Conditional Infectivity of a Human Immunodeficiency Virus Matrix Domain Deletion Mutant

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We constructed a human immunodeficiency virus (HIV) matrix (MA) deletion mutant by deletion of about 80% of the HIV type 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, the *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 were no major differences 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. 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.

The *gag* gene of all retroviruses including the human immunodeficiency virus (HIV) encodes the Gag protein, which constitutes the major internal protein of retrovirus particles. 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 (11, 17, 24, 48, 49). In HIV, Gag is translated initially as a polyprotein, Pr55. A myristic acid is attached to a glycine, the second amino acid residue, after removal of the N-terminal methionine during translation (53, 56): myristylation is essential for HIV assembly (4, 15, 38, 54). 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 (Ψ) and Gag and/or Gag-Pol proteins (1, 13, 20, 28). Assembling virions receive their envelope coats containing HIV envelope (Env) proteins when they bud out from the cell membrane (52). During or after budding, Gag precursors are processed by the viral protease (PR) into four major products, namely, p15 (MA or matrix), p24 (CA or capsid), p7 (NC or nucleocapsid), and p6 (6, 21, 27, 35). Mutations of the p6 domain do not affect assembly but may interfere with the process of budding (11, 14). The NC domain contains two Cys-His motifs which are required for packaging or stability of viral genomic RNA in virus particles (1, 2, 13). The capsid protein, p24, surrounds 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 functions 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 (19, 44, 45, 54). For instance, MA deletion in Mason-Pfizer monkey virus (44, 45) and Rous sarcoma virus (58) 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 (19). Additionally, previous studies identified HIV *gag* mutants which were impaired in Gag transport to the plasma membrane and virus release (10, 54). Besides transport, membrane binding, and particle formation, other MA functions have been postulated. Because some retroviral proteins can bind nucleic acid in vitro (8, 30, 32, 36, 46, 51), it has been proposed that viral genomic RNA may be incorporated preferentially into particles via a specific interaction with MA (8, 36, 46). Also, since recent studies have shown that p17 (MA) was localized in the nuclei of HIV-infected cells (47), 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 (52). 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 (60, 61).

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 residues for the myristylation signal and a few C-terminal residues just before the MA-CA cleavage site. Western immunoblot 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 an amphotropic murine leukemia virus (A-MuLV) envelope expression plasmid into COS7 cells: viruses in superna-

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FIG. 1. The wt HIV-1 Gag polyprotein is synthesized as a 55-kDa precursor (Pr55) which can be cleaved into a proteolytic intermediate consisting of MA plus CA (p41) and ultimately becomes cleaved into the four mature Gag domains: p17 (matrix or MA), p24 (capsid or CA), p7 (nucleocapsid or NC), and p6. wt HIV-1 (HXB2) encodes a matrix domain of 132 codons which rapidly becomes myristylated at its amino-terminal glycine residue after methionine removal. The Δ MA mutant contains a replacement of 106 codons by four codons, retaining myristylation and MA-CA cleavage signals, with a juncture sequence of 5' GAT CGA TGT CGA CGA TCT GAC 3', where the first G and last C nucleotides correspond to HXB2 nt 828 and 1151, respectively. The predicted Δ MA Gag precursor protein is approximately 42 kDa (Pr42), and a MA-CA cleavage product is predicted to have a molecular mass of 28 kD (p28). Both wt and Δ MA constructs were expressed in the HIVgpt backbone.

tants from cotransfected COS7 cells infected target cells at about 10% of wt levels. Cotransfection of this Δ 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, possibly in addition to its proposed role in the incorporation of HIV Env into particles (10, 60).

MATERIALS AND METHODS

Cell culture, transfections, and infections. COS7, HeLa, and CD4⁺ HeLa cells were maintained in Dulbecco's modified Eagle's medium 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, 15 μ g of wild-type or Δ MA gag mutant HIVgpt plasmid DNA was transfected onto COS7 cells by calcium phosphate precipitation (16). To enhance transfection efficiency, 50 μ M 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- μ m-pore-size filters and frozen at -80°C prior to processing. For infections, either 15 or 10 μ g of wt or Δ MA mutant HIVgpt plasmid DNA was cotransfected with either 10 or 5 μ g of DNA of A-MLV or HIV type 1 envelope protein expression plasmids. At 72 h after transfections, filtered supernatants were used to infect CD4⁺ HeLa cells in the presence of 4 μ g of Polybrene per ml. Selection of mycophenolic acid-resistant colonies followed the protocols described previously (54). Colonies were fixed and stained with 50% methanol plus 0.5% methylene blue. Numbers of drug-resistant colonies were converted into titers (CFU/milliliter).

Recombinant plasmids. The parent HIV wild-type plasmid in this study is HIVgpt, which derives from the HXB2 HIV provirus (41). The Δ MA mutant was derived from recombination of two previously generated HIV gag mutants (54). Construction and sequencing followed the protocols described in Maniatis et al. (31). The retained and inserted codons within the matrix domain of Δ MA mutant are shown in Fig. 1.

Plasmids HIVgpt, SV-A-MLV-env, and HIV-env were provided by D. Littman (37). All expression plasmids contained an active simian virus 40 *ori* sequence, permitting plasmid replication in transiently transfected COS7 cells (37).

Protein analysis. Intracellular and virion-associated Gag proteins were analyzed essentially as described previously (54). 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 phenylmethylsulfonyl fluoride [PMSF]) at 4°C for 45 min at $274,000 \times g$ (SW41 rotor at 40,000 rpm) or through a 3-ml cushion of 20% sucrose in TSE at 4°C 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), collected in IPB plus 0.1 mM PMSF, and then subjected to microcentrifugation at 4°C for 15 min at $13,700 \times g$ (14,000 rpm) to remove debris. Cell lysate was homogenized and saved at -80°C . Supernatant and cell samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE [26]) and then to Western immunoblotting. For immunodetection of HIV Gag proteins, a mouse anti-p24 monoclonal antibody (Epitope Inc., Beaverton, Oreg.) was diluted at 1:20,000 as a primary antibody. For immunodetection of HIV envelope proteins, the primary antibody was mouse anti-gp120 (110-1; Genetic Systems, Redmond, Wash.) at a 1:5,000 dilution, and in the case of AMLV, the primary antibody was a goat anti-gp70 antibody at a 1:5,000 dilution. Secondary antibodies were either goat anti-mouse or a swine anti-goat immunoglobulin-alkaline phosphatase conjugates. For immunofluorescence and sucrose density gradient fractionation, protocols were as described previously (19, 23, 54).

RT assays. Supernatants of transfected COS7 cells were harvested and pelleted as described above. Pellets were suspended in $1 \times$ 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 μ M dTTP, 5 μ g of oligo(dT)₁₂₋₁₈ per ml, 10 μ g of poly(A) per ml, and 10 μ Ci of [α - ^{32}P]dTTP per ml. Reactions were allowed to proceed at 37°C for 1 to 2 h. Samples were precipitated with 10% trichloroacetic acid

(TCA), washed three times, and then quantitated 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% SDS, 10 mM EDTA, and 100 μ g of yeast tRNA per ml. Aliquots were removed for protein samples, and then the suspensions were extracted once with phenol-chloroform, once with chloroform, twice with phenol-chloroform, and once with chloroform and then precipitated with ethanol. Pellets were dried and frozen at -80°C .

For isolation of total cellular RNA, cells were rinsed twice with ice-cold $1 \times$ PBS and 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°C for 15 to 18 h at $115,000 \times g$ (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 and were then subjected to ethanol precipitation. Dried pellets were suspended in 100 μ l of 10 mM Tris (pH 7.4) plus 0.1 mM EDTA and frozen at -80°C .

The template for riboprobe transcription was constructed by inserting a HIV *gag* fragment from the *dl*.MA mutant (*Sac*I-680 to the *Sal*I linker at the HIV *Cl*aI-831 site) into pBlue-scribe (Stratagene). Riboprobe transcriptions used 1 μ g of *Eco*RI-linearized template plasmid, 80 μ Ci of [α - ^{32}P]rGTP, 0.5 mM (each) the other ribonucleotides, 40 mM Tris (pH 7.9), 10 mM NaCl, 6 mM MgCl_2 , 2 mM spermidine, 40 U of RNasin (Promega), 1 mM dithiothreitol, and 10 U of T3 polymerase. Reactions were incubated at 37°C . One hour after incubation, 120 U of RNasin, 10 μ g of yeast tRNA, and 300 mM (final concentration) sodium acetate (pH 7.6) 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-base probes. Probe excised from the gel was eluted by incubation at 50°C for 30 min in 250 μ l 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 μ g of yeast tRNA and then subjected to phenol-chloroform extraction and ethanol precipitation.

For RNAse protections, cell or virus RNA pellets were resuspended in 30 μ l of solution containing 80% formamide, 0.4 M NaCl, 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), and 1×10^5 to 5×10^5 cpm of probe. Mixtures were denatured at 85°C for 5 min then incubated at 42°C overnight. Unhybridized probe was eliminated by incubation at 30°C for 30 min with 40 μ g of RNAse A per ml, 2 μ g of RNAse T_1 per ml, 300 mM NaCl, 10 mM Tris (pH 7.5), and 5 mM EDTA. Reactions were stopped by addition of 50 μ g of proteinase K plus SDS to 0.1% and then by incubation at 37°C for 15 min. Protected probes were extracted with phenol-chloroform and ethanol precipitated after addition of 20 μ g of 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 *Eco*RV and run on

a 1% low-melting-point gel. The 2.8-kb restriction fragment (HIV HXB2, nucleotide [nt] 115 to 2979) was excised and melted at 70°C . Five microliters (about 200 ng of DNA) was diluted with water and denatured at 95°C 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 μ M deoxynucleoside triphosphates (no dCTP), and 0.4 M *N*- α -hydroxyethylpiperazine-*N'*- α -ethanesulfonic acid (HEPES; pH 6.6), 20 μ g of $\text{pd}(\text{N})_6$ per ml (Pharmacia), 2 μ g of ultrapure bovine serum albumin, 30 μ Ci of [α - ^{32}P]dCTP, and 5 U of Klenow DNA polymerase in a final volume of 30 μ l. Random primer extension reactions were carried out at 37°C for 30 min, after which DNA probes were purified on Sephadex G-50 (Sigma) spin columns following the addition of 20 μ g of yeast tRNA plus 10 mM EDTA. Genomic DNA isolation, blotting, and hybridizations followed protocols described elsewhere (39, 40, 50).

RESULTS

Assembly and processing of wt 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, *Cl*aI (a *Sal*I linker insertion at nt 831 near the N terminus of MA) and *Pvu*II (a *Sal*I linker insertion at nt 1147 near the C terminus of MA) (54). As shown in Fig. 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 (37), 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 days later. Gag proteins in each sample were separated by SDS-PAGE followed by Western immunoblotting with an anti-p24 monoclonal antibody. As expected, wt Pr55 and p41 Gag precursor proteins were observed in wt-transfected COS7 cells (Fig. 2, lane A), while mature CA (p24) and precursors were present in media pellets (Fig. 2, lane C). Also as expected, no *dl*.MA mutant proteins were detectable with an anti-matrix antibody (data not shown). However, the *dl*.MA mutant released virus Gag proteins that were detectable with the anti-p24 antibody (Fig. 2). Within cells, small amounts of predicted *dl*.MA Gag precursors Pr42 (corresponding to wt Pr55 with the MA deletion) and p28 (corresponding to wt p41 with the MA deletion) were observed (Fig. 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 (Fig. 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 two- to fourfold differences were not investigated. These results indicated that the MA domain deletion does not block Gag protein release or processing in the COS7 cell overexpression system.

Characterization of virus particles. Our previous studies indicated that pelleted culture media Gag proteins isolated as in Fig. 2 represented virus particles (18, 54). To confirm that released mutant Gag proteins Pr42, p28, and p24 were particle associated, viral proteins in the media supernatants were analyzed by sucrose density gradient fractionation and Western immunoblotting as described in Materials and Methods. As shown in the bottom panel of Fig. 3, wt particles banded in

FIG. 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 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 and then by a secondary alkaline phosphatase-conjugated goat anti-mouse antibody at a 1:1,500 dilution, and then of alkaline phosphatase activity was measured. The molecular mass 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.

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.

On the basis of 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 RT activities, particles from four separate wt or mutant samples were assayed by using exogenous templates as described in Materials and Methods. In each case, the counts per minute of incorporated nucleotides into the exogenous template were at least 14-fold higher than background levels (Table 1). To determine virion-associated RT activities, normalized counts per minute values were divided by the densitometer-determined virus-associated Gag protein levels. As shown in Table 1, 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 deleted portion of 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, 9, 12, 13, 22, 33, 34), 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

FIG. 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 (54). Fractions were collected from top to bottom, and Gag proteins were analyzed by immunoblotting as described in Fig. 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. Top, *dl*.MA; bottom, wt.

described in Materials and Methods. Our probe was a 183-base antisense riboprobe which crosses the HIV-1 major splice donor (Fig. 4). As shown, the probe is expected to yield 63- to 64-base fragments when protected by spliced HIV transcripts, or 150- or 156-base fragments when protected by wt or *dl*.MA genomic transcripts, respectively. Using yeast tRNA (Fig. 4B, lanes B and H) or RNA samples from mock-transfected cells

TABLE 1. RT activities of *dl*.MA and wt HIV particles^a

Construct	Total cpm	Total cpm – background cpm	Gag antigen	Total cpm – background cpm/Gag antigen
Mock	1,095			
Mock	1,265			
wt	18,830	17,650	1.55	11,387
wt	18,890	17,710	1.22	14,516
wt	44,595	43,415	2.95	14,717
wt	23,585	22,405	1.06	21,136
<i>dl</i> .MA	19,005	17,825	1.05	16,976
<i>dl</i> .MA	19,795	18,615	1.24	15,012
<i>dl</i> .MA	28,450	27,270	1.27	21,472
<i>dl</i> .MA	17,965	16,785	0.62	27,072

^a COS7 cells were transfected with either 10 or 15 µg of wt or the deletion MA (*dl*.MA) HIVgpt constructs. Three days posttransfection, media supernatants (10 ml) were filtered and virions were pelleted through a 20% sucrose cushion as described in Materials and Methods. Pellets were resuspended in 100 µl of PBS, from which 20 µl was used for RT assays and 50 µl was used for immunodetection of Gag antigen by Western blotting. Total TCA-precipitable product after a 1-h reaction is shown in the TCA column. Background counts per minute for subtraction were from the averaged mock values. Total Gag antigen was determined by densitometry of immunoblot filters, and values listed are arbitrary densitometer units.

FIG. 4. RNA incorporation into virus particles. HIV RNA from cellular and viral RNA isolations was quantified by RNase protection. (A) The RNase protection antisense probe is 183 bases and contains 5' vector sequences plus HIV (HXB2) proviral sequences corresponding to nt 831 to 680, crossing the HIV-1 major splice donor. Either wt or *dl*.MA spliced RNAs should protect probe fragments of 63 to 64 bases, while wt or *dl*.MA genomic viral RNAs are predicted to protect bands of 150 and 156 bases, respectively. The difference in wt and *dl*.MA genomic protected fragments is because the *Sal*I linker sequence near the 5' end of the riboprobe matches the *Sal*I sequence (GTCGAC) at the deletion junction of the *dl*.MA mutant (Fig. 1). (B) Equivalent amounts of matched total cellular (lanes C, D, I, and J) and media (lanes E, F, K, and L) RNAs from mock (lanes I and K), wt (lanes D and F), *dl*.MA (lanes C and E), and NC RNA packaging mutant A14-15 (lanes J and 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 is shown 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 control lanes B, H, I, and K) 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.

(lanes I and K) a background band of approximately 146 bases 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 bases and spliced transcripts smears at 60 to 70 bases.

Not surprisingly, only a very weak genomic RNA signal appeared associated with the A14-15 NC mutant particles (Fig. 4B, 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, counts per minute 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 unit 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 (54), 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 A-MLV envelope protein expression plasmid, A-MLV-env (37). Infection of HeLa or CD4⁺ HeLa cells and selection of drug-resistant colonies followed the procedures described in Materials and

FIG. 5. Infectivity of pseudotyped wt or *dl.MA* virions. COS7 cells were cotransfected with 10 μ g of wt or *dl.MA* HIVgpt plasmids plus 5 μ g of either 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 Materials and Methods. About 2 weeks later, drug-resistant colonies were fixed and stained with 50% methanol plus 0.5% methylene blue. (A and D) Mocks; (B) *dl.MA* pseudotyped with A-MLV Env; (C) wt pseudotyped with A-MLV Env; (E) *dl.MA* pseudotyped with HIV Env; (F) wt pseudotyped with HIV Env.

Methods. Surprisingly, the *dl.MA* mutant displayed a significant infectivity. As shown in Fig. 5, the number of drug-resistant colonies of the mutant (plate B) was about 1/10 of the wt number (plate C) in a parallel experiment. To corroborate results in Fig. 5A to C, parallel infections of CD4⁺ HeLa cells with A-MLV-env pseudotyped mutant and wt HIVgpt viruses were repeated eight 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 (Fig. 6). The DNA probe for hybridization derived from an *EcoRV* restriction fragment which covered the *dl.MA* deletion (Fig. 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 and 553 bp in the 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 Fig. 5, no drug-resistant colonies were observed on either mock-infected (Fig. 5D) or mutant-infected (Fig. 5E) plates, while approximately 1,000 CD4⁺ HeLa cell colonies were observed on the wt-infected plate (Fig. 5F). 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.

TABLE 2. Infectivity of the HIV *dl.MA* mutant pseudotyped with HIV or A-MLV envelope proteins^a

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

^a COS7 cells on 10-cm plates were transfected with 15 μ g of wt or *dl.MA* HIVgpt plasmids plus 10 μ g of either an A-MLV or an 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 Materials and Methods. Titers indicate CD4⁺ HeLa mycophenolic acid-resistant colonies per milliliter, and each *dl.MA* transfection was performed in parallel with a wt HIVgpt transfection from which percent wt titer values derive. Each percent wt value derives from a separate transfection experiment.

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 Materials and Methods, and HIV envelope proteins were immunodetected with a mouse anti-gp120 antibody. As shown in Fig. 7, HIV envelope proteins gp160 and gp120 were observed in wt (lanes B, E, and G) and mutant (lanes C, F, and H) virus samples in three separate experiments. That these proteins 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 and D). While both gp160 and gp120 were associated with wt and *dl.MA* particles, reproducible quantitative differences were apparent; by densitometric quantitation of Western blots, wt particles contained 1.8 ± 0.4 -fold less gp160 and 1.9 ± 0.3 -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 and H) or anti-p24 (lanes I and 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 higher and gp120 was lower than in the wt sample (lanes G and H). In contrast, the incorporation of

FIG. 6. Southern blot analysis of wt- and *dl.MA*-infected cells. COS7 cells were transfected with 10 μ g of wt or *dl.MA* HIVgpt plasmids plus 5 μ g of an 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 Dulbecco's modified Eagle's medium 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 and B) or *HindIII* (lanes C and D), electrophoresed, blotted, and hybridized with a probe from the HXB2 *EcoRV* nt 115 to 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 1,475-bp 3' *HindIII* long terminal repeat fragment (HXB2, nt 8141 to 9616) appears faintly in lanes C and D on the original autoradiograph.

envelope proteins into wt and mutant virions was roughly equal when both were pseudotyped with A-MLV envelope proteins. Comparable amounts of A-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 and N). Our observations implicate an interaction between HIV MA and Env (discussed below) and suggest that A-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 (37).

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 (3, 7, 19, 44, 45, 47, 54, 57, 59). For most mammalian retroviruses, myristylation of MA appears essential for efficient membrane association (4, 42, 43, 55) which, except for type D retrovirus (43), appears to be a prerequisite to assembly (4, 15, 38, 42, 54, 55). However, a number of mutations into the middle region of the HIV *gag* matrix domain have no apparent effect on virus assembly (54, 60). 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 (60).

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 (Fig. 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 was processed appropriately (Fig. 2). It is noteworthy that more subtle mutations in the deleted domain have been demonstrated to impair assembly (10, 54, 60). One of these mutations removed 84 of the 106 residues removed in our mutant and resulted in a 10-fold reduction of virus particle release, as well as intracellular assembly of particles at the endoplasmic reticulum (10). These different results could be explained if smaller 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. Additionally, it is possible that the four foreign residues (CRRS) in the *dl.MA* mutant could have some effect on virus release. It also 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 overexpression system used here and in other matrix deletion studies (10, 60).

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; Fig. 2 to 4). Most surprisingly, when pseudotyped with an A-MLV Env protein, the *dl.MA* particles were 9.7% as infectious as wt particles (Fig. 5; Table 2). This result indicates that an intact MA is not absolutely required for early replication events such as uncoating or reverse transcription in dividing HeLa or CD4⁺ HeLa cells. It also suggests that in target HeLa and HeLa CD4⁺ cells, an intact MA is not essential for nuclear localization or integration events, although we have not directly demonstrated that the expressed proviruses were integrated or nuclear localized. However, given other results (5, 29), it is entirely possible that MA may affect early processes in other dividing or nondividing cell types. In particular, evidence suggests that, unlike most retroviruses, HIV can infect nondividing cell types (29) and that a putative nuclear localization signal in MA may be needed for directing integration complexes to nuclei of such cells (5). Thus, it would be of interest

FIG. 7. Envelope protein incorporation into wt and mutant HIV particles. Envelope protein expression plasmids A-MLV-env or HIV-env were transfected into COS7 cells alone or with wt or *dl.MA* HIVgpt constructs. Proteins from virus pellets were separated by SDS-PAGE, electroblotted, and detected with antibodies to HIV Env (lanes A to H), A-MLV Env (lanes K and L), or HIV Gag (CA; lanes I, J, M, and N). Lanes: A and D, from samples transfected with the HIV-env plasmid alone; B, E, and G, derived from samples transfected with HIV-env and wt HIVgpt; C, F, and H, derived from samples transfected with HIV-env and *dl.MA* HIVgpt. Samples I and J were from virus pellets identical to G and H but were blotted with anti-Gag rather than anti-Env for Env and Gag quantitation purposes. Control lanes K and L were from samples transfected with A-MLV-env plus either wt HIVgpt (lane K) or *dl.MA* HIVgpt (lane L) and were detected with antibodies to A-MLV Env. Samples M and N were from virus pellets identical to those in K and L but were blotted with anti-Gag antibodies. 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 immunodetection of HIV Gag proteins, a mouse anti-p24 monoclonal antibody at a 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 a 1:5,000 dilution, and for the A-MLV envelope proteins, the primary antibody was a goat anti-gp70 antibody at a 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.

to determine whether the *dl.MA* mutant can infect nondividing cells.

In contrast to results with A-MLV-Env pseudotypes, the *dl.MA* virus appeared much less infectious when matched with the HIV Env protein (Fig. 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 interact specifically with MA. Thus, our data support previous genetic implications of a HIV Env-matrix interaction (60, 61). Further support for this notion derives from Fig. 7. As illustrated and expected, particle-associated A-MLV Env protein levels were similar for wt and *dl.MA* virions (Fig. 7). In contrast, in three separate experiments, HIV Env protein levels in wt and mutant viruses differed: gp160 levels were twofold higher in *dl.MA* particle preparations, while gp120 levels were twofold lower in mutant particles (Fig. 7). Obviously, the 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 postassembly functions or if receptor binding and/or fusion events are not related linearly to viral gp120 levels. Our data also suggest that gp160 is incorporated into wt and *dl.MA* particles, although this interpretation must be tempered by the fact that observation of non-virus-associated media gp160 has been reported (23). It is noteworthy that smaller deletions of the HIV MA domain have been shown to reduce gp120 levels on virus particles over 10-fold, and potential virus-associated gp160 levels were difficult to assess (10, 60). The quantitative differences in gp120 levels on our *dl.MA* mutant versus those of others (10, 60) could arise if the MA domains of smaller deletion mutants interfere with Env protein packaging. Alter-

natively, the HIV matrix domain could have an effect on the stability of gp120 in virus particles or, seemingly less likely, on the cellular processing of gp160 to gp120 plus gp41. In the former case, gp120 packaged into particles may fall off into the medium in a process dependent on time and/or the specific nature of the matrix mutation.

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