in a 45 minute spin through a 20% sucrose cushion, we believe that they represent virus-

associated proteins. Based on centrifugation clearing rate estimates, the minimum size of a pelletable particle would be 165 S, and greater than 90% of our media HIVgpt *gag* protein was recoverable by this method (data not shown). Nevertheless, we were surprised to find that even dl.NsiPst, the 56 amino acid deletion protein, was released as a high molecular weight complex, and thus we examined particles released from COS7 cells in more detail. To do so, media supernatant proteins were fractionated by sucrose density gradient centrifugation, assayed by immunoblotting and quantitated by densitometry. Since ClaI and PvuII mutants were infectious (Table 1), Psi$^-$ and A14-15 mutants had been examined previously (1), and limited release of AccI and HindIII mutant proteins prohibited their analysis, we focused our examination on wt, dl.NsiPst, SpeI, ApaI, BglII, and PR$^-$ media proteins. As shown in Figure 3, wt and all five mutant proteins banded in our fractions 7 and 8, at densities between 1.16 and 1.18 g/ml. This particle density reading is consistent with that for retrovirus particles (3, 18), and indicates that the levels of pelletable media *gag* proteins reflect the amount of virus particles released from cells.

To evaluate reliably the effects of *gag* mutations on HIV assembly and particles release, we quantitated Gag protein levels with a scanning densitometer and determined ratios of total Gag proteins levels in the media supernatants versus cells. To compare with wt HIVgpt, ratios obtained with each mutant were divided by wild-type ratios in parallel experiments. The results shown in Table 2 indicate that most mutants were capable of particle assembly and release as their ratios were comparable to that of the wt construct.

Two mutants, ClaI and dl.NsiPst, possessed media to cell Gag ratios that were even higher than the wt ratio: this result could be due either to increased levels of intracellular mutant Gag protein degradation or to increased assembly rates. In contrast, the ratio of the Myr$^-$ mutant was less than 1% of the wild-type ratio, a result consistent with the previous demonstration that myristylation-minus HIV mutants are blocked in virus assembly (5, 34). With the exception of Myr$^-$, the ratios of two matrix mutants, AccI and HindIII, were lower than those of any other mutants. Indeed, AccI Gag proteins were detected predominantly inside cells, with media to cell ratios at 8 to 30% of wt levels (Figure 2, Table 2). Theoretically, AccI *gag* protein release values could be a consequence of protein instability in virus particles or inefficient particle budding. Because we have not observed major differences in particle-associated *gag* protein degradation rates (data not shown), we favor the hypothesis that the AccI matrix mutation inhibits HIV-1 particle release. Support for this notion comes from indirect immunofluorescence studies. After transfection with wt HIVgpt, or Myr$^-$, ClaI, AccI, HindIII, BglII or PR$^-$ constructs, COS7 cells on coverslips were processed for immunofluorescent localization of Gag antigen using a anti-p24*gag* first antibody and a rhodamine-conjugated rabbit anti-mouse second antibody (see Materials and Methods). As illustrated in Figure 4A, wt Gag proteins were present throughout the cytoplasm of transfected cells as a heterogeneously staining haze. Localization patterns of ClaI, HindIII, BglII and PR$^-$ mutant Gag proteins could not be distinguished from wt patterns. In contrast, cells expressing the AccI mutant showed punctate staining asymmetrically located

around cell nuclei (Figure 4B). The pattern of the AccI mutant was different from that with the Myr$^-$ protein, which localized to the cytoplasm of transfected cells (Figure 4C). These results suggest that AccI mutants demonstrated reduced particle assembly because the Gag proteins were trapped intracellularly.

Characterization of virus particles

The results of Table 1 indicate that many of our HIV *gag* mutants were poorly infectious, while data in Table 2 shows that, for most mutants, virion release was unimpaired. An obvious explanation for this difference is that the virus particles assembled by our mutant Gag proteins were defective for one reason or another. Since reverse transcription is essential for HIV replication, *gag* mutants also were characterized by measuring reverse transcriptase (RT) activity levels. Assays were performed as described in the Materials and Methods, and in each case the cpm of incorporated nucleotide was at least 3-fold over background levels (Table 3). While nucleotide incorporation counts yielded some information as to RT levels, to obtain specific activities for each mutant, the ratios of normalized cpms versus densitometer-determined virus-associated Gag protein levels were compared with wild-type levels in parallel experiments. The results of Table 3 show that most mutants showed at least 50% of the wt RT activity. The only exceptions to this rule were the SpeI, BglII, and PR$^-$ mutants. For the BglII and SpeI mutants, which retained 30 to 60% of wt activity (Table 3), reduced RT levels could be due to reduced stability, processing, or

incorporation of *gag-pol* proteins into virions. With our protease-defective mutant, specific RT activities were 30% and 50% of wt levels, consistent with previous reports (35). Although unprocessed *gag-pol* fusion proteins may result in reduction of enzymatic activity (23, 24, 26), it is conceivable that the low enzymatic activity of the protease mutant may be due to the stability of immature virus cores (see below), and inefficient detergent release of Gag-Pol fusion proteins during RT assays.

Previous studies have shown that a functional protease is required for retrovirus infectivity (13, 20, 35), suggesting a requirement for HIV Gag protein processing. We analyzed processing of HIV *gag* mutants by immunoblotting of particle-associated proteins. Figure 5 shows that most mutants were processed as well as wild-type HIVgpt, where p24*gag* is present but precursor forms Pr55*gag* and p41*gag* also were observed. As expected, the clearest exception was the PR$^-$ mutant, where the processing was blocked completely. In this experiment, media AccI and HindIII Gag levels were below our levels of detection, consistent with their reduced release from cells, but results in Figure 2 shows that at least some processing of these proteins occurred. The dl.NsiPst construct with a 56 amino acid-deletion in the capsid domain still could be processed and detected as bands of 49 kDa, 35 kDa and 18 kDa, corresponding to the wild-type Pr55*gag*, p41*gag* and p24*gag* proteins. To assess the processing efficiency of each mutant, we quantitated the p24-associated Gag proteins in media supernatant samples by densitometry and plotted the ratio of each Gag product versus total Gag proteins. As illustrated in Figure 6, and as observed in Figures 2

and 5, most mutants displayed a processing pattern similar to that of wild-type. However, there were several exceptions. In particular, mutants AccI, SpeI, and BglII were processed incompletely, suggesting either that these mutant Gag proteins were resistant to cleavage, that mutant Gag-Pol proteins possessed defective protease moieties, or that mutant Gag-Pol proteins entered virions at reduced efficiencies (see Discussion).

Of all our mutants, only PR$^-$ was completely devoid of mature virus particle associated mature proteins. This allowed us to investigate structural differences between immature and mature HIV particles. Previous studies have demonstrated that immature virions of ASLV and MuLV protease mutants were resistant to nonionic detergent (14a, 42). To investigate whether the HIV protease mutant had a similar property, culture supernatants of transfected cells were treated with 0.5% Triton X-100 followed by centrifugation through 20% sucrose cushions as described in the Materials and Methods. Gag proteins recovered in the pellets were analyzed by immunoblotting. As shown in Figure 7, most protease mutant virions were recovered in the pellet either with or without treatment of Triton X-100 (Figure 7, lanes C and D). In contrast, p24^{gag} in particles produced by wt HIVgpt was solublized by Triton treatment (Figure 7, lanes A and B). However, it was noted that wild type HIVgpt media Pr55^{gag} proteins appeared resistant to Triton X-100 solublization, suggesting either that proteolytic processing is very rapid once it is initiated, or that immature and mature Gag proteins were not present within the same particles.

DISCUSSION

Trono et al. have reported that coexpression of HIV *gag* mutants with wild-type HIV could result in a drastic reduction of virus infectivity, suggesting that mutant Gag proteins were incorporated into wild-type virus particles and interfered with subsequent viral replication (46). We have analyzed the infectivity of HIV *gag* mutants by cotransfection with an amphotropic envelope expression plasmid, SV-A-MLV-env, into COS7 cells. Titers of generated virus particles were determined by infection and selection of drug-resistant colonies. Although HIV and MuLV Env proteins do not share significant homology, Page et al. reported that titers of virions generated by coexpression of HIVgpt and MuLV Env were generally higher than those of virions generated by coexpression of HIVgpt and HIV Env in parallel transfections, indicating that pseudotyping of MuLV Env with HIVgpt is an efficient process (33). However, a recent study demonstrated that the HIV matrix domain may be involved in the incorporation of HIV envelopes into virions (50), and we must note that due to our utilization of a murine envelope protein, we would be unable to detect specific perturbation of putative HIV Gag and Env interaction in our system.

We were surprised to discover the number of mutants that were able to assemble virus particles. To a certain extent, this may be attributable to use of a high expression COS cell system. However, Western immunoblot analysis indicated a failure of mutants AccI and Myr- to produce high levels of virus particles (Figure 2 and Table 2). Our findings with the Myr- mutant corroborate previous reports that

myristylation of HIV Gag is required for virus assembly and infectivity (5, 34). Also, previous work has shown that deletion mutations around the MA AccI region could block virus assembly, as very little RT activity was released into the culture media (50): however, these mutants were not further characterized. Indirect immunofluoresence revealed that mutant AccI Gag proteins appeared trapped around perinuclear area (Figure 3), indicating that mutation at this region may block a normal Gag transport route or cause the protein to mislocalize. Previous studies have suggested that the matrix domain of Moloney murine leukemia virus Gag protein may interact with intracellular membranes prior to transport to cell surface (15). Thus, it is conceivable that the HIV matrix domain also may interact with intracellular membranes under some circumstances.

Our linker-generated mutants ClaI (near the MA amino terminus, after the 15th codon of *gag*) and PvuII (after codon 120, 12 codons from the CA coding region) did not affect virus assembly or processing and had significant exogenous template RT activities, consistent with previous reports (50, 51). Our infectivity analysis showed mutants ClaI and PvuII were somewhat infectious (PvuII), or up to half as infectious as wt (ClaI). However, we cannot exclude the possibility that the matrix domain may be involved in *in vivo* replication processes.

Several lines of evidence have suggested that retroviral capsid domains are key regions responsible for interactions among the *gag* polyproteins (9, 37). Surprisingly, our HIV mutant containing a 56 amino acid CA deletion did not affect virus assembly, budding, or

processing (Figures 2 and 6). The dl.NsiPst mutant RT activity was comparable to that of wild-type (Table 3), and this mutant even exhibited a characteristic wild-type retrovirus density of 1.16-1.18 g/ml (Figure 3). Apparently, the amino-terminal HIV Gag CA this region may be dispensable for HIV core assembly. The other capsid mutation (SpeI) containing only a two amino acid insertion, apparently eliminated viral infectivity (Table 1). Incomplete processing (Figures 2 and 6) and low enzymatic activity (Table 3) may have rendered this mutant noninfectious. However, early post-binding steps in the infection process also may be affected by the SpeI mutation.

Both nucleocapsid mutants A14-15 and ApaI could assemble, release and process Pr55*gag* (Figures 2 and 6), but showed reduced or no infectivity (Table 1). These observations are consistent with previous work, suggesting that NC (p7) does not play a role in core assembly (1). The inability of mutant A14-15 to replicate appears to be due to defective packaging of viral RNA into virions, as previous quantitation of viral RNA by slot-blot hybridization (1) showed a nearly complete block in RNA encapsidation (1). However, preliminary results with our ApaI linker insertion between Cys-His finger motifs suggest that RNA incorporation is not inhibited (data not shown), although the mutation reduced infectivity.

As for the PR$^-$ mutant (BclI), our findings support previous studies (13, 35) which showed that protease-defective mutants are blocked for virus infectivity but not for assembly. The fact that maturation of immature virions by viral protease is required for viral infectivity, is consistent with our findings with mutants AccI,

SpeI, and BglII, in which virions are both incompletely processed and noninfectious or poorly infectious (Table 1 and Figure 6). It is possible that these mutations may lead to conformation changes in Gag precursors, and subsequently interfere with the exposure of cleavage sites to protease. Alternatively, conformation changes of Gag-Pol fusion proteins induced by these mutants may interfere with dimer formation of Gag-Pol molecules, which is required for activation of viral protease (22, 31, 49). The low RT activity of mutants AccI, SpeI, and BglII, also may be a consequence of incomplete processing, defective Gag-Pol protein dimerization, or insufficient incorporation of Gag-Pol into virions (22, 31, 42).

Because the majority of our HIV *gag* mutants assembled noninfectious or poorly infectious particles, these studies strongly support the notion that retroviral Gag proteins function not only driving assembly for the construction of virus particles, but also in a variety of post-assembly, post-processing events. It will be of interest to dissect the effects of these and other mutations on the processes of endogenous template reverse transcription, nuclear localization and integration events.

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Table 1. Infectivity of HIV *gag* mutants.

Construct[a]	Titer	HIVgpt titer[b]	infectivity[c] %
HIVgpt	1,681	1,681	100
Psi-	8	263	3.0
Myr-	0	263	0
ClaI	72	544	13.2
ClaI	3,234	4,916	65.8
ClaI	3,687	4,916	75.0
AccI	0	880	0
AccI	0	1,660	0
HindIII	2	1,392	0.1
HindIII	183	3,428	5.3
PvuII	66	544	12.1
PvuII	174	4,916	3.5
PvuII	508	4,916	10.3
dl.NsiPst	0	544	0
dl.NsiPst	0	660	0
SpeI	0	2,080	0
SpeI	0	2,080	0
ApaI	2	984	0.2
ApaI	251	3,428	7.3
A14-15	0	660	0
A14-15	0	660	0
BglII	0	1,392	0
BglII	33	3,428	1.0
PR-	0	1,392	0
PR-	0	660	0

dl.NsiPst + PR⁻	3	660	0.5

[a] Each construct was cotransfected with SV-MLV-A-env into COS7 cells. In most cases, two days later, cell-free supernatants were used to infect HeLa cells. In two experiments (those with HIVgpt titers of 3,428 and 4,916), cell supernatants were collected after three days rather than two days. Infections and selections for mycophenolic acid-resistant colonies were performed as described in the Materials and Methods.

[b] The value for the HIVgpt titer was an average of 10 independent experiments. The average for HIVgpt titers from three and two day collections were 4,172 and 1,058 ± 611 respectively. Duplicate experiments were performed with different DNAs, at different times, or both.

[c] Infectivities for each mutant were determined by the ratio of its titer versus the wt HIVgpt titer in a parallel experiment shown in the middle column.

Table 2. HIV Gag protein release from cells[a]

Construct	Expt	Total Gag protein in[b]: Media	Cells	Media/Cell ratio[c]	%WT
HIVgpt	1	4.96	3.35	1.46	100
	2	31.88	24.0	1.33	100
	3	9.89	15.2	0.65	100
	4	3.62	6.0	0.60	100
Myr$^-$	1	< 0.32	19.6	0.016	<1
ClaI	2	30.6	9.8	3.12	234
AccI	1	0.72	6.0	0.12	8
	2	3.74	9.2	0.41	30
	3	1.57	16.0	0.10	15
HindIII	2	10.8	10.0	1.08	81
	3	6.66	19.6	0.34	52
PvuII	4	2.91	7.2	0.40	67
dl.NsiPst	2	24.47	7.2	3.40	255
SpeI	3	9.20	4.6	2.0	130
ApaI	3	4.43	7.2	0.62	95
BglII	2	8.32	32.4	1.49	112
	3	34.4	18.6	1.85	286
PR$^-$	2	18.23	9.6	1.90	143

[a] Supernatant and cell samples of wild type and mutant constructs were analyzed by immunoblot techniques as described in the Materials and Methods. Gag proteins from media or cell samples were quantitated by scanning Pr55, p41, and p24 band densities from immunoblots. The results of four separate experiments were compiled.

[b] The sum of arbitrary density units (Pr55 + p41 + p24) indicated the total Gag proteins in the media or inside the cells. In the case of the Myr^- mutant, no Gag proteins could be detected in the media: the value 0.32 indicated the minimal detectable signal by densitometry.

[c] Ratios of total Gag protein in the media versus total Gag inside the cells were indicators of virus particle assembly and release. For comparison with release of wild-type virus, the ratio of each mutant was divided by the ratio of wild-type in each parallel experiment and was multiplied by 100.

Table 3. Reverse transcriptase activities of HIV *gag* mutants[a]

Construct	cpm incorporated	Relative activity (% WT)[b]
HIVgpt	3,090	100
	5,890	100
	1,781	100
	4,868	100
ClaI	5,700	101
	2,621	56
AccI	1,450	66
HindIII	5,420	83
	1,380	54
NsiPst	5,180	113
	3,453	90
SpeI	1,810	45
	1,526	43
A14-15	1,838	63
BglII	3,010	30
	5,710	64
PR$^-$	1,310	30
	1,352	50

[a] Preparation of supernatants and reverse transcriptase (RT) assays were performed as described in the Materials and Methods.

Note that activities (cpm incorporated) were at least 3-fold higher than that of background (396 ± 6 cpm). For each sample, virus-associated Gag protein levels were determined as described in Table 2. Results of four separate transfection experiments are given.

[b] Relative activities were determined as percentages of wild-type (wt) activities as follows: 100 X [(mutant cpm-background/mutant Gag protein)/(wt cpm-background/wt Gag protein)].

Figure 1. HIV *gag* mutations.

(A) Deletion, site-directed, and linker insertion mutations were generated at the designated nucleotide locations in the HIV-1 *gag* gene and introduced into the HIVgpt construct for analysis. HIVgpt, described by Page et al. (33), contains an SV40 *ori* and the drug resistance *gpt* gene in place of *env* coding sequences. The *tat* and *rev* genes remain intact, and expression of HIVgpt in COS7 cells results in release of noninfectious virus particles. As shown, four mutations were created within the matrix domain by linker insertion at restriction sites of ClaI-831, AccI-960, HindIII-1085, and PvuII-1147. In addition, a myristylation minus version of HIVgpt was generated from a mutant kindly provided by L. Ratner (5). Two capsid mutations were generated: a mutation with a deletion from NsiI-1251 to PstI-1418 (dl.NsiPst) and a mutant with a linker insertion at SpeI-1508. Within the nucleocapsid domain, there were two linker-generated mutations: ApaI-2010 and BglII-2096. A third nucleocapsid mutation, A14-15, was a site-specific mutation at the zinc finger motifs and was a gift from R. Young (1). In addition, there were two mutations outside the *gag* coding region, including a protease mutation with a linker insertion at BclI-2429, and a packaging signal (Psi) deletion mutation, also from R. Young (1).

(B) Viral RNA sequences and encoded protein sequences of mutated HIV *gag* regions are shown. In the case of the Psi mutant, 39 bp between the first splice donor site and the *gag* initiation codon were deleted, while fifty-six amino acids were deleted in mutant dl.NsiPst. For all other mutants, inserted or changed amino acids are designated in bold type set.

HIV GAG MUTATIONS

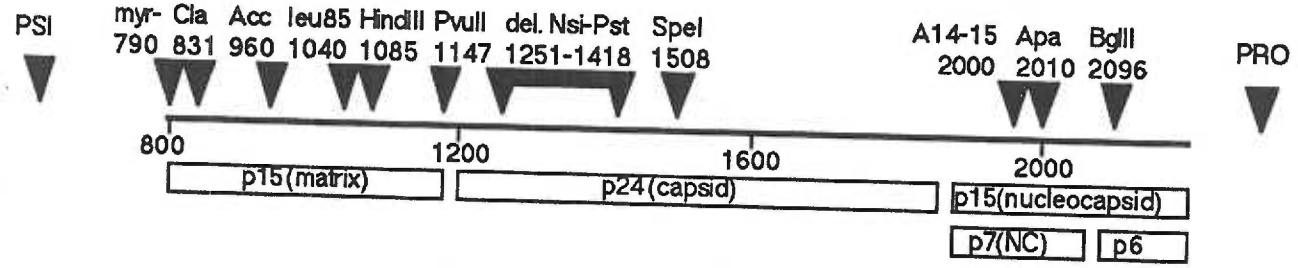


Figure 1B. HIV *gag* mutations.

Psi⁻	----------39 bp deletion------- AG AUG GGT GCG AGA M G A R
Myr⁻	AUG GCU GCG AGA GCG UCA GUA UUA AGC GGG GGA GAA UUA GAU M **G>A** A R A S V L S G G E L D
ClaI	GGA GAA UUA GAU CGA UGU CGA CAU CGA UGG GAA AAA AUU CGG G E L D R **C R H R** W E K I R
AccI	ACA UCA GAA GGC UGU AGC GGG AUC CCG AGA CAA AUA CUG GGA T S E G C **S G I P** R Q I L G
HindIII	GAC ACC AAG GAA GCU CGG AUC CGA GCU UUA GAC AAG AUA GAG D T K E A **R I R A** L D K I E
PvuII	CAA GCA GCA GCA GAU CGU CGA CGA UCU GAC ACA GGA CAC AGC Q A A A **D R R R S** D T G H S
NsiPst	UCA CCU AGA ACU UUA AAU GCA/GAA UGG GAU AGA GUG CAU CCA S P R T L N A E W D R V H P
SpeI	AUA GCA GGA ACU ACU AGU UCU AGU ACC CUU CAG GAA CAA AUA I A G T T S **S S** T L Q E Q I
A14-15	UAU UUC AAU UAU GGC AAA GAA GGG CAC ACA GCC AGA AAU UGC **CY** F N **CY** G K E K H T A R N C UAU UGG AAA UAU GGA AAG GAA GGA CAC CAA AUG AAA GAU UGU **CY** W K **CY** G K E G H Q M K D C
ApaI	AGA AAU UGC AGG GCC CCC CCU CGA GGG GGG GCC CCU AGG AAA R N C R **A P P R G G** A P R K
BglII	AAU UUU UUA GGG AAG AUC CGU CGA CGG AUC UGG CCU UCC UAC N F L G K **I R R D** I W P S Y
PR⁻	AGA CAG UAU GAU CCG UCG ACG GAU CAG AUA CUC AUA GAA AUC R Q Y D **P S T D** Q I L I E I

Figure 2. Expression and release of HIV Gag proteins.

COS7 cells were transfected with the designated plasmids. After 48-72 hrs, supernatants and cells were collected and prepared for protein analysis as described in the Materials and Methods. Supernatant samples (lanes A to G; corresponding to 50% of the total sample) and cell samples (lanes I to O; corresponding to 5 % of the total cell sample) were fractionated by SDS-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose filter. HIV Gag proteins were detected with a mouse anti-p24 monoclonal antibody at a 1:20,000 dilution followed by a secondary alkaline phosphatase-conjugated goat anti-mouse antibody at a 1:1,500 dilution, and detection of alkaline phosphatase activity. Molecular weight markers (lane H) are indicated on the left, and HIV Gag proteins Pr55, p41, and p24 are shown on the right. Lanes were as follows: A and I, wild type; B and J, mock; C and K, ClaI, D and L, AccI; E and M, HindIII; F and N, NsiPst; G and O, BglII.

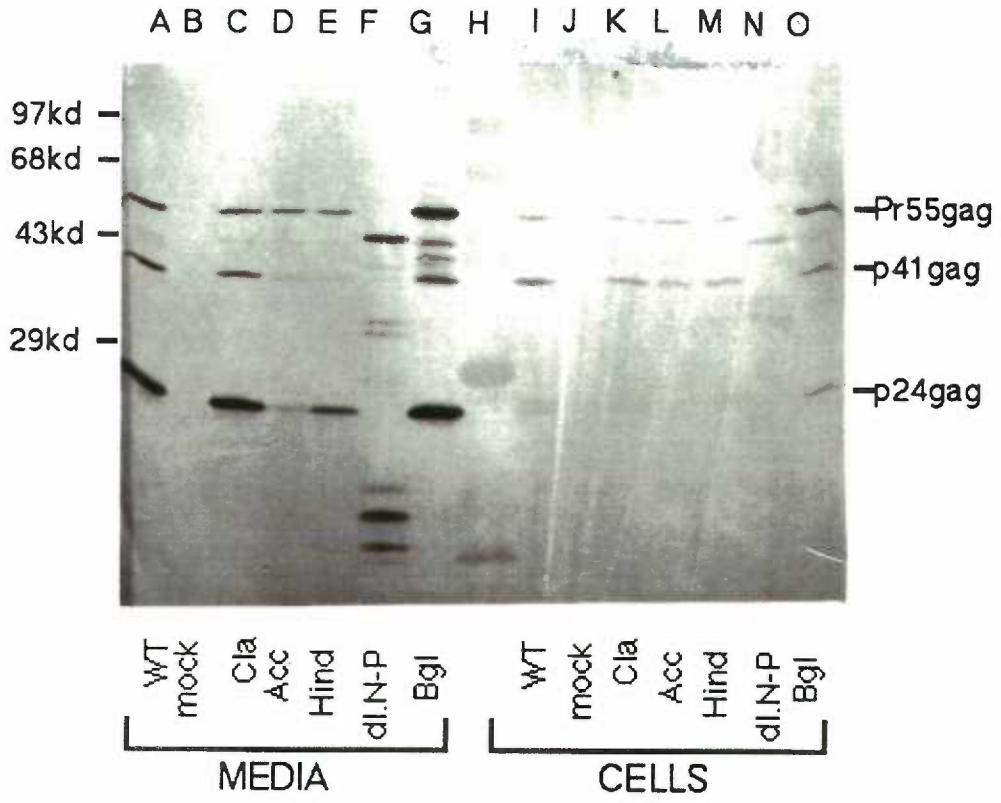
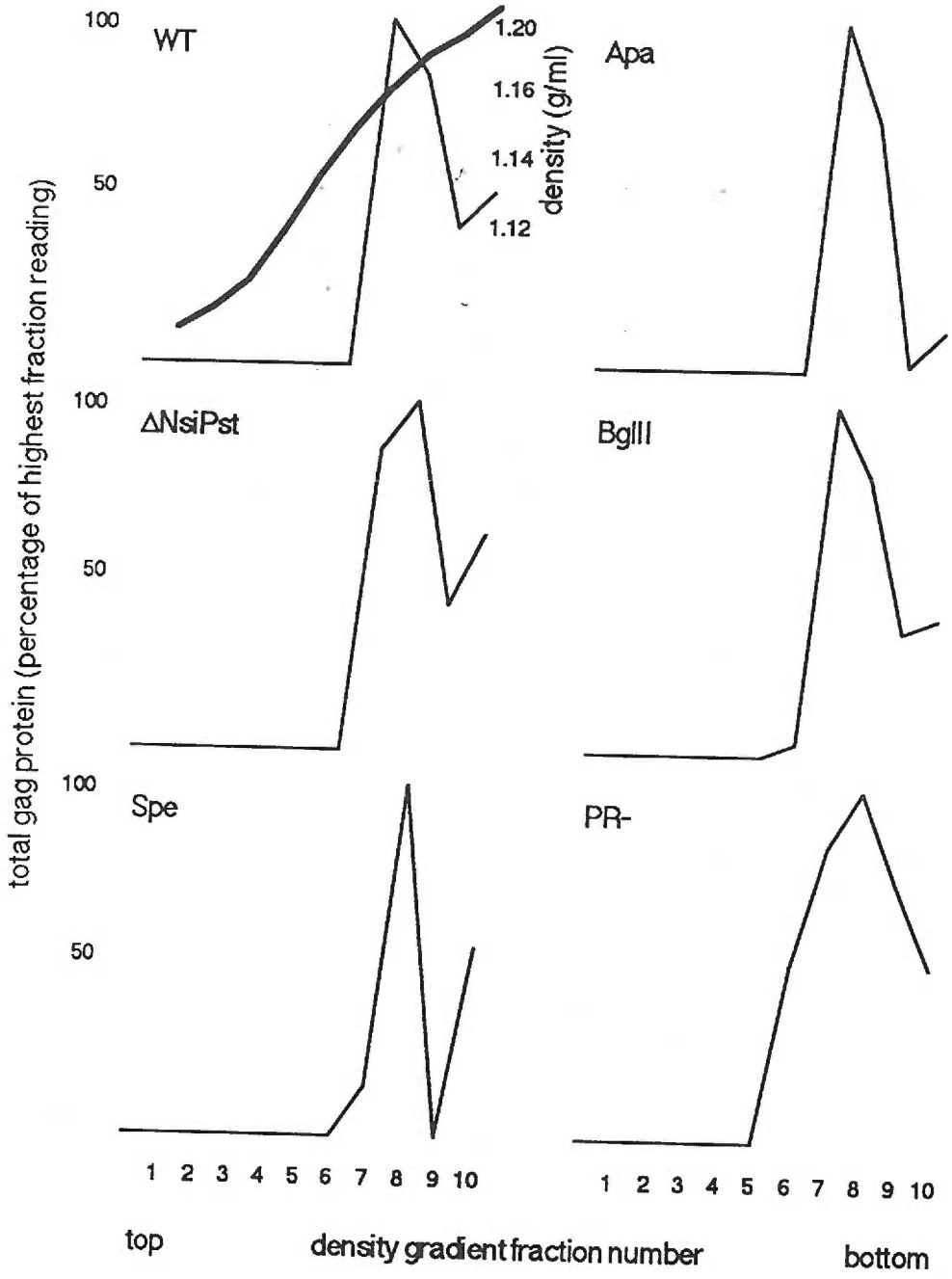

A B C D E F G H I J K L M N O
97kd
68kd
43kd
29kd
Pr55gag
p41gag
p24gag
WT
mock
Cla
Acc
Hind
dl.N-P
Bgl
MEDIA
WT
mock
Cla
Acc
Hind
dl.N-P
Bgl
CELLS

Figure 3. Sucrose density gradient fractionation of HIV particles.

Supernatants from transfected COS7 cells were collected and fractionated by sucrose density gradients (20 to 50%) as described in the Materials and Methods. Fractions were collected from top to bottom, and virus-associated Gag proteins from each fraction were measured by immunoblot techniques and quantitated by scanning densitometry. As shown, the peak Gag protein fractions had densities of 1.16-1.18 g/ml.



Wang and Barklis
Figure 3

Figure 4. Indirect immunofluorescence detection of HIV *gag* proteins in COS7 cells.

COS7 cells grown on cover slips were transfected with wild-type HIVgpt (A), or *gag* AccI (B), or Myr$^-$ (C) mutant constructs. At 48 h after transfection, cells were fixed and permeablized for immunofluorescence assays as described in the Materials and Methods. The primary antibody was a mouse anti-p24 monoclonal antibody at a 1:1,000 dilution, and the secondary antibody was a 1:100 dilution of rhodamine-conjugated rabbit anti-mouse antibody. Mock transfected COS7 cells and cells not exposed to the primary anti-gag antibody yielded no signals (data not shown). The white bars indicate 20 microns.

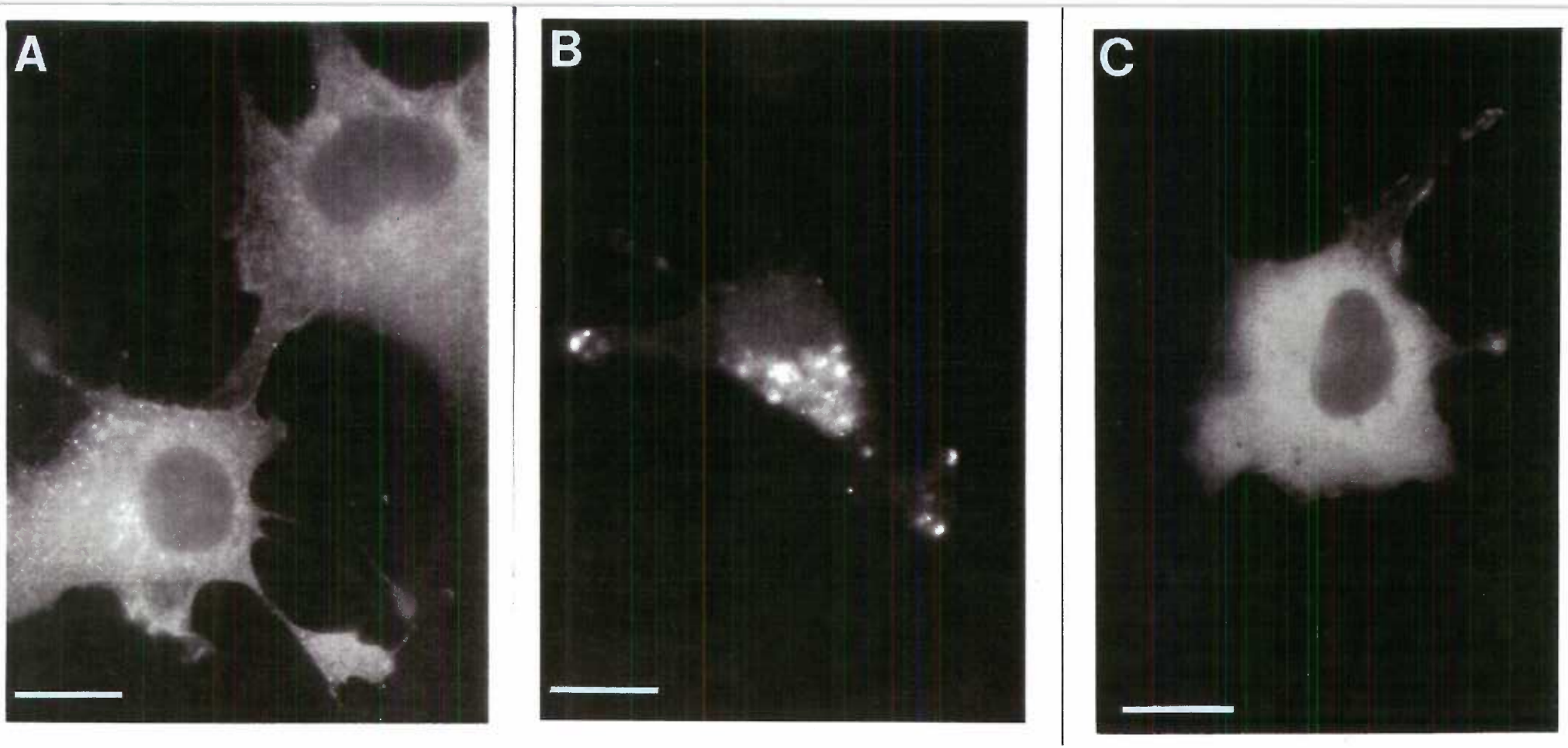

A
B
C

Figure 5. Assembly and processing of HIV *gag* mutants.

COS7 cells were transfected with wild type HIVgpt and mutant plasmids. Forty-eight hours after transfections, supernatants were collected and prepared for protein analysis as described in the Materials and Methods. Samples were fractionated by 10% SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis with anti-p24 antibody as described in legend of Figure 2. Lane designations are as follows: A, wt (HIVgpt); B, Psi^-; C, ClaI; D, Acc; E, HindIII; F, NsiPst; G, SpeI; H, ApaI; I, A14-15; J, BglII; K, Pro^-; L, mock; M, standard. HIV Pr55, p41 and p24 Gag proteins are designated at the left. Note that particle release of AccI and HindIII mutants was low in this experiment and that processing levels were determined from other transfections.

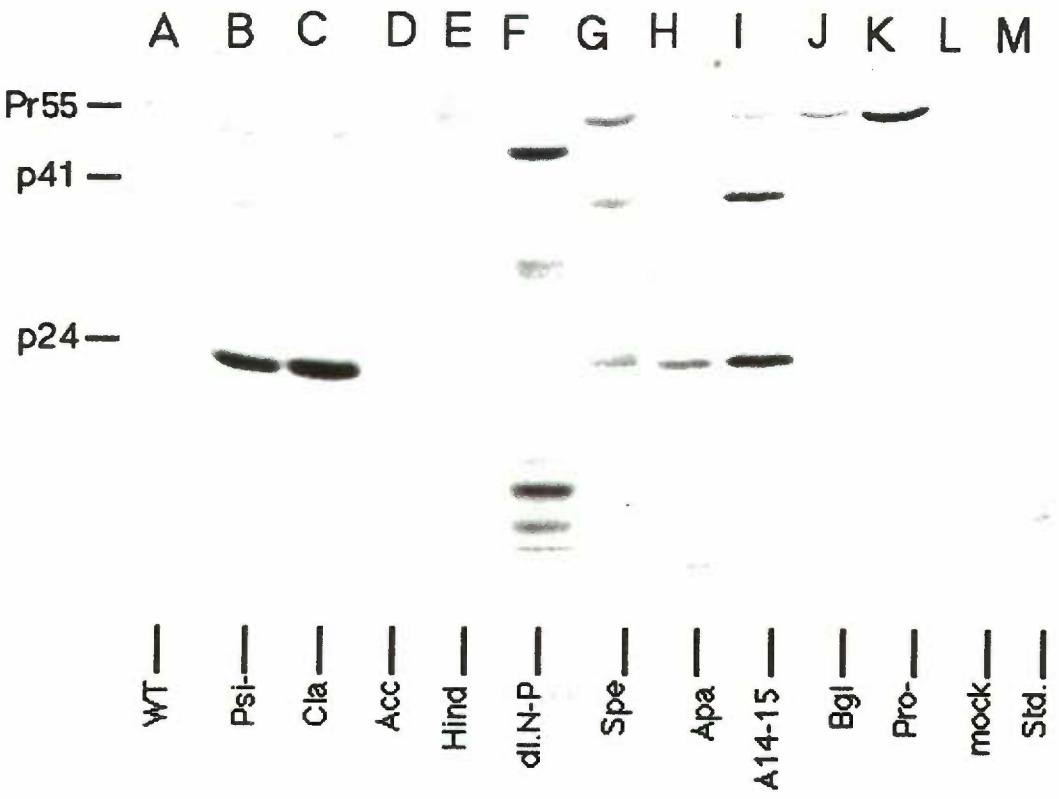
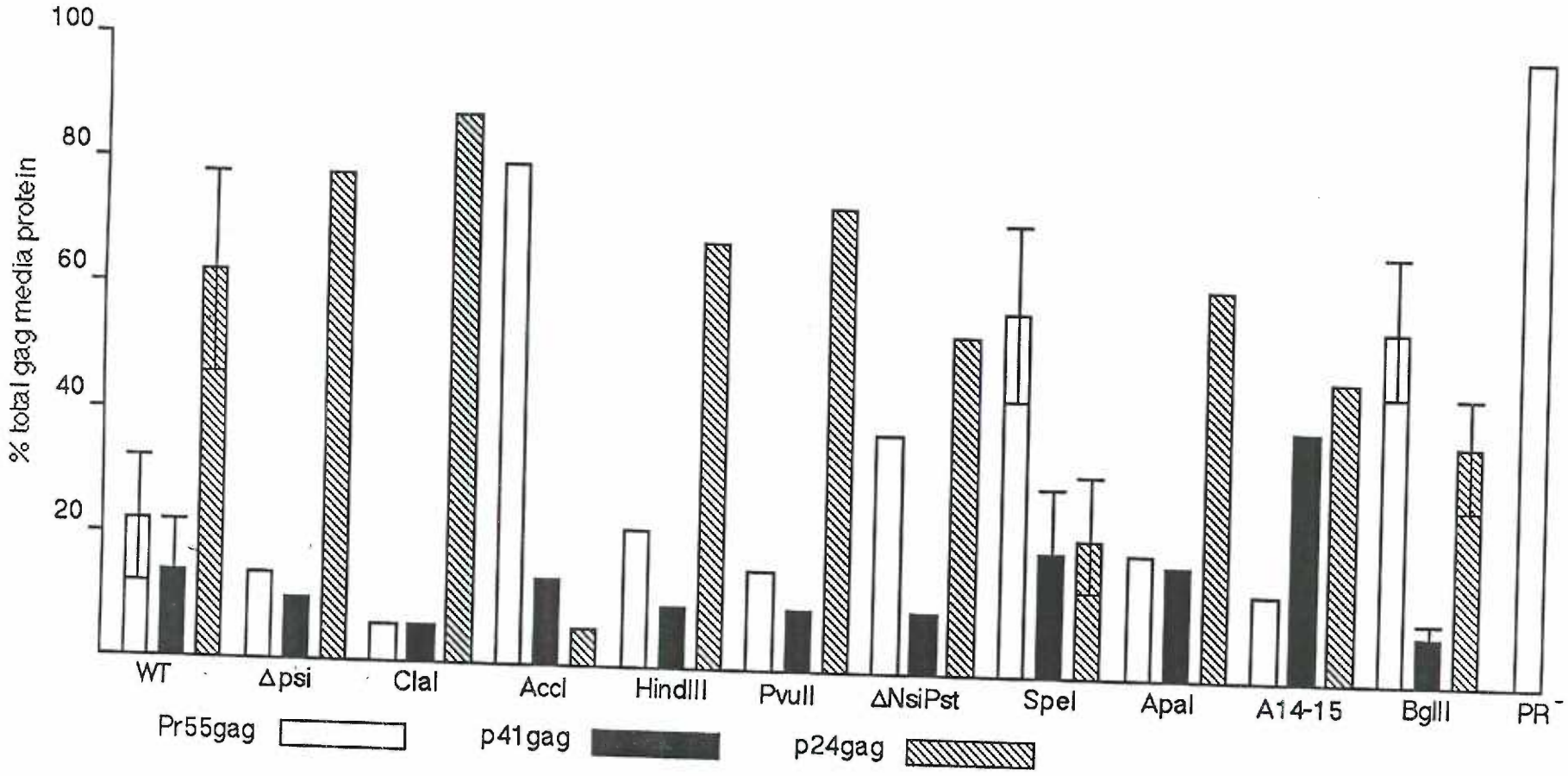

A B C D E F G H I J K L M
Pr55
p41
p24
WT
Psi-
Cla
Acc
Hind
dl.N-P
Spe
Apa
A14-15
Bgl
Pro-
mock
Std.

Figure 6. Processing of HIV *gag* mutants.

Gag proteins in media supernatants were quantitated by a scanning densitometry as described in legend of Table 2. The percentages of Pr55, p41, and p24 were obtained by dividing density units of individual bands by the total Gag protein density units, and multiplying by 100. Wild-type values derived from six independent trials; mutants SpeI and BglII were from three experiments each; and all others derived from one experiment each.

Processing of HIV gag mutants



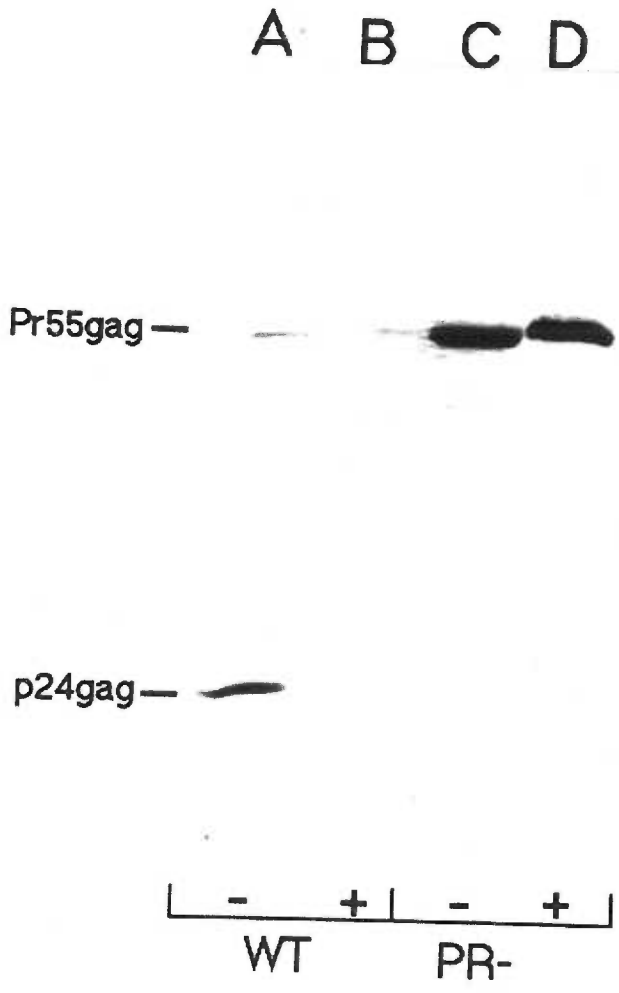

A B C D
Pr55gag
p24gag
- + - +
WT
PR-

Figure 7. Virus particle sensitivity to non-ionic detergent treatment.

Supernatants from wild-type or protease mutant transfected COS7 cells were filtered through a 0.45 µm-pore-size filter. Cell-free supernatants then were mock treated (lanes A, C) or treated with 0.5% Triton-X 100 (lanes B, D). Following treatment, samples were centrifuged through 2 ml 20% sucrose at 274,000 X *g* and 4°C for 45 min. Pellets were solublized in sample buffer and separated on 10% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose filter. Immunodetection of Gag proteins was performed as described in legend to Figure 2. Core protein precursor Pr55*gag* and mature p24*gag* protein bands are indicated on the left.

Manuscript #2

CONDITIONAL INFECTIVITY OF A HUMAN IMMUNODEFICIENCY VIRUS MATRIX DOMAIN DELETION MUTANT

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Running title: Human Immunodeficiency Virus Matrix Mutant

ABSTRACT

We constructed a human immunodeficiency virus matrix (MA) deletion mutant by deletion of about 80% of the HIV-1 Gag MA domain but retaining myristylation and proteolytic processing signals. The effects of this deletion matrix (dl.MA) mutant on HIV particle assembly, processing, and infectivity were analyzed. Surprisingly, this dl.MA mutant still could assemble and process virus particles, had a wild-type (wt) retrovirus particle density, and possessed wt reverse transcriptase activity. RNase protection experiments showed that dl.MA mutant particles preferentially packaged viral genomic RNA. When both mutant and wt particles were pseudotyped with an amphotropic murine leukemia virus envelope protein, mutant infectivity was about 10% of wt level. In contrast, infectivity of the dl.MA mutant was 1,000-fold less than that of wild-type when mutant and wt particles were pseudotyped with the HIV envelope protein. Protein analyses of pseudotyped virions indicated that there was no major difference between mutant and wt viruses in the efficiency of amphotropic murine leukemia virus envelope protein incorporation. In contrast, there was a reduction in the amount of mutant particle-associated HIV envelope protein gp120, while the levels of virus-associated HIV envelope precursor protein gp160 were similar for the two virus types. Our results suggest that an intact HIV matrix domain is not absolutely required for reverse transcription, nuclear localization or integration, but is necessary for appropriate HIV envelope protein function.

INTRODUCTION

The *gag* gene of all retroviruses including the human immunodeficiency virus (HIV) encodes the viral core protein, Gag. In the absence of other viral components, the expressed Gag protein can assemble into a virus-like particle, indicating that Gag is sufficient for particle formation (9, 15, 22, 46, 47). In HIV, Gag is translated initially as a polyprotein, Pr55. A myristic acid is attached to the glycine, the second amino acid residue, after cleavage of the N-terminal methionine during translation (51, 54): myristylation is essential for HIV assembly (3, 13, 36, 52). In the late stages of retrovirus assembly, Gag proteins are transported to the plasma membrane where assembly proceeds. Viral genomic RNAs appear to be encapsidated via an interaction of the RNA packaging signal (Psi) and Gag and/or Gag-Pol proteins (1, 11, 18, 26). Assembling virions receive their envelope coats containing HIV envelope (Env) proteins when they bud out from the cell membrane (50). During or after budding, Gag precursors are processed by the viral protease (PR) into four major products, namely, p15 (MA, matrix), p24 (CA, capsid), p7 (NC, nucleocapsid), and p6 (5, 19, 25, 33). Mutations of p6 domain do not affect assembly but may interfere with the process of budding (9, 12). The NC domain contains two Cys-His motifs which are required for packaging of viral genomic RNA into virus particles (1, 11). The capsid protein, p24, forms the core of the virion, while the amino-terminal Gag product p17 appears to form a matrix under the envelope.

For most retroviruses, a variety of potential function have been ascribed to the Gag MA domain. In many cases, the MA domain has been implicated in protein transport, membrane binding, and particle formation (17, 42, 43, 52). For instance, MA deletion in Mason-Pfizer monkey virus (MPMV) (42, 43) and Rous sarcoma virus (RSV) (56) destroys particle formation. In Moloney murine leukemia virus (M-MuLV), amino acid insertions in the MA domains which do not block myristylation still affect protein transport and assembly into virions (17). Additionally, our previous studies identified a HIV *gag* insertion mutant which was impaired in Gag transport to the plasma membrane and particle formation (52). Besides transport, membrane binding and particle formation, other MA functions have been postulated. Because some retroviral proteins can bind nucleic acid *in vitro* (7, 28, 30 34, 44, 49), it has been proposed that viral genomic RNA may be incorporated preferentially into particles via a specific interaction with MA (7, 34, 44). Also, since recent studies have shown that p17 (MA) was localized in the nuclei of HIV-infected cells (45), it is conceivable that MA also may be involved in steps of virus replication such as reverse transcription, nuclear localization, and integration. Finally, it has been proposed that a specific interaction between Env and Gag may promote the incorporation of Env into virions (50). In the case of M-MuLV such an interaction presumably would involve an association of M-MuLV MA with the M-MuLV Env protein complex gp70 (SU) and p15E (TM) or its precursor Pr80 Env, while with HIV, MA may associate with the HIV Env complex gp120 (SU) plus gp41 (TM) or its precursor, gp160. Indeed, recent genetic

evidence suggests that such an Env-MA interaction occurs for HIV-1 (58, 59).

Here we report a novel HIV MA-deletion mutant, in which about four-fifths of the HIV-1 MA codons were deleted, retaining the N-terminal amino acid residues for the myristylation signal and a few MA protein C-terminal residues just before the MA/CA cleavage site. Western blot analysis and sucrose density gradient fractionation experiments indicated that the dl.MA mutant can assemble virus particles and proteolytically process Gag proteins. Reverse transcriptase (RT) activities of this mutant were comparable to wild-type (wt) levels, and RNase protection experiments showed that mutant particles contained significant levels of viral genomic RNA. To our surprise, this mutant produced infectious virus particles when cotransfected with a murine leukemia amphotropic envelope expression plasmid into COS7 cells: viruses in supernatants from cotransfected COS7 cells infected target cells at about 10% wt levels. Cotransfection of this dl.MA mutant with a HIV Env expression plasmid resulted in the production of noninfectious virus particles. Our results indicate that , aside from myristylation and cleavage signals, the HIV MA domain is not absolutely required for particle formation, RNA incorporation, reverse transcription, nuclear localization or integration. However, the HIV MA domain appears essential to HIV Env function, although our data suggest that MA facilitates gp120 function and/or stability in virus particles, rather than the preferential incorporation of HIV Env into particles.

MATERIALS AND METHODS

Cell culture, transfections, and infections. COS7, HeLa and $CD4^+$ HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (Gibco). Confluent COS7 cells were trypsinized and split 1:10 onto 10-cm dishes 24 h before transfection. For protein analysis, fifteen micrograms of wild-type or dl.MA *gag* mutant HIVgpt plasmid DNA were transfected into COS7 cells by calcium phosphate precipitation (14). To enhance transfection efficiency, 50 µM chloroquine was added to the media during the pre-glycerol shock incubation periods (4 to 6 h). Three days later, supernatants of transfected COS7 cells were filtered through 0.45 µm-pore size filters and frozen at -80^0C prior to processing. For infections, either fifteen or ten micrograms of wt or dl.MA mutant HIVgpt plasmid DNA were cotransfected with either ten or five micrograms DNA of amphotropic murine leukemia virus (A-MLV) or HIV-1 envelope protein expression plasmids. At 72 h after transfections, filtered supernatants were used to infect $CD4^+$ HeLa cells in the presence of 4 µg/ml polybrene. Selection of mycophenolic acid-resistant colonies followed the protocols described previously (52). Colonies were fixed and stained with 50% methanol plus 0.5% methylene blue. Numbers of drug-resistant colonies were converted into titers (colony forming units/ml).

Recombinant plasmids. The parent HIV wild-type plasmid in this study is HIVgpt, which derives from the HXB2 HIV provirus (39).

The dl.MA mutant was derived from recombination of two previously generated HIV *gag* mutants (52). Construction and sequencing followed the protocols described in Maniatis et al. (29). The retained and inserted codons within the matrix domain of dl.MA mutant are shown in Figure 1. Plasmids HIVgpt, SV-A-MLV-env, and HIV-env were provided by D. Littman (35).

Protein analysis. Intracellular and virion-associated Gag proteins were analyzed essentially as described previously (52). Briefly, filtered supernatants derived from transfected COS7 were centrifuged either through a 2 ml cushion of 20% sucrose in TSE (10 mM Tris hydrochloride, 100 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonylfluroide [PMSF]) at 4^0C for 45 min at 274,000 X *g* (SW41 rotor at 40,000 rpm) or through a 3 ml cushion of 20% sucrose in TSE at 4^0C for 2 h (SW28 rotor at 20,000 rpm). Virus pellets were suspended in IPB (20 mM Tris hydrochloride pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, 1% Triton X-100, 0.02% sodium azide) plus 0.1 mM PMSF. Cells were rinsed with ice-cold phosphate-buffered saline (PBS) and collected in IPB plus 0.1 mM PMSF followed by microcentrifugation at 4^0C for 15 min at 13,700 X *g* (14,000 rpm) to remove debris. Cell lysate was homogenized and saved at -80^0C. Supernatant and cell samples were subject to SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 24) followed by Western immunoblotting. For immunodetection of HIV Gag proteins, a mouse anti-p24 monoclonal antibody (Epitope Inc, Beaverton, Oregon) was diluted at 1:20,000 as primary antibody. For immunodetection of HIV envelope

proteins, the primary antibody was mouse anti-gp120 (#110-1, Genetic Systems, Redmond, WA) at 1:5,000 dilution, and in the case of MLV, the primary antibody was a goat anti-gp70 antibody at 1:5,000 dilution. Secondary antibodies were either goat anti-mouse or a swine anti-goat IgG-alkaline phsophatase conjugates. For immunofluorescence and sucrose density gradient fractionation, protocols were as described previously (17, 21, 52).

Reverse transcriptase assays. Supernatants of transfected COS7 cells were harvested and pelleted as described above. Pellets were suspended in 1X PBS and mixed with 50 mM Tris buffer pH 8.3, 20 mM dithiothreitol, 0.6 mM $MnCl_2$, 60 mM NaCl, 0.05% Nonidet P-40, 10 µM dTTP, 5 µg/ml oligo$(dT)_{12-18}$, 10 µg/ml polyA and 10 µCi/ml [α-^{32}P]dTTP. Reactions were allowed to proceed at 37^{0}C for 1-2 h. Samples were precipitated with 10% TCA (trichloroacetic acid) and washed three times followed by liquid scintillation counting.

RNase protection. Three days posttransfection, supernatants of transfected COS7 cells were collected and pelleted as described above. The pellets were suspended in RNase-free water containing 300 mM sodium acetate pH 7.6, 0.1% sodium dodecyl sulfate (SDS), 10 mM EDTA, and 100 µg/ml yeast tRNA. Aliquots were removed for protein samples and then the suspensions were extracted once with phenol/chloroform, once with chloroform, twice with phenol/chloroform, once with chloroform, and precipitated with ethanol. Pellets were dried and frozen at -80^{0}C.

For isolation of total cellular RNA, cells were rinsed twice with ice-cold 1X PBS then suspended in 4 M guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarkosyl (N-Lauryl sarcosine), and 0.1 M β-mercaptoethanol. Cell lysates were pipetted up and down several times to reduce the viscosity and spun through a 3 ml CsCl (6.2 M)/EDTA (0.1 M) cushion at 15^{0}C for 15-18 h at 115, 000 x g (Beckman SW 50.1 rotor at 35,000 rpm). Pellets were rinsed twice with ice-cold 70% ethanol and resuspended in 10 mM Tris pH 7.4 plus 0.1 mM EDTA followed by ethanol precipitation. Dried pellets were suspended in 100 µl 10 mM Tris pH 7.4 plus 0.1 mM EDTA and frozen at -80^{0}C.

The template for riboprobe transcription was constructed by inserting a HIV gag fragment from the dl.MA mutant (SacI-680 to the SalI-linkered ClaI-831 site) into pBluescribe (Stratagene). Riboprobe transcriptions used 1 µg EcoRI-linearized template plasmid, 80 µCi [α-^{32}P]-rGTP, 0.5 mM of each of the other ribonucleotides, 40 mM Tris pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine, 40 U RNasin (Promega), 1 mM DTT, and 10 U T3 polymerase. Reactions were incubated at 37^{0}C. One hour after incubation, 120 U RNasin, 10 µg yeast tRNA, and 300 mM sodium acetate pH 7.6 (final concentration) were added to the reaction mixtures and probes were ethanol precipitated after extraction with phenol/chloroform. The pellets were suspended in sequencing loading dye and run on a 5% denaturing polyacrylamide sequencing gel to purify and isolate the 183 b probes. Probe excised from the gel was eluted by incubation at 50^{0}C for 30 min in 250 µl containing 1 M ammonium acetate pH 7.4, 0.1% SDS, and 1 mM EDTA. The elution

procedure was repeated once and pooled elutions were combined with 20 µg yeast tRNA followed by phenol/chloroform extraction and ethanol precipitation.

For RNase protections, cell or virus RNA pellets were resuspended in 30 µl solution containing 80% formamide, 0.4 M NaCl, 40 mM Pipes pH 6.4, and 1-5 x 10^5 cpm of probe. Mixtures were denatured at 85^0C for 5 min then incubated at 42^0C overnight. Unhybridized probe was eliminated by incubation at 30^0C for 30 min with 40 µg/ml RNase A, 2 µg/ml RNase T1, 300 mM NaCl, 10 mM Tris pH 7.5, and 5 mM EDTA. Reactions were stopped by addition of 50 µg proteinase K plus SDS to 01% followed by incubation at 37^0C for 15 min. Protected probes were extracted with phenol/chloroform and ethanol precipitated after addition of 20 µg yeast tRNA. Pellets were rinsed, dried, and suspended in sequencing buffer. Samples were denatured and run on a 5% acrylamide sequencing gels and exposed to X-ray films. In some cases, protected probes, which reflected the amounts of viral RNAs, were excised from the gel and quantitated by liquid scintillation counting while Gag protein levels were measured in parallel samples.

Southern blot hybridization. For the Southern blot hybridization probe, plasmid HIVgpt was cut with EcoRV and run on a 1% low-melting-point (LMP) gel. The 2.8 kb restriction fragment (HIV HXB2 nt. 115 to nt. 2979) was excised and melted at 70^0C. Five µl (about 200 ng DNA) was diluted with water and denatured at 95^0C for 10 min. The DNA templates were mixed with 100 mM Tris buffer pH 8.0, 10 mM $MgCl_2$, 20 mM β-mercaptoethanol, 40 µM dNTPs (no

dCTP), and 0.4 M Hepes pH 6.6, 20 µg/ml p $(dn)_6$ (Pharmacia), 2 µg ultrapure bovine serum albumin (BSA), 30 µCi [α-^{32}P]dCTP, and 5 U Klenow DNA polymerase in a final volume of 30 µl. Random primer extension reactions were carried out at 37^{0}C for 30 min, after which DNA probes were purified on Sephadex G-50 (Sigma) spin columns following addition of 20 µg yeast tRNA plus 10 mM EDTA. Genomic DNA isolation, blotting, and hybridizations followed protocols described elsewhere (37, 38, 48).

RESULTS

Assembly and processing of wild type and dl.MA mutant particles

We constructed a HIV mutant with a major deletion in the Gag MA domain by recombination of two linker-generated mutants ClaI (a SalI linker insertion at nt. 831 near the N-terminus of MA) and PvuII (a SalI linker insertion at nt. 1147 near the C-terminus of MA) (52). As shown in Figure 1, the recombinant construct resulted in a replacement of 106 codons by four amino acid residues within the 132 codon matrix domain. Amino acid residues near matrix domain N- and C-termini were not deleted to retain myristylation and MA/CA cleavage signals. We introduced the dl.MA mutant into the HIVgpt backbone which, as described previously (35), contains the drug-resistant *gpt* gene in the HIV *env* coding region.

To examine whether wt and mutant particles were assembled and released, we transfected the wt and dl.MA HIVgpt constructs into COS7 cells and collected cells and particles in culture media 3 d later. Gag proteins in each sample were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western immunoblotting using an anti-p24 monoclonal antibody. As expected, wt Pr55 and p41 Gag precursor proteins were observed in wt-transfected COS7 cells (Figure 2, lane A), while mature CA (p24) and precursors were present in media pellets (Figure 2, lane C). Interestingly, the dl.MA mutant assembled and released virus particles. Within cells, small amounts of predicted dl.MA Gag

precursors Pr42 (corresponding to wt Pr55) and p28 (corresponding to wt p41) were observed (Figure 2, lane B), while p24 also was seen, at relatively higher levels than in wt-transfected cells. All dl.MA Gag protein forms were present in pelleted media samples (Figure 2, lane D), with p24 (CA) representing the major species. Comparison of intracellular versus released wt and dl.MA Gag proteins showed no gross impairment of dl.MA release, although potential subtle differences were not investigated. These results indicated that the MA domain deletion does not block Gag protein release or processing in the COS7 cell over-expression system.

Characterization of virus particles

Our previous studies indicated that pelleted culture media Gag proteins isolated as in Figure 2 represented virus particles (16, 52). To confirm that media *gag* mutant proteins were particle-associated, viral proteins in the media supernatants were analyzed by sucrose density gradient fractionation and Western immunoblotting as described in the Materials and Methods. As shown in the bottom band of Figure 3, wt particles banded in gradient fractions 7 and 8, at a density of 1.16 to 1.18 g/ml. Similarly, mutant particles banded in fractions 7 and 8, demonstrating that dl.MA Gag proteins form particles of wt density.

Based on the fact that Gag proteins in dl.MA particles were processed, it seemed likely that mutant Gag-Pol fusion proteins were incorporated into virus particles and retained PR activity. To test for reverse transcriptase (RT) activities, particles from four separate wt

or mutant samples were assayed using exogenous templates as described in the Materials and Methods. In each case, the cpm of incorporated nucleotides into the exogenous template was at least 14-fold higher than background levels (Table 1). To determine virion-associated RT activities, normalized cpm values were divided by the densitometer-determined virus-associated Gag protein levels. As shown in Table 2, mutant activities were comparable to the wt ratio levels in all samples. Since Gag-Pol is incorporated into virions by putative interactions with Gag proteins, the results shown here indicate that the HIV matrix domain does not appear to be directly involved in promoting incorporation of Gag-Pol into virions.

In addition to the aforementioned analysis of wt and mutant particles, we sought to quantify viral RNA levels in wt and mutant virions. Although evidence has shown that mutations disrupting Cys-His motifs within retroviral NC proteins appear to prevent packaging of RNA into virions (1, 8, 10, 11, 20, 31, 32), a role for MA in RNA packaging, if any, remains unclear: the HIV dl.MA mutant gave us the opportunity to investigate whether the HIV MA domain is involved in packaging of viral RNAs. To do so, we quantitated virion-associated and intracellular viral RNA levels by RNase protection experiments as described in Materials and Methods. Our probe was a 183 b antisense riboprobe which crosses the HIV-1 major splice donor (Figure 4). As shown, the probe is expected to yield 63-64 b fragments when protected by spliced HIV transcripts, or 150 or 156 b fragments when protected by wt or dl.MA genomic transcripts, respectively. Using yeast tRNA (Figure 4, lanes B, H) or RNA samples from mock transfected cells (lanes I, K) a background band of

approximately 146 b was visible: this band was present if RNA samples were pretreated with DNase, and was the only band visible when positive control (wt cellular and viral RNA) samples were pretreated with RNase A (data not shown). Other than this background band, the major bands observed in RNAs from COS7 cells transfected with wt, dl.MA or packaging-defective A14-15 NC mutant (1) constructs were genomic bands at 150 or 156 b, and spliced transcripts smears at 60-70, respectively.

Not surprisingly, neither spliced nor genomic RNA appeared associated with the A14-15 NC mutant particles (Figure 4, lane L), which also demonstrated that transfected proviral DNA has not been detected in our virus samples. Our results with RNAs from wt virions also were expected: in this case, genomic viral RNA was incorporated into virions preferentially, relative to spliced RNAs (lane F). In the dl.MA viral RNA sample, genomic RNA also appeared enriched (compare lanes E and C). As a rough quantitation of relative levels of genomic RNAs in wt or dl.MA particles, cpm in viral genomic bands were determined and normalized to densitometry-determined Gag protein levels from identical virus particle pellets. This determination yielded values of 3,226 and 1,979 (cpm/arbitrary Gag protein densitometer units) for wt and dl.MA viruses, respectively. Taken together, these results suggest that the deletion in our dl.MA virus appeared to have relatively minor effects on the specificity and level of genomic viral RNA incorporation.

Conditional infectivity of the dl.MA mutant

Results shown above indicated that the dl.MA mutant was competent in assembly and processing of virus particles. In addition, this mutant had RT activity, contained viral genomic RNA, and exhibited a wt retrovirus particle density. Although our previous studies showed that most assembly-competent HIV *gag* mutants were noninfectious or poorly infectious (52), we examined the infectivity of the dl.MA mutant during one round of infection. For these studies, mutant or wt HIVgpt constructs were cotransfected into COS7 cells with an amphotropic murine leukemia virus envelope protein expression plasmid A-MLV-env (35). Infection of HeLa or $CD4^+$ HeLa cells and selection of drug-resistant colonies followed the procedures described in Materials and Methods. Surprisingly, the dl.MA mutant displayed a significant infectivity. As shown in Figure 5, the number of drug-resistant colonies of the mutant (Plate B) was about one-tenth of wt number (Plate C) in a parallel experiment. To corroborate results in Figure 5 A to C, parallel infections of $CD4^+$ HeLa cells with A-MLV-env pseudotyped mutant and wt HIVgpt viruses were repeated 8 times. As shown in Table 2, similar results were obtained in each paired infection, with dl.MA titers averaging 9.7 $\pm$ 2.4% that of wt titers. Since this result was unexpected , it was important to prove that *gpt* resistant colonies were due to infection by the dl.MA virus. To do so, genomic DNA from wt or dl.MA-infected, *gpt*-selected pools of clones were examined by Southern blot hybridization (Figure 6). The DNA probe for hybridization derived from an EcoRV restriction fragment which covered the dl.MA deletion (see Figure 6). As shown , this probe hybridized to bands at approximately 2.8 (lane A) and 2.5 (lane B) kb for wt and mutant

infected cells, respectively. Because the HindIII restriction site at HIV nt 1085 was deleted in the dl.MA mutant, hybridization of HindIII-digested genomic DNA gave rise to a band of about 900 bp in the dl.MA sample (lane D) and bands of 627 bp and 553 bp in wt case (lane C). These results are in agreement with our predictions, and indicate that *gpt*-resistant cells deriving from dl.MA infections harbor dl.MA proviruses.

In contrast with our results on A-MLV-env pseudotypes, pseudotyping of the dl.MA mutant with HIV envelope proteins resulted in the production of noninfectious or poorly infectious virions. As shown in Figure 5, no drug-resistant colonies were observed on either mock- (Figure 5 D) or mutant-infected (Figure 5 E) plates, while approximately one thousand $CD4^+$ HeLa cell colonies were observed on the wt-infected plate (Figure 5 F). Repetition of this experiment a total of 11 times yielded similar results (Table 2), and relative to wt HIVgpt, the HIV Env-pseudotyped dl.MA virus was only $0.1 \pm 0.3\%$ infectious. Thus, in comparison with wt, the dl.MA virus is 100-fold less infectious when matched with HIV than with A-MLV Env proteins.

To examine the Env protein specific impairment of dl.MA virus function, mutant and wt virions pseudotyped with HIV envelopes were analyzed by Western immunoblotting. Virus pellets of cotransfected COS7 cells were prepared for protein analyses as described in the Materials and Methods, and HIV envelope proteins were immunodetected with a mouse anti-gp120 antibody. As shown in Figure 7, HIV envelope proteins gp160 and gp120 were observed in wt (lanes B, E, G) and mutant (lanes C, F, H) virus samples in three

separate experiments. That these protein were particle-associated was supported by the fact that transfections of COS7 cells with the HIV Env protein expression plasmid alone did not result in the release of pelletable HIV Env proteins (lanes A, D). While both gp160 and gp120 were associated with wt and dl.MA particles, reproducible quantitative differences were apparent: wt particles contained slightly less gp160 and 2- to 5-fold more gp120 than mutant particles. To compare amounts of particle-associated HIV Gag and Env proteins, one set of recovered pellets was split into two equal portions, and immunoblotted with anti-gp120 (lanes G, H) or anti-p24 (lanes I, J). As shown, the level of virion-associated mutant Gag protein (lane J) was comparable to that of wt (lane I), while dl.MA-associated gp160 was slightly higher and gp120 was 3- to 5-fold lower than in the wt sample (lanes G, H). In contrast, the incorporation of envelope proteins into wt and mutant virions was roughly equal when both were pseudotyped with amphotropic murine leukemia virus envelope proteins. Comparable amounts of MLV envelope precursors gp80 and outer surface domains gp70 were observed in wt (lane K) and mutant (lane L) virus particles which contained equivalent amounts of Gag proteins (lanes M, N). Our observations implicate an interaction between HIV MA and Env (discussed below), and suggest that amphotropic MLV Env proteins are able to be incorporated into HIV virus particles without the need for a specific interaction with the HIV Gag matrix domain. Furthermore, our results are consistent with the observations that pseudotyping of A-MLV-env with HIVgpt was an efficient process, and that titers of virions generated by coexpression of HIVgpt with

A-MLV- env tended to be higher than those of virions generated by pseudotyping of HIVgpt with HIV-env in parallel experiments (35).

DISCUSSION

The matrix domain of retroviruses has been postulated to possess a variety of activities necessary for replication, including functions necessary for membrane binding, assembly, viral RNA or Env protein incorporation, and nuclear localization (2, 6, 17, 42, 43, 45, 52, 57). For most mammalian retroviruses, myristylation of MA appears essential for efficient membrane association (3, 40, 41, 53) which, except for type D retrovirus (41), appears to be a prerequisite to assembly (3, 13, 36, 40, 52, 53, 55). However, a number of mutations into the middle region of the HIV Gag matrix domain have no apparent effect on virus assembly (52, 58). Some of these mutants appeared defective in Env-associated functions, implicating the HIV Gag matrix in a specific Env-Gag protein interaction necessary for Env protein incorporation into virions (58).

Our results concern the characterization of HIV MA deletion which removes 80% of the matrix domain, leaving only the putative myristylation and MA/CA cleavage signals (Figure 1). Although immunofluorescence analyses suggested that the mutant protein was enriched on perinuclear membranes (data not shown), it was released from cells at approximately wt levels and processed appropriately (Figure 2). It is noteworthy that more subtle mutations in the deleted domain have been demonstrated to impair assembly (52, 58). These different results could be explained if subtle mutations induced interactions which cause Gag proteins to be trapped intracellularly, while potentially deleterious regions of

interaction were removed altogether in the dl.MA mutant. In this regard, it is important to emphasize that small but real effects of the dl.MA mutation on virus assembly could be masked by virtue of the COS7 cell over-expression system.

Although we have yet to obtain amounts of material sufficient for electron microscopic analysis, the virus particles produced by the dl.MA mutant appeared wt by a variety of criteria. They are the appropriate density, are processed, possess wt RT activities, and specifically incorporate viral genomic RNAs at approximately wt levels (Table 1, Figures 2-4). Most surprisingly, when pseudotyped with an amphotropic murine leukemia virus envelope (A-MLV-env) protein, the dl.MA particles were 9.7% as infectious as wt particles (Figure 5, Table 2). This result indicates that an intact MA is not absolutely required for early replication events such as uncoating, reverse transcription, integration or nuclear localization, in dividing HeLa or $CD4^+$ HeLa cells. However, given other results (4, 27), it is entirely possible that MA may affect early processes in other dividing or non-dividing cell types.

In contrast to results with A-MLV-env pseudotypes, the dl.MA virus appeared virtually noninfectious when matched with the HIV Env protein (Figure 5, Table 2). This suggests that, unlike A-MLV-env, which appears to function in HIV particles by a matrix-independent mechanism, HIV Env proteins must specifically interact with MA. Thus, our data support previous genetic implications of a HIV Env-matrix interaction (58, 59). Further support for this notion derives from Figure 6. As illustrated and expected, particle-associated A-MLV Env protein levels were similar for wt and dl.MA

virions (Figure 6). In contrast, in three separate experiments, HIV env protein levels in wt and mutant viruses differed: gp160 levels were equal or slightly higher in dl.MA particle preparations, while gp120 levels were 2- to 5-fold lower in mutant particles (Figure 6). Obviously, the 2- to 5-fold reduction of gp120 in dl.MA particles is considerably less than the infectivity differences we observed (Table 2). This disparity could be accounted for if the MA domain affects gp120 post-assembly functions, or if receptor binding and/or fusion events are not related linearly to viral gp120 levels. Interestingly, our data also suggest that gp160 is incorporated into wt and dl.MA particles with equal efficiency, although this interpretation must be tempered by the fact that observation of nonvirus-associated media gp160 has been reported (23). However, assuming the Figure 6 differences in dl.MA and wt gp160 to gp120 ratios are valid, then MA does not have a great effect on Env incorporation into particles. Rather, the HIV matrix domain could have a effect on the stability of gp120 in virus particles or, seemingly less likely, on the cellular processing of gp160 to gp120 plus gp41.

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Table 1. Reverse transcriptase activities of dl. MA and wild type HIV particles.

construct	cpm	cpm-mock	Gag antigen	(cpm-mock)/Gag
mock	1095	-----	----	-----
mock	1265	-----	----	-----
wt	18830	17650	1.55	11387
wt	18890	17710	1.22	14516
wt	44595	43415	2.95	14717
wt	23585	22405	1.06	21136
dl.MA	19005	17825	1.05	16976
dl.MA	19795	18615	1.24	15012
dl.MA	28450	27270	1.27	21472
dl.MA	17965	16785	0.62	27072

COS7 cells were transfected with either 10 or 15 µg of wild type (wt) or the deletion MA (dl.MA) HIVgpt constructs. Three days post-transfection, media supernatants (10 ml) were filtered and virions were pelleted through a 20% sucrose cushion as described in the Materials and Methods. Pellets were resuspended in 100 µl PBS from which 20 µl was used for reverse transcriptase (RT) assays and 50 µl was used for immunodetection of gag antigen by Western blotting. Total trichloroacetic acid (TCA) precipitable product after a

1 h reaction is as shown in the cpm column. Background cpm for subtraction were from the averaged mock values. Total *gag* antigen was determined by densitometry of immunoblot filters, and values listed were arbitrary densitometer units.

Table 2. Infectivity of the HIV dl.MA mutant pseudotyped with HIV or amphotropic murine leukemia virus (A-MLV) envelope proteins.

PSEUDOTYPED WITH A-MLV ENV			PSEUDOTYPED WITH HIV ENV		
dl.MA titer	wt titer	%wt	dl.MA titer	wt titer	%wt
456	3,200	14.3	2	196	1.02
40	512	7.8	0	192	0
244	3,296	7.4	0	224	0
168	1,712	9.8	0	4,912	0
152	2,268	6.7	0	624	0
1,568	15,296	10.3	0	6,192	0
1,136	11,230	10.1	2	4,274	0.05
952	8,768	10.8	2	3,376	0.06
average % wt = 9.7 ± 2.4%			0	2,784	0
			0	1,748	0
			0	4,416	0
			average % wt = 0.1 ± 0.3%		

COS7 cells on 10 cm plates were transfected with 15 µg of wt or dl. MA HIVgpt plasmids plus 10 µg of either an amphotropic murine leukemia virus (A-MLV) or HIV envelope protein expression plasmid. Three days after transfections, COS7 media supernatants were collected, filtered, and used to infect CD4+ HeLa cells, after which infected cells expressing the bacterial *gpt* gene were selected as described in the Materials and Methods. Titers indicate CD4+ HeLa mycophenolic acid resistant colonies per ml, and each dl.MA transfection was performed in parallel with a wt HIVgpt transfection from which % wt titer values derive. Each % wt value derives from a separate transfection experiment.

Figure 1. Wt and dl.MA constructs.

The wt HIV-1 Gag polyprotein is synthesized as a 55 kd precursor (Pr55), which can be cleaved into a proteolytic intermediate (p41), and ultimately becomes cleaved into the four mature Gag domains: p17 (matrix, MA), p24 (capsid, CA), p7 (nucleocapsid, NC) and p6. Wild type HIV-1 (HXB2) encodes a matrix domain of 132 codons which rapidly becomes myristylated at its amino-terminal glycine residue after methionine removal. The dl.MA mutant contains a replacement of 106 codons by four codons, retaining myristylation and MA/CA cleavage signals. The predicted dl.MA Gag precursor protein is approximately 42 kd (Pr42), and a MA-CA cleavage product is predicted to have a molecular weight of 28 kd (p28). Both wt and dl.MA constructs were expressed in the HIVgpt backbone.

Figure 1. Wild type and deletion MA gag constructs.
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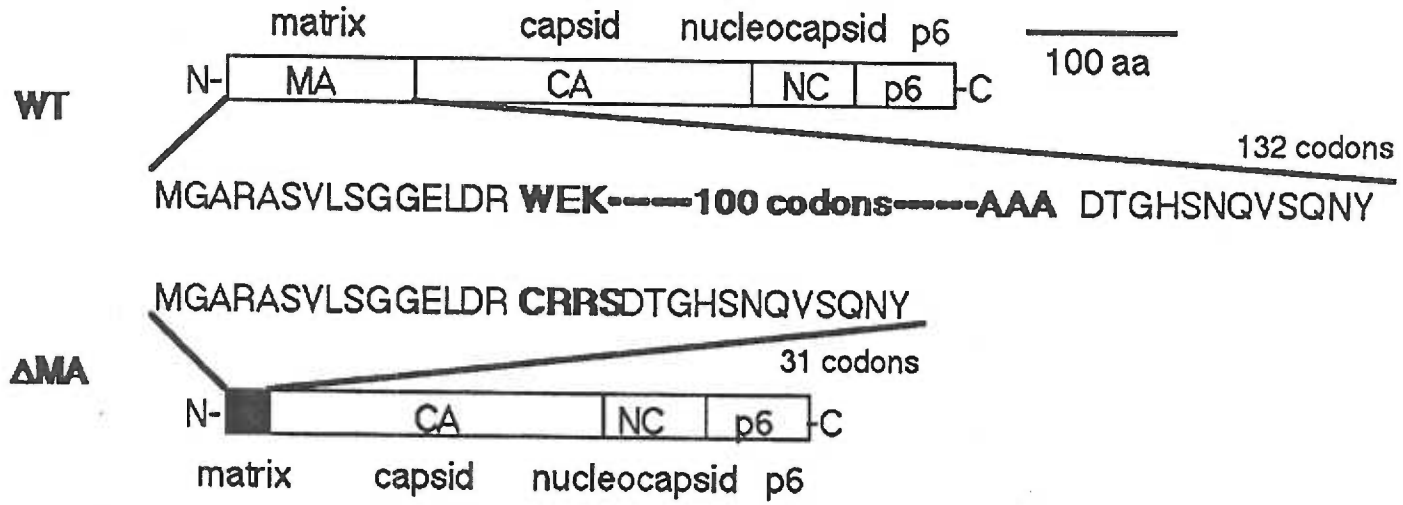


Figure 2. Expression and release of wt and mutant HIV Gag proteins.

COS7 cells were transfected with wt or dl.MA HIVgpt plasmids. After 72 h, supernatants and cells were collected and prepared for protein analysis as described in the Materials and Methods. Supernatant samples (lanes C and D, corresponding to 50% of the total sample) and cell samples (lanes A and B, corresponding to 5% of the total cell sample) were fractionated by SDS-PAGE and were electroblotted onto a nitrocellulose filter. HIV Gag proteins were detected by immunoblotting with a mouse anti-p24 monoclonal antibody at a 1:20,000 dilution, followed by a secondary alkaline phosphatase-conjugated goat anti-mouse antibody at a 1:1,500 dilution, and detection of alkaline phosphatase activity. The molecular weight markers are indicated on the right and wt HIV Gag proteins Pr55, p41, and p24, and mutant Gag proteins p42, p28 and p24 are on the left.

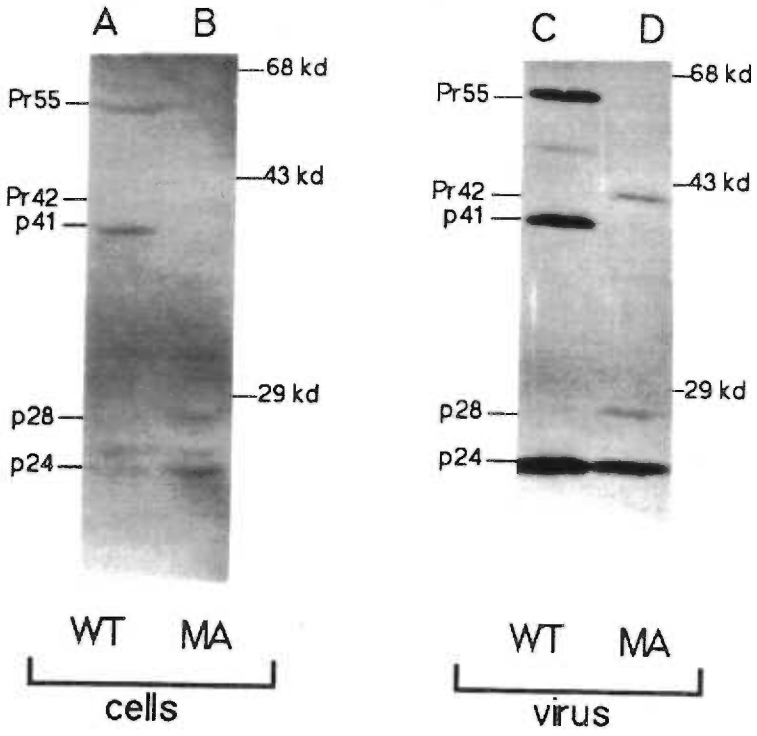
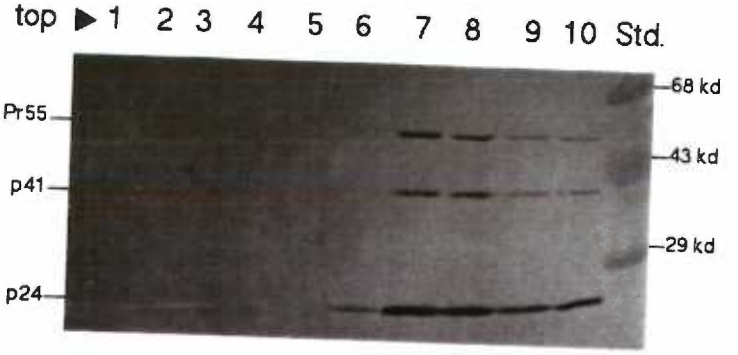

A
B
C
D
68 kd
43 kd
29 kd
Pr55
Pr42
p41
p28
p24
WT
MA
cells
virus

Figure 3. Analysis of HIV particles by sucrose density gradient fractionation.

Supernatants from transfected COS7 cells were collected and fractionated on 20 to 50% sucrose density gradients as described previously (52). Fractions were collected from top to bottom, and Gag proteins were analyzed by immunoblotting as described in Figure 2. Protein molecular weight markers are indicated on the right and wt or mutant Gag proteins are shown on the left. The peak Gag protein fractions 7 and 8 had densities of 1.162 and 1.180 g/ml respectively.

ΔMA sucrose gradient



WT sucrose gradient

Figure 4. RNA incorporation into virus particles.

HIV RNA from cellular and viral RNA isolations was quantified by RNase protection. Panel A: The RNase protection antisense probe is 183 b and contains 5' vector sequences plus HIV (HXB2) proviral sequences corresponding to nt 831-680, crossing the HIV-1 major splice donor. Either wt or dl.MA spliced RNAs should protect probe fragments of 63-64 b, while wt or dl.MA genomic viral RNAs are predicted to protect bands of 150 and 156 b respectively. Panel B: Equivalent amounts of matched total cellular (lanes C, D, I, J) and viral (lanes E, F, K, L) RNAs from mock (lanes I, K), wt (lanes D, F), dl.MA (lanes C, E) and NC RNA packaging mutant A14-15 (lanes J, L) transfections were used in RNase protection assays. Each sample (C to F and I to L) also contained 10 mg of yeast tRNA, and samples in lanes B and H contained tRNA only. After hybridization, RNase digestion and protected probe isolation, products were separated on 5% acrylamide sequencing gels and visualized by autoradiography. Note that undigested probe was seen in lanes A and G; that genomic and spliced RNA signals in control reactions were sensitive to RNase but not DNase treatment of samples prior to hybridization; and that a background band which migrates slightly faster than the wt genomic protected fragment (and is visible in tRNA control lanes B and H) was insensitive to sample RNase treatment. Probe, genomic and spliced bands are as indicated. When dl.MA and wt genomic RNA protection bands from lanes E and F were excised, counted and normalized for Gag protein levels from parallel Western blots, cpm/arbitrary Gag densitometer units were 1,979 for dl.MA and 3,226 for wt.

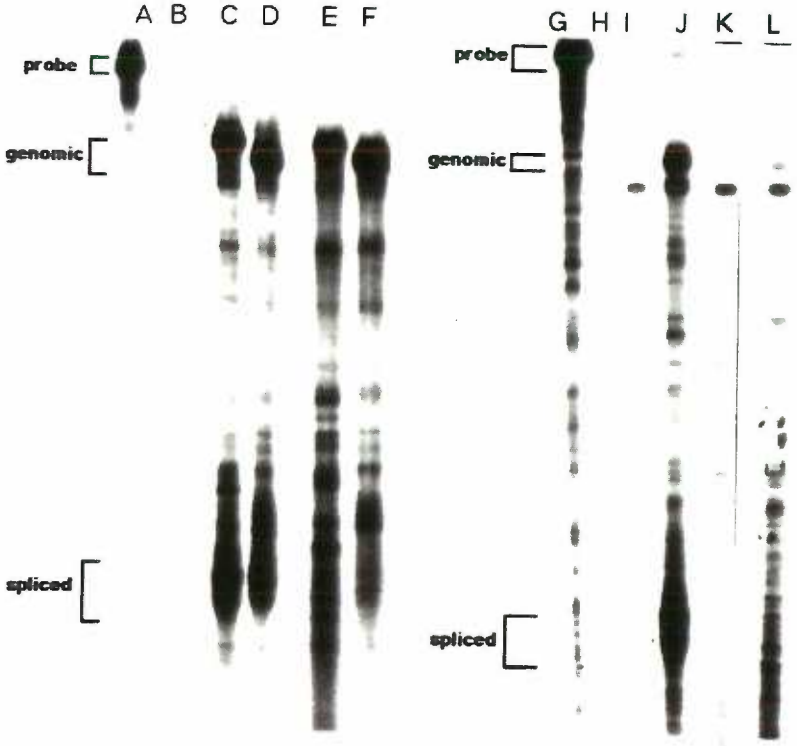

A B C D E F
probe
genomic
spliced
G H I J K L
probe
genomic
spliced

Figure 5. Infectivity of pseudotyped wt or dl.MA virions.

COS7 cells were cotransfected with 10 μg of wt or dl.MA HIVgpt plasmids plus 5 μg of either amphotropic murine leukemia virus (A-MLV) or HIV envelope protein expression plasmids. Seventy-two hours after transfection, supernatants were collected, filtered , and used to infect $CD4^+$ HeLa cells. Infection and selection of drug-resistant colonies followed the procedures as described in the Materials and Methods. About two weeks later, drug-resistant colonies were fixed and stained with 50% methanol plus 0.5% methylene blue. Plates A and D, mocks; plate B, dl.MA pseudotyped with A-MLV Env; plate C, wt pseudotyped with A-MLV Env; plate E, dl. MA pseudotyped with HIV Env; plate F, wt pseudotyped with HIV Env.

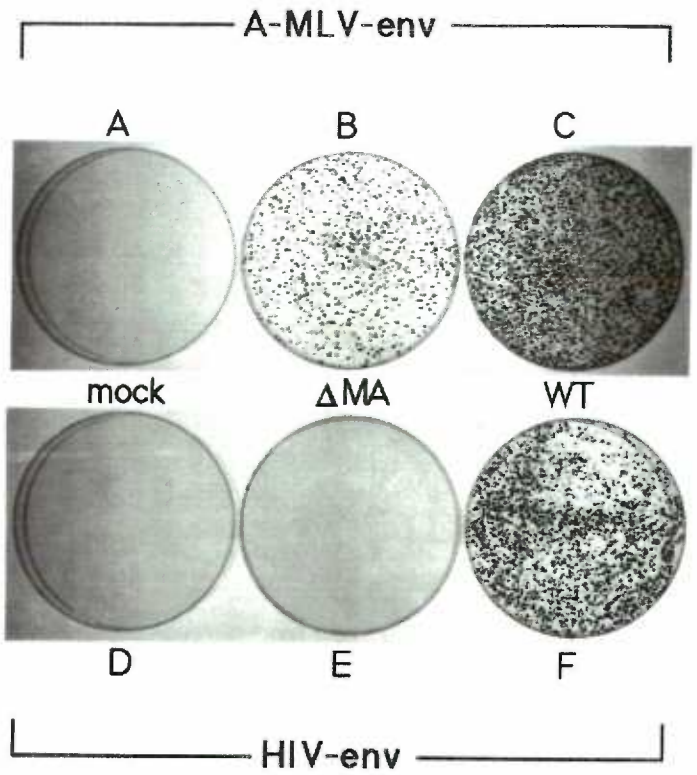

A-MLV-env
A
B
C
mock
ΔMA
WT
D
E
F
HIV-env

Figure 6. Southern blot analysis of wt- and dl.MA-infected cells.

COS7 cells were transfected with 10 μg of wt or dl.MA HIVgpt plasmids plus 5 μg of an amphotropic murine leukemia virus (A-MLV) envelope protein expression plasmid. 72 h posttransfection, supernatants were collected, filtered, and used to infect $CD4^+$ HeLa cells. Infection and selection of drug-resistant colonies were performed as described in Materials and Methods, and drug-resistant colonies were trypsinized, dispersed, and refed every 3 to 4 days with DMEM plus 10% fetal calf serum until cells grew to confluence. Preparations of cellular genomic DNA for Southern blot hybridization followed the protocols described in Materials and Methods. DNA from wt (lanes A and C) or dl.MA (lanes B and D) infected cells was digested with EcoRV (lanes A, B) or HindIII (lanes C, D), electrophoresced, blotted, and hybridized with a probe from the HXB2 EcoRV nt 115-2979 fragment. As shown, size standards are on the left, and a map of 5' proviral EcoRV and HindIII sites, with deletion and probe locations is on the right. Note that the predicted 1475 bp 3' HindIII LTR fragment (HXB2 nt 8141-9616) appears faintly in lanes C and D on the original autoradiograph.

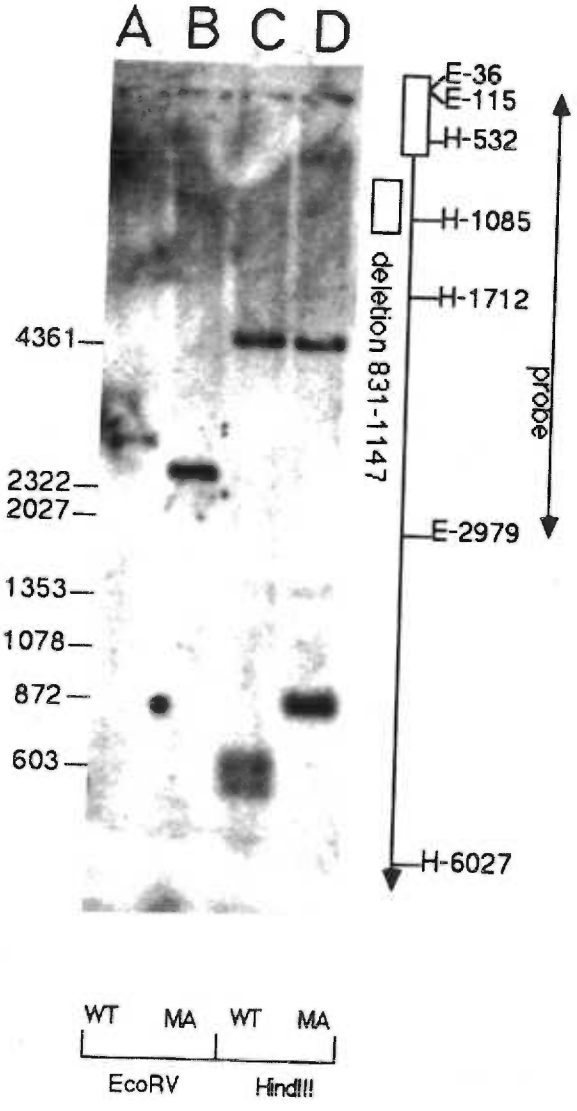
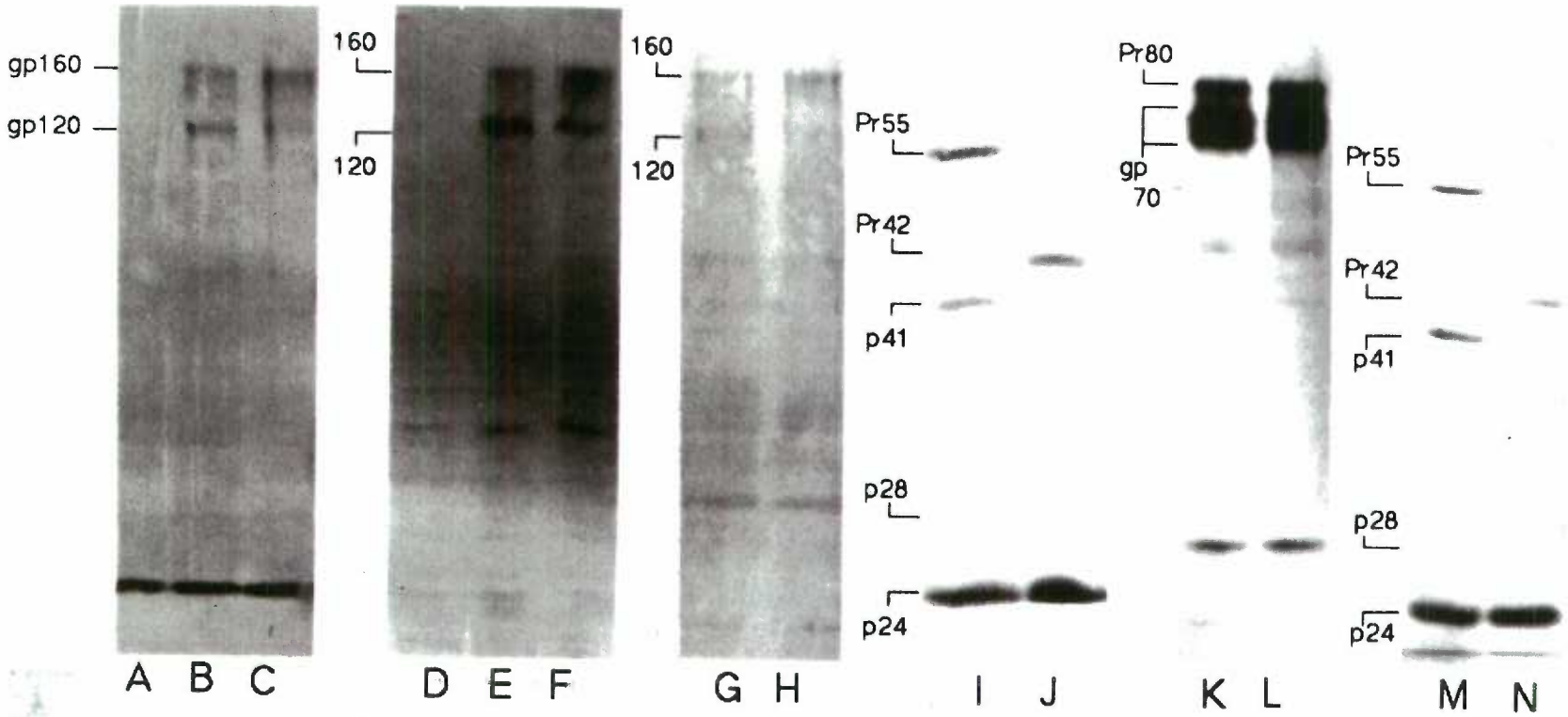

A B C D
4361
2322
2027
1353
1078
872
603
E-36
E-115
H-532
H-1085
H-1712
deletion 831-1147
probe
E-2979
H-6027
WT
MA
WT
MA
EcoRV
HindIII

Fig. 7. Envelope protein incorporation into wt and mutant HIV particles.

Envelope protein expression plasmids A-MLV-env (lanes K to N) or HIV-env (lanes A to J) were transfected into COS7 cells alone (lanes A, D) or with wt (lanes B, E, G, I, K, M) or dl.MA (lanes C, F, H, J, L, N) HIVgpt constructs. Proteins from virus pellets were separated by SDS-PAGE, electroblotted, and detected with antibodies to HIV-env (lanes A-H), A-MLV-env (lanes K, L), or HIV gag (CA; lanes I, J, M, N). Lanes G, I; H, J; K, M; and L, N were from identical virus pellets, but were blotted with either anti-Env or anti-Gag antibodies for Env: Gag quantitation purposes. For each sample, three to six plates were transfected. Three days later, supernatants were collected, filtered, and pooled. Preparations of supernatants for protein analyses were performed as described in Materials and Methods. For immunodeteciton of HIV gag proteins, a mouse anti-p24 monoclonal antibody at 1:20,000 dilution was used as the primary antibody. For immunodetection of HIV envelope proteins, the primary antibody was a mouse anti-gp120 antibody at 1:5,000 dilution; and for the MLV amphotropic envelope proteins, the primary antibody was a goat anti-gp70 antibody at 1:5,000 dilution. Secondary antibodies were either goat anti-mouse or swine anti-goat alkaline phosphatase-conjugated antibodies at 1:1,500 dilutions. HIV envelope proteins gp160 and gp120, MLV envelope proteins gp80 and gp70; wt Gag proteins Pr55, p41, and p24, and dl.MA Gag proteins p42, p28, and p24 are as indicated.


gp160
gp120
160
120
160
120
Pr55
Pr42
p41
p28
p24
Pr80
gp
70
Pr55
Pr42
p41
p28
p24
A B C D E F G H I J K L M N

Manuscript #3

ASSEMBLY OF HIV GAG-B-GALATOSIDASE FUSION PROTEINS INTO VIRUS PARTICLES

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Running title: Assembly of HIV fusion proteins into particles

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ABSTRACT

We have studied the assembly of human immunodeficiency virus (HIV-1) Gag-B-galactosidase (Gag-B-gal; GBG) fusion proteins into HIV particles in the presence of HIV Gag proteins. Release of fusion proteins from cells was measured by assay of media versus cellular B-gal activities, and was dependent on co-expression of unfused Gag proteins. Gag-B-gal incorporation into virus particles was demonstrated by detergent treatment and density gradient fractionation studies, and was dependent on protein-protein interactions requiring the C-terminal two-thirds of the HIV CA domain. The central MA domain appeared unimportant for fusion protein incorporation; a nonmyristylated GBG protein was incorporated but at a relatively reduced level; while the NC and p6 domains slightly affected the assembly of fusion proteins into particles. Subcellular fractionation studies showed that all fusion proteins including the nonmyristylated one, were associated with cellular membranes to a certain extent. However, assembly into particles did not correlate with levels of membrane association. Similarly, virion incorporation levels of Gag-B-gal proteins did not correlate with their immunofluorescence localization patterns. However, we observed that while most fusion proteins displayed a perinuclear ring with heterogeneous staining throughout cells, short fusion proteins appeared enriched on the intracellular membranes, and fusion proteins with intact MA but deleted NC domains showed an enhanced surface staining without a clear perinuclear ring.

Altogether, our data suggest that the CA domain is the primary determinant for assembly of HIV fusion proteins into virus particles.

INTRODUCTION

During the HIV replication cycle, viral proteins Gag, Gag-Pol, and Env are transported to the plasma membrane where Gag and Gag-Pol precursor polyproteins assemble into virions and bud out from the cell surface along with membrane-anchored Env proteins (4, 31). Without other viral components, HIV Gag precursors still can assemble into virus-like particles (5, 8, 14, 18, 28, 29), and in wt HIV particles, Gag and Gag-Pol proteins form the internal core structure. HIV Gag is translated initially as a precursor Pr55, which is cotranslationally modified by removal of the N-terminal methionine and attachment of a myristic acid to the second amino acid glycine (32, 35). During or after virus budding, the Pr55 Gag precursor is processed proteolytically by the *gag-pol* encoded protease (PR) into (from N- to C-terminal ends) matrix (MA; p17), capsid (CA; p24), nucleocapsid (NC; p7), and p6 (11, 16, 19, 26). The MA domain retains the myristate moiety and forms a matrix under the viral membrane; CA is the major core protein; NC contains the Cys-His motif, which is required for viral RNA packaging (1); and the p6 domain may be involved in the process of budding (5, 6). The viral protease and enzymatic functions (reverse transcriptase, RNase H, and integrase) required for viral replication derive from the Gag-Pol fusion protein, which also is myristylated and translated by ribosomal frameshifting at a frequency of about 5 to10% (12, 36). The NC domain within Gag-Pol is truncated and the p6 domain is replaced by a different domain referred to as p6* (25). Although

Pr55 is essential for HIV assembly and release (5, 8, 14, 18, 28, 29), the process of particle formation is still unclear. During assembly, Gag precursors appear to self-assemble into virions via Gag-Gag interactions and Gag-Pol may be incorporated into virions via its N-terminal Gag determinants. However, despite recent work (24, 30), the regions responsible for the Gag-Pol assembly into virions have not been defined precisely.

Previously, we set up a system to study assembly of M-MuLV Gag-B-gal (GBG) fusion proteins into virus particles in which the B-gal gene was fused to various regions of the M-MuLV *gag* gene and the fusion constructs were expressed in the presence or absence of wild-type (wt) M-MuLV Gag proteins. We found that fusion proteins retaining the M-MuLV myristylation signal and intact MA and CA domains were assembled into wt M-MuLV particles, while other fusion proteins were not incorporated (13). However, fusion proteins also were released in a non-viral, low density vesicle form, and retention of an intact M-MuLV MA domain on a Gag-B-gal fusion protein was sufficient to target proteins into this low density fraction, even in the absence of wt M-MuLV Gag proteins (13). Furthermore, mutations of the myristylation signal caused fusion proteins to localize to the cytosol and prevented their assembly, while fusions within the M-MuLV MA became trapped on intracellular membranes (13). We interpreted these data as showing that the M-MuLV matrix domain is required for appropriate intracellular protein transport, and that CA-CA interactions mediate M-MuLV fusion protein entry into wt particles (13).

Since the previous fusion protein system provided a convenient assay with which to analyze the assembly process, we established a similar system to study assembly of HIV Gag-B-gal (GBG) fusion proteins into HIV-1 particles. HIVGBG was constructed by fusion of B-gal at the end of the HIV NC domain, and HIVGBG proteins could be incorporated into virions when cotransfected into COS7 cells with an assembly-competent HIV Gag protein expression plasmid. In contrast to the M-MuLV GBG system, HIVGBG proteins were not released from the cells in the absence of wt HIV Gag proteins. Analysis of a series of fusion proteins shows that the HIV CA domain appears required for fusion protein incorporation into virions. In contrast to the M-MuLV system, the nonmyristylated HIVGBG protein was incorporated into particles, albeit at reduced levels relative to the myristylated protein. Subcellular fractionation studies demonstrated that virion incorporation of the fusion proteins was relatively independent of membrane binding strength, and did not correlate with immunofluorescence staining patterns. We believe this system will be useful in mapping of Gag protein domains which facilitate Gag-Pol protein entry into HIV particles.

MATERIALS AND METHODS

Plasmids and constructs. Plasmid HIVgpt (21) and its derivative mutants PR$^-$, dl.NP, dl.MA, AccI, and Myr$^-$ were as described previously (33, 34). To construct HIVGBG, a BamHI-SalI fragment containing the B-galactosidase coding region from a modified BAG vector (13) was inserted in place of the HIV-1 BglII (HXB2 nt 2096) to SalI (HXB2 nt 5786) fragment in HIVgpt (21). The sequence at the junction area is AG ATC TCG GGG GAT CCC GTC. The fusion site is a BamH1 linker adjacent to the eighth codon (GTC) of the B-gal coding region, and the underlined nucleotide is HIV nt 2096. HIVGBG derivative Gag-B-gal constructs, GBG2289, 1900, 1248, 1147, and 831 were generated by utilization of BamHI linkers and were fused with B-gal at the designated HIV nt position, yielding constructs otherwise identical to HIVGBG. BamHI sites at the HIV *gag* gene were generated either by polymerase chain reaction (PCR) or linker insertions. We also made a fusion construct by replacing the NC domain (nt 1900 to 2067) of HIVGBG with 9 codons (AAT TCC TGC AGC CCG GGG GAT CCG CGG GGT ACT). Since this recombinant construct was nearly identical to GBG1900, and both gave similar results in all assays, we have designated both as the GBG1900. For 831, a BglII site was created adjacent to 831-ClaI and fused with B-gal by ligation of the compatible cohesive ends. GBGdlCA, with a deletion of most of CA, was generated by replacement of the HIV nt 1248 to 1900 with 8 codons : GCA GCC CCG GAT CCC CCG GGC TGC AGG AAT. The underlined nucleotides indicate the HIV nt positions 1248 and 1900. The *gag* mutations within dl.NP, dl.MA, AccI, and Myr$^-$

were introduced into the HIVGBG construct, yielding the GBGdINP, GBGdIMA, GBGAccI, and GBGMyr⁻ respectively. Methods for cloning and sequencing were according to Maniatis et al. (17). HIVgpt and HIVGBG constructs are illustrated in Figure 1 and the sequences at the fusion junctions are shown in the figure legend.

Cell culture and transfections. COS7 cells were maintained as described previously (33). At 24 h before transfections, confluent COS7 cells were split at 1:10 onto 10-cm plates or at 1: 80 onto cover slips. Fifteen µg of plasmid DNA of Gag-B-gal constructs were transfected into COS7 cells by calcium-phosphate precipitation (7). When HIVGBG constructs were cotransfected with helper constructs, 8 µg of each plasmid DNA were used. Three days after transfections, culture media and cells were collected for protein or enzyme analysis. For protein stability studies, at 48 h post-transfection, cells from 10-cm plates were trypsinized, dispersed and split equally onto five 60-mm dish plates and grown for 18 h in DMEM plus 10% fetal calf serum. Culture media then was removed and plates were refed with media containing 100 µg/ml cycloheximide, after which cells were collected at various time points for B-gal assays.

Protein and enzyme assays. Supernatants from transfected COS7 cells were collected, filtered through 0.45 µm-pore-size filters and centrifuged at 4^0C for 1-2 h at 274,000 X *g* (SW41 rotor at 40,000 rpm). Pellets were suspended in phosphate-buffered saline (PBS). Cells were rinsed twice with ice-cold PBS and collected in 1 ml PBS, pelleted and resuspended in PBS. B-gal assays followed the protocol

of Norton and Coffin (20). In some cases, samples were not treated with 0.1% sodium dodecyl sulfate (SDS). In other cases, filtered supernatants were divided into two equal portions; one was treated with 0.5% Triton-X 100 and the other one was mock-treated. Supernatants then were centrifuged through a 20% sucrose cushion in TSE (10 mM Tris hydrochloride, 100 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) at 4^0C for 45 min at 274,000 X *g*. Pellets were recovered in PBS and analyzed by B-gal assay as usual. Total cellular proteins were quantitated by the microassay procedure of Bradford (2). Sucrose density gradient fractionation experiments were performed as described previously (13, 33).

Immunoblotting and immunofluorescence. For detection of virion-associated gag proteins, viral pellets were suspended in 100 to 200 µl IPB (20 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 0.02% sodium azide). Samples then were mixed with an equal volume of 2X sample buffer plus 5% β-mercaptoethanol, boiled for 4 to 5 min, and subjected to SDS-PAGE (15) followed by electroblotting onto nitrocellulose membranes. Electroblotted membranes were immunodetected with a primary mouse anti-p24*gag* monoclonal antibody at 1:20,000 dilution followed by secondary goat anti-mouse alkaline phosphatase-conjugated IgG antibody. Detection of alkaline phosphatase activity by color reaction solution was as described previously (13, 33). Indirect immunofluorescence assays for Gag-B-gal fusion proteins or HIV Gag proteins were performed by standard

methods (27). Briefly, cells grown on cover slips were fixed for 20 min in ice-cold PBS containing 3.7% formaldehyde. Cells were washed once with PBS, once with Dulbecco's modified Eagle's medium (DMEM) plus 10% heat-inactivated calf serum, and then permeablized at room temperature for 10 min in PBS plus 0.2% Triton-X 100. Cells were incubated at 37^{0}C with primary antibody for 1 h, and with the secondary antibody for 30 min. Following each incubation, cells were treated by three 5 to 10 min washes with DMEM/Calf. The primary antibody was either a mouse anti-B-gal antibody (Promega) at 1:3,000 dilution, or a mouse anti-p24*gag* monoclonal antibody (Epitope, Inc) at 1:1,000 dilution, and the secondary antibody was a goat anti-mouse rhodamine-conjugated IgG antibody (Tago) at 1:100 dilution. After the last DMEM/Calf wash, cover slips were washed with PBS three times and mounted in 50% glycerol in PBS.

Subcellular fractionation Subcellular fractionation followed the protocol described by Jones et al. (13). Briefly, cells were washed twice with ice-cold PBS and harvested in 1 ml PBS. Cells then were pelleted, resuspended in 1.5 ml swelling buffer (2 mM $NaHCO_3$, 1 mM $MgCl_2$, and 0.1 mM PMSF) and set on ice for 5 min followed by douncing 200 times with a Wheaton dounce type A pestle. Lysates were cleared of unbroken cells, nuclei, and debris by centrifugation at 1,000 X *g* for 5 min. Supernatant then was fractionated by centrifugation at 174,000 X *g* at 4^{0}C for 15 min, after which supernatants (S2) and pellets (P2), representing crude cytosol and membrane fractions, were collected.

RESULTS

Assembly of Gag-B-galactosidase fusion proteins into HIV particles

We previously established a system to study the incorporation of Gag-B-galactosidae fusion proteins into Moloney murine leukemia virus (M-MuLV) particles (13). In that system, the B-gal gene was fused to various portions of M-MuLV *gag* gene. Gag-B-gal constructs were expressed in the presence or absence of wt Gag proteins, and incorporation of Gag-B-gal fusions proteins into M-MuLV virus particles was analyzed by assay of cell media versus intracellular B-gal activities. We adapted this approach to set up a HIV Gag-B-gal fusion protein system for study of Gag-B-gal fusion protein incorporation into HIV particles. We initially chose to examine a construct in which B-gal was fused to *gag* at the C-terminus of the HIV-1 NC domain. The resultant recombinant, designated HIVGBG, lacked p6 but contained intact MA, CA, and NC domains (Figure 1). To provide wt HIV Gag proteins, the HIVgpt expression plasmid (21; Figure 1) also was used, as were previously described (33) HIVgpt constructs expressing mutants which were deficient in myristylation (Myr^-), impaired in assembly (AccI), or protease defective (PR^-; Figure 1).

For analysis, we followed the protocol shown in Figure 2. As illustrated, the HIVGBG construct was transfected into COS7 cells with or without helper (HIVgpt) constructs and the amounts of released

Gag-B-gal fusion proteins were monitored by the ratios of the media versus intracellular B-gal enzyme activities. Interestingly, in the absence of a helper construct, the ratio of media to cellular B-gal activities produced by the HIVGBG construct was 0.05 (Table 1), indicating very low levels of B-gal release. This result was in contrast to that observed for M-MuLV Gag-B-gal fusion proteins, which were released at high levels in the absence of helper proteins (13). However, cotransfection of HIVGBG with a helper HIVgpt plasmid led to a 10-fold increase in B-gal release (Table 1), suggesting that HIVGBG Gag-B-gal proteins became incorporated into HIV particles produced by the HIVgpt constructs. This interpretation was supported by our observation that the assembly-competent but protease-defective HIVgpt PR^- mutant also could help the release of HIVGBG B-gal activity (Table 1). In contrast, the assembly-defective HIVgpt mutant, Myr^-, did not facilitate B-gal activity release. Similarly, the HIVgpt AccI mutant which is impaired in particle assembly, showed a reduced ability to induce B-gal release.

The results shown in Table 1 implied that Gag-B-gal fusion proteins were incorporated into the HIV particles. To support this conclusion, we performed the following experiments. Virus pellets were prepared and B-gal assays were performed by standard methods except that the additional 0.1% SDS, which is required for substrate to gain maximum access to B-gal in particles, was omitted from one set of the identical samples in the parallel assays. As shown in Table 2, B-gal activity levels of the SDS-treated samples were higher than those of untreated samples in parallel experiments.

Comparing with the ratios of untreated versus treated (-SDS/+SDS) B-gal activities, SDS treatment affected results more in cotransfections with HIVgpt PR$^-$ than in cotransfections experiments with wt HIVgpt. These results are in agreement with the fact that the PR$^-$ mutant forms particles which are more stable than wt HIV-1 particles (9). We have shown that immature PR$^-$ particles are more resistant to disaggregation by non-ionic detergent (33). Thus, we also would predict that non-ionic detergent treatment should affect the sedimentation of B-gal activity associated with wt particles to a greater extent than PR$^-$ particles. As expected, Triton X-100 treatment of particles produced by HIVGBG plus HIVgpt (wt) cotransfections drastically reduced the amount of pelletable B-gal activity to 22 to 34% of untreated levels (Table 2). In contrast, most B-gal activity remained associated with PR$^-$ particles after Triton treatment (Table 2). This result supports the notion that HIVGBG Gag-B-gal fusion proteins were incorporated into wt and PR$^-$ particles.

As one additional test, we examined the density of B-gal activity released from cotransfected COS7 cells. To do so, supernatant from COS7 cells cotransfected with HIVGBG and HIVgpt constructs was fractionated through a 20-50% sucrose density gradient. Fractions were collected and analyzed for B-gal activity and for Gag protein levels by immunoblot detection of p24^{gag}. As shown in Figure 3, B-gal activity bands at the same density as did the HIV Gag protein (1.16 g/ml), indicating that Gag-B-gal fusion proteins are neither free in the supernatant nor randomly associated with

membranes. Combined with the data from SDS and Triton treatment experiments (Table 2), this result supports the conclusion that HIV Gag-B-gal fusion proteins were assembled into HIV particles.

Requirements for fusion protein incorporation into virus particles

Assembly of HIVGBG into HIV particles permitted us to investigate the Gag domains required for Gag-B-gal fusion protein incorporation into virus particles. For our analysis, a series of HIV Gag-B-gal fusion constructs was generated as described in the Materials and Methods. HIVGBGAccI and HIVGBGMyr$^-$ were similar to their HIVgpt counterparts except that their backbones were of the HIVGBG form (Figure 1). Besides HIVGBG, which was fused at HIV nt 2096, B-gal also was fused to other sites along the HIV-1 *gag* gene: constructs 2289, 1900, 1248, 1147, and 831 were generated by B-gal fusion at the the end of p6 (2289), CA (1900), and MA (1147), or to amino-terminal portions of CA (1248) or MA (831). (The nucleotide sequences at the junction regions of the fusion constructs are shown in the legend to Figure 1.) Additionally, the internal deletion construct HIVGBGdlCA, was a deletion of most of the CA domain from HIVGBG. The other two HIVGBG internal deletion constructs, dlNP and dlMA, were engineered by transferring the deletions from HIVgpt constructs. HIVgptdlNP was a HIV *gag* mutant with a 56 amino acid deletion within the CA domain (33), and HIVgptdlMA was a deletion of most of the MA domain (34). These two HIV *gag*

mutants have been demonstrated to be capable of assembling and processing proteins in virus particles (33, 34).

Assembly of Gag-B-gal fusion proteins into particles was assayed as described above (Figure 2). Gag-B-gal constructs were cotransfected with or without a wt HIVgpt helper plasmid, and in two cases HIVGBG was cotransfected with variant constructs (HIVgptdlNP or HIVgptdlMA). As shown in Table 3 and described above, HIVGBG, which contains HIV MA, CA and NC domains, can incorporate into wt HIV (HIVgpt) particles. HIVGBGdlNP proteins were assembled into wt HIV particles, and HIVgptdlNP was able to incorporate HIVGBG proteins into mutant particles as demonstrated by 18.5 and 9.4-fold increases in B-gal release in the presence of HIVgpt helper plasmids. Similarly, HIVGBGdlMA entered the wt HIV particles and HIVgptdlMA could incorporate HIVGBG into mutant particles, as shown by 8.4 and 11.4-fold increases in B-gal release ratios. These results were not surprising since we have shown that the N-terminal portion of CA (dlNP) and most of MA can be deleted (dlMA) without affecting virus assembly, processing, and reverse transcriptase (RT) activities. Thus it appears that these amino-terminal regions of HIV Gag probably are not important for fusion protein entry into HIV particles. Likewise, it does not seem as though p6 or NC regions play major roles in this process. Deletion of the NC domain in the 1900 construct (Figure 1) effected a diminution of B-gal release, but the release ratio in the presence versus absence of wt HIVgpt helper was still 5.6 (Table 3). Addition of the p6 domain in the HIVGBG2289 construct actually reduced basal and HIVgpt-

associated release levels, possibly due to inhibitory effects of the p6 domain on fusion protein interactions.

Although previous studies showed that Gag-Pol myristylation was dispensable for HIV-1 Gag-Pol protein entry into particles (24, 30), our results with the HIVGBGMyr$^-$ construct were equivocal: release of this Gag-B-gal protein was increased only 3-fold by coexpression with wt HIVgpt. This discrepancy between Gag-Pol and Gag-B-gal results may be due to putative interactions between wt Gag proteins and Pol region domains on Myr$^-$ Gag-Pol proteins. Alternatively, or in addition, the embedded p6* region on Gag-Pol proteins may help circumvent the need for the myristate moiety on Gag-Pol proteins.

Whereas NC and P6 domains appeared of questionable importance, and MA and amino-terminal CA regions appeared unimportant to Gag-B-gal fusion protein incorporation into HIV particles, B-gal activity release required the presence of the carboxy-terminal two-thirds of the CA domain. As shown in Table 3, constructs with deletions covering the C-terminal two-thirds of CA (GBGdlCA, GBG1248, GBG1147, GBG831) expressed proteins which failed to show large increase of B-gal release in the presence of wt particles. Correspondingly, all myristylation signal positive Gag-B-gal proteins which retained this region, including the HIVGBGAccI protein, were released at high levels when co-expressed with HIVgpt.

The results above implied that CA interactions were essential for Gag-B-gal assembly into HIV particles, based on ratios of released versus intracellular B-gal activities. However, because different half-lives of intracellular and extracellular B-gal activities would affect the ratios, it was important to examine the stability of B-gal activities. Not surprisingly, we found that extracellular virus-associated B-gal appeared to be stable during incubations at 37^{0}C for over 24 h (data not shown). To measure the half-life of intracellular B-gal activities, Gag-B-gal fusion constructs were transfected into COS7 cells and treated with cycloheximide (100 µg/ml) to stop protein synthesis, after which intracellular B-gal activities were measured over a time course. As shown in Figure 4, fusion proteins GBG2289, HIVGBG, GBG1900, dlNP, and dlMA, which were able to be incorporated into virus particles, had long B-gal activity half-lives of around 24-25 h. Fusion proteins Myr$^-$, 1248, and dlCA had relatively short half-lives of 15.2, 16.8, and 21.4 h respectively. This would result in an underestimate of how much intracellular B-gal was present over the time course of the experiment, and hence would result in an overestimate of how much was actually released into media. In contrast. the GBG831 and 1147 proteins had relatively long half-lives, which could have led to a slight underestimate in their released B-gal activities. However, it appears unlikely that these half-life differences could adjust B-gal release ratios to normal levels.

Intracellular localization of Gag-B-gal fusion protein

Because HIV assembly occurs at the plasma membranes of infected cells, any defect in fusion protein membrane association or transport to the cell surface could account for impaired assembly into virus particles. Thus to control for these effects, we performed subcellular fractionation experiments to determine whether Gag-B-gal proteins were associated with cellular membranes. To do so, transfected COS7 cells were collected and fractionated to isolate the crude membrane (P2) and cytosol (S2) fractions which were subjected to B-gal and protein assays as described in the Materials and Methods. Ratios of P2 versus S2 B-gal activities and specific activities are shown in Table 4: higher ratios are indicative of greater membrane association. Following this protocol, we previously have found that the P2/S2 specific activity ratio of free B-gal expressed in 3T3 fibroblasts was approximately 0.1, indicating that most free B-gal protein was associated with the cytosol. In contrast, M-MuLV Gag-B-gal fusion proteins that were assembled into M-MuLV virions had specific activity ratios of 5 to 15. Our results (Table 4) show that all HIV Gag-B-gal fusion proteins were associated with membranes to some extent, with ratios of specific activities from 1.39 to 14 (Table 4). We found that, unlike M-MuLV Myr$^-$ proteins (10), HIVGBG Myr$^-$ associated with intracellular membranes, in agreement with previous observations (3). HIVGBG831 had a low P2/S2 ratio, similar to the case with short M-MuLV GBG fusions which retained P2/S2 specific activity ratios of 0.5 to 1.0 (13). Unexpectedly, we found that GBG2289 had low ratios in three independent experiments. However, we found no correlation between membrane association levels and a fusion protein's ability to be incorporated into virions. For instance,

both GBG2289 and 831 proteins had low P2/S2 ratios, but 2289 can and 831 can not assemble into virions (Table 3). Conversely, HIVGBG and 1248 had similar membrane association ratios (Table 4), but unlike HIVGBG, HIVGBG1248 proteins were unable to be incorporated into particles.

To obtain additional information on fusion protein subcellular localizations, indirect immunofluorescence experiments were performed using a mouse anti-B-gal antibody as described in the Materials and Methods. As illustrated in Figure 5, the majority of proteins (HIVGBG, dlCA, dlNP, and even Myr^-; Figure 5b, d, e, j respectively) showed similar patterns with heterogeneous cytoplasmic staining as well as a slight perinuclear ring. However, this pattern did not correlate with levels of fusion protein incorporation into particles, as HIVGBG and dlNP were released from cells at high levels while dlCA and Myr^- were not (Table 3). GBG2289 (Figure 5a) looked similar to the others but had a hazy, less heterogeneous staining pattern, perhaps indicative of its lower level of membrane association. HIVGBG831 (Figure 5i) localized to a perinuclear area, similar to what we have seen with HIVgptAccI (33), and with short M-MuLV GBG fusions (13). The apparent membrane localization but low P2/S2 ratio for the HIVGBG831 protein (Table 4) is similar to what we saw with short M-MuLV fusion proteins, and it is possible that such proteins are bound loosely to membranes and dissociate during fractionation. It also is possible that the HIVGBG831 protein did not assemble into virions because it was trapped on perinuclear membranes. However,

GBGdlMA often had a similar staining pattern, although in many cells (as in Figure 5h) it showed punctate spots spreading throughout the cell.

Interestingly, fusion proteins with intact MA but deleted NC domains (GBG1900, 1147, 1248) all appeared similar, with an absence of a clear perinuclear ring, and patterns reminiscent of surface staining (Figure 5c, 5f, 5g). Approximately 70-90% of GBG1900-, 1147-, and 1248-transfected cells looked like this, while the others appeared similar to HIVGBG (Figure 5b). In contrast, only 10-20% of HIVGBG-transfected cells appeared to have this putative surface-staining pattern. Since this pattern correlated with the NC deletion, we examined HIV Gag protein staining patterns on COS7 cells transfected with either wt HIVgpt or the HIVgptA14-15 mutant (33), which expressed a viral RNA packaging-defective Gag protein with mutation in the NC Cys-His motifs. As presented in Figure 5k, the wt HIVgpt Gag localization pattern appeared similar to that of HIVGBG (Figure 5b), whereas the A14-15 mutant showed a flat homogeneous pattern with no pronounced perinuclear ring in 70-90% of the cells examined. Thus it seems possible that the Cys-His motifs of the HIV NC domain may influence the transport or compartmentalization of the HIV Gag or Gag-Pol proteins.

DISCUSSION

We established a system to study the assembly of HIV Gag-B-galactosidase (Gag-B-gal) proteins into virions. This system permitted us to examine regions within Gag responsible for fusion protein incorporation into virus particles. Although Gag-B-gal proteins do not mimic Gag-Pol proteins precisely since, by necessity, they lack Pol domains, their analysis can be instructive with regard to Gag and Gag-Pol protein interactions. Expression of HIV Gag-B-gal proteins in COS7 cells did not result in the release of B-gal activity without co-expression of HIV Gag protein (Table 1). In contrast, we previously found that M-MuLV Gag-B-gal fusion proteins were released from cells in the absence of Gag proteins, demonstrating a difference in M-MuLV and HIV virus assembly mechanisms. That released HIVGBG proteins were assembled into virus particles was suggested by several lines of evidence. Firstly, assembly-competent viruses HIVgpt wt and PR^- facilitated B-gal release, while the assembly-defective mutants, Myr^- and AccI, did not (Table 1). Secondly, maximal activity of media material required SDS during assay incubations (Table 2), suggesting that activity was within an enveloped structure. Similarly, Triton X-100, which dissociates mature wt particles but has less of an effect on PR^- particles (33), greatly reduced pelletability of B-gal associated with wt but not PR^- mutant particles (Table 2). Finally, sucrose density gradient fractionation showed that media HIVGBG B-gal activity and wt Gag proteins banded together with a density of 1.16 mg/ml (Figure 3).

Our assays of fusion proteins expressed in the absence or presence of helper HIV Gag proteins demonstrated the importance of the C-terminal two-thirds of the CA domain for fusion protein assembly. Indeed, all myristylation signal-positive fusion proteins possessing this region were assembly competent (2289, HIVGBG, 1900, dlNP, dlMA, HIVGBGAccI) while those lacking the C-terminal two-thirds of the CA domain (GBGdlCA, GBG1248, GBG1147, GBG831) appeared impaired in their abilities to be assembled into virions (Table 3). These results suggest that HIV CA may be an appropriate target for antiviral approaches intended to interfere with assembly. Less clear were results with nonmyristylated Gag-B-gal proteins. Our GBGMyr$^-$ could enter virions, but at reduced levels which were halfway between results with nonmyristylated HIV Gag-Pol, which enters virus particles (24, 30), and nonmyristylated M-MuLV Gag-B-gal proteins, which do not (10). Possibly HIV Gag-Pol p6* or Pol regions compensate for the missing myristate moiety (24, 30).

Comparison of B-gal release data (Table 1, 3) with subcellular fractionation results (Table 4) indicates that assembly of fusion proteins into particles was independent of fusion protein membrane binding affinities. Relative to B-gal expressed in 3T3 cells (10, 13), all HIV Gag-B-gal proteins, including HIVGBGMyr$^-$, bound with some affinity to COS7 cellular membranes (Table 4). However, while both HIVGBG2289 and 831 were bound loosely to membranes (Table 4), HIVGBG2289 was released from cells while 831 was not. Similarly, HIVGBG, 1900, dlCA, dlNP, 1248, and 1147 all had B-gal specific

activity P2/S2 ratios of 5.9-8.5, while half were released and half remained cell-associated when co-expressed with wt Gag proteins (Table 3).

Levels of helper-induced Gag-B-gal release also did not correlate with immunofluorescence localization staining patterns. These experiments showed that staining patterns could be grouped broadly into four different groups which had little bearing on release levels: HIVGBG, 2289, dlCA, dlNP and Myr$^-$ had heterogeneous staining patterns with slight perinuclear rings, similar to that of HIVgpt (Figure 5); 1900, 1248 and 1147 appeared surface localized (Figure 5c, f, g); 831 showed bright fluorescence in an asymmetric perinuclear location (Figure 5i); and dlMA showed a similar pattern to that of 831 but also with bright spots of fluorescence extending throughout about half of the cells (Figure 5h). One possible but untested explanation of the low levels of 831 protein release is that it is trapped irreversibly on intracellular membranes. This subcellular compartmentalization could be a consequence of mislocalization or may indicate that association with intracellular membranes is a natural but dead end pathway. Alternatively, association with intracellular membranes may be involved with the natural HIV Gag or Gag-Pol transport pathway, although experiments have shown that such a route must be independent of the effects of brefeldin (23) and monensin (22). Also potentially relating to Gag and Gag-Pol intracellular transport, were the enhanced surface staining patterns of fusion proteins with intact MA but deleted NC domains (HIVGBG1900, 1248 and 1147). That the Gag staining

pattern of the HIV NC Cys-His mutant HIVgptA14-15 also appeared surface-enhanced (Figure 51) suggests that the wt HIV Gag Cys-His motif may increase Pr55*gag* affinity for intracellular membranes. Whether this difference is relevant to the mechanism of HIV assembly is unknown.

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Table 1. Incorporation of a HIV Gag-B-galactosidase fusion protein into wild-type and mutant HIV particles.

B-gal construct	helper construct	N	(media B-gal/cell B-gal) - helper	+ helper	ratio
HIVGBG	HIVgpt(wt)	40	0.05 ± 0.03	0.53 ± 0.31	13.2 ± 9.0
HIVGBG	HIVgpt(PR^-)	5	0.04 ± 0.03	0.84 ± 0.65	19.3 ± 12.1
HIVGBG	HIVgpt(Myr^-)	8	0.07 ± 0.04	0.09 ± 0.05	1.4 ± 0.6
HIVGBG	HIVgpt(AccI)	6	0.06 ± 0.03	0.14 ± 0.05	2.7 ± 1.0

COS7 cells were transfected with 15 μg of HIVGBG plasmid alone or cotransfected with 8 μg of HIVGBG plasmid plus 8 μg of designated wt or mutant HIVgpt helper construct and the procedure for assay of Gag-B-gal fusion protein incorporation into virus particles was as illustrated in Figure 2 and described in the Materials and Methods. Media and cell samples were aliquoted for B-gal enzyme assays. Results are presented as the ratios of total normalized B-gal enzyme activities released to the media versus inside cells with standard deviations. N indicates the number of times the experiment was performed and data in the ratio column were obtained by comparing the ratios of B-gal activities in the presence versus absence of helper constructs. Note that ratios were averaged from individually tabulated experimental ratios and were not ratios of averages from the previous two columns.

Table 2. Association of B-galactosidase activity with virus particles.

helper construct	(B-gal activity) -SDS	+SDS	(-SDS/+SDS)x100
HIVgpt(wt)	1.40	3.66	39
HIVgpt(wt)	3.17	13.47	24
HIVgpt(PR-)	0.30	2.90	10
HIVgpt(PR-)	2.99	17.08	18

helper construct	(B-gal activity) +Triton	-Triton	(+Triton/-Triton)x100
HIVgpt(wt)	1.02	3.92	26
HIVgpt(wt)	1.12	3.34	34
HIVgpt(wt)	1.34	6.20	22
HIVgpt(PR-)	5.40	7.66	70
HIVgpt(PR-)	0.76	0.88	86
HIVgpt(PR-)	11.89	17.29	69

COS7 cells were cotransfected with 8 µg of HIVGBG plus an equal amount of helper constructs HIVgpt wt or PR^-. Three days later, cell medias were collected and filtered through 0.45 µm-pore-size filters. As described in the Materials and Methods, identical virus pellets were treated with or without 0.1% SDS in the B-gal assays, or identical supernatants were treated with or without 0.5% Triton-X 100 prior to centrifugation through a 20% sucrose cushion and collection of virus pellets. In the first two columns total B-gal units are given, and relative B-gal activities were determined as percentages of SDS-treated (-SDS/+SDS) or Triton-untreated (+Triton/-Triton) B-gal activities.

Table 3. Requirements for fusion protein incorporation into HIV particles.

B-gal construct	helper construct	N	(media B-gal/cell B-gal) - helper	+ helper	ratio
HIVGBG2289	HIVgpt(wt)	8	0.04 ± 0.02	0.22 ± 0.07	5.7 ± 2.8
HIVGBG	HIVgpt(wt)	40	0.05 ± 0.03	0.53 ± 0.31	13.2 ± 9.0
HIVGBG1900	HIVgpt(wt)	6	0.06 ± 0.03	0.31 ± 0.13	5.6 ± 2.9
HIVGBGdlCA	HIVgpt(wt)	6	0.08 ± 0.02	0.10 ± 0.04	1.3 ± 0.4
HIVGBGdlNP	HIVgpt(wt)	4	0.01 ± 0.01	0.25 ± 0.12	18.5 ± 9.7
HIVGBG	HIVgptdlNP	4	0.05 ± 0.01	0.46 ± 0.12	9.4 ± 2.6
HIVGBG1248	HIVgpt(wt)	9	0.11 ± 0.05	0.13 ± 0.09	1.3 ± 0.6
HIVGBG1147	HIVgpt(wt)	6	0.05 ± 0.01	0.06 ± 0.03	1.2 ± 1.0
HIVGBGAccI	HIVgpt(wt)	4	0.04 ± 0.01	0.21 ± 0.04	5.9 ± 2.3
HIVGBGdlMA	HIVgpt(wt)	4	0.04 ± 0.01	0.27 ± 0.11	8.4 ± 4.6
HIVGBG	HIVgptdlMA	4	0.06 ± 0.02	0.61 ± 0.09	11.4 ± 4.6
HIVGBG831	HIVgpt(wt)	12	0.05 ± 0.02	0.08 ± 0.07	1.9 ± 1.3
HIVGBGMyr⁻	HIVgpt(wt)	4	0.05 ± 0.02	0.13 ± 0.04	3.0 ± 0.6

COS7 cells were transfected with Gag-B-gal expression constructs (Figure 1) with or without the designated HIVgpt helper constructs as described in the legend to Table 1. Three days post-transfection, media and cells were collected and processed for B-gal assays as described in the Materials and Methods. The ratios of B-gal activity levels in the presence of helper constructs versus those without helper constructs were obtained as described in the legend to Table 1. N indicates numbers of experiments , and in each case, at least 4 independent trials were carried out with the indicated standard deviations. Note that the data for HIVGBG plus HIVgpt (wt) is identical to that shown in Table 1.

Table 4. Membrane association of fusion proteins.

B-gal construct	B-gal activity			B-gal/mg protein		
	P2	S2	P2/S2	P2	S2	P2/S2
HIVGBG2289	0.30	0.43	0.70	0.53	0.38	1.39
HIVGBG	5.07	1.02	4.97	6.35	0.78	8.14
HIVGBG1900	10.44	3.45	3.03	12.00	1.91	6.28
HIVGBGdlCA	6.80	3.67	1.85	9.69	1.63	5.94
HIVGBGdlNP	0.33	0.15	2.20	5.79	0.89	6.50
HIVGBG1248	8.41	2.09	4.02	58.40	6.92	8.44
HIVGBG1147	3.76	1.03	3.65	5.50	0.83	6.63
HIVGBGdlMA	0.41	0.09	4.56	0.70	0.05	14.00
HIVGBG831	7.00	8.24	0.85	8.97	3.73	2.40
HIVGBGMyr-	0.31	0.44	0.70	0.66	0.35	1.89

Three days after transfections with the indicated constructs, COS7 cells were collected and fractionated into P2 and S2 fractions as described in Materials and Methods. Fractions were assayed for total B-gal activities (units) and proteins levels (mg). The ratios of P2 versus S2 B-gal activities are indicated in the third column while the right-most column shows the ratios of P2 versus S2 specific B-gal activities. Note that free B-gal gives a specific activity P2/S2 ratio of approximately 0.1 (13), and that higher ratios are indicative of higher levels of membrane association.

Figure 1. Recombinant HIVgpt and HIVGBG constructs.

HIVgpt constructs: The wt *gag* domains MA (matrix; p17), CA (capsid; p24), NC (nucleocapsid; p7) and p6/p6* embedded in Gag-Pol fusion proteins of the parent construct HIVgpt (21) are illustrated. As described previously (33), the protease-defective (PR^-) mutant was constructed by a 12 bp linker insertion in the PR coding region; dl.NP was generated by a deletion from NsiI-1251 to PstI-1418 within CA; dl.MA (34) was constructed by a deletion from ClaI-831 to PvuII-1147 and replacement with 4 amino acids within the MA; AccI was a MA mutant with a 12 bp linker insertion at AccI-960; Myr^-, in which the second amino acid glycine of Gag was changed to alanine, blocks the Gag myristylation (3, 33). **HIVGBG constructs**: Gag-B-galactosidase fusion constructs were made by replacing regions from the indicated *gag* nt locations to the HIVgpt SalI site (HXB2 nt 5786) with B-gal. For 2289, HIVGBG, 1900, 1248, 1147 and 831, the fusion junctions are as follows, where the underlined nt indicates the HIV nt position designated in the construct name (or nt 2096 for HIVGBG), and the final GTC is the eighth codon of B-gal: 2289, CTG GAT CCC GTC; HIVGBG, AG ATC TCG GGG GAT CCC GTC; 1900, CA AAT TCC TGC AGC CCG GGG GAT CCC GTC or fusion as in HIVGBG and deletion of HIV nt 1900-2067 as described in the Materials and Methods; 1248, GCA GCC CCG GAT CCC GTC; 1147, CG GAT CCC GTC; 831, CGA TTA GAT CCC GTC. For dl.CA, dl.NP, dl.MA, AccI, Myr^- constructs, the fusion site was as in HIVGBG, but mutations or deletions from previously described HIVgpt mutants (33, 34), or as described in the Materials and Methods for dl.CA, were transferred to the HIVGBG backbone.

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Figure 1

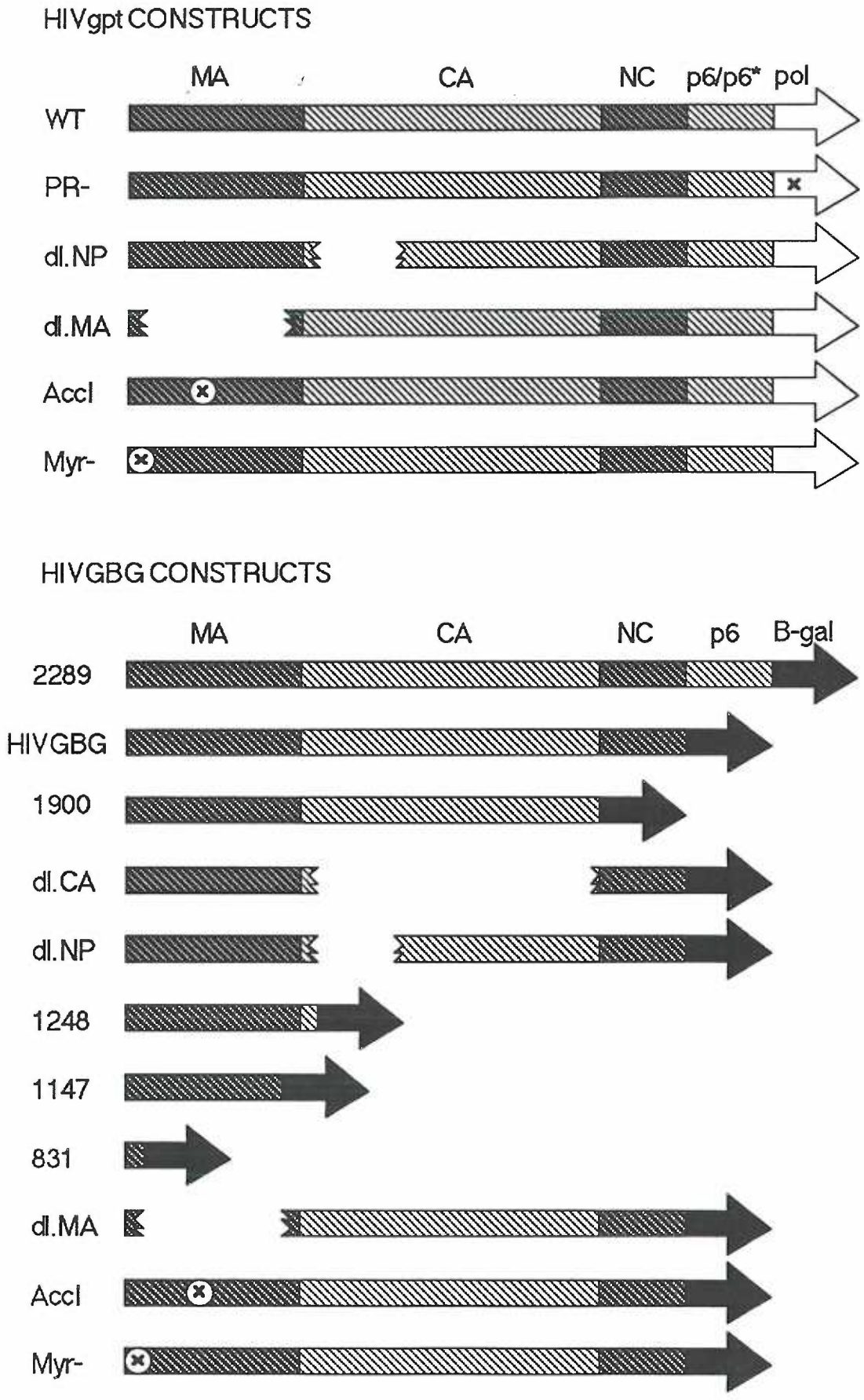

Figure 2. Assay of Gag-B-gal fusion protein incorporation into HIV-1 particles.

To assays fusion protein incorporation into HIV particles, COS7 cells are transfected with fusion protein expression constructs with or without HIVgpt-derived Gag protein expression "helper" constructs. At 72 h, media and cells are collected, and assayed for B-gal activity as described in the Materials and Methods. For each transfection, ratios of B-gal activity levels in the media versus in the cells indicated the level of released HIV Gag-B-gal fusion proteins. B-gal release ratios in the presence versus absence of Gag proteins expressed from helper constructs were compared in parallel transfections. Higher release levels in the presence of a helper construct are indicative of Gag-B-gal fusion protein incorporation into virus particles.

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Figure 2

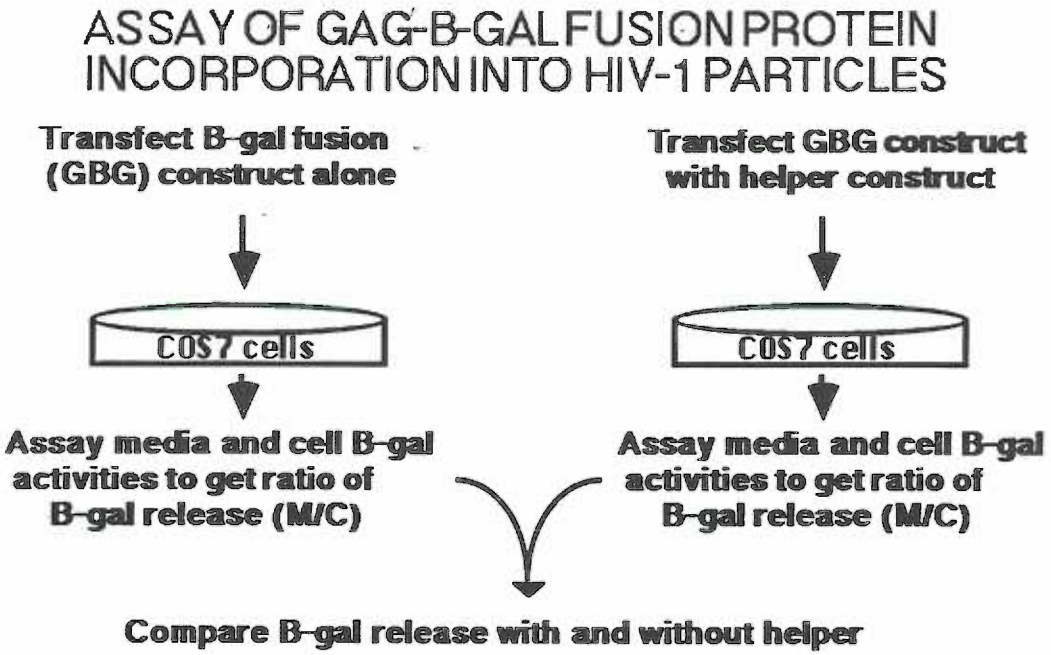

Figure 3. Sucrose density gradient fractionation of culture media from cells cotransfected with HIVGBG and HIVgpt.

COS7 cells were cotransfected with HIVGBG and HIVgpt (Figure 1). Three days later, cell supernatants were collected, filtered and centrifuged at 4^0C for 45 min at 200,000 X *g*. Pellets were gently suspended in PBS and layered onto a 20-50% sucrose gradient followed by ultracentrifugation at 4^0C overnight at 201,000 X *g*. Fractions were collected from top to bottom, and for each fraction, B-gal activities were measured and particle-associated Gag proteins were determined by immunoblotting and quantitated by scanning densitometry. As shown, B-gal activity and Gag protein peak fractions had a density of 1.16 g/ml.

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Figure 3

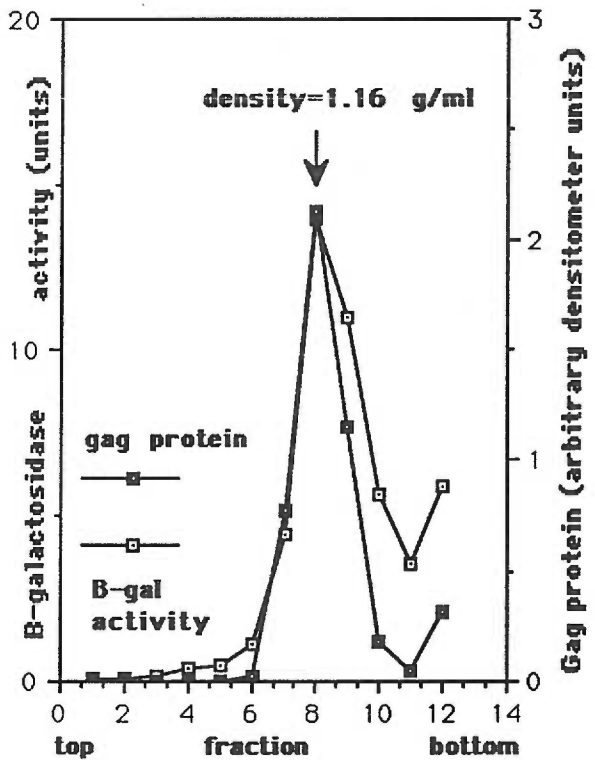

Figure 4. Stability of Gag-B-gal fusion proteins.

COS7 cells grown on 10-cm plates were transfected with designated HIVGBG constructs. Two days later, transfected cells on each plate were trypsinized, dispersed, and split equally onto five 60-mm plates containing DMEM plus 10% fetal serum media. 18 h later, cell medias were removed and cells were refed with media plus 100 µg/ml cycloheximide. Cells then were collected at designated time intervals and B-gal activities were measured as described in the Materials and Methods. B-gal activities from untreated plates were defined as zero time, and results of treated samples were plotted relative to time as percent zero time activity on a long scale y axis. Lines were generated by the least squares method and protein half lives were determined from these plots.

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Figure 4.

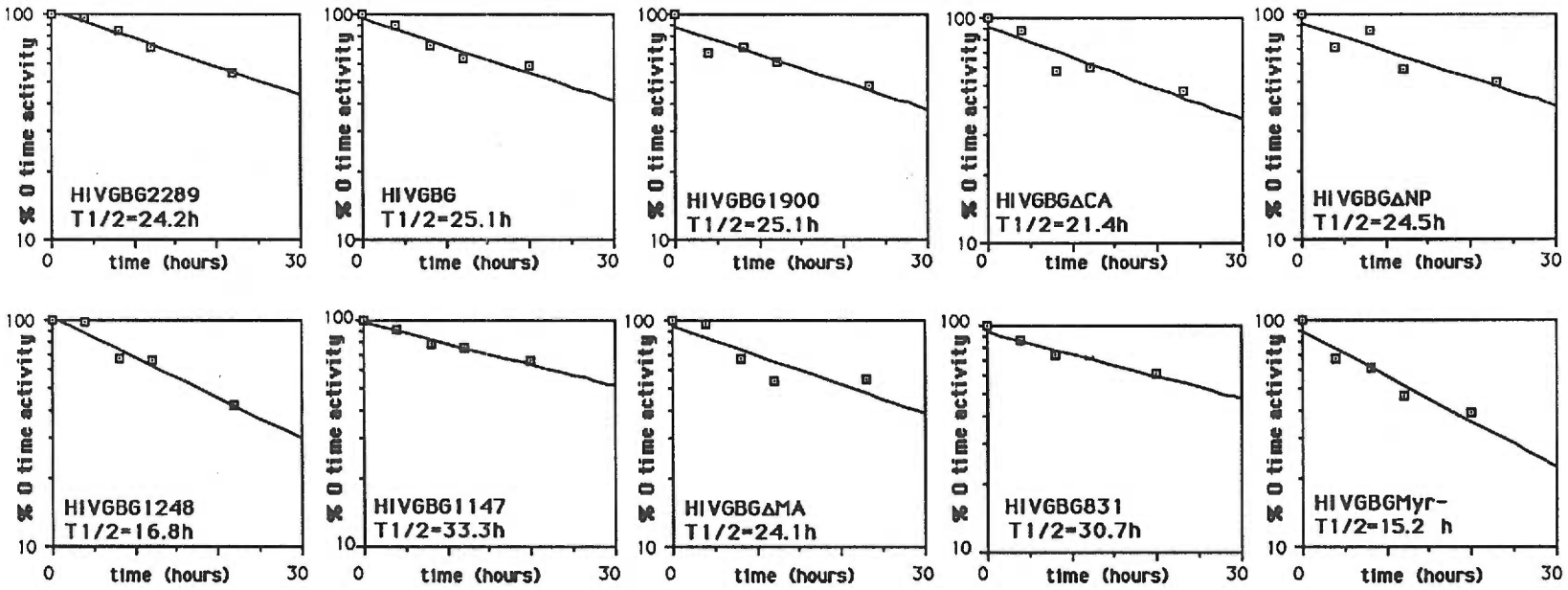
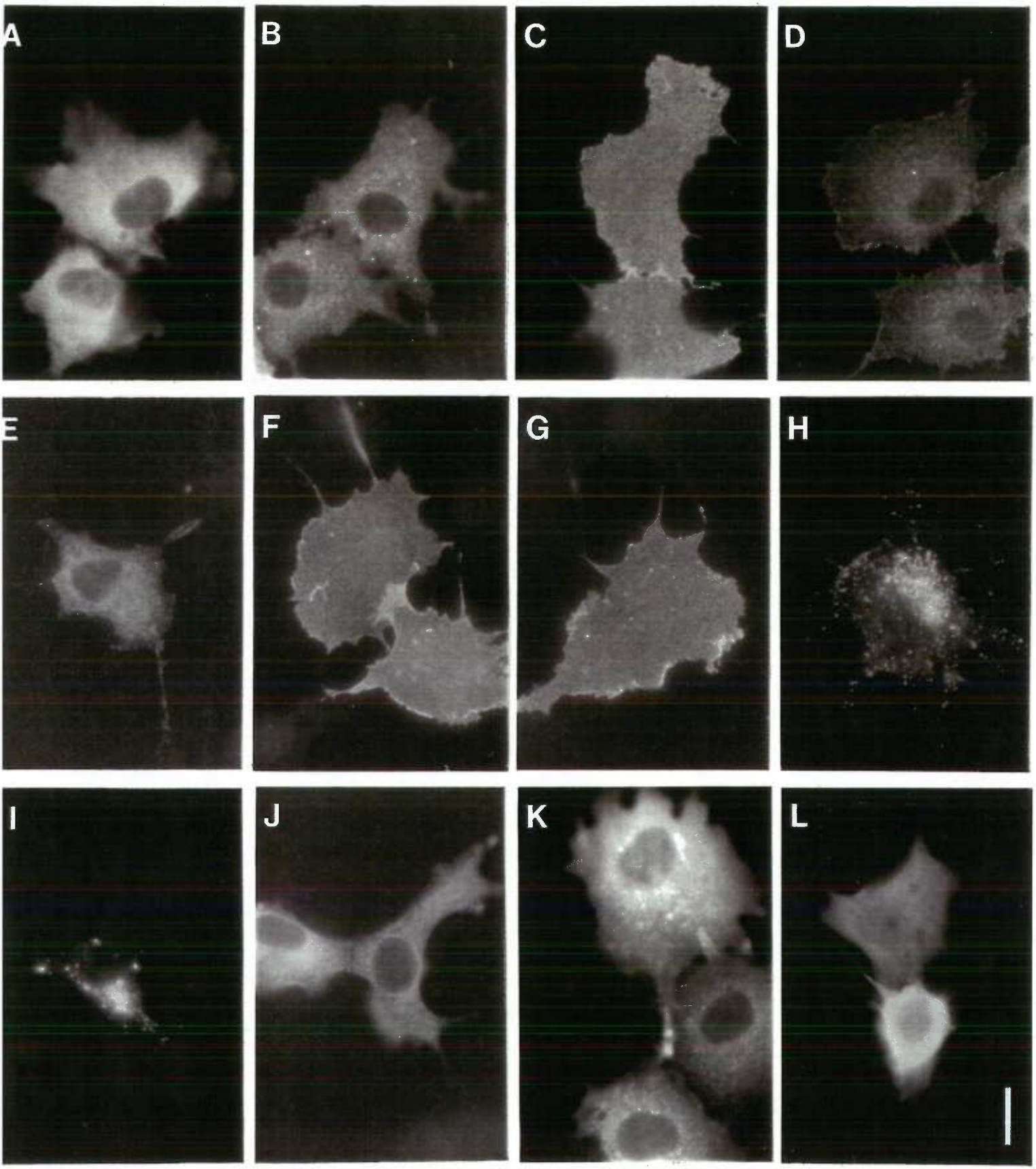


Figure 5. Indirect immunofluorescence detection of HIV Gag-B-gal fusion proteins, or HIV Gag proteins in COS7 cells.

COS7 cells grown on cover slips were transfected with HIV Gag-B-gal expression constructs GBG2289 (A); HIVGBG (B), GBG1900 (C); GBGdlCA (D); GBGdlNP (E); GBG1248 (F); GBG1147 (G); GBGdlMA (H); GBG831 (I); GBGMyr- (J); or with HIVgpt expression constructs HIVgpt wt (K) or HIVgptA14-15 (L). Two days after transfections, cells were fixed and permeabilized for immunofluorescence assays as described in the Materials and Methods. Panels A through J were detected with a mouse anti-B-gal antibody at 1:3,000 dilution, while panels K and L were detected with a mouse anti-p24*gag* monoclonal antibody at 1:1,000 dilution. The secondary antibodies were goat anti-mouse rhodamine-conjugated IgG antibodies at a 1:100 dilution. The white bar in panel L indicates 20 microns.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Discussion and Conclusions

HIV, the etiological agents of AIDS, primarily infects $CD4^+$ T cells and monocyte-macrophages and leads to a progressive immunosuppression of the host. Severe immune defects render HIV-infected individuals susceptible to opportunistic infections or neoplasms, two common lethal complications of AIDS. Many efforts have been made to study HIV, and some novel features in the control of its replication have been found. However, like other retroviruses, the mechanism of HIV assembly is still unclear. HIV appears to assemble at host cell plasma membranes during the late stage of the virus replication cycle. The HIV Gag precursor, Pr55 still can self-assemble into virus-like particles in the absence of other proteins. Subsequent proteolytic processing of Pr55 by the viral protease gives rise to four major mature products: matrix (MA), capsid (CA), nucleocapsid (NC) and p6 domains (77, 95, 121). The *pol* gene, which encodes protease and the essential replicative enzymes is translated initially as a Gag-Pol fusion protein and is incorporated into virions during assembly. It has been known that myristylation of HIV Gag is essential for particle formation (16, 70). However, little is known about the process of Gag and Gag-Pol assembly into virus particles.

Gag is synthesized on free polysomes but eventually becomes associated with the plasma membrane where oligomerization and assembly of Gag proteins into particles proceeds (178). For most mammalian retroviruses (excluding D type retroviruses), myristylation appears to be a prerequisite for particle formation, as nonmyristylated *gag* mutants do not assemble or release particles.

Myristylation may stabilize the association of Gag proteins with cellular membranes and promote Gag transport to the plasma membrane. Support for this theory comes from the studies of type D retroviruses: nonmyristylated mutants are blocked particle transport but are not affected in particle assembly (145). Not surprisingly, our HIV Myr$^-$ mutant demonstrated defective of virus assembly, consistent with the previous reports (16).

Although many retroviral Gag proteins required myristylation, the fact that RSV and other members of lentivirus subfamily, visna and equine infectious anemia virus (EIAV) produce Gag precursors that are not myristylated suggests that aside from myristylation, some other Gag domains, possibly MA (146), may play a role in the process of Gag transport. In support of this hypothesis, our HIVgpt mutant AccI, which contains a 4 amino acid insertion at the middle part of MA was impaired in particle assembly and release, and appeared trapped around the perinuclear area by immunofluorescence assays. In addition, b-gal fusion within the MA domain (GBG831) resulted in a fusion protein which displayed a perinuclear (RER and Golgi) staining pattern, similar to the previous results with analogous M-MuLV Gag-b-gal fusion proteins (83). These observations suggest that the MA domain may contain a signal for proper Gag localization and transport. Failure of our AccI mutant to assemble and release particles may be due to disruption of an appropriate protein conformation that is required for the subsequent transport. It is unknown whether there is any host cellular protein involved in the process of Gag transport. However, recent studies have shown that enterovirus capsid proteins can be coprecipitated

with heat shock protein 70 (HSP 70), implying that in some cases, cellular factors may be associated with viral structural proteins and involved in the process of capsid transport or assembly (109, 110). Paradoxically, our dl.MA mutant was still able to assemble and release virus particles. One possible explanation is that linker-insertion mutation (AccI) induced interactions that caused the Gag proteins to be trapped intracellularly while a deletion of most of MA domain removed the potential deleterious regions.

Although transport of Gag to the plasma membranes is a prerequisite for HIV particle formation and release, the process of Gag assembly is still unclear. Self-assembly of Gag precursors appears to be via Gag-Gag interactions and the Gag-Pol polyprotein is thought to be incorporated into virions by virtue of its Gag protein determinants. *In vitro* studies (48) and previous studies of MLV assembly (67, 83, 104, 156) suggested that the CA is the most important region involved in the process of Gag-Gag interactions.

In our studies, we found that most of our HIV *gag* mutants, except Myr$^-$ and AccI mutants, could assemble and release virions, and these mutant virions had wt particle densities. Moreover, deletion of most MA domain (dl.MA) or a deletion of 56 codons within the CA domain (dl.NsiPst) did not affect the assembly, processing of virus particles. These results suggested that either HIV *gag* is not completely sensitive to mutations or that most of our mutations were not introduced within the key regions for particle formation. Not surprisingly, deletion of the HIV CA domain (dl.CA) abolished the particle assembly (data not shown). Perhaps the major homology region (MHR) that has been found within the C-terminal

one-third of mammalian retrovirus capsid domains may be a region responsible for retrovirus core assembly (191). In agreement with this hypothesis, recent studies have demonstrated that mutations within the C-terminal one-third of HIV CA block particle formation (78, 138).

Although two groups reported that myristylation of the HIV Gag-Pol polyprotein is not necessary for its assembly into virions (129, 162), the functional domain required in assembly for Gag-Pol was not delineated. Our results from studies of HIV Gag-b-gal incorporation into HIV virions showed that fusion proteins lacking the C-terminal two-thirds of the CA domain (GBG831, 1147, 1248) were excluded from the virions, suggesting that assembly of HIV Gag-Pol into virions may be determined by the CA domain. This result is similar to the previous data obtained from studies of M-MuLV (74). Our GBGdlMA can be incorporated into wt particles and dlMA can help the release of b-gal activity into media, suggesting that HIV MA is not involved directly in assembly of Gag-Pol fusion proteins into virions. Our evidence that dl.MA possessed a wt RT activity corroborates this conclusion. Our fusion protein lacking the NC domain (GBG1900) was slightly reduced in b-gal activity release. Preliminary results also showed that a *gag* mutant (dl.NC) with a deletion of the HIV NC domain was slightly defective for particle assembly and release, and it had a reduced level in helping HIVGBG b-gal activity release (data not shown). This evidence suggests that the HIV NC domain may play some part in particle assembly although it must be a relatively minor one.

In contrast to the previous reports that myristylation of HIV Gag-Pol is not required for incorporation into virions (129, 162), our nonmyristylated Gag-b-gal fusion construct (GBGMyr$^-$), appeared to be incorporated at a reduced level, halfway between the nonmyristylated Gag-Pol (129, 162) and the nonmyristylated M-MuLV Gag-b-gal (74). This discrepancy may be due to the differences among the systems employed. For instance, Pol protein domains or the p6* domain within the Gag-Pol proteins may be involved in Gag-Pol incorporation into virions. However, consistent with previous observations (16), our subcellular membrane fractionation studies showed that GBGMyr$^-$ was still associated with membranes, although to a lower extent than most myristylated fusion proteins. Nevertheless, the membrane association levels of HIV Gag-b-gal fusion proteins did not correlate with the levels of their incorporation into particles, since the long fusion protein (GBG2289) still could assemble into virions, although it had a low membrane association level.

Processing of Gag and Gag-Pol precursors occurs during or after virus budding and it is essential for virus infectivity (90, 132). During virus assembly, interactions among Gag-Pol polyproteins brings about the dimerization of the protease peptide, a prerequisite for activation of the viral protease (119, 124). Most of our HIV assembly-competent mutants were able to process particle proteins at wt levels except mutants AccI, SpeI, BglII, and PR$^-$. All four mutants had low RT levels, which may be due to insufficient Gag-Pol incorporation and/or impaired processing of the Gag-Pol proteins. Consistent with the idea that incorporation is important; the dl.NsiPst

and dl.MA mutants processed viral proteins as well as wt, displayed wt RT activity levels; efficiently incorporated the Gag-b-gal into virus particles, and their Gag-b-gal derivative constructs (GBGdlNP and GBGdlMA) also were capable of assembly into wt particles. However, in some cases we can not rule out the possibility that the low RT levels may be due to the resistance of immature mutant particles to the nonionic detergent used in RT assays. Consequently, the substrates may not have complete access to viral particle enzymes during the RT assay. This idea agrees with the data obtained from assays of the PR$^-$ mutant.

Another factor that could cause incomplete processing of Gag mutant proteins may be due to conformational changes that conceal the cleavage sites. Alternatively, the conformational changes induced by the Gag mutations may disrupt the dimer formation of protease, which is essential for activation of the viral protease (119, 124). One recent study of processing of a variety of HIV *gag* mutants has shown that some *gag* mutants impaired in processing can be proteolytically cleaved *in trans* by the viral protease (105), indicating that the cleavage sites within the Gag mutant proteins are not inaccessible, but that *gag* mutants may disrupt the function of the protease (PR) in the context of Gag-Pol.

Previous studies have shown that deletion mutations within the HIV MA did not affect virus assembly and processing but reduced HIV gp120 incorporation into virus particles (195), suggesting the involvement of the HIV MA in incorporation of HIV Env. However, we found that the reduced levels of dl.MA particle-associated gp120 could not account for the infectivity differences

when compared with the case of wt. This suggests that either the MA domain affects the gp120 postassembly function and/or the binding or postbinding events are not related linearly to gp120 levels. Thus, HIV MA may have an effect on the HIV Env stability or function through its interaction with the envelope protein complex (gp120 plus gp41).

Besides assembly, retroviral Gag proteins appear to be involved in other phases of the replication cycle. In MLV, some mutations within the *gag* did not block particle assembly or release but impaired virus infectivity (67, 104), and the MLV CA domain has been shown to be associated with the proviral preintegration complex (13). Similarly, some of our HIV *gag* mutants did not affect particle formation but eliminated the virus infectivity, possibly at early post-entry replication steps. These observations indicate that *gag* may play a role in the early stages of HIV life cycle.

Most of our HIV *gag* mutants could assemble, process virus particles, had significant RT activities, and contained viral RNA, but were noninfectious or poorly infectious in single round infectivity assays. RNase protection experiments (Zhang et al, unpublished results) showed that most mutant contained significant amounts of viral genomic RNAs with the exceptions of packaging-defective mutants A14-15 and Psi^-. These results strongly support the notion that *gag* is functionally involved in the other phases of virus life cycle. Interestingly, an insertion of only two amino acids at the central part of the CA domain (SpeI) did not affect virus assembly but eliminated infectivity, possibly due in part to incomplete processing of Gag proteins or to low RT levels. In contrast, mutants

within the MA were infectious. Our dl.MA mutant still could package viral RNA and demonstrated 10% of wt infectivity level when pseudotyped with amphotropic M-MuLV envelope proteins. These observations indicate that an intact HIV MA is not essential for reverse transcription, nuclear localization, and integration in dividing cells. However, HIV-1 can infect the nondividing cells and possess a putative nuclear localization signal in its MA domain (17, 18). Thus, it would be of interest to test the infectivity of our dl.MA in nondividing cell systems.

Conclusions of the thesis:

(1) HIV *gag* is functionally involved in the postassembly, postprocessing stages of viral infection.

(2) HIV *gag* mutants can affect proteolytic processing, and immature HIV particles were resistant to nonionic detergent treatment.

(3) MA is not absolutely required for HIV viral RNA packaging, reverse transcription, nuclear localization, or integration.

(4) HIV MA may be involved in Gag transport.

(5) HIV MA interacts with HIV Env during viral replication.

(6) HIV MA is not essential for particle formation and Gag-Pol assembly into virions.

(7) HIV CA is the key region for particle assembly and HIV Gag-b-gal incorporation into virions.

(8) HIV NC has a minor of effect on Gag-b-gal incorporation.

(9) Nonmyristylated HIV Gag-β-gal fusion proteins are assembled into particles at reduced levels.

Future Directions

1. Follow-up experiments on HIVgpt entry

Although most HIV *gag* mutants had significant RT activities on exogenous templates, they were noninfectious or poorly infectious, indicating that they are replication-defective for one reason or another. For instance, pseudotyped mutants may be impaired in binding to the cellular receptors or in entry. Endogenous reverse transcription may be defective or full-length double stranded linear viral DNAs would be unable to be completed due in part to impairment in template switching. Mutants could be blocked at nuclear transport or integration after entry into cells.

The binding affinity of each assembly-competent mutant to target cells could be assessed by immunofluorescence. Pseudotyped virions would be incubated with HeLa cells for 30 min at different temperatures. Unbound virions will be washed out and bound virions could be detected with fluorescein isothiocyanate (FITC)-conjugated anti-MLV Env antibody and the amount of bound virions is analyzed by a fluorescence-activated cell sortor (FACS) (29). Alternatively, cells can be incubated with radiolabeled virions and binding affinity of each mutant will be determined by the ratio of radioactivity levels in cell samples versus in supernatant samples after separation of virions from cells. Most radioactivities will remain in supernatant samples of mutants which are impaired in binding or adsorption (10).

To determine RT activities in viruses, strong-stop (-) and (+) viral DNAs from detergent-disrupted virus DNA synthesis reactions (125a) can be quantitated. RT activities of mutants in infected cells could be analyzed by polymerase chain reaction (PCR) to determine unintegrated viral DNA levels. To do so, cell samples could be collected at different time intervals after incubation with pseudotyped virions, and separated into nuclear and cytoplasmic fractions: intracellular viral DNA or RNA then can be amplified by PCR or RT-PCR, and different R/U5 and LTR/*gag* primer pairs should distinguish late from early reverse transcription products (197). By this methodology, effects of *gag* mutations on viral RT or nuclear localization in infected cells can be differentiated. While mutants are competent in RT and nuclear localization in infected cells, they may be impaired in integration into cell genome and unable to transduce the drug-resistant gene. Mutants which are defective in integration after entry may possess integration activity *in vitro*. Integration activity of mutants can be assayed in a cell-free system using the øX174 replicative-form I (RFI) DNA as the target (48a).

2. Follow-up experiments on the dl.MA mutant

The evidence that the dl.MA mutant was infectious when pseudotyped with amphotropic MLV envelope proteins indicates that an intact HIV MA domain is not required for reverse transcription, nuclear localization, or integration in dividing cells. The dl.MA mutant still could incorporate the HIV Env into particles but the pseudotyped virions were noninfectious, suggesting that HIV MA domain may interact with HIV Env and affect the stability of

envelope proteins or functions of the envelope protein complex on binding or postbinding events.

To test infectivity of dl.MA mutant in nondividing cells, HeLa cells arrested by treatment with the DNA polymerase α inhibitor aphidicolin, could be incubated with dl.MA pseudotyped virions followed by a PCR-based HIV-1 DNA integration assay (100a). High-molecular-weight cellular DNA, isolated at 48 h postinfection and digested with restriction enzymes, can be religated and used as templates for PCR with opposing primers from the HIV-1 LTR (100). By analysis of PCR products, integrated proviral DNA is differentiated from the unintegrated or circular viral DNA. If no integrated mutant proviruses are detected, protocols described above for viral entry assays can be followed to dissect the mutant's ability for RT or nuclear localization in growth-arrested cells.

The dl.MA mutant may affect stability of HIV envelope protein complex and cause the gp120 shedding more easily from mutant than from wt virions. To address this question, wt and mutant pseudotyped virions can be incubated at 37^0C and at different time points samples will be collected and particle-associated HIV envelope proteins are analyzed by western immunoblot. The interaction between dl.MA Gag proteins and HIV Env proteins may lead to changes of surface envelope protein conformations and the topology. Since the HIV envelope protein is heavily glycosylated, the carbohydrates on the envelope protein can be used as markers to study the effects of the MA deletion on the HIV Env protein structure or conformation. Wt and mutant pseudotyped virus samples will be treated with galactose oxidase which oxidizes the

surface accessible terminal galactosyl residues, and oxidized sugars will be labeled with biotin hydrazides (45a). Samples then are treated with proteases (trypsin and/or V8 protease) and subjected to SDS-PAGE and Western immunoblotting using alkaline phosphatase-conjugated streptavidin to detect biotinylated peptides. To compare with the results of wt samples, the signals from the mutant samples may be fainter, undetectable, or different due to changes in conformation or topology of HIV Env proteins.

Cross-linking and sucrose density gradient fractionation experiments also can be performed to analyze the structure of HIV Env protein on wt and mutant pseudotyped virions. Virus particles can be cross-linked with 3, 3- dithiobis-sulfosuccinimidyl propionate (DTSSP), and lysed viral samples subjected to sucrose density gradient centrifugation (46a). Samples from fractions then are incubated in the presence or absence of reducing agent and analyzed by Western immunoblotting. The amounts of envelope protein high-molecular-complex forms (trimers or dimers) from mutant virions may be reduced in comparison to those of wt samples.

Although HIV envelope proteins on the mutant virions may exhibit correct structure and conformation, the dl.MA mutant could impair the functions of HIV envelope proteins during binding or fusion. Binding of pseudotyped virions to $CD4^+$ HeLa cells can be assayed by either immunofluorescence or with radiolabeled virions as described above. Virions bound to target cells are detected by either FITC-conjugated anti-HIV gp120 antibody or the cell-associated radioactivity level. For controls, aliquots of virions are used to infect $CD4^-$ HeLa cells in parallel experiments. Membrane

fluorescence dequenching methods (161a) might be employed to assay fusions between pseudotyped mutant virions and $CD4^+$ HeLa cells. Intact mutant and wt pseudotyped virions labeled with a self-quenching dye, octadecylrhodamine B-chloride (R-18), are absorbed to $CD4^+$ and $CD4^-$ (for controls) HeLa cells at 4^0C followed by washing of nonadsorbed virus and incubation at 37^0C for different period of time. Cells will be analyzed for fluorescence in a fluorometer. The kinetics and extent of fusion between the virions and target cells are based on the relief of the self-quenching of fluorescence. If mutant virions still can bind to the $CD4^+$ receptors and fuse with the cell membrane, strategies as described above for viral entry assays will be performed. PCR or RT-PCR reactions will be carried out to detect the mutant DNA or RNA in infected cells.

3. Follow-up experiments on HIVGBG system

HIV Gag-β-gal fusion constructs with deletions covering the C-terminal two-thirds of the CA domain were impaired in assembly into virus particles, indicating that the HIV CA domain is the most important region for fusion protein incorporation into virions. Further mutations within the CA domain should be engineered to define the regions critical for fusion proteins incorporation. The proposed MHR region within the CA domain among mammalian retroviruses has been predicted to be an amphiphilic α helix secondary structure. For HIV, the Glu-291 and Arg-299 residues are conserved and lie on the hydrophilic surface which may be involved in protein-protein interactions. The Glu-291 will be changed to a nonpolar amino acid Leu, and/or the basic Arg-299 will be mutated

to an acidic amino acid Gln. Constructed *gag* point mutations will be introduced into the HIVGBG construct and tested for the mutant fusion protein assembly into virions. The Tyr-262 and Lys-272 residues also are conserved in both HIV and SIV, and may be involved in the process of assembly. The hydrophilic Tyr-262 and Lys-272 will be changed to hydrophobic amino acids Phe and Leu respectively.

Although the HIVGBG fusion construct is not identical to the natural Gag-Pol fusion protein, several lines of our evidence suggest that incorporation of HIVGBG into particles reflect the level of Gag-Pol protein assembly into virions. Both the HIVGBG and Gag-Pol are similar in their sizes and can not release into the culture media without co-expression of HIV wt Gag proteins. Immunofluorescence assays of HIVGBG and its derivative fusion constructs revealed that patterns of fusion protein localization were similar to those of HIV wt and mutant Gag proteins. Subcellular fractionation experiments also showed that HIV Gag-β-gal fusion proteins were associated with the cellular membranes. Thus, HIVGBG fusion proteins are similar to the Gag and Gag-Pol proteins in the transport route. To confirm that the results of HIVGBG apply to the Gag-Pol fusion protein, mutations within the HIVGBG construct will be introduced into a HIVgpt mutant construct which expresses the unprocessed Gag-Pol fusion protein. The HIVgpt mutant construct was generated by introducing a forced frame shifting mutation into the HIVgpt PR^- mutant. Recombinant Gag-Pol fusion constructs with mutations in the *gag* gene then can be expressed in COS7 cells in the presence or absence of wt Gag proteins. To provide HIV Gag proteins, a HIVgpt mutant with a terminator

codon at the end of the *gag* gene has been constructed. Assembly of HIV mutated Gag-Pol fusion proteins into virions will be assessed by ratios of the RT activity release in the presence of helper constructs versus in the absence of helper constructs. Immunofluorescence and subcellular fractionation assays of recombinant HIV Gag-Pol fusion proteins in COS7 cells will be performed.

Clinical Relevance

Our resuts showed that most HIV *gag* mutants, with the exceptions of Myr$^-$ and AccI mutants, still could assemble and release virus particles. Intracellular trapping of AccI mutant proteins suggested that the mutated region within the MA domain was involved in Gag transport. Although most mutants could process Gag proteins, exhibited wt retrovirus particle densities and had significant RT activities, they were noninfectious or poorly infectious. Most of the HIV MA domain could be deleted without major effects on assembly of particles and processing of Gag proteins. The fact that dl.MA virions were infectious when matched with A-MLV Env proteins suggests that an intact HIV MA domain is not essential for reverse transcription, nuclear localization, or integration. Assays of HIV Gag-β-gal fusion protein assembly into virus particles indicate that the HIV CA domain is the primary determinant for fusion protein incorporation into virions.

Since our data were obtained from analyses of HIV *gag* mutations that were introducecd into a replication-defective provirus, it is unknown whether our results apply to replication-competent viruses. HIV *gag* mutants may still assemble and release

virus particles when they are introduced into a wt HIV proviral plasmid. Because HIVgpt is pseudotyped more efficiently with A-MLV Env than with HIV Env, and because the *gag* mutations may disrupt the interactions between the Gag and HIV Env proteins, the titers of mutants will be expected to be much lower than those of their pseudotyped counterparts. The amounts of particle-assocaited Gag versus Env proteins would be analyzed by Western immunoblotting. Assembly-competent mutants which still can incorporate HIV Env proteins may be noninfectious due to interference of envelope protein stabilities and functions. Protocols for viral entry assays described above will be followed. Entry-defective mutants will be further assessed by binding and fusion assays. If necessary, the structure and topology of HIV envelope proteins on mutant virions will be studied by the methods described above.

The anti-p24*gag* has been used to screen HIV-infected persons for clinical diagnosis of AIDS and the number of $CD4^+$ T cells is the marker for follow-up during the clinical course. Our results regarding the mechanism of HIV assembly appear not relevant clinically to the diagnosis or prognosis of AIDS. Moreover, HIV Gag proteins are not exposed on the viral or cell surface and unlike Env proteins, which can serve as the target for immunization. Our results that some noninfectious HIV *gag* mutants were assembly-competent also doesn't contribute to the development of vaccines.

However, our findings that some mutants impaired in the process of virus assembly can provide information to develop treatments for AIDS. For instance, the dl.MA mutant was

noninfectious when matched with HIV envelope proteins, suggesting that the HIV MA domain can affect the functions of HIV envelope proteins. This indicates that the HIV MA can be a potential target for interfering the production of infectious virions. The regions within the MA domain that interact with the HIV Env can be defined by genetic approaches. Chemical compounds will be screened for specific interactions with the defined regions within the MA domain and tested for interfering HIV envelope protein incorporation. Similarly, the CA domain which is required for Gag assembly and Gag-Pol fusion protein incorporation also could be a potential target for inhibiting particle assembly. In addition, the evidence that mutants impaired in viral RNA packaging are noninfectious suggested that prevention of viral RNA incorporation is another strategy for blocking HIV replication.

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