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Incorporation of Human Immunodeficiency Virus Type 1 Gag Proteins into Murine Leukemia Virus Virions

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The retroviral Gag polyprotein is necessary and sufficient for assembly and budding of viral particles. However, the exact inter- and intramolecular interactions of the Gag polyproteins during this process are not known. To locate functional domains within Gag, we generated chimeric proviruses between human immunodeficiency virus type 1 (HIV-1) and murine leukemia virus (MuLV). In these chimeric proviruses, the matrix or capsid proteins of MuLV were precisely replaced with the matrix or capsid proteins of HIV-1. Although the chimeric proviruses were unable to efficiently assemble into mature viral particles by themselves, coexpression of wild-type MuLV Gag rescued the HIV proteins into virions. The specificity of the rescue of HIV proteins into MuLV virions shows that specific interactions involving homologous matrix or capsid regions of Gag are necessary for retroviral particle formation.

The retroviral Gag polyprotein contains sufficient information to direct assembly of retroviral particles (for reviews, see references 8 and 38). After budding from infected cells, cleavage of the Gag polyprotein by the virally encoded protease releases the mature proteins matrix (MA), capsid (CA), and nucleocapsid (NC). The murine leukemia virus (MuLV) *gag* gene encodes an additional small protein, found between MA and CA, called p12 (or X) which has no known function (18). The human immunodeficiency virus type 1 (HIV-1) Gag polyprotein also contains a fourth protein, p6, found at the carboxy terminus, which may play a role in virus release from infected cells (10). The addition of myristic acid at the amino terminus of Gag is required for MuLV and HIV-1 virion assembly (3, 30).

Previous studies have shown that deletions or mutations in the MA or CA protein of several retroviruses can affect assembly of particles (11–13, 15, 16, 27, 28, 32, 35–37). The only sequence homology in MA or CA among distantly related retroviruses is a 20-amino-acid region in CA called the major homology region, in which there are six invariant amino acids (34, 37). Regardless, there likely exists functionally homologous domains within the retroviral Gag protein critical for particle assembly.

We wished to define the differences between roles general to divergent retroviruses that MA and CA play in particle morphogenesis and infectivity and roles of MA and CA that are specific to HIV. Therefore, we have taken the approach of creating chimeric proviruses that contain domains of both HIV-1 and MuLV in the Gag polyprotein. We developed a polymerase chain reaction (PCR)-based technique that allowed us to precisely replace the MA and/or CA proteins of MuLV with the MA and/or CA proteins of HIV-1 in an MuLV-based complete provirus.

We find that replacement of the MuLV MA or CA with the HIV-1 MA or CA did not inhibit viral RNA or chimeric protein synthesis. However, these proviruses are unable to efficiently produce viral particles. On the other hand, introduction of wild-type MuLV could rescue HIV-1 Gag proteins into mature particles. The rescue of HIV-1 Gag proteins into MuLV virions was specific and was dependent on the presence of MuLV MA or CA in the Gag polyprotein. These results suggest that MA and CA mediate Gag protein interactions in *trans* during particle assembly.

MATERIALS AND METHODS

Nomenclature. Chimeric proviruses are named as follows. MuLV(CA_{HIV}) indicates that the CA protein of an MuLV provirus has been replaced by the CA protein of HIV-1. The rest of the provirus is MuLV. Cell lines containing these chimeric proviruses are named with the cell type followed by the name of the chimeric provirus [for example, 3T3-MuLV (CA_{HIV}) are NIH 3T3 cells containing the MuLV(CA_{HIV}) provirus].

All MuLV sequences are from the pAM clone (22) which contains the amphotropic 4070A envelope and Moloney Gag-Pol and long terminal repeat sequences. All HIV sequences are derived from pLAI (25), a molecular clone of the LAI strain of HIV-1.

Cells, transfections, and infections. NIH 3T3tk cells (called 3T3 here) and the canine osteosarcoma cell line, D17, were grown in Dulbecco modified Eagle medium (DMEM) with 10% calf serum. All transfections were done by calcium phosphate coprecipitation (4). Approximately 10^5 cells were transfected with 5 μg of each chimeric provirus plus 0.5 μg of a selectable marker plasmid that encoded either HSV-1 thymidine kinase (FepTK, a gift of Elliot Epner) or G418 resistance (RSVneo, a gift of Maxine Linial). After incubation overnight at 35°C and in 3% CO_2, the cells were washed twice with phosphate-buffered saline (PBS) and refed with DMEM–10% calf serum containing hypoxanthine-aminopterin-thymidine or 0.4 μg of G418 per ml. Cell clones were isolated approximately 1 week after transfection.

Superinfection of the chimeric-expressing 3T3 clones or D17 cells with wild-type MuLV or spleen necrosis virus (SNV) was carried out in the presence of 10 μg of Polybrene per ml for 2 h.

Construction of chimeric proviruses. Chimeric proviruses were made by a PCR strategy to be described in more detail elsewhere. An example of the strategy is described here for one of the chimeric proviruses (Fig. 1). Primer sequences and

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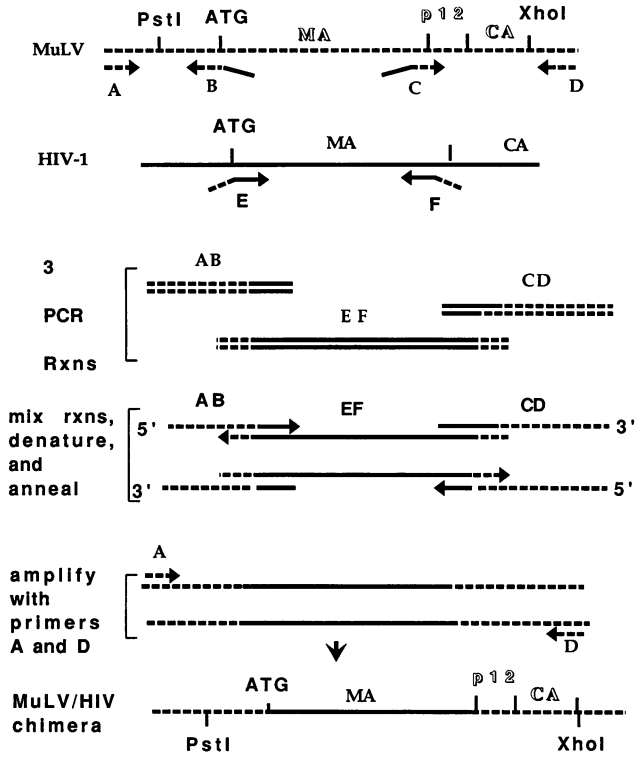

FIG. 1. PCR technique to create an HIV-MuLV chimeric provirus. Shown here is an exchange of MA of MuLV with the MA of HIV-1. The dashed lines represent MuLV sequences, while the solid lines represent HIV sequences. The outlined protein names MA, p12, and CA are MuLV proteins, and the solid protein names MA and CA are HIV-1 proteins. The small arrows labelled A, B, C, D, E, and F represent oligonucleotides. The bent arrows indicate that the oligonucleotide is homologous to MuLV in one half and homologous to HIV in the other half. AB, CD, and EF represent PCR-amplified fragments. Details of the protocol are described in Materials and Methods.

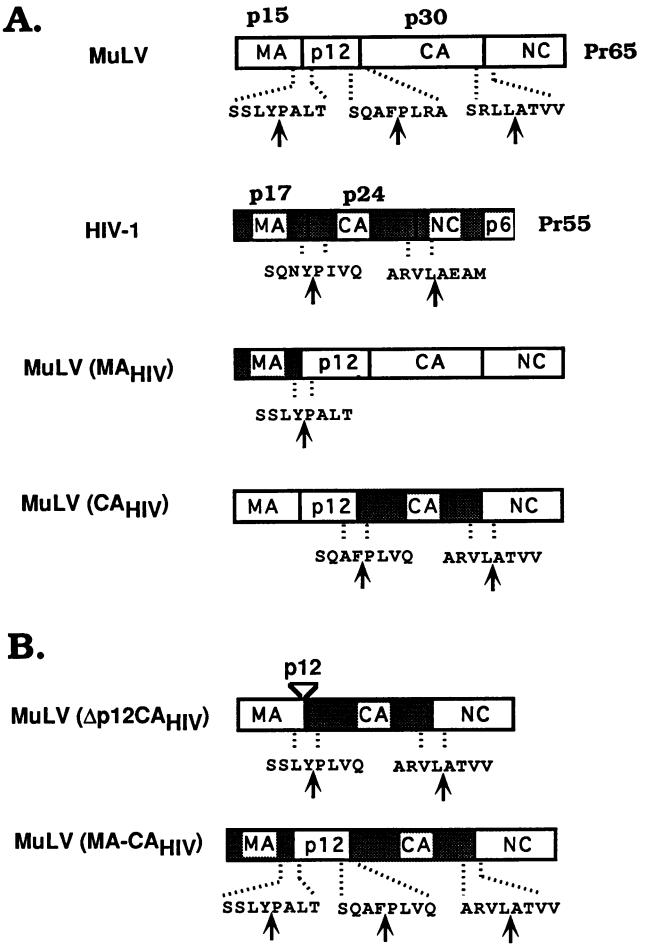

FIG. 2. MuLV, HIV-1, and chimeric MuLV–HIV-1 Gag polyproteins. (A) The Gag precursor polyprotein of MuLV, Pr65, consists of MA (p15), p12, CA (p30), and NC. The Gag precursor polyprotein of HIV-1, Pr55, consists of MA (p17), CA (p24), NC, and p6. MuLV(MA_{HIV}) contains the MA of HIV-1 precisely substituted for the MA of MuLV. MuLV(CA_{HIV}) contains the CA of HIV-1 precisely substituted for the MA of MuLV. The remaining proviral sequences (not shown) of MuLV(MA_{HIV}) and MuLV(CA_{HIV}), including the long terminal repeats, are that of MuLV. The four amino acids on each side of the cleavage sites of HIV-1 and MuLV are shown. An arrow denotes the actual cleavage site. The four amino acids on each side of the proposed cleavage sites of MuLV(MA_{HIV}) and MuLV(CA_{HIV}) are also shown. (B) MuLV(MA-CA_{HIV}) and MuLV(Δp12CA_{HIV}) chimeric proviruses. In MuLV(MA-CA_{HIV}), the MA and CA proteins of HIV-1 replace the MA-p12-CA proteins of MuLV. The cleavage site between HIV-1 MA and CA is the normal HIV-1 cleavage site. MuLV (Δp12CA_{HIV}) was created from MuLV(CA_{HIV}) by precisely deleting the p12 sequences with the oligonucleotides. The proposed cleavage site between MuLV MA and HIV-1 CA is also shown.

details of the PCR method for each of the chimeric proviruses (Fig. 2) are available from the authors. PCR primers were designed such that the 3′ ends were complementary to MuLV and the 5′ ends were complementary to HIV (or vice versa). In the exchange illustrated in Fig. 1, the target DNA is the MuLV genome, and the insert DNA is the HIV genome. In this case, we have precisely replaced the MA-encoding region of MuLV with the MA-encoding region of HIV-1.

Oligonucleotides A and D are completely homologous to MuLV DNA and are just 5′ and 3′ to the restriction sites *Pst*I and *Xho*I, respectively. Oligonucleotides B and E are complementary to one another, as are oligonucleotides C and F. B has 13 nucleotides (nt) homologous to MuLV at the 3′ end and 12 nt homologous to HIV at the 5′ end (there is overlap at the ATG). C, E, and F also contain 11 to 14 nt homologous to one DNA (target or insert) and then 11 to 14 nt homologous to the other DNA. The nucleotide at which the homology ends in each oligonucleotide is the point at which the junction between the target and the insert will occur. The sequences of the oligonucleotides are as follows (MuLV sequences are in uppercase letters, and HIV-1 sequences are in lowercase letters): A, 5′-CGCTTCTGTTCGCGCGCTTC-3′; D, 5′-GTGGTGT AATCCCAGTCTGG-3′; B, 5′-CTCTCGCACCcatattctcag-3′; C, 5′-cagccaggtcagcTCCCTTTATCC-3′; E, 5′-ctgagaatatg GGTGCGAGAG-3′; and F, 5′-GGATAAAGGGAgctgacct ggct-3′.

Three separate PCRs were set up with the following oligonucleotides and DNA: (i) oligonucleotides A and B and MuLV DNA, (ii) oligonucleotides C and D and MuLV DNA, and (iii) oligonucleotides E and F and HIV DNA. The PCR products were then run out on an agarose gel, and the band corresponding to the amplified fragment was purified from the gel (Fig. 1, products AB, EF, and CD). A second round of PCR containing

the products AB, EF, and CD in the same tube was then set up. Because primers B and E are complementary to each other, fragments AB and EF have 24 bp of homology to one another at one end of the DNA molecules (likewise for CD and EF). Therefore, when a second round of PCR was set up, some small percentage of AB was hybridized to EF, and some small percentage of CD was hybridized to EF. Although these hybridizations at one end are rare, they were amplified when they did occur because the second round of PCR contains only primers A and D.

After the second round of PCR, the product was purified and cut with restriction enzymes that are between primers A and D (*Pst*I and *Xho*I in the example shown in Fig. 1). This restriction fragment was then cloned back into the MuLV complete genome. We then sequenced the entire insert to select clones in which the PCR has not introduced additional mutations. About 50% of the clones contain no mutations that change amino acids in the amplified fragments.

The PCR mixtures contain 1 ng of restriction enzyme-cut DNA, 1 μM each oligonucleotide, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM deoxynucleoside triphosphates, 20 mM Tris (pH 8.4), 10 μg of gelatin per ml, and 1.0 U of *Taq* polymerase. The PCR machine is programmed for 1 cycle at 93°C for 4 min, 5 cycles at 93°C for 1 min, 42°C for 1 min, 75°C for 30 s or longer (30 s for each 400 bp to be amplified), 25 cycles at 93°C for 1 min, 60°C for 1 min, and 75°C for 30 s or longer (30 s for each 400 bp to be amplified).

Analysis of viral proteins. For isolation of viral cytoplasmic proteins, cells were harvested by being scraped into cold PBS (0.15 M NaCl, 7 mM NaCl, 3 mM KCl), washed twice in the same buffer, and then resuspended in 100 μl of reticulocyte suspension buffer (0.01 M Tris-HCl [pH 7.4], 0.01 M NaCl, 5 mM $MgCl_2$, 0.25% Nonidet P-40) per 2×10^6 cells. The nuclei were pelleted by centrifugation at $1,000 \times g$ for 5 min. An equal volume of 2× loading dye (0.1 M Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol, 0.2 M dithiothreitol) was added to the supernatant. After being boiled for 10 min, 100 μl was run on an SDS–15% polyacrylamide gel.

For isolation of secreted viral proteins, 30 ml of cell culture media was collected, and cell debris was pelleted by centrifugation at $3,000 \times g$ for 20 min. The supernatant was spun through 3 ml of a 20% (wt/vol) sucrose cushion made in standard buffer (0.1 M Tris-HCl [pH 8], 0.1 M NaCl, 1 mM EDTA) at 23,000 rpm for 2 h in an SW28 rotor. The viral pellets were resuspended in 100 μl of reticulocyte suspension buffer, and an equal volume of 2× loading dye was added. After being boiled for 10 min, 100 μl was run on an SDS–15% polyacrylamide gel.

Following electrophoresis, the proteins were transferred to nitrocellulose by semidry transfer (Hoeffer). After incubation overnight at 4°C in blocking reagent (5% nonfat dry milk and 0.5% Tween 20 in PBS), the membrane was incubated in primary antibody (anti-HIV sera diluted at 1:250 in blocking reagent or anti-MuLV sera diluted at 1:1,000) for 1.5 h at room temperature and then incubated in horseradish peroxidase-conjugated anti-human or anti-goat immunoglobulin G (Cappell) diluted at 1:5,000. The horseradish peroxidase-conjugated antibodies were detected by using the enhanced chemiluminescence kit by Amersham according to the manufacturer's instructions. HIV antiserum is pooled serum from HIV-infected humans. Goat serum reactive with MuLV virion proteins was obtained from Quality Biotech Inc.

Sucrose density gradient analysis. Thirty milliliters of culture supernatant was harvested and concentrated by centrifugation through a 20% sucrose cushion (see above). The pelleted material was then resuspended in 100 μl of standard buffer and loaded onto a premade 4-ml 20 to 60% linear sucrose gradient. After centrifugation at 32,000 rpm for 2 h 15 min in an SW55 Ti rotor, 20 equal fractions of 200 μl were collected, starting at the bottom of the tube. The density of each fraction was determined with a refractometer.

ELISA and RT assays. The amount of $p24^{gag}$ in viral pellets and cell-free media was determined by enzyme-linked immunosorbent assay (ELISA; Coulter Immunology) by using the instructions and standards supplied by the manufacturer. Samples were diluted in DMEM with 10% calf serum before analysis. Reverse transcriptase (RT) assays were done by using 10 μl of cell-free media and 50 μl of cocktail (50 mM Tris-HCl [pH 8], 0.6 mM $MnCl_2$, 4 mM dithiothreitol, 60 mM NaCl, 0.05% Nonidet P-40, 5 μg of poly(A) per ml, 1.5 μg of oligo(dT) per ml, 10 μCi of [^{32}P]dTTP). After a 2-h incubation at 37°C, 50 μl of each sample was spotted onto DE81 paper, washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and rinsed in 95% ethanol. The dried blot was exposed to film overnight at −80°C.

RNA extraction and Northern blotting. Total RNA was isolated from the 3T3 cells as previously described (5). For Northern (RNA) blot analysis, 10 μg of RNA was loaded onto a 1% agarose-formaldehyde gel and blotted to nylon filters by standard procedures (29). The blots were hybridized with DNA probes labelled with [^{32}P]dCTP by random priming.

RESULTS

HIV-MuLV chimeric Gag proteins can be expressed in 3T3 cells. Both MuLV and HIV are type C retroviruses that bud at the plasma membrane. In order to define regions of the Gag polyprotein required for particle assembly, we used a novel PCR-based method to construct chimeric retroviruses in which the MA or CA of MuLV was precisely replaced by the corresponding coding regions of HIV (Fig. 2A). The PCR primers were designed so that in most cases the four amino acids on each side of the cleavage site between Gag proteins remained identical to the normal MuLV cleavage site, and in other cases conservative changes were made near the cleavage site (Fig. 2A). These chimeric *gag* genes were engineered back into an amphotropic MuLV provirus. The provirus in which the MA of MuLV has been replaced by the MA of HIV-1 (LAI strain) is called MuLV(MA_{HIV}), and the provirus in which the CA of MuLV has been replaced by the CA of HIV-1 is called MuLV(CA_{HIV}) (Fig. 2A).

To determine the effects of swapping these regions of Gag on virus production, MuLV(MA_{HIV}) and MuLV(CA_{HIV}) were each cotransfected into 3T3 cells with a selectable marker, and stable clones were established. Northern blot analysis was used to characterize the proviral RNA transcribed from the chimeric proviruses (Fig. 3). Full-length genomic RNA and spliced *env* RNA transcribed by clones transfected with MuLV (MA_{HIV}) and MuLV(CA_{HIV}) comigrate with viral RNA bands from amphotropic MuLV-infected 3T3 cells (Fig. 3). The RNA blot was stripped and reprobed with an HIV *gag*-specific probe. As expected, this probe recognizes only the unspliced genomic RNA and not the spliced *env* RNA.

We next asked whether the chimeric RNAs were translated into viral proteins of the expected sizes. The MuLV Gag precursor protein is approximately 65 kDa. Since in MuLV (MA_{HIV}) the matrix gene of MuLV ($p15^{gag}$) has been replaced with the HIV matrix gene ($p17^{gag}$), we expected the precursor Gag protein to be about 67 kDa. In the MuLV(CA_{HIV}) provirus, the MuLV capsid gene ($p30^{gag}$) has been replaced by the HIV gene ($p24^{gag}$), so we expected to find a precursor protein of about 59 kDa. Cytoplasmic proteins were harvested

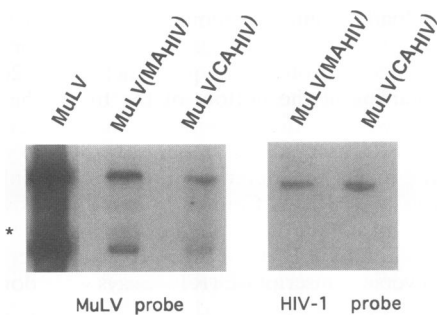

FIG. 3. Northern blot analysis of MuLV(MA$_{HIV}$) and MuLV (CA$_{HIV}$) RNAs. Total RNA was isolated from both chimeric-expressing 3T3 clones and also MuLV-infected 3T3 cells. The RNA blot was probed with a *Bam*HI-*Xho*I fragment from the MuLV *env* gene (left side) or a PCR-generated 500-bp DNA fragment from the HIV Gag MA-CA region (right side). The position of the 28S rRNA is marked (*).

from both of the chimeric virus-expressing 3T3 clones for examination by Western blotting (immunoblotting). After probing the protein blot with human anti-HIV sera, we found chimeric Gag precursors of the expected sizes (Fig. 4A). Therefore, although it had been reported that regions in the HIV *gag* gene suppress expression of heterologous RNAs (6, 21, 31, 33), HIV matrix and capsid proteins can be expressed in the context of a chimeric HIV-MuLV provirus as part of the Gag polyprotein.

Chimeric proviruses secrete only low levels of viral proteins but can be rescued by helper virus. We next asked whether the chimeric Gag proteins are capable of directing assembly of mature virions. If so, we expected to find processed Gag proteins in the cell culture media. Viral particles were harvested from the media of 3T3-transfected MuLV(MA$_{HIV}$) and 3T3-MuLV(CA$_{HIV}$) clones and centrifuged through a 20% sucrose cushion. The pelleted material was subjected to Western blotting, and the protein blot was probed with anti-HIV serum. Although the cell supernatant was weakly positive for RT activity (data not shown), we could not detect HIV-specific proteins from the MuLV(MA$_{HIV}$) viral pellet (Fig. 4B, lane 2). In the MuLV(CA$_{HIV}$) viral pellet, we did detect a partially processed Gag protein of about 45 kDa (Fig. 4B, lane 4). Therefore, the MuLV(CA$_{HIV}$) clone secretes some Gag protein, but it is apparently unable to be fully processed.

A subset of mutations in the Gag protein of Rous sarcoma virus and MuLV that affect particle assembly can be rescued into virions by wild-type Gag protein (11, 32, 37). Therefore, we asked whether MuLV could complement the assembly defects in our two chimeric Gag proteins and allow them to be incorporated into mature virus particles. The clones were infected with ecotropic MuLV virus at a multiplicity of 1 infectious unit per cell, and RT activity in the cell supernatant was measured 3 days after infection. Since the level of RT activity in the MuLV(MA$_{HIV}$) supernatant is very low and undetectable in the MuLV(CA$_{HIV}$) media, a dramatic increase in RT activity indicated efficient superinfection with MuLV (data not shown).

One week after superinfection, viral proteins were harvested from the MuLV(MA$_{HIV}$) cell supernatants by centrifugation and were used in Western blotting. After the protein blot was probed with anti-HIV serum, a protein of 17 kDa that comigrates with the matrix protein found in the HIV viral pellet is readily detected (Fig. 4B, lanes 1 and 3). This dramatic increase in p17^{gag} in the viral pellet after infection of MuLV

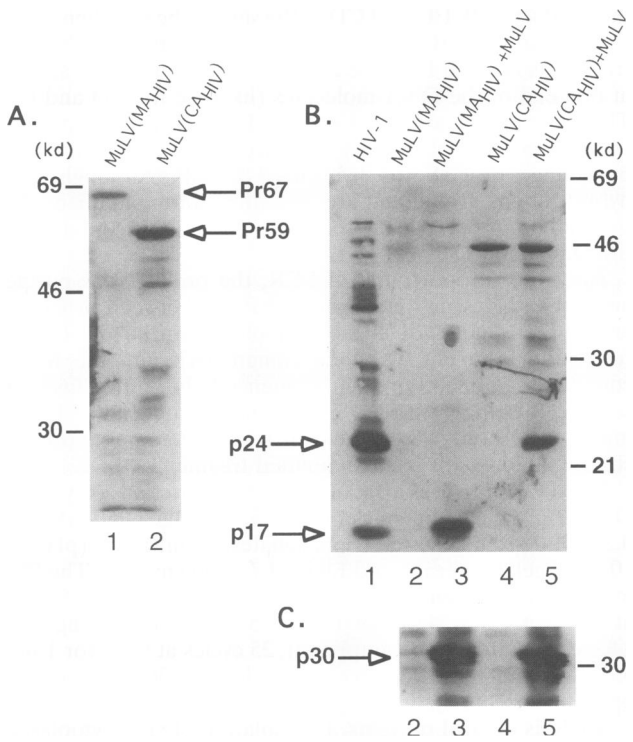

FIG. 4. Western blot analysis of MuLV(MA$_{HIV}$) and MuLV (CA$_{HIV}$) intracellular and secreted proteins. (A) Cytoplasmic proteins were harvested from MuLV(MA$_{HIV}$) (lane 1)- and MuLV(CA$_{HIV}$) (lane 2)-expressing 3T3 clones, and the protein blot was probed with human anti-HIV sera. (B) Secreted proteins were harvested from cell culture supernatants by centrifugation through a 20% sucrose cushion. The protein blot was probed with human anti-HIV sera. Lanes: 1, viral proteins harvested from HIV-infected H9 cells; 2, 3T3-MuLV (MA$_{HIV}$); 3, MuLV-superinfected 3T3-MuLV(MA$_{HIV}$); 4, 3T3-MuLV (CA$_{HIV}$); 5, MuLV-superinfected 3T3-MuLV(CA$_{HIV}$). (C) The protein blot in panel B was stripped and reprobed with anti-MuLV serum. The positions of the viral proteins and molecular mass markers are noted.

(MA$_{HIV}$) with MuLV demonstrates that wild-type virus can rescue MuLV(MA$_{HIV}$) and allow it to efficiently assemble into mature, fully processed viral particles.

Viral proteins were also harvested from the superinfected 3T3-MuLV(CA$_{HIV}$) cells for Western blotting. In the presence of the wild-type Gag proteins, we could now readily detect processed HIV capsid protein (p24^{gag}) in the particulate fraction (Fig. 4B, lane 5). This protein comigrates with the capsid protein (p24^{gag}) found in the HIV-1 virus lane (Fig. 4B, lane 1). The protein blot was stripped and reprobed with anti-MuLV sera (Fig. 4C). High levels of p30^{gag} were detected in lanes 3 and 5, showing that large amounts of wild-type MuLV virus are released from the superinfected cells.

Quantitation of p24^{gag} from the 3T3-MuLV(CA$_{HIV}$) and the MuLV-infected 3T3-MuLV(CA$_{HIV}$) viral pellets was done by ELISA. The results show a 100-fold increase in the amount of HIV-1 p24^{gag}, or MuLV(CA$_{HIV}$) Gag, released from the superinfected cells (Table 1). We conclude from these results that production of both the MuLV(MA$_{HIV}$) and MuLV(CA$_{HIV}$) chimeric viruses is efficiently rescued by wild-type MuLV Gag.

MuLV(CA$_{HIV}$) proteins are released in low-density particles but are found in dense particles after rescue by wild-type uLV. Properly assembled HIV and MuLV viral particles band in a

TABLE 1. Levels of $p24^{gag}$ released from chimeric provirus-expressing cells[a]

Cell line	Provirus	$p24^{gag}$ (pg/ml) induced by indicated superinfecting virus[b]			Fold increase
		No virus	MuLV	SNV	
3T3		ND[c]	2.5	ND	ND
3T3	MuLV(CA_{HIV})	56	5,572	ND	100
3T3	MuLV($\Delta p12CA_{HIV}$)	3.5	91	ND	26
D17	MuLV(CA_{HIV})	77	1,832	ND	24
D17	MuLV(MA-CA_{HIV})	29	89	ND	3
D17	MuLV(CA_{HIV})	53	ND	171	3.2
D17	MuLV(MA-CA_{HIV})	71	ND	297	4

[a] Viral particles were harvested from 5 ml of cell-free supernatant by centrifugation through a 20% sucrose cushion. The amount of $p24^{gag}$ in the viral pellet was determined by ELISA as described in Materials and Methods.

[b] Cells were infected with either MuLV or SNV at an input of 1 infectious unit per cell, and virus was harvested at 1 week postinfection. 3T3 cells were infected with ecotropic MuLV, and D17 cells were infected with xenotropic MuLV. Superinfections were verified by the quantity of RT activity in the supernatant at the end of the experiment.

[c] ND, not done.

linear sucrose gradient at a density of about 1.16 g/ml (1, 26). Several MuLV and HIV Gag proteins with mutations in capsid which affect assembly are found in low-density fractions of a sucrose gradient (11, 12, 15, 36, 37). To determine whether the assembly defect in MuLV(CA_{HIV}) results in production of low-density particles, we banded pelleted material from 3T3-MuLV(CA_{HIV}) culture supernatants on a 20 to 60% linear sucrose gradient. Fractions were collected and assayed for $p24^{gag}$ by ELISA. With wild-type HIV, we found the peak of $p24^{gag}$ protein in a fraction with a density of about 1.17 g/ml (not shown). The major peak of $p24^{gag}$ with the MuLV (CA_{HIV}) virus, though, was found in a fraction with a much lower density, about 1.13 g/ml (Fig. 5). This shows that the secreted MuLV(CA_{HIV}) Gag protein is not assembled into dense virus particles and suggests a defect in proper virion assembly.

We next asked whether the MuLV-rescued MuLV(CA_{HIV}) virus is found in a dense particle or remains in a low-density form. Pelleted material from the supernatant of superinfected 3T3-MuLV(CA_{HIV}) cells was banded on a sucrose gradient. The peak of $p24^{gag}$ protein is now found in a fraction with a density of 1.16 g/ml, identical to wild-type HIV and wild-type MuLV (Fig. 5A and data not shown). There is also a "shoulder" of material at the lower density that might indicate the presence of both types of particles in the culture. Nonetheless, these data show that wild-type Gag complements the assembly defect in the MuLV(CA_{HIV}) Gag, allowing it to be assembled into a mature, dense particle. The low-density form of MuLV (CA_{HIV}) particles is therefore a result of improper assembly and not simply due to the presence of the HIV CA protein.

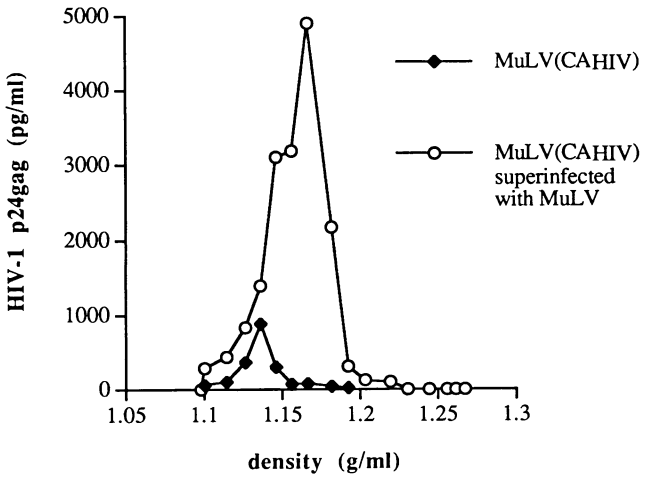

FIG. 5. Sedimentation profiles of MuLV(CA_{HIV}). (A) Sedimentation profiles of MuLV(CA_{HIV}) virus and MuLV-rescued MuLV (CA_{HIV}) virus. Viral particles were isolated from 30 ml of cell-free media by centrifugation through a 20% sucrose cushion and loaded onto a 20 to 60% linear sucrose gradient. The particles were banded by centrifugation, and equal-volume fractions were collected from the gradients. The density of each fraction was determined with a refractometer. The amount of $p24^{gag}$ in each fraction was determined by ELISA.

We also examined the density of particles produced by MuLV(MA_{HIV}). Although there were not enough particles released by MuLV(MA_{HIV}) to detect by Western blotting (Fig. 4), there were enough particles to detect by an assay for RT activity. In contrast to MuLV(CA_{HIV}), MuLV(MA_{HIV}) particles were the same density as wild-type MuLV (about 1.16 g/ml [Fig. 6]). Therefore, MuLV rescues only the quantity of MuLV(MA_{HIV}) particles, while it rescues both the quantity and the quality of MuLV(CA_{HIV}) particles.

The rescue of MuLV(MA_{HIV}) and MuLV(CA_{HIV}) is specific for MuLV. After superinfecting 3T3-MuLV(MA_{HIV}) and 3T3-MuLV(CA_{HIV}) with MuLV, we found that assembly of both the chimeric viruses was efficiently rescued. We considered two explanations for the rescue. Firstly, specific Gag-Gag protein interactions within the MuLV MA or CA regions could mediate rescue, or secondly, the chimeric Gag polyproteins could coassemble and release nonspecifically along with the efficiently produced wild-type virus. To determine the viral specificity of rescue of HIV proteins into virions, we attempted to rescue the chimeric Gag polyproteins into virions with another retrovirus, SNV. The Gag protein of SNV, although clearly related to that of MuLV, is divergent in sequence and shares only about 30% amino acid identity with MuLV (24). Since NIH 3T3 cells are not susceptible to SNV infection, we first had to introduce MuLV(MA_{HIV}) and MuLV(CA_{HIV}) into D17 cells, which are readily infected by SNV. Cells were cotransfected with each chimeric construct plus the plasmid pRSVneo and selected with the drug G418. RT- or $p24^{gag}$-positive colonies were pooled and expanded.

To ensure that the MuLV(MA_{HIV}) and MuLV(CA_{HIV}) proviruses could be rescued from D17 cells by wild-type MuLV, the chimeric Gag-expressing cells were superinfected with xenotropic MuLV at a multiplicity of infection of 1. At 1

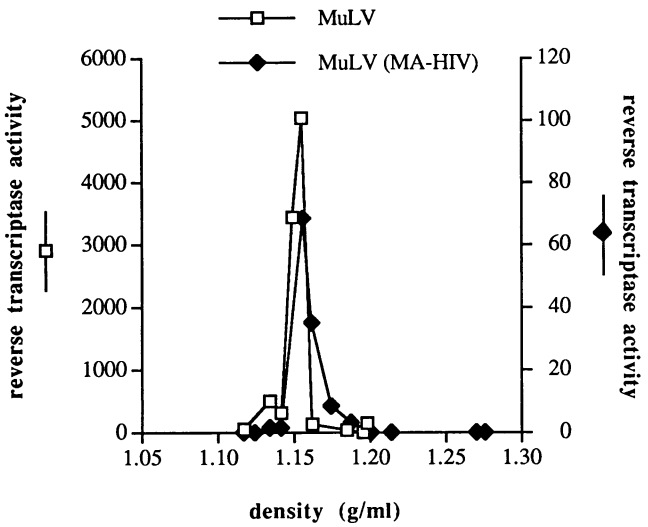

FIG. 6. Sedimentation profiles of MuLV(MA_{HIV}) and wild-type MuLV. Sucrose gradients were done as described for Fig. 5; 10 μl of each fraction was assayed for RT activity as previously described (9). The film was quantified by image analysis (14), and results are expressed in arbitrary units. The open squares are wild-type MuLV and use the scale of the *y* axis on the left. The filled diamonds are MuLV(MA_{HIV}) and use the scale of the *y* axis on the right.

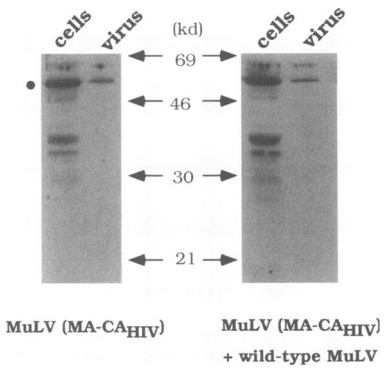

FIG. 7. Western blot of proteins produced by MuLV(MA-CA_{HIV}). Cytoplasmic and secreted proteins were harvested from D17-MuLV (MA-CA_{HIV}) cells and culture supernatant before and after superinfection with wild-type MuLV (xenotropic). The protein blots were probed with human anti-HIV sera. The unprocessed Gag protein is marked by a black dot, and the positions of the molecular mass markers are noted. Lanes cells, cytoplasmic protein; lanes virus, protein from culture supernatants that were pelleted through a 20% sucrose cushion as described in Materials and Methods. The panel on the right was infected with MuLV at a multiplicity of about 1.

week postinfection, cell culture supernatants were collected from the superinfected cells and virions were isolated. The amount of $p24^{gag}$ in the MuLV(CA_{HIV}) viral pellet was increased 24-fold after superinfection (Table 1). This is a smaller increase in release of MuLV(CA_{HIV}) virus than was found with the superinfected 3T3 cells. This may be because MuLV replicates more efficiently in 3T3 cells than in D17 cells. Rescue of MuLV(MA_{HIV}) by MuLV was confirmed by Western blotting (data not shown). Therefore, in D17 cells, as in 3T3 cells, production of the MuLV(MA_{HIV}) and MuLV (CA_{HIV}) viruses can be rescued by wild-type MuLV.

The D17-MuLV(MA_{HIV}) and D17-MuLV(CA_{HIV}) cells were then infected with SNV, and virus was harvested from cell supernatants at 1 week postinfection. The amount of MuLV (CA_{HIV}) virus (or pelletable $p24^{gag}$) increased only threefold after infection with SNV (Table 1). Some of this increase in MuLV(CA_{HIV}) Gag protein may be due to cell lysis observed in the SNV-infected cells. No HIV-specific bands could be detected on the Western blot of the MuLV(MA_{HIV}) or SNV-infected MuLV(MA_{HIV}) viral pellets, indicating that SNV could not rescue the MuLV(MA_{HIV}) Gag (data not shown). Reprobing the blot with SNV-specific antibodies showed high levels of SNV production. Only MuLV, and not SNV, can rescue MuLV(MA_{HIV}) and MuLV(CA_{HIV}) proviruses. This suggests that rescue requires specific homologous Gag protein interactions.

The MA or CA region of MuLV, but not $p12^{gag}$, is required for rescue of the chimeric viruses by wild-type MuLV. Both of the chimeric proviruses contain the $p12^{gag}$ protein of MuLV between MA and CA. To determine whether this small region is important for rescue, the proviral construct MuLV ($\Delta p12CA_{HIV}$) was made by deleting $p12^{gag}$ from MuLV (CA_{HIV}) (Fig. 2B). If protein interactions in the $p12^{gag}$ region are required for rescue by wild-type Gag, we would not expect this virus to be rescued by infection with MuLV. The MuLV($\Delta p12CA_{HIV}$) proviral construct was transfected into 3T3 cells along with the TK plasmid, and hypoxanthine-aminopterin-thymidine-resistant $p24^{gag}$-positive clones were isolated. Infection of the 3T3-MuLV($\Delta p12CA_{HIV}$) cells with wild-type MuLV increased the level of pelletable $p24^{gag}$ 26-fold (Table 1). This indicates that Gag protein interactions within the $p12^{gag}$ region are not required for rescue.

We next determined whether the presence of a homologous MA or CA region of Gag was absolutely required for rescue by creating another proviral construct in which both the MA and the CA genes of MuLV were replaced with HIV genes (Fig. 2B). D17 cells were transfected with this construct, MuLV (MA_{HIV}), and $p24^{gag}$-positive cells were pooled. After the D17-MuLV(MA-CA_{HIV}) cells were superinfected with MuLV, the amount of pelletable $p24^{gag}$ was only increased fourfold (Table 1). The absence of a large increase in $p24^{gag}$ in the superinfected D17-MuLV(MA-CA_{HIV}) viral pellet indicates that this chimeric provirus cannot be rescued by MuLV. SNV also did not increase the amount of pelletable $p24^{gag}$ of D17-MuLV(MA-CA_{HIV}) cells (Table 1).

We verified this result by Western blotting of HIV proteins from D17-MuLV(MA-CA_{HIV}) cells and from viral pellets before and after infection with MuLV (Fig. 7). The presence of an unprocessed Gag protein is detectable in the cell fraction at about the predicted size of 63 kDa (Fig. 7). However, unlike cells containing MuLV(CA_{HIV}) or MuLV(MA_{HIV}), superinfection of D17-MuLV(MA-CA_{HIV}) had no effect on the quantity of released Gag protein (Fig. 7). The presence of wild-type MuLV after superinfection was verified by RT activity (data not shown). Since MuLV could not complement production of the MuLV(MA-CA_{HIV}) virus but can rescue MuLV(MA_{HIV}) and MuLV(CA_{HIV}), we conclude that protein-protein interactions involving the MA or CA regions must occur to complement the assembly defect of the chimeric Gag proteins.

DISCUSSION

To define regions of the retroviral Gag polyprotein important in particle morphogenesis, we have constructed HIV-MuLV chimeric viruses in which the MA or CA regions of the MuLV Gag polyprotein have been precisely replaced by the corresponding HIV MA or CA regions. The chimeric provi-

ruses were unable to assemble high levels of viral particles on their own. However, coexpression of wild-type copies of MuLV by superinfection of cells containing the chimeric proviruses led to the incorporation of HIV MA or HIV CA into MuLV virions. Because the rescue of assembly of these chimeric viruses was dependent on the presence of MuLV MA or CA in both the chimeric provirus and in the superinfecting virus, these results suggest that virus assembly is mediated by interactions that can use either MA or CA in *trans*.

Although viral pseudotypes that contain Env proteins of one retrovirus on the core of another retrovirus are readily formed, mixed particles containing core proteins from two divergent retroviruses do not (20). However, our results suggest that mixed particles can be made by expressing Gag proteins in the context of a chimeric Gag precursor. There are two results that argue that HIV-1 CA is incorporated into MuLV virions in our experiments. Firstly, the partially processed MuLV(CA_{HIV}) Gag protein secreted from the NIH 3T3 clones was fully processed after superinfection, indicating assembly into mature particles. Secondly, the MuLV(CA_{HIV}) Gag protein that is found in low-density fractions of a sucrose gradient bands at the proper density after superinfection. This indicates that HIV-1 CA was incorporated into normal virions which presumably contain both HIV-1 and MuLV CA proteins.

Several studies with MuLV and Rous sarcoma virus suggest that only MA and CA are necessary for particle formation (7, 11, 15, 38), although the entire Gag gene is important in infection to mediate RNA packaging, initiation of reverse transcription, integration, and other events (2, 7, 13, 23, 32). It is not clear why the chimeric HIV-1–MuLV Gag polyproteins do not assemble into virions on their own. Regardless of the defect, it does not preclude them from being rescued into virions by wild-type MuLV (Fig. 4). Assembly of the wild-type Gag polyprotein into particles might catalyze the correct folding of the chimeric Gag polyprotein. Alternatively, if efficient assembly requires transient interactions between MA on one molecule and CA from a homologous virus on another molecule, then wild-type virus could rescue the chimeric Gag polyproteins into virions by supplying the missing MuLV MA or CA domains in *trans*. This would explain why most mutations or deletions in CA eliminate particle assembly (11, 32, 35). Moreover, the lack of specific contacts between wild-type MuLV Gag and the chimeric MuLV(MA-CA_{HIV}) Gag could explain why this virus is not rescued.

In contrast to chimeric proviruses constructed by using convenient restriction sites (17), the PCR method described here has allowed us to construct precise replacements of domains within the Gag polyprotein between HIV-1 and MuLV. Therefore, it is possible that these domains will retain their specific functions in the hybrid virions. These rescued chimeric viruses may be useful in understanding the specificity of HIV-1 Env incorporation into virions (39) and in mapping specific phenotypes of HIV, such as the ability to integrate into nondividing cells (19).

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ADDENDUM IN PROOF

The MuLV(MA_{HIV}) provirus is infectious.

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