JOURNAL OF VIROLOGY, Nov. 1992, p. 6304–6313
0022-538X/92/116304-10$02.00/0

Vol. 66, No. 11

The Nonmyristylated Pr160$^{gag\text{-}pol}$ Polyprotein of Human Immunodeficiency Virus Type 1 Interacts with Pr55^{gag} and Is Incorporated into Viruslike Particles

JINSEU PARK AND CASEY D. MORROW*

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294-0007

Received 4 June 1992/Accepted 5 August 1992

The expression of the *pol* gene of human immunodeficiency virus type 1 occurs via a ribosomal frameshift between the *gag* and *pol* genes. The resulting protein, a Gag-Pol polyprotein, is produced at a level 5 to 10% of that of the Gag protein. The Gag-Pol polyprotein is incorporated into virions and provides viral protease, reverse transcriptase, and integrase, which are essential for infectivity. It is generally believed that the Gag-Pol polyprotein is incorporated into virions via interaction with the Gag protein, although the details of the mechanism are unknown. To further study this problem, we have constructed a human immunodeficiency virus type 1 proviral genome which overexpresses the Gag-Pol polyprotein (Pr160$^{gag\text{-}pol}$). Transfection of this proviral genome (pGPpr−) into COS-1 cells resulted in the expression of full-length Pr160$^{gag\text{-}pol}$ polyprotein. Although the majority of the Pr160$^{gag\text{-}pol}$ was confined to the cells, low levels of reverse transcriptase activity were detectable in the cell supernatants. The cotransfection of pGPpr− with a second plasmid which expresses only the Pr55^{gag} precursor (pGAG) resulted in a significantly higher level of Pr160$^{gag\text{-}pol}$ in the medium of transfected cells. Sedimentation analysis using sucrose density gradients demonstrated that most Pr160$^{gag\text{-}pol}$ was found in fractions corresponding to the density of virion particles, indicating that the Pr160$^{gag\text{-}pol}$ polyprotein was released in association with a Pr55^{gag} viruslike particle. To further characterize the requirements for the release, a mutation was constructed to express an unmyristylated Pr160$^{gag\text{-}pol}$ polyprotein. Coexpression with Pr55^{gag} demonstrated that the unmyristylated Pr160$^{gag\text{-}pol}$ was also incorporated into virion particles. Subcellular fractionation experiments revealed that the distributions of the Pr160$^{gag\text{-}pol}$myr− and Pr160$^{gag\text{-}pol}$ in the membrane and cytosol were similar under low- or high-ionic-strength conditions. Taken together, these results suggest that myristylation of the Pr160$^{gag\text{-}pol}$ polyprotein is not required for the interaction with the Pr55^{gag} necessary for packaging into a viruslike particle.

The genome of human immunodeficiency virus type 1 (HIV-1) contains three major genes, *gag*, *pol*, and *env*, which are common to all retroviruses (24, 31, 35, 44). The *gag* proteins are produced initially as a precursor protein, Pr55^{gag}, which is cleaved into the major *gag* proteins (p17 [MA], p24 [CA], and p15 [NC] [p9/p6]) by the HIV-1 protease during virion morphogenesis (5, 14, 23). The *pol* proteins are synthesized as a Gag-Pol polyprotein precursor (Pr160$^{gag\text{-}pol}$) which results from a ribosomal frameshift between the *gag* and *pol* genes (10, 15, 48). Because of a low frequency of frameshifting, this Pr160$^{gag\text{-}pol}$ polyprotein is synthesized at a level approximately 5 to 10% of the level of the Pr55^{gag} polyprotein, and it provides viral protease (p10), reverse transcriptase (p66/51), and integrase (18).

The Pr160$^{gag\text{-}pol}$ polyprotein plays a central role in the HIV-1 life cycle. Since it is difficult to detect this polyprotein in infected cells, previous studies have relied on the use of mutants that positioned the *gag* and *pol* genes in the same open reading frame (22, 25, 39). From the results of these studies, it was found that the expression of Pr160$^{gag\text{-}pol}$ resulted in the activation of the viral protease and subsequent proteolytic processing of the polyprotein. Cells overexpressing the Pr160$^{gag\text{-}pol}$ polyprotein did not produce immature virions or infectious virus (22, 25, 39). In contrast, cells expressing the *gag* precursor Pr55^{gag} produced and released immature or mature virions, depending on the presence of protease, even in the absence of the *env* gene product (12, 17, 40, 46).

Since the expression of Pr160$^{gag\text{-}pol}$ resulted in the activation of the viral protease and extensive proteolytic processing, it has been difficult to study the molecular features of the intact polyprotein. Early studies reported that the transfection of HIV-1 proviral or subviral genomes with mutations to inactivate the protease resulted in the production of immature virus particles containing low but significant levels of reverse transcriptase activity (22, 27, 28). These results suggested that Pr160$^{gag\text{-}pol}$ possesses reverse transcriptase activity, although the levels are considerably lower than that for the mature reverse transcriptase. The Gag-Pol polyprotein from murine leukemia virus has also been found to possess a low but significant level of reverse transcriptase activity (8).

The structural features of Pr160$^{gag\text{-}pol}$ which are essential for the interaction with Pr55^{gag} and targeting into virions are unknown. Both Pr55^{gag} and Pr160$^{gag\text{-}pol}$ are modified by the cotranslational addition of *N*-myristic acid to the amino-terminal glycine residue (4, 13, 25). Previous studies have found that myristylation of the Pr55^{gag} protein of HIV-1 is required for the assembly and release of immature virus particles (12, 13). The role that myristylation of Pr55^{gag} plays in assembly is not clear, although it has been suggested that myristylation facilitates the stable association of proteins with the membrane components (4). The myristylation of proteins, though, is not a general signal for the targeting of proteins to the plasma membrane. Indeed, all retroviral

* Corresponding author.

gag-related polyproteins are not myristylated, and many other myristylated proteins are not bound to the plasma membrane but are found in the cytosol, in the secretory pathway, or in the nucleus (1, 2, 6, 26, 43).

To further study the targeting of the $Pr160^{gag\text{-}pol}$ into HIV-1 virions, we constructed a proviral genome containing double mutations to position the *gag* and *pol* genes in the same translational reading frame and to inactivate the viral protease. The mutant proviral genome was transfected into cells, and the expression and release of $Pr160^{gag\text{-}pol}$ in the presence and absence of $Pr55^{gag}$ were determined. Oligonucleotide site-directed mutagenesis was used to create a mutation which removed the myristylation signal from the $Pr160^{gag\text{-}pol}$ polyprotein. The results of these experiments suggest that $Pr160^{gag\text{-}pol}$ is incorporated into virions and released from cells when coexpressed with $Pr55^{gag}$. Furthermore, the myristylation of $Pr160^{gag\text{-}pol}$ is not required for the protein-protein interactions between $Pr160^{gag\text{-}pol}$ and $Pr55^{gag}$ necessary for inclusion of Gag-Pol into viruslike particles.

MATERIALS AND METHODS

Materials. All chemicals, unless otherwise noted, were purchased from Sigma Chemical Co. Tissue culture reagents were obtained from GIBCO/BRL. Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs. The plasmid, pCH110, was purchased from Pharmacia, and the anti-β-galactosidase antibody was obtained from Promega. The reagents for polymerase chain reaction were purchased from Perkin-Elmer Cetus. Sequenase enzyme was obtained from U.S. Biochemicals. The [^{35}S]methionine (Translabel) was purchased from ICN; [α-^{35}S]ATP was obtained from Amersham, Inc.; [9,10-^{3}H(N)]myristic acid and [α-^{35}S]TTP were purchased from Dupont-NEN. The immunoassay for HIV-1 p24 was obtained from Abbott Laboratories. The DNA oligonucleotides used in this study were obtained from the University of Alabama at Birmingham (UAB) Cancer Center Oligonucleotide Synthesis Core facility. The antibodies specific for HIV-1 p24/25 proteins were obtained from the NIH AIDS Repository (41), while sera from AIDS patients were obtained through the Center for AIDS Research, UAB.

Methods: tissue culture cells. The COS-1 cells were maintained in Dulbecco modified Eagle medium (DMEM) with 5% newborn calf serum–5% fetal calf serum (complete medium).

Construction of mutations in the HIV-1 proviral genome. The majority of the techniques for isolation and manipulation of plasmids DNAs were done by standard protocols (21). For site-directed oligonucleotide mutagenesis, DNAs containing nucleotides 1 to 2201 of the wild type or the genome containing a mutation at the site of ribosomal frameshifting (FS2) were used (25). The DNAs containing an *Eco*RI-*Bal*I restriction fragment were ligated into the *Eco*RI-*Sma*I site of the replicative form of M13mp19 and transformed into the *dut*-minus *ung*-minus mutant host CJ236. The resultant uracil-rich single stranded DNA was used as the template for oligonucleotide site-directed mutagenesis (19).

Two different oligonucleotide site-directed mutants were constructed with the wild-type or FS2 templates. The first mutant was designed to inactivate the viral protease by a substitution of an asparagine for the aspartic acid (amino acid 25) by using the following oligonucleotide: 5′-GAAGC TCTATTAAATACAGGAGCAG-3′. A second mutant was constructed to substitute an alanine for the glycine amino acid at the second position of the *gag* gene by using the following oligonucleotide: 5′-GGAGAGAGATGGCTGCG AGAGCGTC-3′. Previous studies have established that expression of *gag* genes containing this mutation prevents cotranslational myristylation (4, 13). The conditions for mutagenesis were as previously described (19). Following mutagenesis and transformation into *Escherichia coli* DH5αF′, the desired recombinant phages were screened for the presence of the correct mutation by the dideoxynucleotide chain termination method for sequencing DNA (36). The resulting phages were designated M13HXBPRO(−) and M13FS2MYR(−).

Construction of HIV-1 proviral genomes for expression of *gag* and *gag-pol* precursor proteins. The plasmid pHXB2 gpt contains a complete HIV-1 proviral genome which is infectious even though it does not encode a Vpu protein (30). We will refer to this plasmid as pHXB2. The construction of pHXB2FS2, in which the *gag* and *pol* genes are in the same translational reading frame, has also been described in detail (25). To reconstruct the protease or myristylation mutations into these proviral genomes, polymerase chain reaction was used to amplify DNA flanking the region of the mutation (34). For the construction of plasmids containing the protease mutation, a *Bss*HI (nucleotide 255)-to-*Bcl*I (nucleotide 2009)-amplified DNA fragment from the replicative form of M13HXBPRO(−) was ligated into the proviral genomes; for the mutation to prevent myristylation of the Gag and Gag-Pol polyproteins, a *Bss*HII (nucleotide 255)-to-*Cla*I (nucleotide 375)-amplified DNA fragment from the replicative form of M13FS2MYR(−) was ligated into similarly digested pHXB2 and pHXB2FS2. The plasmids containing the mutation were screened by restriction digestions, followed by direct sequencing using the dideoxy sequencing method (36). The proviral genomes with a mutation in the protease gene are identified as pro−, while those with a mutation designed to prevent myristylation are denoted by myr−.

To construct HIV-1 proviral genomes which would express unprocessed $Pr55^{gag}$ polyprotein, pHXB2 was digested with *Bcl*I and the resulting ends were filled in by using avian myeloblastosis virus reverse transcriptase. The plasmid was digested with *Bal*I, the resulting blunt ends were ligated with T4 ligase, and the plasmid was transformed into *E. coli*. The resulting plasmid, named pGAG, contains a deletion between nucleotides 2009 and 4133; this deletion results in the loss of the coding region for the majority of the *pol* gene so that no functional protease, reverse transcriptase, or integrase is expressed. The construction of the mutant to remove the myristylation signal in pGAG was performed by the *Bss*HII-*Cla*I DNA fragment exchange as described for the plasmid containing the wild-type proviral genome. The resulting plasmid was named pGAGmyr−.

DNA transfections and metabolic labelings. The COS-1 cells were passaged 24 h prior to transfection and were 50% confluent at the time of transfection. Transfections were performed with 5 μg of CsCl-purified plasmid DNA per ml by using DEAE-dextran (molecular mass, 500,000 Da) at 500 μg/ml as a facilitator (20, 25). After 3 h with the DNA–DEAE-dextran mixture, the cells were washed and placed in complete medium with chloroquine (20 μg/ml) for an additional 2 h. Following a 10% dimethyl sulfoxide shock for 2 min, the cells were washed with DMEM and incubated at 37°C for 48 h in complete medium.

For metabolic labeling, the transfected COS-1 cells were washed with DMEM and incubated for 1 h in DMEM minus methionine. The medium was then removed and replaced

with DMEM minus methionine supplemented with [^{35}S]methionine (Translabel) at 50 μCi/ml. For labeling with [^{3}H]myristic acid, the cells were incubated in complete medium with [^{3}H]myristic acid (100 μCi/ml) for 4 h as previously described (33). After the labeling period, the medium was removed and the cells were washed once with DMEM. The cells were lysed by incubation in radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 150 mM NaCl, 25 mM Tris-HCl [pH 7.34]) on ice for 5 min. Following removal of high-molecular-weight DNA and cell debris by centrifugation, the specified antibodies were added and allowed to react at 4°C for 12 h. The immunoprecipitates were collected by addition of protein A-Sepharose (20 μl of a 1:10 dilution [wt/vol] in RIPA buffer) for 1 h at room temperature, followed by centrifugation and three washes with RIPA buffer. To release the immunoprecipitates, the beads were boiled in gel sample loading buffer (50 mM Tris-HCl [pH 6.8], 5% SDS, 5% β-mercaptoethanol, and 0.1% bromophenol blue) for 5 min and then briefly centrifuged. The immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography and subsequent autoradiography using Kodak X-Omat AR film with intensifier at −70°C. In some instances, the radioactivity was also quantitated with a Phosphorimager system (Molecular Dynamics).

Density gradient analysis of virus from transfected cells. The supernatants from the COS-1 cells mock transfected or transfected with plasmids containing the HIV-1 proviral genomes were collected at 2 days posttransfection. The medium was clarified by centrifugation at 1,000 × *g* for 5 min, followed by filtration through a 0.2-μm-pore-size Acrodisc (Gelman Sciences). The supernatant was layered on 4 ml of 20% sucrose cushion and centrifuged in an SW41 rotor at 120,000 × *g* for 2 h. The pellet was resuspended in 200 μl of phosphate-buffered saline (PBS) and stored at −20°C for further assay. In some cases, the pelleted material was layered on top of a 20 to 60% sucrose gradient. After centrifugation at 100,000 × *g* for 18 h in an SW41 rotor, 24 equal fractions were collected starting from the bottom of the tube. Sucrose densities for each fraction were determined with a refractometer.

Subcellular fractionations. All cell fractionations were performed at 4°C by previously described techniques (4, 32). The transfected COS-1 cells were metabolically labeled with 100 μCi of [^{35}S]methionine per ml for 2 h. The labeled cells were washed with cold PBS and scraped into swelling buffer (10 mM KCl, 20 mM Tris-HCl [pH 7.8], 1 mM EDTA, 0.1% 2-mercaptoethanol, 1 μM aprotinin). After 20 min at 4°C, the cells were lysed with 50 strokes of a tight-fitting B glass Dounce homogenizer. The nuclei were pelleted by centrifugation for 10 min at 1,000 × *g* after the final salt concentration was adjusted to 0.15 M NaCl. The supernatant from this spin was centrifuged at 100,000 × *g* for 30 min to prepare membrane and soluble fractions. The resulting supernatant fraction and membrane fraction were designated S-100(−) and P-100(−), respectively, to indicate low-ionic-strength conditions. The membrane fraction, P-100(−), was resuspended in swelling buffer, and half of the fraction was saved for immunoprecipitation. The remaining half fraction was adjusted to 1 M NaCl and centrifuged again at 100,000 × *g* for 30 min. The new fractions were designated P-100(+) and S-100(+), respectively, to indicate the high-ionic-strength conditions. The cytosolic fractions were adjusted with 5× RIPA buffer to make a 1× RIPA buffer final concentration. The membrane fractions were resuspended directly in 1× RIPA buffer. The HIV-1 proteins were then immunoprecipitated with anti-p24/25 antibody.

Reverse transcriptase assays. For the analysis of virion-associated reverse transcriptase activity, 25-μl samples from the pelleted particles were placed in a well of a 96-well plate and 75 μl of a reaction cocktail containing 50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 5 mM $MgCl_2$, 150 mM KCl, 0.1% Triton X-100, 0.5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 1.25 μg of poly(A) · oligo(dT), and 5 μCi of [α-^{35}S]TTP (25). The reaction was allowed to proceed for 90 min at 37°C, and then the mixture was directly spotted onto NA-45 paper (Schleicher & Schuell). The samples were washed once with 0.2 M $NaPO_4$ (pH 7.5) and air dried, and the radioactivity was quantitated with an AMBIS densitometer.

p24 antigen capture assay. The p24 antigen assay was performed by using a kit by Abbott Laboratories according to the manufacturer's instructions. An assay with known amounts of p24 was used to generate a standard curve in which to extrapolate the samples. All samples were diluted so that the values obtained were in the linear portion of the standard curve.

RESULTS

Characterization of HIV-1 provirus with a defective protease. To characterize the Gag-Pol polyprotein, we constructed a mutation in the infectious HIV-1 proviral genome (pHXB2) (30) which resulted in the inactivation of HIV-1 protease (pHXB2pro−) (Fig. 1). The expression of HIV-1-specific proteins in COS-1 cells transfected with the wild-type or mutant proviral genome was analyzed 48 h posttransfection by immunoprecipitation using anti-p24 antibody or anti-reverse transcriptase antibody (Fig. 2). In the cells transfected with pHXB2 (wild type), the proteolytically processed product p24 and the *gag* gene precursor, p55, were immunoprecipitated. In contrast, the unprocessed $Pr55^{gag}$ precursor and a larger protein consistent with the molecular mass of the $Pr160^{gag\text{-}pol}$ polyprotein were detected in cells transfected with protease mutant pHXB2pro−. The proteins in the lysates were also immunoprecipitated with antibodies specific for reverse transcriptase. In the cells transfected with pHXB2 (wild type), $Pr160^{gag\text{-}pol}$ and p66 (reverse transcriptase) as well as several probable proteolytic processing intermediates were evident, while only $Pr160^{gag\text{-}pol}$ was detected from the cells transfected with pHXB2pro− (Fig. 2A). The medium from the transfected cells was next analyzed for release of HIV-1 virus particles. For these studies, we first clarified the medium by a low-speed centrifugation, followed by ultracentrifugation through a 20% sucrose cushion. The pelleted viral proteins were then analyzed by immunoprecipitation with anti-p24 or anti-reverse-transcriptase antibodies. We readily detected the proteolytically processed *gag* product, p24, in the pellets of cells transfected with pHXB2. In contrast, predominantly unprocessed $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ were immunoprecipitated from the pellets of cells transfected with pHXB2pro−. At present, we do not know whether the additional proteins immunoprecipitated with the anti-Gag antibodies are a result of de novo synthesis (23) or proteolytic processing by cellular proteases (14). Using anti-reverse transcriptase antibodies, we detected the mature reverse transcriptase, p66, in virions derived from transfection of pHXB2 (wild type), while only $Pr160^{gag\text{-}pol}$ was detected in virions from pHXB2pro− (Fig. 2B). These results indicated that the $Pr160^{gag\text{-}pol}$ expressed from pHXBpro− was incorporated

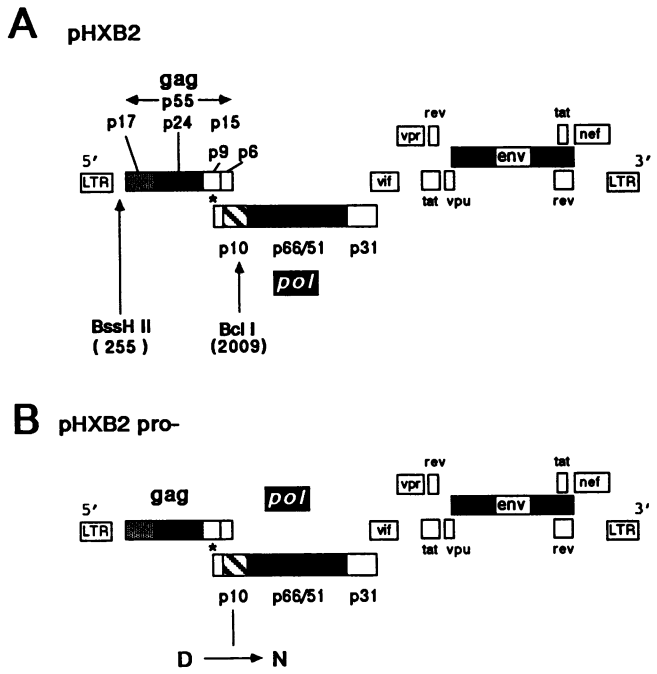

FIG. 1. HIV-1 proviral genome and location of the mutation in the protease gene. (A) HIV-1 proviral genome. The organization of the HIV-1 proviral genome is shown with the relevant restriction sites, *Bss*HII at nucleotide 255 and *Bcl*I at nucleotide 2009. The expression of the *gag* gene results in the synthesis of a precursor protein, pr55, which is subsequently processed by the viral protease, p10, to release the mature *gag* proteins (p17, p24, p9, and p6). The translational reading frame of the *pol* gene is in a minus one position from the *gag* gene, and expression occurs via a ribosomal frameshift to produce a *gag-pol* polyprotein which is subsequently processed by the viral protease to produce mature *gag* and *pol* gene products (viral protease [p10], reverse transcriptase [p66/p51], and integrase [p31]). The asterisk refers to the position at which ribosomal frameshift occurs (nucleotide 1635). (B) pHXBpro−. A point mutation was introduced by site-directed mutagenesis to change the aspartic acid in the active site of protease to the asparagine. LTR, long terminal repeat.

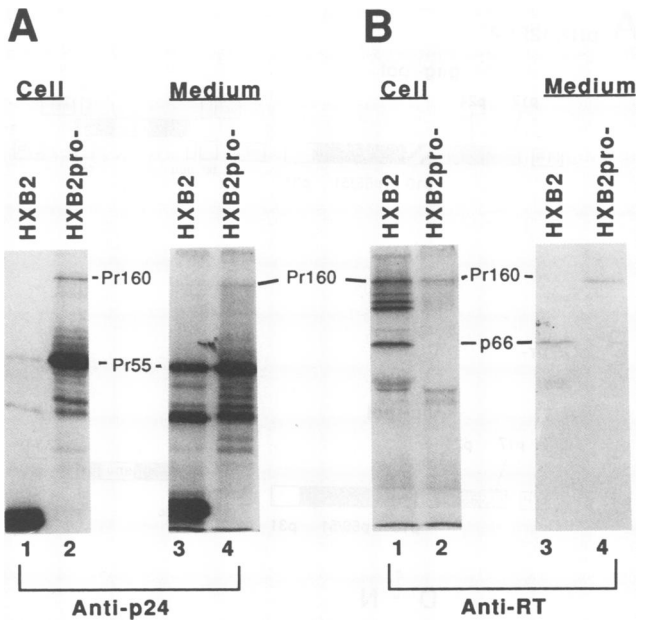

FIG. 2. Analysis of HIV-1-specific proteins from COS-1 cells transfected with wild-type or mutant proviral genomes. COS-1 cells were transfected with the designated plasmids. At 48 h posttransfection, the cells were metabolically labeled with [^{35}S]Translabel (100 μCi/ml) for 15 h. The cell lysates were analyzed by immunoprecipitation with rabbit anti-p24 antibody (A) or anti-reverse transcriptase antibody (B) followed by SDS–10% polyacrylamide gel electrophoresis and autoradiography. The media from COS-1 transfected cells which had been metabolically labeled with [^{35}S]Translabel were clarified by a low-speed centrifugation followed by ultracentrifugation through a 20% sucrose cushion. The pellets were resuspended in RIPA buffer and analyzed by immunoprecipitation.

into virion particles which could be pelleted through a sucrose cushion. The virus particles from the cells transfected with wild-type or mutant provirus were next analyzed for in vitro reverse transcriptase activity. As expected, the virus from cells transfected with pHXB2 had substantial enzymatic activity, while the pelleted viruslike particles from cells transfected with pHXB2pro− contained reverse transcriptase activity at a level generally 1/10 of that of the wild type (data not shown).

Expression and characterization of Gag and Gag-Pol polyprotein from pGAG and pGAGPOL. To investigate the requirements for incorporation of the Pr160$^{gag\text{-}pol}$ polyprotein into virion particles, we constructed mutants which independently expressed either the Pr55^{gag} or the Pr160$^{gag\text{-}pol}$ polyprotein. Previously, a proviral genome (pHXB2FS2) was created by insertion of a single-base adenine at the site of ribosomal frameshifting to position the *gag* and *pol* genes in the same translational reading frame (25). To inactivate the protease, a second mutation in which the aspartic acid was changed to asparagine was constructed in pHXB2FS2 (9, 18); the resulting plasmid was named pGPpr− (Fig. 3). A proviral mutant which expressed the Pr55^{gag} polyprotein in the absence of *pol* gene products was constructed by deletion of the wild-type proviral DNA such that no functional protease, reverse transcriptase, or integrase would be expressed (pGAG). Previous studies have established that myristylation of the Pr55^{gag}, and possibly Pr160$^{gag\text{-}pol}$, was essential for the formation of infectious virions (4, 13). To evaluate the effect of myristylation in our system, two proviral genomes in which the second amino acid glycine was mutated to an alanine (pGAGmyr− and pGPpr−myr−) were constructed. Previous studies have found that this mutation prevents the myristylation of the *gag*-related proteins from the HIV-1 proviral genome (4, 13).

The expression of polyproteins was analyzed by metabolic labeling of COS-1 cells with [^{35}S]methionine and immunoprecipitation of HIV-1-specific proteins from cell lysates with anti-p24 antibody 2 days posttransfection (Fig. 4A). The cells transfected with pGAG and pGAGmyr− synthesized the Pr55^{gag} polyprotein, while cells transfected with pGPpr− or pGPpr−myr− expressed the Pr160$^{gag\text{-}pol}$ polyprotein. These results demonstrated that unprocessed Pr55^{gag} and Pr160$^{gag\text{-}pol}$ polyproteins were exclusively expressed from separate proviral genomes and that removal of the myristylation signal from pGAG or pGPpr− did not affect the expression of individual polyproteins. For the analysis of myristylation of the Pr55^{gag} and Pr160$^{gag\text{-}pol}$ polyproteins, the COS-1 cells were transfected with myr+ or myr− pGAG and pGPpr− proviral mutants and metabolically labeled with [^{3}H]myristic acid and proteins in cell lysates were immunoprecipitated with serum from AIDS patients (Fig. 4B). The

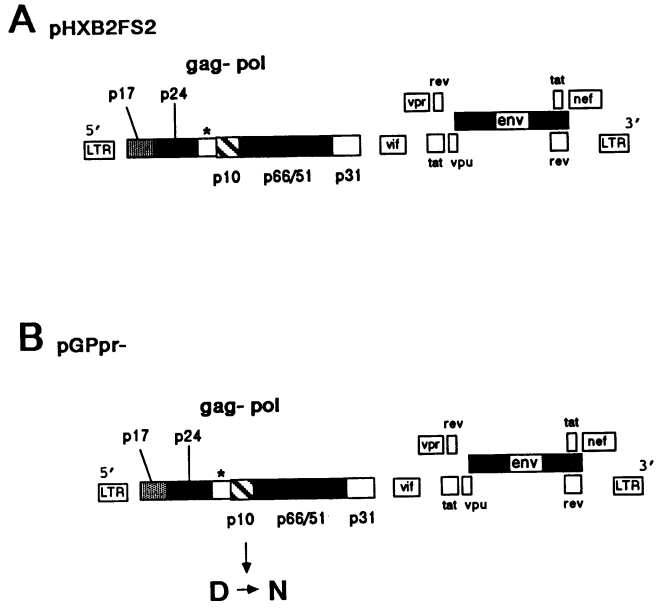

FIG. 3. HIV-1 proviral genomes which express $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$. (A) The proviral genome in which the *gag* and *pol* genes are positioned in the same translational reading frame is depicted. pHXB2FS2 was constructed by insertion of a single adenine residue at the proposed site of ribosomal frameshifting at nucleotide 1635, which results in a *gag-pol* polyprotein identical to that from the wild-type genome (25). (B) pGPpr− was constructed by introducing a second mutation in the protease gene into pHXB2FS2. By site-directed mutagenesis on the protease gene, the codon for aspartic acid in the active site of protease was changed to asparagine. (C) pGAG was made by deletion of the *BclI-BalI* fragment from pHXB2 (wild type), which deletes most of *pol* gene (nucleotides 2009 to 4133). The mutations to change the codon for the second amino acid in pGPpr− and pGAG from glycine to alanine are described in the text. LTR, long terminal repeat.

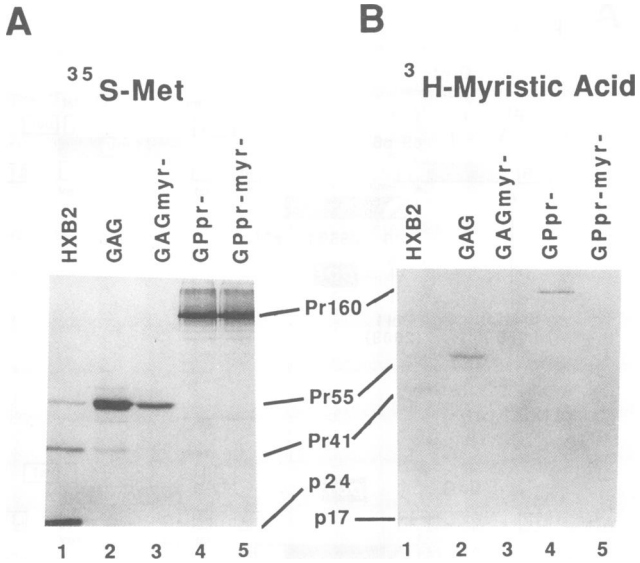

FIG. 4. Analysis of $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ proteins expressed in COS-1 cells transfected with wild-type or mutant proviral genomes. (A) COS-1 cells were transfected with the designated plasmids for 48 h and then metabolically labeled with [^{35}S]Translabel (50 μCi/ml) for 4 h. The intracellular proteins were then immunoprecipitated with rabbit anti-p24 antibody and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The relevant proteins are noted. Lane 1, pHXB2 (wild type); lane 2, pGAG; lane 3, pGAGmyr−; lane 4, pGPpr−; lane 5, pGPpr−myr−. (B) Myristylation of HIV-1 proteins expressed in COS-1 cells transfected with wild-type or mutant proviral genomes. COS-1 cells transfected with the designated plasmids were metabolically labeled with [^{3}H]myristic acid. Following labeling, the cell extracts were immunoprecipitated with pooled sera from AIDS patients and analyzed by SDS–12% polyacrylamide gel electrophoresis and subsequent autoradiography. The relevant proteins are noted. Lanes are as in panel A.

$Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ polyproteins expressed by myr+ proviral mutants could readily be detected by metabolic labeling with [^{3}H]myristic acid, while those polyproteins expressed by the myr− proviral mutants, which lack a myristylation signal, were not detected. Thus, the removal of the myristylation signal in both pGAG and pGPpr− effectively eliminated the cotranslational addition of myristic acid to these proteins.

Incorporation of $Pr160^{gag\text{-}pol}$ into viruslike particles. To determine whether the $Pr160^{gag\text{-}pol}$ polyprotein can be incorporated into virion particles and released, experiments in which pGAG and pGPpr− were cotransfected into COS-1 cells were performed. At 48 h posttransfection, the cells were metabolically labeled, and the intracellular proteins were analyzed by immunoprecipitation with anti-p24 antibodies (Fig. 5A). The expression of the $Pr55^{gag}$ precursor and $Pr160^{gag\text{-}pol}$ was evident in the cells transfected with pHXB2pro−. The $Pr55^{gag}$ precursor was present in cells transfected with pGAG or pGAGmyr−, and the $Pr160^{gag\text{-}pol}$ precursor was present in cells transfected with pGPpr− or pGPpr−myr−. The media from the transfected cells were analyzed by ultracentrifugation through a 20% sucrose cushion followed by immunoprecipitation with anti-p24 antibodies (Fig. 5B). We detected both $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ from cells transfected with pHXB2pro−. Only $Pr55^{gag}$ was present in the medium from cells transfected with pGAG, and consistent with the results of previous studies, very low levels of the unmyristylated $Pr55^{gag}$ were released from the transfected cells (13). No $Pr160^{gag\text{-}pol}$ was detected in the medium of cells transfected with pGPpr− or pGPpr−myr−. The cotransfection of pGAG and pGPpr− resulted in levels of $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ immunoprecipitated from the medium of cells nearly identical with that obtained from the transfection with pHXB2pro−. Similar levels of the $Pr160^{gag\text{-}pol}$ polyprotein were also detected from the medium of cells cotransfected with pGAG and pGPpr−myr−. Using the Phosphorimager, we quantitated the amounts of radioactivity in the bands corresponding to $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$. Setting the ratio of $Pr55^{gag}$ to $Pr160^{gag\text{-}pol}$ found in pHXBpro− at 100%, we determined that the ratio of the $Pr55^{gag}$ to $Pr160^{gag\text{-}pol}$ for the cotransfected pGAG/pGPpr− and pGAG/pGPpr−myr− ranged from 67 to 75%. Interestingly, we also observed a slight increase in the levels of $Pr55^{gag}$myr− in the medium of cells cotransfected with

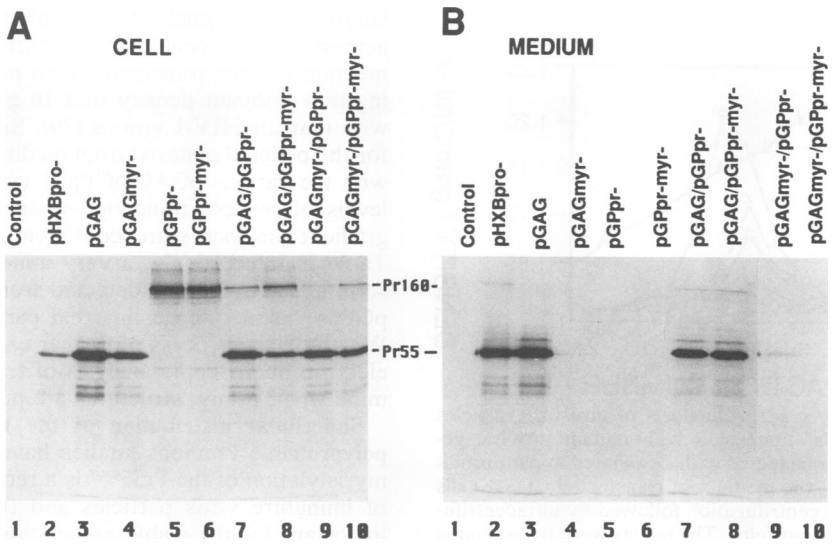

FIG. 5. Coexpression of $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$. COS-1 cells were transfected with individual plasmids or cotransfected with the designated plasmids. After 48 h, the cells were metabolically labeled with [^{35}S]Translabel. After 15 h, the cell lysates or supernatants were analyzed by immunoprecipitation with anti-p24 antibodies and SDS-polyacrylamide gel electrophoresis with subsequent autoradiography. (A) Intracellular proteins immunoprecipitated. The appropriate viral proteins are noted. (B) Labeled particles in the medium were ultracentrifuged through a 20% sucrose cushion, lysed in RIPA buffer, and analyzed by immunoprecipitation with rabbit anti-p24 antibody. The relevant proteins are noted. Control, mock transfected.

pGAGmyr−/pGPpr− compared with those in the medium of cells transfected with pGAGmyr− alone. Furthermore, the medium of cells cotransfected with pGAGmyr−/pGPpr− contained approximately three times the amount of $Pr55^{gag}$ (as determined by the Phosphorimager) than that from cells cotransfected with pGAGmyr−/pGPpr−myr−. These results suggest that $Pr160^{gag\text{-}pol}$ has the capacity to interact with $Pr55^{gag}$myr− resulting in the release of low levels of $Pr55^{gag}$ into the medium.

Analysis of reverse transcriptase activity from virions obtained from cotransfected cells. To further characterize the complementation of the proviral genomes expressing $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$, we analyzed the released virus particles for reverse transcriptase activity. For these studies, viruses in the medium from cotransfected cells were pelleted through a 20% sucrose cushion prior to analysis for in vitro enzyme activity (Table 1). The pellets from medium of cells transfected with pHXB2pro− contained significant levels of reverse transcriptase activity. In contrast, the pellets from cells transfected with pGAG alone gave background levels of activity. A low but significant level of activity was detected in the medium of cells transfected with pGPpr− alone. The normalized reverse transcriptase activity (counts per minute per nanogram of p24) from cells cotransfected with pGAG/pGPpr− was lower than that of cells transfected with the control plasmid pHXB2pro− but clearly above the background levels obtained from analysis of the medium of cells transfected with pGAG. The normalized level of reverse transcriptase activity obtained from cultures cotransfected with pGAG/pGPpr−myr− was comparable to that for cells transfected with pGAG/pGPpr−. This demonstrated that $Pr160^{gag\text{-}pol}$myr− was released from the cells in association with $Pr55^{gag}$ which could be pelleted by ultracentrifugation. The level of reverse transcriptase activity obtained from the medium of cultures cotransfected with pGAGmyr−/pGPpr− varied from experiment to experiment; sometimes these values were no different from that for pGPpr− alone, while in a few instances increased activity was detected. In all cases tested, though, the levels obtained from the medium of cells transfected with pGAGmyr−/pGPpr− were lower than those from the medium of cells transfected with pGAG/pGPpr−myr−. The requirement for myristylation was evident in that cotransfection of pGAGmyr−/pGPpr−myr−

TABLE 1. Analysis of reverse transcriptase activity from media of COS-1 cells transfected with mutant proviral genomes[a]

DNA	Reverse transcriptase activity	
	Total cpm[b]	cpm/ng of p24[c]
None	529	ND
pHXBpro−	54,428	117,122
pGAG	583	ND
pGPpr−	6,341	ND
pGAGmyr−	459	ND
pGPpr−myr−	835	ND
pGAG/pGPpr−	154,163	83,784
pGAGmyr−/pGPpr−	3,944	ND
pGAG/pGPpr−myr−	52,002	60,467
pGAGmyr−/pGPpr−myr−	687	ND

[a] COS-1 cells were transfected with the designated plasmids or cotransfected with equal amounts of the designated plasmids. At 48 h posttransfection, the culture media were clarified by a low-speed centrifugation followed by ultracentrifugation through a 20% sucrose cushion as described in Materials and Methods. The values are from a single experiment. Two subsequent experiments using the same DNA preparations gave a similar pattern of results.

[b] The reverse transcriptase activities of the ultracentrifuged pellets were measured as described in Materials and Methods. The values represented are for the total sample (counts per minute of [^{35}S]TTP incorporated using the standard assay).

[c] The amounts of p24 antigens in the medium of cells transfected with the designated plasmids were determined by antigen capture assay. The amounts of reverse transcriptase were normalized to p24 antigen detected in the culture medium. ND, not determined.

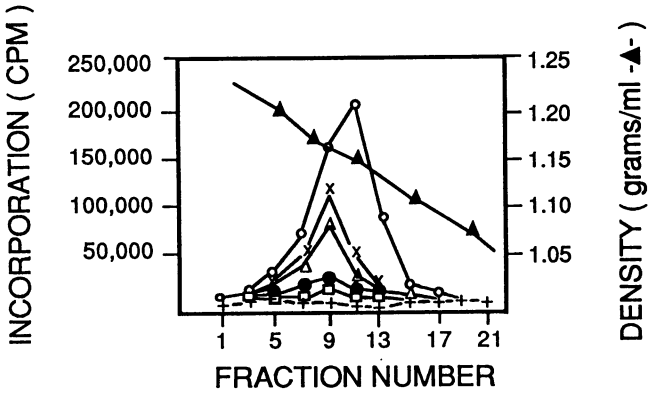

FIG. 6. Sucrose density gradient analysis of viruslike particles released from COS-1 cells transfected with mutant proviral genomes. COS-1 cells were transfected with designated mutant proviral genomes. After 3 days, the medium of transfected COS-1 cells was clarified by low-speed centrifugation followed by ultracentrifugation to pellet the viruslike particles. The pellets were resuspended in PBS and layered into a 20 to 60% linear sucrose density gradient. Following centrifugation, 24 fractions were collected from the bottom of the tube and odd-numbered fractions were analyzed for reverse transcriptase as described in Materials and Methods. The density of each fraction was determined by a refractometer. The gradient samples are presented from the bottom (left) to the top (right). ○, pHXB2pro−; ×, pGAG/pGPpr−; △, pGAGmyr+/pGPpr−myr−; ●, pGAGmyr−/pGPpr−myr+; □, pGPpr−; +, pGAG.

resulted in no reverse transcriptase activity detected in the medium over the background level.

To further characterize the association of $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ in the released particles, the pelleted material from the medium of transfected cells was analyzed by sucrose density gradients (Fig. 6). A single peak of enzyme activity was detected for the particles obtained from the medium of cells transfected with pHXB2pro− corresponding to a buoyant density of 1.16 g/ml, which is consistent with that for HIV-1 virions (29). Similar peaks were found for the pelleted material from medium of cells cotransfected with the various pGAG/pGPpr− combinations. The overall levels of reverse transcriptase activity detected from the gradient analysis were consistent with that presented in Table 1. Interestingly, a very minor peak of reverse transcriptase activity was detected from cells transfected with pGPpr− alone, suggesting that particles might be present. We are in the process of further exploring this result by an electron microscopic analysis of transfected cells to determine what, if any, structures are present.

Subcellular distribution of the $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ polyproteins. Previous studies have demonstrated that the myristylation of the $Pr55^{gag}$ is a requirement for the release of immature virus particles and that the myristylation is important for the stable association of the $Pr55^{gag}$ protein with intracellular membranes (4, 12, 13). To extend these studies, we performed similar experiments for the $Pr55^{gag}$ expressed from transfection of pGAG and pGAGmyr− (Fig. 7A). For an internal control, we cotransfected the cells with a plasmid which expresses β-galactosidase (pCH110). We utilized the Phosphorimager to quantitate the amount of β-galactosidase in the soluble and pellet fractions. Consistent with the results of previous studies, we found that over 80% of the β-galactosidase partitioned to the soluble fraction, indicative of a cytoplasmic location (16). Upon the initial fractionation of the transfected cells into cytoplasmic and membrane fractions, a small difference was observed in the distribution between the wild-type and nonmyristylated $Pr55^{gag}$ proteins. The pellets were subjected to a high-salt wash to remove loosely bound $Pr55^{gag}$, followed by recen-

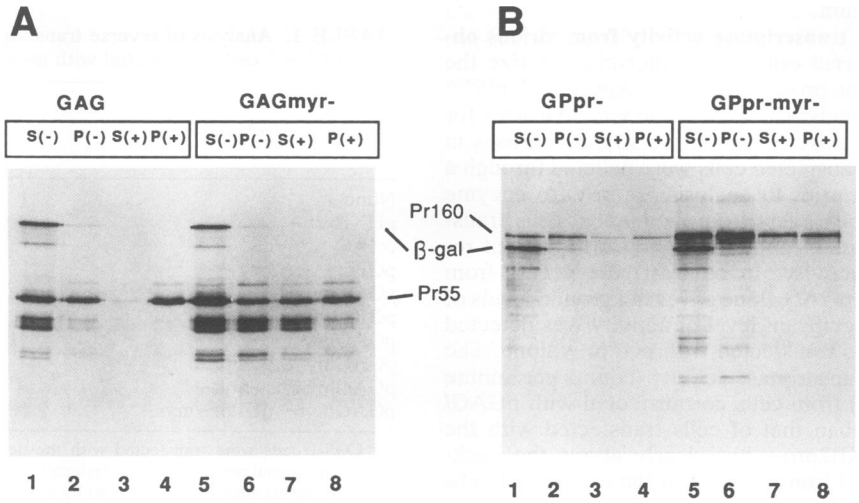

FIG. 7. Subcellular localization of $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ polyproteins in COS-1 cells transfected with mutant proviral genomes. (A) COS-1 cells were cotransfected with pCH110 and pGAG (lanes 1 to 4) or pCH110 and pGAGmyr− (lanes 5 to 8) plasmids. At 48 h posttransfection, transfected cells were labeled with [^{35}S]methionine for 2 h. The labeled cells were fractionated into cytosolic and membrane fractions under low-ionic-strength conditions (0.15 M NaCl in swelling buffer) as described in Materials and Methods. The resulting fractions were designated S(−) (cytosolic) and P(−) (membrane). The P(−) fractions were resuspended in swelling buffer and recentrifuged at 100,000 × *g* for 30 min under high-ionic-strength conditions (1 M NaCl in swelling buffer). The resulting fractions were designated S(+) (soluble) and P(+) (pellet). Each fraction was immunoprecipitated with rabbit anti-p24 antibody and monoclonal anti-β-galactosidase antibody and analyzed by SDS-polyacrylamide gel electrophoresis. The $pr55^{gag}$ and β-galactosidase proteins are noted. (B) COS-1 cells were cotransfected with pGPpr− and pCH110 plasmids (lanes 1 to 4) or pGPpr−myr− and pCH110 (lanes 5 to 8), and the expressed $Pr160^{gag\text{-}pol}$ and β-galactosidase were analyzed by subcellular fractionation and immunoprecipitation as described for panel A. The relevant viral proteins are noted.

trifugation and immunoprecipitation. In this case, a greater difference was observed between the wild-type and nonmyristylated $Pr55^{gag}$ proteins as evident by partitioning of wild-type $Pr55^{gag}$ to predominantly the membrane (pellet) fraction (80% by Phosphorimager), while nonmyristylated protein was found equally in the soluble and pellet fractions. We next performed the same subcellular fractionation experiments with wild-type and nonmyristylated $Pr160^{gag\text{-}pol}$ polyproteins (Fig. 7B). A small difference was observed in the distribution of $Pr160^{gag\text{-}pol}$ found in the high-salt washes between the cells transfected with pGPpr− and pGPpr−myr−. Quantitation of the amounts by Phosphorimager revealed that 60% of the $Pr160^{gag\text{-}pol}$ and 50% of the $Pr160^{gag\text{-}pol}$myr− associated with the membranes after a high-salt wash. Interestingly, this slight difference observed in the association with intracellular membranes correlated with the difference in the levels of reverse transcriptase measured from the medium of cells transfected with pGAG/pGPpr− and pGAG/pGPpr−myr−. Taken together, these results demonstrate that myristylation of the $Pr160^{gag\text{-}pol}$ is not absolutely required for the stable association with intracellular membranes or association with $Pr55^{gag}$ needed for inclusion in a viruslike particle.

DISCUSSION

In this study, we describe the construction and characterization of HIV-1 proviral genomes containing mutations which result in the overexpression of the $Pr160^{gag\text{-}pol}$ polyprotein. We demonstrate that most of the $Pr160^{gag\text{-}pol}$ was confined within the cells; a low but significant level of $Pr160^{gag\text{-}pol}$ was released from transfected cells, as evident by reverse transcriptase activity in the supernatant of cells. When coexpressed with $Pr55^{gag}$, the $Pr160^{gag\text{-}pol}$ polyprotein was incorporated into viruslike particles and released from transfected cells. The myristylation of $Pr160^{gag\text{-}pol}$ was not required for incorporation into particles. There was no significant difference between the myristylated and nonmyristylated $Pr160^{gag\text{-}pol}$ with respect to the subcellular localization and stable association with intracellular membranes.

Previous studies have described the characterization of a HIV-1 proviral genome containing single amino acid changes to inactivate the viral protease (13, 27, 28). In these studies, as with those presented here for pHXB2pro−, transfection of proviral genomes containing these mutations resulted in the release of particles containing the $Pr160^{gag\text{-}pol}$ polyprotein. From these early studies, it was not clear whether expression of $Pr160^{gag\text{-}pol}$ alone induced particle formation. We demonstrated that complete HIV-1 proviral genomes with mutations to inactivate the viral protease and to position the *gag* and *pol* genes in the same translational reading frame resulted in the exclusive expression of the $Pr160^{gag\text{-}pol}$ protein. The majority of the $Pr160^{gag\text{-}pol}$ was confined within the cell, although a low level was released from the transfected cells. At present, we do not know whether this is due to cell death or whether $Pr160^{gag\text{-}pol}$ alone can form particles at a low frequency. Our results are consistent, though, with those of previous studies with murine leukemia virus (11) and Rous sarcoma virus (3, 7, 42) which reported that proviral genomes which overexpressed Gag-Pol resulted in the intracellular accumulation of the polyprotein without release of virus. As suggested by Craven et al., this defect could be due to steric interference by the *pol* domain in particle formation (7). It is also possible that *gag* protein p15, which is disrupted in the $Pr160^{gag\text{-}pol}$ polyprotein, is important for virion formation. Further experiments are under way to investigate this question.

The $Pr160^{gag\text{-}pol}$ polyprotein was efficiently released from the cells when coexpressed with the $Pr55^{gag}$ protein. From the sucrose density gradient analysis, $Pr160^{gag\text{-}pol}$ was associated with particles formed from the $Pr55^{gag}$ protein. The fact that the $Pr160^{gag\text{-}pol}$ precursor contains enzymatic activity but approximately 1/10 that of p66/51 mature reverse transcriptase in viruses derived from transfection of pHXB2 is consistent with previous reports from other groups (13, 22, 27, 28). Previous studies have described the enzymatic activity for the Gag-Pol polyprotein of murine leukemia virus in a range similar to that for HIV-1 (8), while enzymatic activity of the Gag-Pol polyprotein from Rous sarcoma virus was negligible (7, 42). Since each of these viruses relies on a different strategy for the expression of Gag-Pol and contains mature reverse transcriptases with different physical properties, it is probably not surprising that the Gag-Pol polyproteins from each virus might have a different capacity for reverse transcription. The role that the enzymatic activity of the Gag-Pol polyprotein might play in reverse transcription of the viral genome is unknown. It is tempting to speculate that the Gag-Pol polyprotein might facilitate the initiation of reverse transcription, possibly by positioning the cellular $tRNA^{Lys}$ primer on the viral genome and initiating the reverse transcription. Further studies are needed to test this possibility.

The mechanism by which $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ are targeted to the appropriate intracellular membrane and released from the cell is, at present, unknown. Previous studies have demonstrated that the cotranslational addition of *N*-myristic acid to $Pr55^{gag}$ is essential for the release of immature virus particles (12, 13). In previous studies, the nonmyristylated Gag of murine leukemia virus was excluded from virus assembly (37). In contrast, Wills et al. have demonstrated that myristylation of the Gag protein is not the sole determinant for targeting to the plasma membrane and release of virions (47). The results of our studies demonstrate that the $Pr160^{gag\text{-}pol}$ of HIV-1 can be rescued into particles regardless of the myristylation state. The role that myristylation of the $Pr160^{gag\text{-}pol}$ polyprotein plays in intracellular transport and membrane association is not clear. We observed no significant differences between the partitioning of myristylated and unmyristylated $Pr160^{gag\text{-}pol}$ into the cytoplasmic or membrane fractions, although identical procedures found a difference between the myristylated and unmyristylated $Pr55^{gag}$. This difference may be due in part to the ability of the myristylated $Pr55^{gag}$ to form particles (13). That is, the $Pr55^{gag}$ probably forms a tighter association with the plasma membrane as a consequence of the virus assembly and release process. In support of this idea is the fact that the subcellular distribution of the nonmyristylated $Pr55^{gag}$, which is not released from cells, was similar to that for the myristylated or unmyristylated $Pr160^{gag\text{-}pol}$. It is possible that $Pr160^{gag\text{-}pol}$ is incapable of the stable association with the plasma membrane and must interact with $Pr55^{gag}$ at the plasma membrane or in the cytoplasm for incorporation into virions. Although we do not know the nature of the protein-protein interactions required for the incorporation of $Pr160^{gag\text{-}pol}$ into virions, we anticipate that the interaction between $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ might involve the matrix and capsid regions shared between the two polyproteins (16, 38, 45). Further experiments using the complementation system described in the report will be needed to identify the essential regions for $Pr55^{gag}$-$Pr160^{gag\text{-}pol}$ interactions involved in the incorporation of $Pr160^{gag\text{-}pol}$ into virions.

ACKNOWLEDGMENTS

We thank Etty Benveniste, Beatrice Hahn, and David Ansardi for helpful comments and suggestions. We also thank Eric Hunter and John Dubay for assistance with the reverse transcriptase assays and use of the AMBIS densitometer and Jeff Engler for the use of the Phosphorimager. We thank Nancy Vaida for preparation of the manuscript.

The antiserum to HIV-1 p24/p25^{gag} (76C) (from Kathelyn Steimer, Chiron Corp.) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS Program, NIAID. All transfections were carried out in the UAB Center for AIDS Research Central Virus Core Facility supported by the Center Core Grant (AI-27767). The support for the DNA oligonucleotides was from the UAB Comprehensive Cancer Center Core Grant (NCI CA 13148). This work was supported by Public Health Service grant from the National Institutes of Health (AI 27290) to C.D.M.

REFERENCES

1. **Aitken, A., P. Cohen, S. Santikarn, D. H. Williams, A. G. Calder, A. Smith, and C. B. Klee.** 1982. Identification of the NH_2-terminal blocking group of calcineurin B as myristic acid. FEBS Lett. **150**:314–318.
2. **Buss, J. E., M. P. Kamps, and B. M. Sefton.** 1984. Myristic acid is attached to the transforming protein of Rous sarcoma virus during or immediately after synthesis and is present in both soluble and membrane-bound forms of the protein. Mol. Cell. Biol. **4**:2697–2704.
3. **Bennett, R. P., S. Rhee, R. C. Craven, E. Hunter, and J. W. Wills.** 1991. Amino acids encoded downstream of *gag* are not required by Rous sarcoma virus protease during *gag*-mediated assembly. J. Virol. **65**:272–280.
4. **Bryant, M., and L. Ratner.** 1990. Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. Proc. Natl. Acad. Sci. USA **87**:523–527.
5. **Cann, A. J., and J. Karn.** 1989. Molecular biology of HIV-1: new insights into the virus life-cycle. AIDS **3**(Suppl. 1):S19–S34.
6. **Carr, S. A., K. Biemann, S. Shoji, D. C. Parmalee, and K. Titani.** 1982. *n*-Tetradecanoyl is the NH_2-terminal blocking group of the catalytic subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle. Proc. Natl. Acad. Sci. USA **79**:6128–6131.
7. **Craven, R. C., R. P. Bennett, and J. W. Wills.** 1991. Role of the avian retroviral protease in the activation of reverse transcriptase during virion assembly. J. Virol. **65**:6205–6217.
8. **Crawford, S., and S. P. Goff.** 1985. A deletion mutant in the 5′ part of the *pol* gene of Moloney murine leukemia virus blocks proteolytic processing of the *gag* and *pol* polyproteins. J. Virol. **53**:899–907.
9. **Darke, P. L., C. T. Leu, L. J. Davis, J. C. Heimbach, R. E. Diehl, W. S. Hill, R. A. F. Dixon, and I. S. Sigal.** 1989. Human immunodeficiency virus protease: bacterial expression and characterization of the purified aspartic protease. J. Biol. Chem. **264**:2307–2312.
10. **Dickson, C., R. Eisenman, H. Fan, E. Hunter, and N. Teich.** 1984. Protein biosynthesis and assembly, p. 513–640. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), Molecular biology of tumor viruses—RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Felsenstein, K. M., and S. P. Goff.** 1988. Expression of the *gag-pol* fusion protein of Moloney murine leukemia virus without *gag* protein does not induce virion formation or proteolytic processing. J. Virol. **62**:2179–2182.
12. **Gheysen, D., E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. De Wilde.** 1989. Assembly and release of HIV-1 precursor pr55^{gag} virus-like particles from recombinant baculovirus-infected insect cells. Cell **59**:103–112.
13. **Gottlinger, H. G., J. G. Sodroski, and W. A. Haseltine.** 1989. Role of capsid precursor processing and myristylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86**:5781–5785.
14. **Gowda, S. D., B. S. Stein, and E. G. Engleman.** 1989. Identification of protein intermediates in the processing of the p55 HIV-1 *gag* precursor in cells infected with recombinant vaccinia virus. J. Biol. Chem. **264**:8459–8462.
15. **Jacks, T., M. D. Power, F. R. Masiarz, P. A. Luciw, P. J. Barr, and H. E. Varmus.** 1988. Characterization of ribosomal frameshifting in HIV-1 *gag-pol* expression. Nature (London) **331**:280–283.
16. **Jones, T. A., G. Blaug, M. Hansen, and E. Barklis.** 1990. Assembly of *gag*-β-galactosidase proteins into retrovirus particles. J. Virol. **64**:2265–2279.
17. **Karacostas, V., K. Nagashima, M. A. Gonda, and B. Moss.** 1989. Human immunodeficiency virus-like particles produced by a vaccinia virus expression vector. Proc. Natl. Acad. Sci. USA **86**:8964–8967.
18. **Kohl, N. E., E. A. Emini, W. A. Schleif, L. J. Davis, J. C. Heimbach, R. A. F. Dixon, E. M. Scolnick, and I. S. Sigal.** 1988. Active human immunodeficiency virus protease is required for viral infectivity. Proc. Natl. Acad. Sci. USA **85**:4686–4690.
19. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
20. **Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb.** 1984. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. Nucleic Acids Res. **12**:5707–5717.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Mergener, K., M. Facke, R. Welker, V. Brinkmann, H. R. Gelderblom, and H.-G. Krausslich.** 1992. Analysis of HIV particle formation using transient expression of subviral constructs in mammalian cells. Virology **186**:25–39.
23. **Mervis, R. J., N. Ahmad, E. P. Lillehoj, M. G. Raum, F. H. R. Salazar, H. W. Chan, and S. Venkatesan.** 1988. The *gag* gene products of human immunodeficiency virus type 1: alignment within the *gag* open reading frame, identification of posttranslational modifications, and evidence of alternative *gag* precursors. J. Virol. **62**:3993–4002.
24. **Muesing, M. A., D. H. Smith, C. D. Cabradilla, C. V. Benton, L. A. Lasky, and D. J. Capon.** 1985. Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. Nature (London) **313**:450–458.
25. **Park, J., and C. D. Morrow.** 1991. Overexpression of the *gag-pol* precursor from human immunodeficiency virus type 1 proviral genomes results in efficient proteolytic processing in the absence of virion production. J. Virol. **65**:5111–5117.
26. **Paul, A. V., A. Schultz, S. E. Pincus, S. Oroszlan, and E. Wimmer.** 1987. Capsid protein VP4 of poliovirus is N-myristylated. Proc. Natl. Acad. Sci. USA **84**:7827–7831.
27. **Peng, C., N. T. Chang, and T. W. Chang.** 1991. Identification and characterization of human immunodeficiency virus type 1 *gag-pol* fusion protein in transfected mammalian cells. J. Virol. **65**:2751–2756.
28. **Peng, C., B. K. Ho, N. T. Chang, and T. W. Chang.** 1991. Role of human immunodeficiency virus type 1-specific protease in core protein maturation and viral infectivity. J. Virol. **63**:2550–2556.
29. **Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo.** 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science **224**:497–500.
30. **Ratner, L., A. Fisher, L. L. Jagodzinski, H. Mitsuya, R. S. Liou, R. C. Gallo, and F. Wong-Staal.** 1987. Complete nucleotide sequences of functional clones of the AIDS virus. AIDS Res. Hum. Retroviruses **3**:57–69.
31. **Ratner, L., W. V. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature (London) **313**:277–284.
32. **Rein, A., M. R. McClure, N. R. Rice, R. B. Luftig, and A. M.**

Schultz. 1986. Myristoylation site in pr65^{gag} is essential for virus particle formation by Moloney murine leukemia virus. Proc. Natl. Acad. Sci. USA **83**:7246–7250.

33. **Rhee, S. S., and E. Hunter.** 1987. Myristylation is required for intracellular transport but not for assembly of D-type retrovirus capsids. J. Virol. **61**:1045–1053.
34. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
35. **Sanchez-Pescador, R., M. D. Power, P. J. Barr, K. S. Steimer, M. M. Stempien, S. L. Brown-Shimer, W. W. Gee, A. Renard, A. Randolph, J. A. Levy, D. Dina, and P. A. Luciw.** 1985. Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV-2). Science **227**:484–492.
36. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
37. **Schultz, A. M., and A. Rein.** 1989. Unmyristylated Moloney murine leukemia virus Pr65^{gag} is excluded from virus assembly and maturation events. J. Virol. **63**:2370–2373.
38. **Schwartzberg, P., J. Colicelli, M. L. Gordon, and S. P. Goff.** 1984. Mutations in the *gag* gene of Moloney murine leukemia virus: effects on production of virions and reverse transcriptase. J. Virol. **49**:918–924.
39. **Shioda, T., and H. Shibuta.** 1990. Production of human immunodeficiency virus (HIV)-like particles from cells infected with recombinant vaccinia viruses carrying the *gag* gene of HIV. Virology **175**:139–148.
40. **Smith, A. J., M.-I. Cho, M.-L. Hammarskjöld, and D. Rekosh.** 1990. Human immunodeficiency virus type 1 Pr55^{gag} and Pr160$^{gag\text{-}pol}$ expressed from a simian virus 40 late replacement vector are efficiently processed and assembled into viruslike particles. J. Virol. **64**:2743–2750.
41. **Steimer, K. S., J. P. Puma, M. D. Powers, M. A. Powers, C. George-Nascimento, J. C. Stephans, J. A. Levy, R. Sanchez-Pescador, P. A. Luciw, P. J. Barr, and R. A. Hallewell.** 1986. Differential antibody responses of individuals infected with AIDS-associated retroviruses surveyed using viral core antigen p25^{gag} expressed in bacteria. Virology **150**:283–290.
42. **Stewart, L., and V. M. Vogt.** 1991. *trans*-acting viral protease is necessary and sufficient for activation of avian leukosis virus reverse transcriptase. J. Virol. **65**:6218–6231.
43. **Streuli, C. H., and B. E. Griffin.** 1987. Myristic acid is coupled to a structural protein of polyoma virus and SV40. Nature (London) **326**:619–622.
44. **Wain-Hobson, S., P. Sonigo, O. Danos, S. Cole, and M. Alizon.** 1985. Nucleotide sequence of the AIDS virus, LAV. Cell **40**:9–17.
45. **Weldon, R. A., C. R. Erdie, M. G. Oliver, and J. W. Wills.** 1990. Incorporation of chimeric Gag protein into retroviral particles. J. Virol. **64**:4169–4179.
46. **Wills, J. W., and R. C. Craven.** 1991. Form, function, and use of retroviral *gag* proteins. AIDS **5**:639–654.
47. **Wills, J. W., R. C. Craven, R. A. Weldon, Jr., T. D. Nelle, and C. R. Erdie.** 1991. Suppression of retroviral MA deletions by the amino-terminal membrane-binding domain of p60^{src}. J. Virol. **65**:3804–3812.
48. **Wilson, W., M. Braddock, S. E. Adams, P. D. Rathjen, S. M. Kingsman, and A. J. Kingsman.** 1988. HIV expression strategies: ribosomal frameshifting is directed by a short sequence in both mammalian and yeast systems. Cell **55**:1159–1169.