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Efficient Particle Formation Can Occur if the Matrix Domain of Human Immunodeficiency Virus Type 1 Gag Is Substituted by a Myristylation Signal

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Lentiviruses, such as human immunodeficiency virus type 1 (HIV-1), assemble at and bud through the cytoplasmic membrane. Both the matrix (MA) domain of Gag and its amino-terminal myristylation have been implicated in these processes. We have created HIV-1 proviruses lacking the entire matrix domain of *gag* which either lack or contain an amino-terminal myristate addition sequence at the beginning of the capsid domain. Myristate- and matrix-deficient [myr(−)MA(−)] viruses produced after transient transfection are still able to assemble into particles, although the majority do not form at the plasma membrane or bud efficiently. Myristylation of the amino terminus of the truncated Gag precursor permits a much more efficient release of the mutant virions. While myr(−)MA(−) particles were inefficient in proteolytic processing of the Gag precursor, myristylation enabled efficient proteolysis of the mutant Gag. All matrix-deficient viruses are noninfectious. Particles produced by matrix-deficient mutants contain low levels of glycoproteins, indicating the importance of matrix in either incorporation or stable retention of Env. Since matrix-deficient viruses contain a normal complement of viral genomic RNA, a role for MA in genomic incorporation can be excluded. Contrary to previous reports, the HIV-1 genome does not require sequences between the 5′ splice donor site and the *gag* start codon for efficient packaging.

The human immunodeficiency virus type 1 (HIV-1) *gag* gene is expressed as a polyprotein precursor, $Pr55^{Gag}$. Genetic studies have demonstrated that *gag* is the only gene required for virus particle formation and incorporation of the viral genome (15, 20, 26–29, 43, 54, 56). Like C-type retroviruses, HIV assembles and acquires its lipid bilayer at the cell plasma membrane. After viral budding, the Gag precursor (Pr^{Gag}) molecules are cleaved by the viral protease and the particles assume a condensed morphology (33). Active protease is required for this transition from noninfectious, immature virions to mature virions. The virus-encoded protease processes $Pr55^{Gag}$ into p17 (matrix, or MA), p24 (capsid, or CA), p7 (nucleocapsid, or NC), and p6/9 (a proline-rich protein).

In the uncleaved HIV-1 Gag precursor, those domains responsible for assembly or genome incorporation have not been fully defined. Studies of oncoviruses have indicated that the matrix region of Gag may be important in both of these processes (10, 31, 32, 52, 53, 62). In the case of HIV-1, deletions within the matrix region do not abolish assembly (13, 59, 63), although a large deletion from the central region of the HIV-1 MA appears to hinder efficient release of particles from cells (13). In many retroviruses, including murine leukemia virus, HIV, spleen necrosis virus, and simian immunodeficiency virus, it has been suggested that membrane targeting by the Gag precursor requires the addition of myristic acid to the N-terminal glycine of Gag. Mutations that block the myristylation of the Gag proteins of these viruses result in the loss of extracellular particle formation (4, 9, 15, 24, 29, 30, 47, 50, 58, 60). Whether myristylation of retroviral Gag proteins outside the context of matrix is sufficient to ensure efficient particle assembly and release is not known. In the case of HIV-1, it is clear that a great deal of the matrix domain may be dispensable for assembly (13, 59).

In this paper, two matrix-deficient mutants of HIV-1 are characterized. One mutant lacks the entire matrix domain [myr(−)MA(−)]; the other also lacks matrix but contains a reconstituted myristylation site at the 5′ end of the truncated Gag precursor [myr(+)MA(−)]. The results show that while matrix is not required for HIV-1 viral assembly and release, myristylation of the Gag polyprotein precursor serves to facilitate efficient particle release. Both mutant viruses appear to be noninfectious and poorly incorporate or retain viral Env. Furthermore, viral proteins in the virions produced by the myr(−)MA(−) mutant are inefficiently processed, although they are not deficient in the *pol* gene product. This suggests that myristylation or stable membrane association facilitates cleavage by the viral protease. Comparison of the RNA contents of mutant and wild-type virions revealed that the matrix region is not required for genome encapsidation and that sequences located between the 5′ major splice donor site and the N terminus of Gag are not required in *cis* for genome incorporation.

MATERIALS AND METHODS

Cells. CD4-LTR/βgal cells (34), HeLaTat cells (a gift of Victor Garcia, St. Jude Children's Hospital, Memphis, Tenn.), and SW480 cells, a colon carcinoma line (35), were maintained in Dulbecco's modified Eagle's medium with 10% bovine calf serum. CD4-LTR/βgal cells were maintained with 0.4 mg of G418 per ml and 0.1 mg of hygromycin per ml. COS7 cells (16) and 293T cells (48), derived from 293 cells (22) (a human kidney embryonic cell line), were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. CEM×174

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cells (a human T- and B-cell hybrid cell line) were grown in RPMI 1640 containing 10% fetal bovine serum.

Transfections and infections. DNA transfection of SW480, HeLaTat, and 293T cells was performed by a modified calcium phosphate precipitation method (6). Typically, 2×10^5 cells were plated per 25-cm^2 flask 1 day before transfection. Transfections were performed with 5 to 8 μg of plasmid DNA. COS7 cells were transfected, with 10 μg of plasmid DNA, by the DEAE-dextran method (12). One day before transfection, 10^6 cells were plated per 75-cm^2 flask. The transfected culture supernatants were harvested 48 and 72 h posttransfection. Supernatants from transfected cells were filtered through 0.2-μm-pore-size filters and supplemented with 20 to 100 μg of DEAE-dextran per ml or 2 μg of Polybrene per ml for infection of CD4-LTR/βgal cells or CEM×174 cells, respectively.

Expression plasmids. Plasmids containing the full-length infectious HIV-1 proviruses p125-1 and pBRU3 (12) were provided by M. Emerman (Fred Hutchinson Cancer Research Center). Plasmid pBRU3 has been renamed HIV_{Lai} but is referred to as pBRU3 in this paper. The numbering of the HIV-1 nucleotide sequence referred to herein appear in the EMBL and GenBank database under accession number K02013. Plasmid pΔSC was constructed by site-directed oligonucleotide mutagenesis as described by Eghtedarzadeh and Henikoff (11). An intermediate construct, pBRUNP, used in the mutagenesis as a template, was constructed by subcloning a 778-bp *Nar*I-*Pst*I fragment (nucleotides [nt] 183 to 961) from pBRU3 into pVZ1 (37). Plasmid pΔSC, containing an 80-nt deletion between the 5′ major splice donor site and the *Cla*I site (nt 376), was constructed by using an oligonucleotide 30-mer (5′-AGGCGACTGGTGAG/GATCGATGGGAAAA AA-3′; the slash indicates the site where the deletion was created) to introduce an 80-nt deletion (nt 295 to 374) into pBRUNP. The mutated *Nar*I-*Pst*I fragments were then used to replace the analogous wild-type sequence of pBRU3 to generate pΔSC. An *Aat*II-*Nar*I fragment which contains the simian virus 40 origin of replication (400 nt of *Hin*dIII-*Kpn*I fragment from the vector sequence of p125-1) was cloned into pBRU3 and pΔSC. These simian virus 40 *ori*-containing plasmids were used in all subsequent plasmid constructions and all COS7 cell transfections.

Plasmids pΔSC-T and pΔSCmyr were constructed by PCR mutagenesis (25) (Fig. 1). Plasmid pΔSC-T, containing two consecutive in-frame stop codons (as shown in boldface letters) at the C terminus of the MA region of pΔSC, was constructed with pΔSC as a template. Oligonucleotides BRU366 (5′-GGA GAATTAGATCGATGGG-3′) and BRU689 (5′-TG**CTTACT** AGTCTTTTTTCTT-3′) were used to amplify a 324-nt fragment, whereas oligonucleotides BRU669 (5′-AAGAAAA AAGAC**TAGTAA**GCA-3′) and BRU1568 (5′-CTTTTTCCT AGGGGCCCT-3′) were used to generate a 900-nt fragment. Both PCR-generated fragments were gel purified and used as templates for a second round of PCR using oligonucleotides BRU366 and BRU1568 to create a 1,203-nt cassette. This PCR-amplified cassette (nt 366 to 1568), containing two in-frame stop codons TAG and TAA at the C terminus of the p17 region (nt 681 to 686), was digested with *Cla*I and *Apa*I and used to replace the analogous wild-type sequences of pΔSC to generate pΔSC-T. Plasmid pΔSCmyr, containing an authentic HIV-1 myristylation site (ATG GGT GCG AGA GCG TCA; amino acids MGARAS) at the capsid initiation site, was constructed by the addition of 15 nucleotides (GGT GCG AGA GCG TCA) (as shown in underlined letters) 3′ of the capsid ATG with pΔSC as a template. Oligonucleotide BRU366 19-mer (5′-GGAGAATTAGATCGATGGG-3′) and oligonucleotide BRU761 32-mer (5′-TGACGCTCTCGCAC CCATTTGCCCCTGGATGT-3′) were used to generate a 410-nt fragment, whereas oligonucleotide BRU762 33-mer (5′-GGTGCGAGAGCGTCAGTACATCAGGCCATATCA-3′) and oligonucleotide BRU1568 18-mer were used to generate an 821-nt fragment. Both PCR-generated fragments were gel purified and used as templates for a second round of PCR using oligonucleotides BRU366 and BRU1568 to create a 1,203-nt cassette containing the 15-nt insertion. This PCR-amplified fragment (nt 366 to 1568) was digested with *Cla*I and *Apa*I and used to replace the analogous wild-type sequences of pΔSC to generate pΔSCmyr.

All mutations were verified by double-stranded dideoxynucleotide DNA sequence analysis (U.S. Biochemicals reagent kit for DNA sequencing) of the final plasmids.

p24 antigen capture and reverse transcriptase (RT) assays. Assays for HIV-1 p24 capsid proteins were performed using p24 antigen enzyme-linked immunosorbent assay (ELISA) kits (Coulter, Inc., Hialeah, Fla.). In each experiment, a standard curve was generated by serial dilutions of the capsid antigen standard provided by the manufacturer.

RT activity was measured as previously described (17). Briefly, the culture medium from transfected cells was centrifuged at low speed to clear cell debris. In some experiments, samples were further centrifuged through 20% sucrose in standard buffer (SB) (0.1 M NaCl, 0.01 M Tris [pH 7.4], 0.001 M EDTA) before analysis. Samples (10 μl) were mixed with RT mixture [50 mM Tris (pH 7.8), 7.5 mM KCl, 2 mM dithiothreitol, 5 mM $MgCl_2$ for HIV RT or 0.6 mM $MnCl_2$ for murine leukemia virus RT, 5 μg of poly(A) per ml, 1.57 μg of oligo(dT) per ml, 0.05% Nonidet P-40] containing [^{32}P]TTP. After 90 min of incubation at 37°C, reaction mixtures were spotted into DE81 paper, which was then washed several times with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Filters were subjected to autoradiography or analyzed with a Molecular Dynamic Phosphorimager.

Western immunoblot analysis. Transfected cells were washed twice with phosphate-buffered saline (PBS), lysed in reticulate suspension buffer (10 mM Tris, pH 7.4; 10 mM NaCl; 5 mM $MgCl_2$; 0.5% Nonidet P-40), and then centrifuged to remove nuclei. Sample buffer (42) was added to supernatants before loading. For analysis of viral lysates, culture supernatants collected from transfected cells were cleared of cell debris by low-speed centrifugation. Viral particles were then pelleted through a 20% sucrose cushion at 23,000 rpm for 2 h at 4°C (SW28 rotor; Beckman Instruments, Inc.). Pelleted samples were resuspended in SB and 2× sample buffer (42). Viral proteins were fractionated on sodium dodecyl sulfate–12.5 to 15% polyacrylamide gel electrophoresis (SDS–12.5 to 15% PAGE) gels. The proteins were electrotransferred to nitrocellulose filters and were incubated at 4°C in block solution (PBS–0.5% Tween 20–5% dried milk) for more than 5 h prior to antibody incubation. Serum from an AIDS patient was used at a dilution of 1:200 as a primary antibody. The antibody-antigen complex was visualized by using sheep anti-human immunoglobulin G–horseradish peroxidase conjugate at a dilution of 1:1,500 (Amersham, Arlington Heights, Ill.) and the enhanced chemiluminescence Western blotting analysis system (Amersham).

Metabolic labeling and immunoprecipitation. SDS-PAGE and techniques for cell labeling were similar to those previously described (5). Transfected cells at 48 to 72 h posttransfection were labeled with [^{35}S]methionine (>800 Ci/mmol; NEN Research Products) or [^{3}H]myristic acid (55 Ci/mmol; Amersham Life Science) for 12 or 4 h and then scraped off plates for further analysis. [9,10(*n*)-^{3}H]myristic acid in benzene

was vacuum dried and dissolved in dimethyl sulfoxide prior to addition. The complete labeling medium for [^{3}H]myristic acid labeling consisted of Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 1% dimethyl sulfoxide, and [^{3}H]myristic acid (1 mCi/ml). The medium for [^{35}S]methionine labeling consisted of Dulbecco's modified Eagle's medium lacking methionine, supplemented with 1% dialyzed bovine serum and [^{35}S]methionine (250 μCi/ml). After labeling, supernatants were collected and cleared of cell debris by low-speed centrifugation. Cells and viral supernatants were lysed in radioimmunoprecipitation assay buffer (0.12 M NaCl, 20 mM Tris [pH 8.0], 1% Nonidet P-40, 0.2% sodium deoxycholate, 0.2% SDS) with the proteinase inhibitors 100 μg of phenylmethylsulfonyl fluoride per ml, 50 U of aprotinin per ml, 1 μg of pepstatin A per ml, and 1 μg of leupepsin per ml and centrifuged for 10 min at 10,000 × *g*. Serum from an AIDS patient (4 μl per reaction) and 50 μl of protein A-Sepharose were added to supernatants for 90 min at room temperature. Samples were washed twice with radioimmunoprecipitation assay buffer, once in high-salt buffer (2 M NaCl, 10 mM Tris [pH 7.4], 1% Nonidet P-40, 0.5% sodium deoxycholate), and once more in radioimmunoprecipitation assay buffer. Sample buffer (42) was then added to the washed protein A-Sepharose beads, and the mixture was heated at 100°C for 4 min. The beads were removed by centrifugation, and proteins were separated in an SDS–12.5% acrylamide gel. Gels were fixed in 50% methanol–10% acetic acid, treated with Amplify (Amersham), and exposed to preflashed X-ray film (X-Omat AR; Kodak).

Preparation of viral and cellular RNA. RNA was extracted from purified virus as previously described (3). Total-cell RNA was prepared from cells 48 to 72 h posttransfection by the acid guanidinium-phenol-chloroform method (7). All solutions used in the preparation of RNA were treated with 0.1% diethylpyrocarbonate and autoclaved prior to use.

RNase protection assay. The methods for RNase protection were similar to these previously described (3) with the following modifications. A 449-bp *Nar*I-*Hin*dIII fragment (nt 183 to 631) from pBRU3 was cloned into pVZ1 (37). T3 polymerase was used to express antisense riboprobe from linearized plasmid (linearized with *Bss*HII [nt 257]), and the band ^{32}P-labeled antisense riboprobe was excised from 5% acrylamide gels and eluted in 2 M ammonium acetate–1% SDS–25 μg of tRNA per ml in diethylpyrocarbonate-water at 37°C overnight. About 5 × 10^5 cpm of probe was then mixed with test RNA in a 15-μl reaction mix containing 80% formamide–0.4 M NaCl–10 mM MOPS [3-(*N*-morpholino)propanesulfonic acid]–1 mM EDTA, heated at 95°C for 2 min, and hybridized at 50°C overnight. After digestion with RNase T_1 and RNase A for 1 h at 30°C, reaction mixtures were adjusted to 0.5% SDS, proteinase K was added to 0.5 mg/ml, and the mixture was incubated for 15 min at 37°C and then phenol chloroform extracted and ethanol precipitated. The precipitated reaction mixtures were analyzed in 5% acrylamide gels containing 50% urea.

Sucrose gradient analysis. Density determination was done as previously described (38). Briefly, COS7 cells were transfected with either pBRU3, pΔSC, or pΔSCmyr. Two days posttransfection, culture supernatants were cleared of cell debris by low-speed centrifugation. These clarified viral supernatants were centrifuged through 20% sucrose in SB for 2 h. Pellets were then resuspended in SB, overlaid on a 20 to 60% sucrose gradient made in SB, and centrifuged in an SW55Ti rotor (Beckman Instruments) at 32,000 rpm for 135 min. Gradient fractions were collected from the bottom, and densities were determined with a refractometer.

Electron microscopy. Thin-section electron microscopy was performed as previously described (2). Cells and supernatant from COS7 cells transfected with either pBRU3, pΔSC, or pΔSCmyr were collected 2 days posttransfection. Culture supernatants were pelleted through 20% sucrose. Cell and viral pellets were fixed 1:1 in half-strength Karnovsky's fixative for at least 1 h for further processing. For immunoelectron microscopy studies, pellets were fixed in 2% paraformaldehyde, washed in block solution (PBS containing 0.5% Tween 20 and 5% dried milk), overlaid with anti-gp120 monoclonal antibody (23) 1:50 in block solution for 1 h at room temperature, washed with block solution three times for 10 min each time, overlaid with electron microscopy-grade gold-labeled goat anti-mouse immunoglobulin G (10-nm gold particles; BioCell Research Laboratories) diluted 1:10 in block solution, and incubated for 1 h at room temperature. Samples were then washed with block solution three times for 10 min each time and stained with uranyl acetate. All samples were examined in a JEOL 100SX transmission electron microscope operating at 80 kV.

RESULTS

Viral proteins are released into supernatants of myr(−)MA(−) mutant-transfected cells. Plasmid pΔSC, a myr(−)MA(−) mutant, is derived from the HIV-1 proviral construct pBRU3 and lacks 80 nt which overlap the NH_2 terminus encoded by the *gag* gene and includes the previously defined HIV-1 packaging signal (1, 8, 36). This deletion eliminates the wild-type Gag initiation codon (at nt 336) and sequences encoding the amino-terminal 13 amino acids of the Gag and Gag-Pol polyproteins (Fig. 1). The next methionine codon within the HIV-1 Gag reading frame occurs at nt 759, 10 amino acids 3′ to the NH_2 terminus of the CA domain. Initiation of protein synthesis at this methionine is expected to yield a truncated Gag protein lacking the entire MA domain, including the NH_2-terminal myristic acid addition site. There is no consensus myristylation site (Met Gly X X X Ser/Thr [55,

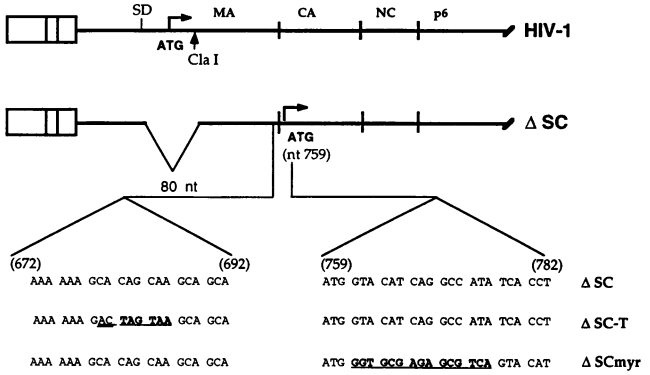

FIG. 1. Matrix-deficient mutants. Plasmid ΔSC is derived from the full-length HIV-1 proviral clone pBRU3, which was mutated to remove 80 nt from the 5′ major splice donor (SD) site to the *Cla*I site (nt 376). Plasmid ΔSC-T is derived from pΔSC and contains two consecutive stop codons (TAGTAA) at the C terminus of the matrix region and two additional nucleotide changes which facilitated screening for mutants. Plasmid ΔSCmyr is derived from pΔSC and contains a myristylation site (GARAS) immediately downstream of the capsid ATG. The changes introduced through mutagenesis are underlined; the introduced stop codons and myristylation site are shown in boldface type. The additional two nucleotide changes (double underlined) in pΔSC-T add a restriction site.

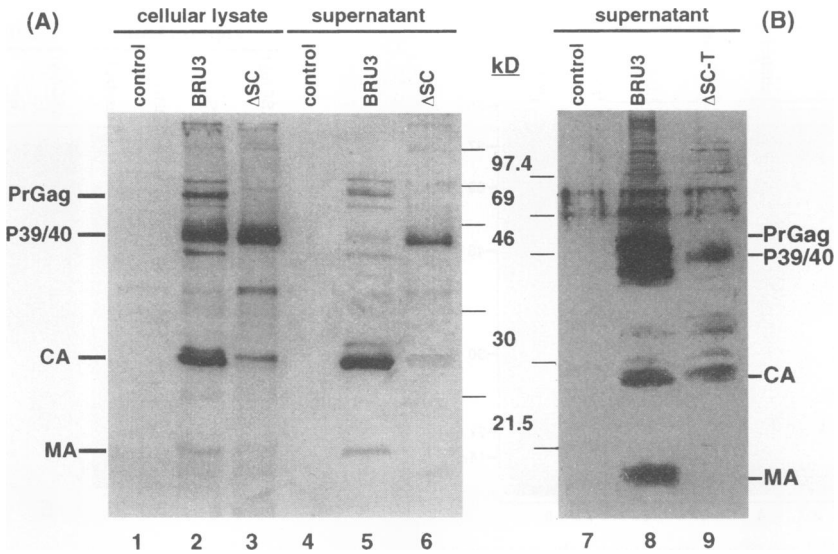

FIG. 2. Western blot analysis of cell- and virion-associated viral proteins. COS7 cells were transfected with pBRU3, pΔSC, pΔSC-T, or salmon sperm DNA (control). Viral supernatants were collected 48 to 72 h posttransfection every 12 to 24 h and pelleted through 20% sucrose cushions. Cell lysates were prepared 72 h posttransfection as described in Materials and Methods. Western blot analysis was done using serum from an AIDS patient. (A) Lanes 1 to 3, intracellular virus-specific proteins from transfected cells; lanes 4 to 6, virion-associated viral proteins from culture supernatants of transfected cells. Proteins were separated in an SDS–15% polyacrylamide gel. (B) Comparison of virion-associated viral proteins in pBRU3 and pΔSC-T. Western blot analysis was done using serum from an AIDS patient. Shown are viral lysates from transfected cells. Proteins were separated by SDS–12.5% PAGE.

57]) near the CA methionine. To examine the phenotype of this mutant, both pΔSC and pBRU3 were expressed in COS7 cells. Two days posttransfection, supernatants were collected, cleared of cell debris, and pelleted through 20% sucrose to reduce contamination by nonassembled HIV proteins. The pelletable p24 and RT levels expressed from the mutant were approximately 20-fold lower than those from the wildtype, although transfection efficiencies were similar as measured by cotransfection with a β-galactosidase-expressing construct (data not shown). The ratio of RT to p24 in the mutant was not less than that in the wild type. This result suggests that the MA domain plays no special role in the incorporation of $Pr^{Gag-Pol}$ into particles. Production of mutant virions is not confined to COS7 cells, since transfection of human SW480 cells using pBRU3 and pΔSC gave similar results, except that the expression levels in SW480 cells from both constructs were lower than in COS7 cells (data not shown).

The myr(−)MA(−) mutant Gag precursor is poorly processed. During HIV morphogenesis, the 55-kDa Gag precursor is cleaved to mature MA, CA, and NC proteins concomitant with budding. The cleaved products and cleavage intermediates are also detectable in the cell lysates, presumably because of associated virions. In order to determine the status of the Gag proteins in ΔSC-transfected cells and in released viral particles, Western blot analysis of transfected COS7 cell extracts was performed. In both viral and cytoplasmic lysates of cells transfected with the wild-type construct, expression of $Pr55^{Gag}$, p39/40 (a truncated Gag protein), p24 CA, and p17 MA was observed as expected (21) (Fig. 2A, lanes 2 and 5). In the case of pΔSC-transfected cells, a truncated Gag-related precursor protein with an apparent molecular mass of 39 to 40 kDa and a 24-kDa protein corresponding in size to CA were observed (Fig. 2A, lanes 3 and 6). No full-length wild-type precursor of the 55-kDa or p17 MA protein was observed in lysates from mutant-transfected cells. These results suggest that the ATG at nt 759 can serve as an initiation codon in the absence of the wild-type ATG and indicate that the truncated ΔSC precursor Gag is processed to an apparently wild-type-size CA protein. Although similar levels of Gag precursor (Pr55 or p39/40) were seen in each case in cytoplasmic lysates (Fig. 2A, lanes 2 and 3), proteolytic processing was less efficient in the mutant construct, resulting in less CA relative to precursor in viral particles compared with levels in the wild type (Fig. 2A, lanes 5 and 6).

The mutant Gag precursor does not initiate within the MA region. The pΔSC provirus did not appear to express any protein migrating in the region of p17 MA in Western blot analysis of cells transfected with the mutant. However, to rule out the possibility that the mutant Gag precursor could be initiated from an initiation codon other than the AUG methionine within the CA coding region, a second mutant, pΔSC-T, was constructed by creating two in-frame termination codons at nt 681 and 684 at the COOH terminus of the matrix region of pΔSC but upstream of the presumptive initiation codon at nt 759 (Fig. 1). If the synthesis of Gag and Gag-Pol polyproteins from the ΔSC genome initiates from the AUG at nt 759 in CA, the pattern of viral proteins produced by the pΔSC-T genome should be identical to those from the pΔSC genome. COS7 cells were transfected with plasmids BRU3 and ΔSC-T, and lysates from viral supernatants were prepared 2 to 3 days later. Viral proteins were analyzed by Western blotting (Fig. 2B). The expected pattern of wild-type Gag proteins (Pr55, CA, and p17 MA) was seen in the viral supernatant from pBRU3-transfected cells (lane 8). In contrast, viral supernatants from pΔSC-T-transfected cells contain only p39/40 and p24 (Fig. 2B, lane 9). This result indicates that the initiation codon at nt 759 is utilized in pΔSC for the synthesis of Pr^{Gag} and $Pr^{Gag-Pol}$ polyproteins.

A myristylated derivative of ΔSC (ΔSCmyr) produces viral particles efficiently. To examine the role of myristylation of the Gag precursor outside the context of the MA region, we constructed a myristylation-positive, matrix-deficient [myr

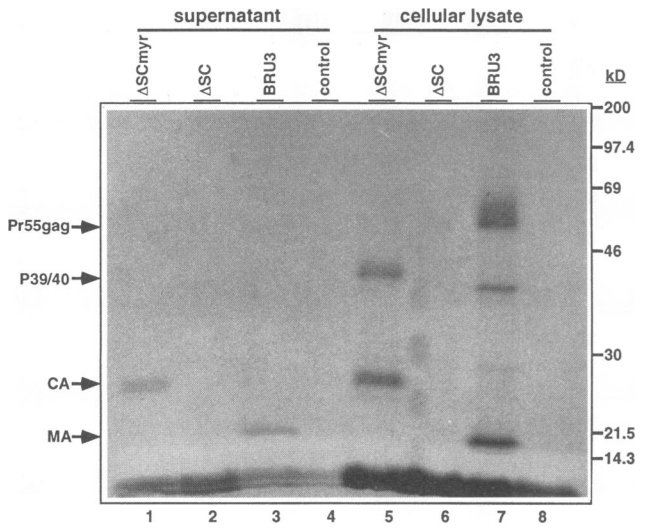

FIG. 3. Myristylated HIV-1-specific proteins from cells and supernatants expressing wild-type and mutant proviruses. COS7 cells were transfected with salmon sperm DNA (control), pBRU3, pΔSC, or pΔSCmyr. Two days posttransfection, cells were labeled with [^{3}H]myristic acid (1 mCi/ml) for 4 h. Cell lysates and viral lysates (supernatant) were immunoprecipitated with serum from an AIDS patient and then analyzed by SDS–12.5% PAGE. The dried gel was exposed to presensitized film for 14 days.

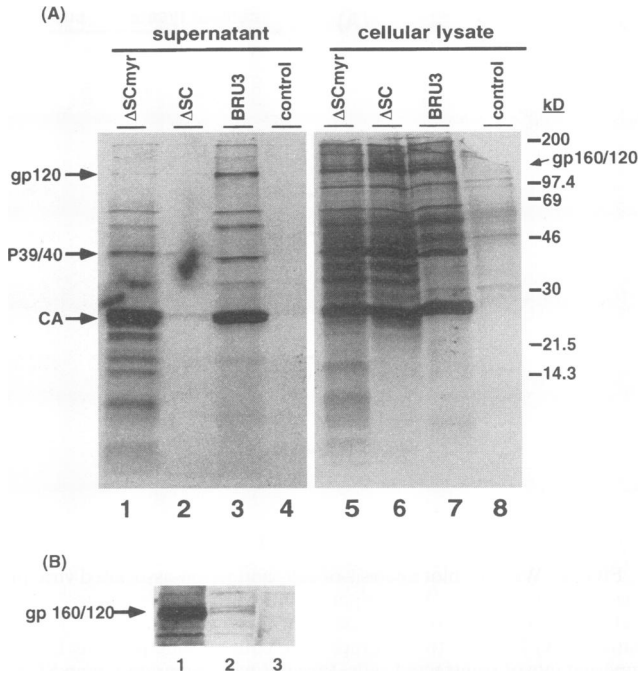

FIG. 4. ^{35}S immunoprecipitation of cells and supernatants expressing wild-type and mutant proviruses. (A) 293T cells were transfected with salmon sperm DNA (control), pBRU3, pΔSC, or pΔSCmyr. Two days posttransfection, cells were labeled with [^{35}S]methionine (250 μCi/ml) for 12 h. Viral supernatants were pelleted through 20% sucrose cushions. Cell lysates and viral lysates (supernatant) were immunoprecipitated with serum from an AIDS patient and then analyzed by SDS–15% PAGE. (B) Comparison of virion-associated viral glycoproteins in pBRU3 and pΔSC. SW480 cells were transfected with salmon sperm DNA (control) (lane 3), pBRU3 (lane 1), or pΔSC (lane 2). Immunoprecipitation was performed as described for panel A except that cells were labeled for 4 h. Shown are viral lysates from transfected cells. Proteins were separated by SDS–12.5% PAGE.

(+)MA(−)] mutant, pΔSCmyr (Fig. 1). A myristylation site was created in pΔSC by inserting the wild-type HIV-1 myristylation signal (5 amino acids, GARAS) immediately 3′ to the ATG at the NH_2 terminus of pΔSC capsid protein, creating the sequence MGARAS at the 5′ end of CA. To determine whether the addition of these 5 amino acids is sufficient to induce myristylation of the mutant precursor Gag, COS7 cells were transfected with wild-type and mutant proviruses and metabolically labeled with [^{3}H]myristic acid. Cell and viral lysates were obtained and immunoprecipitated with serum from an AIDS patient, and the ^{3}H-labeled Gag proteins encoded by mutant and wild-type genomes were analyzed by SDS-PAGE. As shown in Fig. 3, lane 7, the labeled myristylated proteins encoded by wild-type HIV-1 are $Pr55^{Gag}$ and p17 MA, as expected. There is an additional myristylated protein seen at about 39 or 40 kDa which is myristylated and presumably a processed intermediate of $Pr55^{Gag}$ truncated at the carboxyl end. In lane 5, it can be seen that ΔSCmyr-transfected cells synthesize both myristylated $Pr39/40^{Gag}$ and a 24-kDa protein, which is the expected size of myristylated CA. In contrast, none of the Gag proteins expressed from ΔSC are labeled (lane 6). Only processed myristylated proteins are detected in culture supernatants collected from the same transfection: p17 matrix in the case of wild type (lane 3) and a 24-kDa protein in the case of ΔSCmyr (lane 1).

To determine the extent to which myristylated Gag protein can be assembled and released as viral particles in the absence of the matrix protein, 293T cells were transfected with wild-type and mutant proviruses and metabolically labeled with [^{35}S]methionine. Cell lysates and pelleted viral supernatants were obtained and immunoprecipitated with serum from an AIDS patient. The relative amount of virion Gag proteins (Pr39/40 and p24) to intracellular Gag proteins was determined for each virus. ΔSCmyr- and ΔSC-transfected cells released particles 31 and 1% as efficiently as wild-type-transfected cells, respectively (Fig. 4A and Table 1). The myristylation of MA-deficient Gag did not change the pattern of mutant Gag expression but allowed processing of the precursor in particles more efficient than that allowed by the myr (−)MA(−) mutant, pΔSC (Fig. 4A, compare lanes 1 and 3). Pelleted culture supernatants from transfected cells were also

TABLE 1. Infectivity of mutant viruses

Virus	Virion Gag protein/intracellular Gag protein[a]	Pelletable RT activity (RIU/ml)[b]	Infectious unit(s)/ml[c]
BRU3	0.77	7.0×10^6	2.7×10^4
ΔSC	0.01	3.2×10^5	<0.3
ΔSCmyr	0.24	2.3×10^6	<0.3

[a] Transfected 293T cells were labeled with [^{35}S]methionine for 12 h. Supernatants were collected and pelleted at 23,000 rpm with an SW28 rotor (Beckman) for 2 h through 20% sucrose cushions. Pelletable Gag proteins (Pr39/40 and p24) were quantitated with a Phosphoimager.

[b] Virus was generated by transient transfection of HeLaTat cells with equivalent amounts of DNA. Supernatants were pelleted through 20% sucrose cushions as described above before analysis for RT activity. RT activity was quantitated and measured with a Phosphorimager as described in Materials and Methods. RIU, relative image units.

[c] Virus titer was obtained by infection of CD4-LTR/βgal cells by the multinuclear activation of a galactosidase indicator assay (34). For virus BRU3, the range of wild-type viral titer obtained from repeated experiments is 3×10^3 to 6.7×10^4; a representative result is presented here.

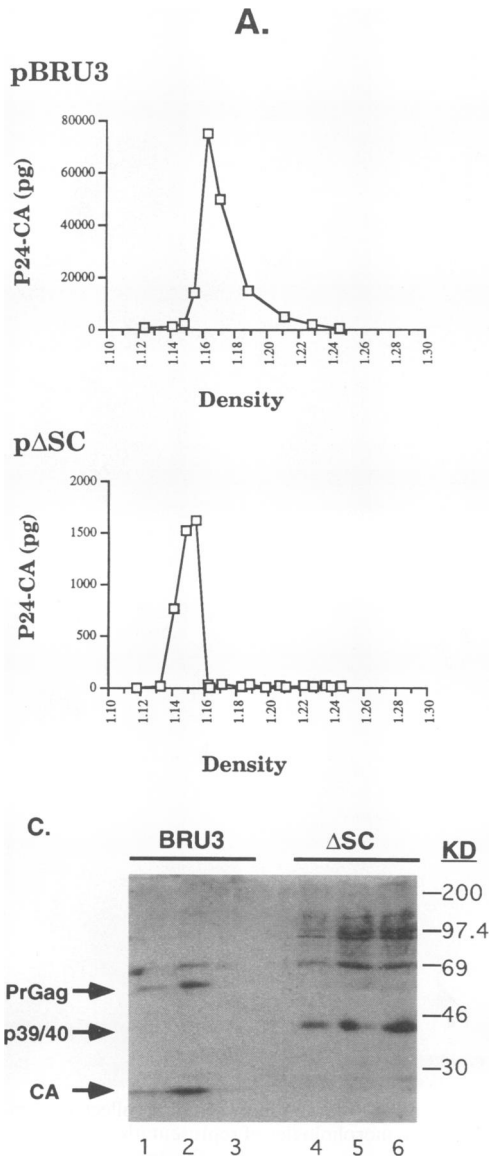

FIG. 5. Sucrose gradient analysis of ΔSC and ΔSCmyr mutant and wild-type virions. Culture supernatants were pelleted through 20% sucrose cushions, resuspended in standard buffer, and spun on 20 to 60% sucrose gradients. Each fraction was assayed for CA (p24) with p24 ELISA kits (A) or for RT activity, recorded as relative image units with a Phosphorimager (B). Sedimentation was from right to left. Density is presented as grams per milliliter. (C) Western blot analyses of the peak fractions collected from sucrose gradients. Each sample was a pool of four peak fractions from either wild-type or mutant viruses which were concentrated by centrifugation for 3 h at 100,000 × *g* before loading. Western blot analysis was performed using serum from an AIDS patient.

assayed for RT activity. The efficiency of mutant virion release when measured by RT activity in the supernatants was similar to levels measured in the immunoprecipitation assay (Table 1). We were unable to compare pelletable CA protein by a standard ELISA because of the poor reactivity of the myristylated mutant capsid with antibody provided in the p24 ELISA (data not shown). These results suggest that myristic acid addition to the Gag precursor enables efficient particle assembly and release even in the absence of the matrix domain.

Myristylation-positive and -negative Gag precursors assemble into viral particles. To determine whether expression of the mutant provirus leads to bona fide virion particles of the correct density, viral supernatants pelleted through 20% sucrose were further separated with 20 to 60% sucrose gradients. The densities of the gradient fractions were determined, and each fraction was assayed by the p24 ELISA for ΔSC viral fractions and by RT activity determination for ΔSCmyr viral fractions and compared with wild-type BRU3 HIV. Peak p24 CA content of ΔSC virions and RT activity of ΔSCmyr virions appeared in fractions close to densities obtained for wild-type HIV (1.17 g/ml). We detected densities of 1.15 g/ml for ΔSC virions and 1.16 g/ml for ΔSCmyr virions (Fig. 5A and B). Since the levels of p24 in ΔSC viral fractions are low, we further analyzed the peak fractions of ΔSC virions by Western blotting. We could detect the presence of Gag precursor protein Pr55 and p24 CA in the wild-type peak fractions and primarily Pr39/40 with a low level of CA in the mutant peak fractions (Fig. 5C). To further demonstrate that the released mutant viral proteins were confined within viral particles, pelleted culture supernatants were treated with trypsin. Resistance to trypsin treatment would suggest that particles are contained within a viral membrane (61). Most of the mutant virions that pelleted through 20% sucrose were resistant to trypsin treatment, indicating that they are membrane associated (data not shown).

In order to analyze the morphology of mutant particles, thin-section electron microscopy was employed. The myr(+)MA(−) mutant particles (Fig. 6B) have an unusual morphology compared with that of the wild type (Fig. 6A). Mutant virions are electron lucent and appear to be composed of two concentric rings. The morphology of myr(+)MA(−)

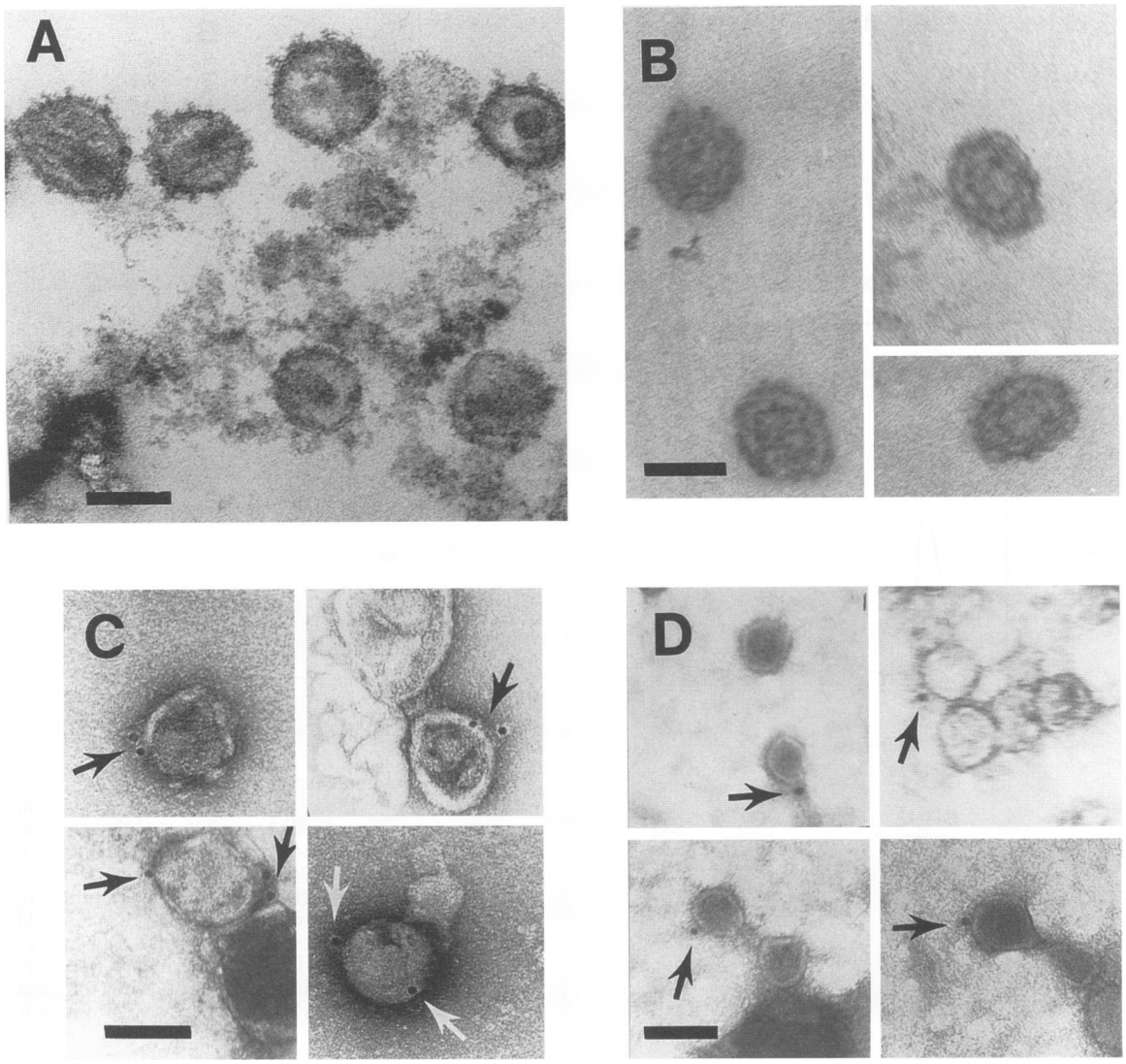

FIG. 6. Electron microscopic analysis of mutant and wild-type virus. (A, B, and E to H) Preparation of samples collected from transfected COS7 cells for thin-section electron microscopy is described in Materials and Methods. The morphologies of representative cell-free wild-type (A) and ΔSCmyr mutant (B) particles are shown. (C and D) Immunoelectron microscopy of viral particles labeled with a monoclonal antibody (110-2) specific for HIV-1 Env gp120. Arrows point to gold-labeled secondary antibody complexes. (C) Wild-type viruses; (D) ΔSC viruses. (E to H) Budding or intracellular particles of mutant and wild-type viruses; (E) wild-type HIV-1-transfected cells; (F) ΔSCmyr-transfected cells; (G and H) ΔSC-transfected cells. Bars = 100 nm.

virions could be due to the myristylation of the capsid proteins which are unable to condense after proteolytic processing. Pelleted ΔSC supernatants also contained virus-like structures (data not shown).

To examine whether envelope glycoproteins are associated with viral particles, immunoelectron microscopy was used. Thin sections made from viral pellets were treated first with a monoclonal antibody specific for HIV-1 Env gp120 (40) and then with a secondary antibody conjugated with colloidal gold. We detected individual wild-type and mutant viral particles which were labeled with the antibody-colloidal gold conjugate (examples shown in Fig. 6C and D for wild type and ΔSC, respectively). No labeled particles could be detected in the negative control (avian leukosis virus) (data not shown). In addition, many of the wild-type particles were labeled in several places. Immunoprecipitation of pelleted mutant viral supernatants with serum from an AIDS patient showed less than 10% envelope incorporation compared with that in wild-type virions (Fig. 4). These results suggest that although the HIV-1 MA domain is not absolutely required for envelope incorporation, MA may either increase the efficiency of incorporation or facilitate a more stable association.

Since myristylated Gag precursor has been shown previously to be required for membrane association, it was of interest to determine whether budding particles could be detected at the cell membrane for both mutant viruses. Thin-section electron microscopy was performed on COS7 cells 3 days after transfection with pBRU, pΔSC, or pΔSCmyr. In the case of wild-type- or pΔSCmyr-transfected cells (Fig. 6E and F), budding particles can easily be detected. Some intracellular particles were also detected in pΔSCmyr-expressing cells (data not shown). However, in pΔSC-expressing cells only few

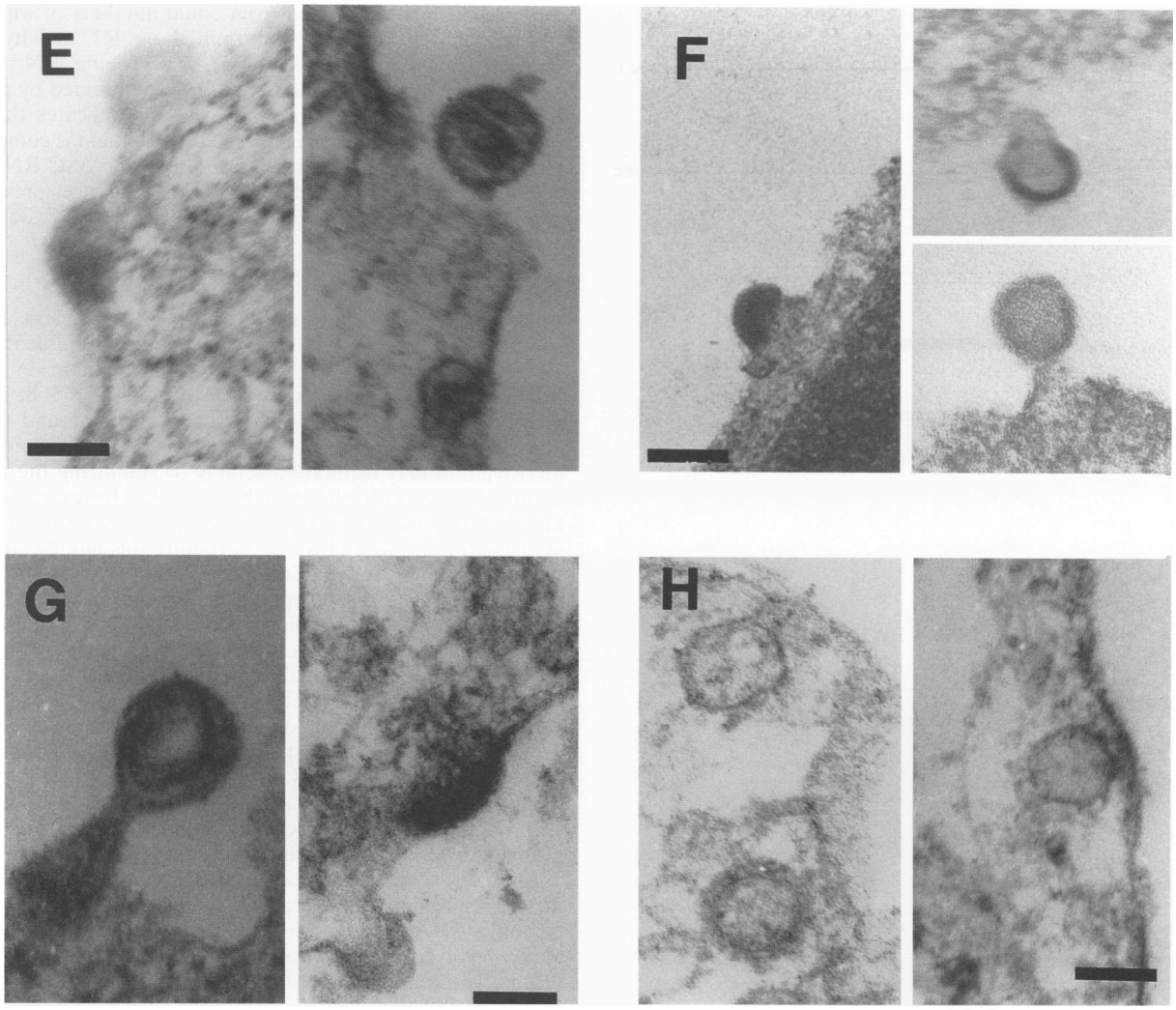

FIG. 6—*Continued.*

membrane-associated viral particles and electron-dense regions in the membrane reminiscent of wild-type HIV-1 assembly sites are detected (Fig. 6G). Since we found that all transfected cultures contain approximately the same amount of Gag precursors, the efficiency of normal viral budding is very low in pΔSC-transfected cells. We also observed intracellular virus-like particles, at a higher frequency than budding particles, in the ΔSC-transfected cells (Fig. 6H). We interpret these data as an indication of a predominantly aberrant assembly mechanism for the mutant particles, which nonetheless leads to the appearance of extracellular particles. However, we cannot rigorously exclude the possibility that only the few budding ΔSC particles are released from cells.

Matrix-deficient virions are noninfectious. To examine whether matrix-deficient virions are infectious, titers of culture supernatants from transfected cells were determined by the multinuclear activation of a β-galactosidase indicator assay (MAGI) (34). In order to score positives in this assay, only a single round of infection is required. HeLa CD4-LTR/βgal cells contain a β-galactosidase gene under control of the HIV-1 long terminal repeat. No β-galosidase expression can be detected in the absence of the HIV-1 *tat* gene product. Infection of CD4-LTR/βgal cells with wild-type HIV-1 or virus encoding the *tat* gene leads to activation of β-galactosidase, which can be monitored with a specific chromophore substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). COS7 cells were transfected with pBRU3, pΔSC, or pΔSCmyr. Viral supernatants were harvested 2 days later, filtered to remove cells, and used to infect CD4-LTR/βgal cells. Virus produced by wild-type pBRU3-transfected cells yielded an average of 4×10^4 infectious units of virus per ml. In contrast, no infectious particles could be detected from infections with mutant supernatants in repeated attempts (Table 1). In addition, infection of a human lymphocyte line (CEM×174) with mutant supernatant yielded no detectable viral proliferation after 6 weeks of cultivation (data not shown). The noninfectious phenotype of the mutants is probably due in part to the poor incorporation or retention of HIV-1 envelope. However, we cannot exclude other roles for MA at early steps in infection.

Genomic RNAs bearing a putative Ψ deletion are packaged efficiently. The RNA content of ΔSC mutant particles was examined to determine whether particles lacking matrix protein can specifically package viral genomic RNA and whether Gag proteins which assemble at aberrant locations may access and incorporate genomic RNA. Culture supernatants were collected from pΔSC- or wild-type-transfected cells and cen-

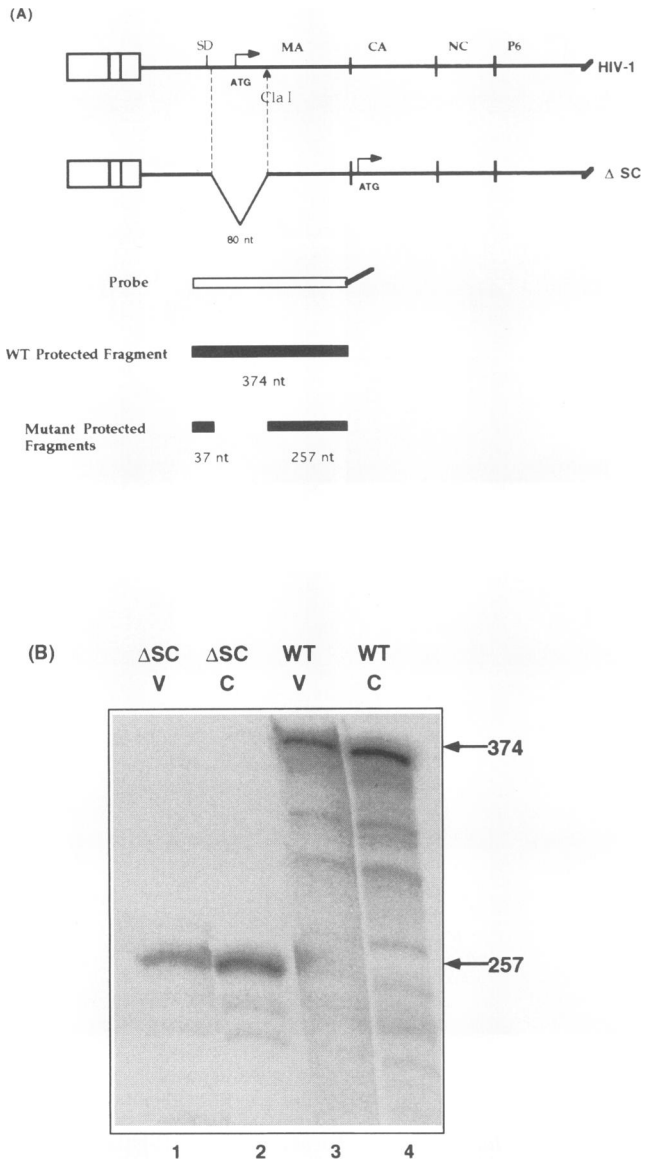

FIG. 7. Analysis of the cell and viral RNAs by an RNase protection assay. COS7 cells were transfected with either pBRU3 or pΔSC. Viral supernatants were collected 48 to 72 h posttransfection and pelleted through 20% sucrose cushions. Resuspended viral pellets were assayed for RT activity, and the same numbers of viral particles were assayed as determined by RT activity. (A) Origin of the ^{32}P-labeled probe and the expected sizes of the protected products; (B) lanes 2 and 4, RNA from cells transfected with either pΔSC or pBRU3; lanes 1 and 3, RNA extracted from supernatants of cells transfected with pΔSC or pBRU3. WT, wild type.

trifuged through 20% sucrose cushions. A uniformly labeled probe which should protect a 374-nt fragment from wild-type HIV-1 was used for the RNase protection assay (Fig. 7A). Since the probe spans the region deleted in ΔSC, two fragments of 37 and 257 nt should be protected in this case. Under the conditions of the assay, the smaller fragment cannot be detected. The results of the RNase protection are shown in Fig. 7B. In both wild-type-transfected cells and viral pellets, the predominant protected RNA is 374 nt, and in pΔSC-transfected cells and viral pellets, a 257-nt fragment is seen. In this experiment, RNA extracted from equal numbers of wild-type and mutant particles (as determined by RT activity) was assayed, and similar levels of HIV-1 specific RNA were detected per particle. Similar results were obtained by primer extension analysis (data not shown). This indicates that the truncated Gag precursor lacking the MA domain is competent for genomic RNA packaging. In addition, the ΔSC RNA can be packaged efficiently, although it lacks sequences previously implicated in HIV-1 genomic RNA packaging (1, 8, 36).

DISCUSSION

Analysis of the expression of two MA-deficient HIV-1 genomes in transfected cells has allowed us to examine the role of myristylation of Gag and the matrix region in virion formation and release. N myristylation of Pr^{Gag} has been shown to be essential for viral particle formation and release (4, 9, 15, 24, 29, 30, 47, 50, 58, 60). It has been speculated that myristylation may facilitate assembly by stabilizing the association of Gag with the membrane (4). However, type D Mason-Pfizer monkey virus Gag polyproteins do not require myristylation for assembly but do require this modification for release (51). Our study indicates that although myr(−)MA(−) Gag polyproteins are competent to assemble, albeit poorly, myristylation affects the efficiency of assembly or release. The function of myristylation could be to ensure that a high local concentration of Gag is maintained at the plasma membrane, permitting efficient assembly, and/or to create a hydrophobic surface on the exterior of the nascent particles, thereby providing the force for extrusion through the plasma membrane. Whether myristylation provides interaction with other membrane locations is unclear. The assembly of myr(−)MA(−) Gag may be a serendipitous event relying on a high localized level of mutant Gag molecules.

Both *cis* sequences located in genomic RNA and *trans* factors are required for specific and efficient packaging of viral genomic RNA during retroviral assembly. The *trans*-acting factor involved in retroviral genomic RNA packaging is the Gag polyprotein precursor in all the retroviral systems studied (reviewed in reference 39). While no specific regions of the Gag polyprotein have been unequivocally identified as required for specific recognition of the genomic RNA (14), the NC domain is clearly important (1, 18, 19, 44–46). Several studies using bovine leukemia virus (BLV) proteins have suggested that the BLV MA protein may be involved in specific binding to the 5′ end of the BLV genome (31, 32), although a direct role of BLV MA in packaging has not been demonstrated. In the case of avian retroviruses, an intact Pr^{Gag} molecule appears to be required for packaging (14, 46). For HIV-1 it has been shown that the Gag precursor protein specifically binds in vitro to the 5′ end of the viral genomic RNA (41). Although some of these results implicate an intact Gag precursor and perhaps MA in packaging, our system provides the first in vivo evidence as to whether the MA region is required in *trans* for viral RNA packaging. We have shown that the mutant virions contain wild-type levels of genomic RNA. Although we cannot rule out the possibility that the truncated p39/40 ΔSC precursor can bind to its deleted RNA genome but not to wild-type RNA, it is most likely that the MA domain of HIV-1 is not required for genomic RNA encapsidation.

The *cis* sequence is defined as the packaging sequence, Ψ, and has been identified at the 5′ end of the retroviral genome in both avian and murine retroviruses by deletion mutagenesis (reviewed in reference 39). Several deletion mutations in the 5′ leader regions, located between the HIV-1 5′ major splice

donor site and the Gag initiator codon of genomic RNA, which lead to the production of noninfectious virions have been created (1, 8, 36). A later report showed that one of the same HIV deletions generated a small amount of infectious particles (49). However, it has been presumed that these deletions eliminated or impaired the packaging of viral genomic RNA. The deletion that we have described is larger than and overlaps these deletions. Thus, we were surprised to find that there is as much mutant RNA per mutant particle as is found with wild-type virus. This strongly suggests that other regions in the genome can serve as Ψ signals.

The ability of the myr(−)MA(−) HIV-1 Gag polyprotein to assemble and the reported inability of myr(−)MA(+) Gag to assemble (4, 15, 24, 29, 58) suggest that the HIV MA region has a negative effect on particle assembly and that the removal of MA allows particles to assemble, possibly without a stable membrane interaction. We propose a model of retroviral assembly in which the path of assembly is influenced by the matrix domain. In the case of a type C retrovirus or lentivirus, the presence of MA constraints results in assembly occurring only after Gag associates with the plasma membrane. Prior to this membrane association event, the Gag MA domain prevents premature particle assembly. In contrast, Mason-Pfizer monkey virus MA is not usually constraining and assembly occurs intracellularly. N-terminal myristylation of Gag appears to ensure that a high local concentration of Gag is maintained at a cell membrane, thus potentiating the frequency of Gag-Gag interactions necessary to drive efficient assembly. In those cases where the MA region prohibits intracellular assembly, myristylation may relieve constraint by anchoring the Gag N terminus to the plasma membrane so intermolecular interactions with other Gag proteins can occur.

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