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Requirements for Incorporation of Pr160$^{gag\text{-}pol}$ from Human Immunodeficiency Virus Type 1 into Virus-Like Particles

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The roles of the human immunodeficiency virus precursor polyproteins Pr55^{gag} and Pr160$^{gag\text{-}pol}$ in viral core assembly were studied in CMT3-COS cells. To do this, the precursors were expressed separately by using a simian virus 40 late replacement vector system described previously. Consistent with previously published data, our results show that the Pr55^{gag} precursor, when expressed alone, was able to form particles which had an immature morphology and that particle formation required the presence of a myristate addition signal at the amino terminus of the precursor. In contrast, the Pr160$^{gag\text{-}pol}$ precursor was not able to form particles when expressed alone, although it still underwent proteolytic processing. Coexpression of the two precursor polyproteins from separate vectors in the same cell resulted in processing of the Pr55^{gag} in *trans* by the protease embedded in Pr160$^{gag\text{-}pol}$ and the formation of virus-like particles containing the products of both precursors. Proteolytic processing occurred independently of the presence of a functional myristate addition signal on either precursor. On the other hand, removal of myristate from one or the other precursor had nonreciprocal effects on virus particle formation. Cells expressing Pr55^{gag} lacking myristate and Pr160$^{gag\text{-}pol}$ containing it did not produce particles. Cells expressing a myristylated Pr55^{gag} and unmyristylated Pr160$^{gag\text{-}pol}$ still produced virus-like particles which contained nearly normal amounts of Pr160$^{gag\text{-}pol}$. The results suggest that the incorporation of Pr160$^{gag\text{-}pol}$ into particles is largely determined by intermolecular protein-protein interactions between the two precursor polypeptides.

The *gag* and *pol* genes of human immunodeficiency virus (HIV) are initially translated into two polyprotein precursors, which are subsequently proteolytically cleaved to form the core of the virus (12, 37). The first precursor, Pr55^{gag}, is encoded exclusively by the *gag* open reading frame, whereas the second, Pr160$^{gag\text{-}pol}$, is encoded by a combined *gag-pol* open reading frame which is created by a ribosomal frameshift mechanism (15, 41). In virus-infected cells, the assembly of these precursors into viruses takes place at the cytoplasmic side of the plasma membrane (5, 7, 37). It has also been shown that expression of the two precursors from expression vectors in the absence of other viral structural proteins leads to the budding of virus-like particles (6, 11, 17, 22, 31, 32). Studies carried out in vitro with purified Pr55^{gag} or its p24 capsid domain have also demonstrated the ability of these molecules to self-assemble (3).

Pr55^{gag} is posttranslationally modified by myristic acid addition at its amino terminus (9, 23, 34, 38). Cleavage by the viral protease gives rise to four final products, which are called (N to C terminal) p17, p24, p9, and p6 (9, 34, 35). The amino-terminal domain of the precursor, p17, forms the viral matrix protein (MA) (34). Like the matrix proteins of other retroviruses, it is found associated with the inner side of the viral membrane (5). Mature p17 retains the myristic acid moiety from Pr55^{gag} at its amino terminus and is also phosphorylated (23).

The central portion of Pr55^{gag} is the p24 domain (23, 34). This protein forms the cone-shaped core or capsid (CA) structure characteristic of HIV. The protein is phosphorylated and constitutes a major antigenic determinant of the virus. Protein p9 is found associated with the viral genomic RNA and constitutes the nucleocapsid (NC) protein of the virus (35). A protein equivalent to p6 is not found in most other retroviruses. p6 appears to play a role in facilitating the release of virus particles from cells (8).

Pr160$^{gag\text{-}pol}$ contains the p17 and p24 domains of Pr55^{gag} followed by a truncated p9 domain, since the site of ribosomal frameshifting occurs in the region of the mRNA that encodes the carboxy terminus of p9 (15, 41). The amino terminus of this protein is identical to that of Pr55^{gag}, and therefore it is also myristylated. Pr160$^{gag\text{-}pol}$ is completely lacking p6, but after p9, the initial part of the *pol* open reading frame forms a different, small, 68-amino-acid domain which has been called p6* (26). It has been suggested that p6* plays a role in regulation of viral protease activity (26). Embedded within the remainder of the precursor (N to C terminal) are the viral protease, reverse transcriptase, and integrase (2, 4, 10, 18, 20, 21, 33).

It has been suggested that viral particle assembly is crucial for the activation of the viral protease, which cleaves the precursors into their mature forms (27, 36, 40). When Pr55^{gag} and Pr160$^{gag\text{-}pol}$ begin to assemble into a particle at the cytoplasmic side of the plasma membrane, interactions between them are believed to lead to dimerization and activation of the protease encoded within Pr160$^{gag\text{-}pol}$ (24). One model of assembly, based on some data obtained with HIV and a wide variety of data obtained with other retrovirus systems, suggests that the intermolecular interactions between different molecules of Pr55^{gag} drive the particle formation and budding process. Pr160$^{gag\text{-}pol}$ would then be incorporated into the budding particle through its amino-terminal domains, which mimic those of Pr55^{gag} (40). Although this model explains how the viral enzymes could be

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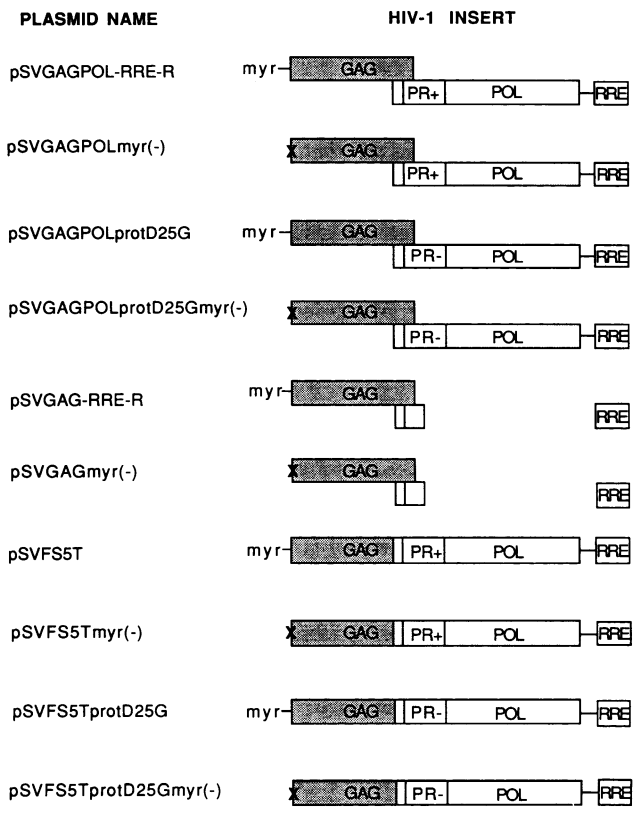

FIG. 1. Schematic representation of alterations made to the HIV-1 sequences present in plasmid pSVGAGPOL-RRE-R. Only the insert portions of the plasmids are presented. The name of each construct is given on the left. The *gag* and *pol* open reading frames as well as the RRE are shown. The protease (PR) gene contained within the *pol* gene has been indicated. The enzyme is in either the active (PR+) or inactive (PR−) form. Constructs in which *gag* and *pol* are fused contain a deletion of five T residues within the overlap region between the two genes which places them into the same open reading frame. Constructs containing a functional myristic acid addition signal are indicated by myr− at the amino-terminal end of the *gag* open reading frame; Myr$^-$ constructs are indicated by an X at this position. All mutations were confirmed by dideoxynucleotide sequencing. See Materials and Methods for details.

incorporated into virus particles, little detail is actually known about the precise roles played by each precursor in the process.

In the present study, we addressed the separate roles played by Pr55^{gag} and Pr160$^{gag\text{-}pol}$ in viral core assembly in transfected CMT3-COS cells. This study used our previously described simian virus 40 (SV40) expression vector system, which was modified to produce Pr55^{gag} and Pr160$^{gag\text{-}pol}$ individually from separate plasmids. We have also examined the requirement for myristic acid at the amino terminus of each precursor.

MATERIALS AND METHODS

Plasmid construction. A schematic representation of the HIV type 1 (HIV-1) sequences present in the plasmids used to express Pr55^{gag} and Pr160$^{gag\text{-}pol}$ in this study are shown in Fig. 1.

The construction of the wild-type *gag-pol* expression plasmid pSVGAGPOL-RRE-R and its properties have been described previously (32). This plasmid contains the *gag*, *pol*, and *vif* regions of HIV-1 under the control of the SV40 late promoter, followed by rabbit beta-globin splicing and polyadenylation signals. The plasmid also contains the gene for SV40 T antigen and the SV40 origin of replication.

pSVGAG-RRE-R is similar to pSVGAGPOL-RRE-R except that most of the *pol* open reading frame has been deleted. To construct it, a *Sal*I-*Bcl*I fragment containing the *gag* open reading frame was isolated from the BH10 clone of HIV-1. The fragment was then cloned into a unique *Xho*I site of the Rev-responsive element (RRE)-containing vector pSVBB1 after both vector and insert were repaired with T4 DNA polymerase. pSVBB1 was made by inserting the *Bgl*II-*Bam*HI fragment containing the RRE into the unique *Bam*HI site of a modified pBABY plasmid (29). This *Bam*HI site is immediately downstream of the SV40 promoter region.

Alterations in the wild-type construct (pSVGAGPOL-RRE-R) were made by exchanging one or more mutant restriction fragment cassettes generated by polymerase chain reaction (PCR) for equivalent wild-type sequences. In some instances, because of the lack of unique restriction sites in the wild-type construct, the mutant fragments were first introduced into a "workshop" vector. The mutation was then transferred as a larger restriction fragment from this plasmid into the wild-type construct. When two mutations were targeted to the same restriction fragment cassette, a second set of PCR reactions were performed to introduce the second mutation into the background of the first. For each construct, the mutation was verified by performing double-stranded dideoxynucleotide DNA sequencing on the exchanged cassette within the final plasmid.

pSVGAGPOLmyr(−) is identical to pSVGAGPOL-RRE-R except that it contains a point mutation in the myristic acid addition signal. The mutation changes a G residue (corresponding to HXB2 nucleotide 791) to a T and causes the second amino acid residue encoded by the *gag* open reading frame to change from a glycine to a valine. The mutation was created by first generating a mutant cassette by splicing by overlap extension (SOE)-PCR (14). The amplified mutated cassette (1,089 bp) containing the myristylation mutation was digested with *Bss*HII and *Spe*I and exchanged for the equivalent *Bss*HI-*Spe*I fragment from pSVGAGPOL-RRE-R.

pSVGAGPOLprotD25G is identical to pSVGAGPOL-RRE-R except that it contains a point mutation in the HIV-1 protease. The mutation changes the A residue (corresponding to HXB2 nucleotide 2359) to a G and the aspartic acid residue at the active site of the enzyme to a glycine. The mutation was created by first generating a mutant cassette by SOE-PCR. The amplified cassette (1,238 bp) containing the protease mutation was digested with *Spe*I and *Bcl*I and inserted into pGEM-11Zf(−)HIV between the corresponding *Spe*I and *Bcl*I sites. pGEM-11Zf(−)HIV contains an *Sfi*I-*Bam*HI fragment from pSVGAGPOL-RRE-R inserted into the vector pGEM-11Zf(−) (Promega) between the *Sfi*I and *Bam*HI sites and was used as an intermediate cloning vector. The mutation was then removed from the resultant plasmid as a *Bss*HII-*Bam*HI fragment and exchanged for the corresponding fragment in pSVGAGPOL-RRE-R.

pSVFS5T contains a deletion of five T residues (corresponding to HXB2 nucleotides 2083 to 2087) from the overlap region between *gag* and *pol*. This mutation created a 100% frameshifting event by placing *gag* and *pol* into the same open reading frame. The mutation was created by first generating a mutant cassette by SOE-PCR. The amplified

mutant cassette (1,233 bp) containing the frameshifting mutation was digested with *Spe*I and *Bcl*I and inserted in pGEM-11Zf(−)-HIV between the corresponding *Spe*I and *Bcl*I sites. The mutation was then removed from the resultant plasmid as a *Bss*HII-*Bam*HI fragment and exchanged for the corresponding fragment in pSVGAGPOL-RRE-R.

pSVGAGmyr(−) is identical to pSVGAG-RRE-R except it contains a defective myristic acid addition signal. It was constructed by exchanging the *Bss*HII-*Spe*I mutant cassette described above for the construction of pSVGAGPOL myr(−) with the equivalent sequences from pSVGAG-RRE-R.

pSVGAGPOLprotD25Gmyr(−) is identical to pSVGAG POLprotD25G except it also contains a defective myristic acid addition signal. It was constructed by exchanging the *Bss*HI-*Spe*I mutant cassette described above for the construction of pSVGAGPOLmyr(−) with the equivalent sequences from pSVGAGPOLprotD25G.

pSVFS5Tmyr(−) is identical to pSVFS5T except it contains a defective myristic acid addition signal. It was constructed by exchanging the *Bss*HII-*Spe*I mutant cassette described above for the construction of pSVGAGPOLmyr (−) with the equivalent sequences from pSVFS5T.

pSVFS5TprotD25G contains both the mutation in the active site of the protease and the frameshifting deletion described above. It was created as described above for pSVGAGPOLprotD25G except that pSVFS5T was used as a template to create the mutant cassette carrying the protease point mutation.

pSVFS5TprotD25Gmyr(−) is identical to pSVFS5Tprot D25G except that it also contains a defective myristic acid addition signal. It was constructed by exchanging the *Bss*HII-*Spe*I mutant cassette fragment described above for the construction of pSVGAGPOLmyr(−) with the equivalent sequences from pSVFS5TprotD25G.

The plasmid used to supply *rev* in *trans*, pCMVrev, has been described previously as pRev1 (19, 32). It contains a *rev* cDNA fragment under the control of the promoter-enhancer region from the simian cytomegalovirus (CMV) IE94 gene (−650 to +30). The HIV insert is followed by splicing and polyadenylation signals from the rabbit beta-globin gene.

Cells and transfections. CMT3-COS cells were transfected by the DEAE-dextran method described previously (13). The cells were harvested 60 to 72 h posttransfection. In experiments involving cotransfection of an SV40-based vector and pCMVrev, 5 μg of the SV40-based vector and 2 μg of pCMVrev were used per 100-mm dish of cells. When two SV40 vectors were cotransfected, 5 μg of each was used together with 2 μg of pCMVrev. In the case of *gag* and *gag-pol* cotransfections, this represented an input *gag*/*gag-pop* molar ratio of 1:0.74, since these plasmids were 9,600 and 12,939 bp in size, respectively.

Western immunoblot analysis. Western blots were done on Immobilon P membranes (Millipore Corp.) as described previously (32). In some experiments, after the primary antibody was washed off, the blots were incubated in phosphate-buffered saline (PBS) containing 1% evaporated milk, 0.025% Tween 20, and 2.5 μCi of ^{125}I-labeled staphylococcal protein A (New England Nuclear) instead of the second antibody. These blots were washed three times for 15 min each time in PBS containing 0.5% Tween 20 and then overnight in the same buffer. The blots were allowed to air dry and were then autoradiographed.

Ultrastructural studies. Sectioning, staining, and electron microscopy were kindly performed by Moon-Il Cho as previously described (32).

Reverse transcriptase assays. Particle-associated reverse transcriptase was assayed by standard procedures (39). Briefly, particles from 8 ml of cleared medium were pelleted through 4 ml of 20% (wt/vol) sucrose in 25 mM Tris (pH 7.2)–140 mM NaCl–5 mM KCl–1 mM EDTA at 100,000 × *g* for 2 h at 4°C. The pellet was resuspended in 200 μl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), and a 10-μl aliquot was assayed.

Immunoprecipitation of labeled cells and medium. CMT3-COS cells were transfected with the appropriate plasmid(s). At 48 h posttransfection, the medium was removed, and the cells were washed with 5 ml of minimum essential medium (GIBCO) lacking either L-methionine or both L-methionine and L-cysteine. Cells were incubated at 37°C for 4 h in 1.5 ml of the appropriate medium containing [^{35}S]methionine alone or both [^{35}S]methionine and [^{35}S]cysteine (150 to 200 μCi per plate). The labeling medium was replaced with 3 ml of serum-free Iscove's modified Dulbecco's medium, and the cells were incubated for an additional 4 h, after which the cells and medium were harvested.

At the time of harvest, the medium was removed from the cells and cleared of cellular debris by centrifugation at 1,500 rpm in an IEC tabletop centrifuge for 10 min at 4°C. The clarified medium was transferred to a fresh tube and stored at −20°C until it was assayed. The cells were scraped from the plates with a rubber policeman into 10 ml of PBS, pelleted at 1,500 rpm in an IEC tabletop centrifuge for 10 min at 4°C, and washed once with an additional 1 ml of PBS. Cell pellets were stored at −20°C until assayed.

Equivalent amounts of medium and cellular material (usually one-third of the plate) were used for immunoprecipitations. The medium was thawed and Triton X-100 was added to 1% (final concentration) prior to use. The cell pellets were resuspended in 1.2 ml of lysis buffer A (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA [pH 8.0], 1% Triton X-100, 0.04% deoxycholate), vortexed briefly, and cleared of debris by centrifugation for 1 min at a 4°C in a microcentrifuge. Each sample was initially treated for 30 min with 25 μl of swollen 10% (wt/vol) protein A-Sepharose (Pharmacia) in PBS on ice, with occasional mixing. The protein A-Sepharose was removed by brief centrifugation, and the supernatants were transferred to clean tubes. The samples were then incubated with 5 μl of HIV-1-positive human serum for 2 h, on ice, after which 25 μl of 10% (wt/vol) protein A-Sepharose was added. Incubation was continued for 1 h on ice with occasional mixing. The samples were then subjected to centrifugation, and the pellets were washed four times in lysis buffer A, one time in lysis buffer A plus 500 mM NaCl, and one time in TNE (10 mM Tris-HCl [pH 8.0], 25 mM NaCl, 1 mM EDTA [pH 8.0]) before being analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the gel was stained with Coomassie blue, destained with a solution containing 40% methanol and 10% acetic acid, washed briefly in distilled water, and soaked in Amplify (Amersham) solution for 45 min. The gel was then dried on a piece of Whatman filter paper and subjected to fluorography.

RESULTS

Our previously published work demonstrated that expression of the *gag* and *pol* regions of HIV from an SV40-based vector produced virus-like particles which closely resembled

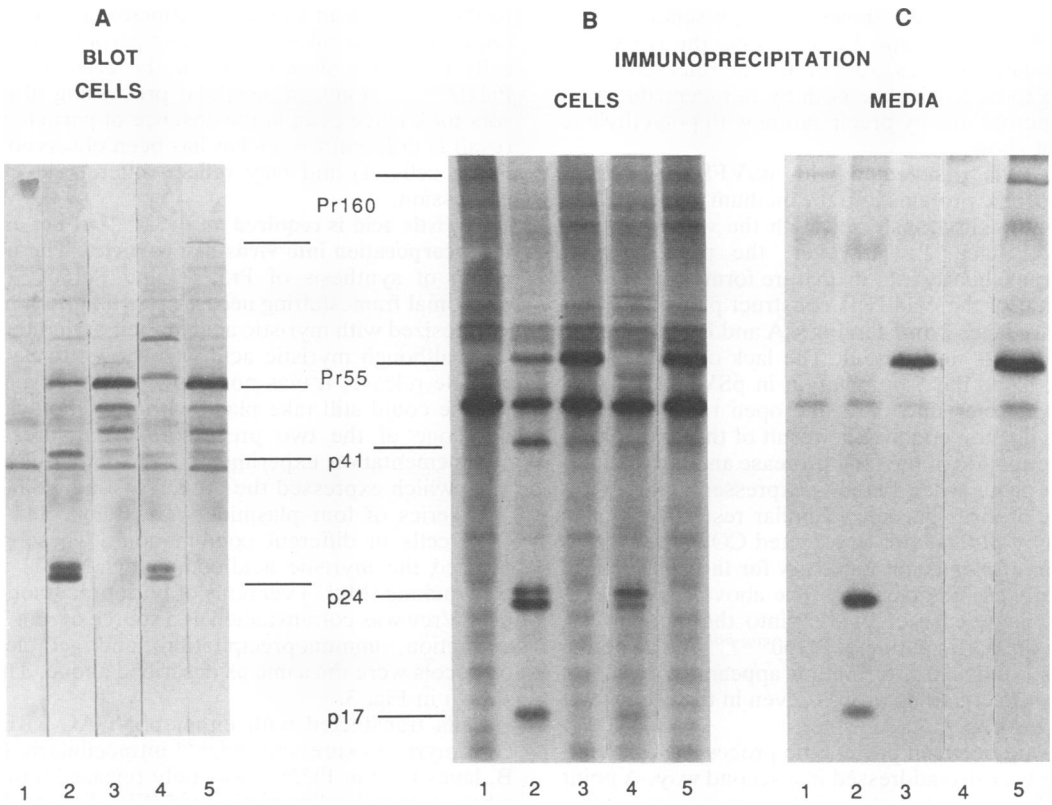

FIG. 2. Western blot and immunoprecipitation analyses of HIV-1 proteins expressed from modified plasmids. Parallel plates of CMT3-COS cells were transfected with pBABY (lanes 1), pSVGAGPOL-RRE-R (lanes 2), pSVGAG-RRE-R (lanes 3), pSVFS5T (lanes 4), or pSVGAGPOLprotD25G (lanes 5). In all cases, the cells were also transfected with pCMVrev. At 72 h posttransfection, one set of cells was harvested, separated on a 15% polyacrylamide gel, and analyzed by Western blot with an HIV-1-positive human serum (A). At 48 h posttransfection, the other set of cells was metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine. The cells (B) and medium (C) were then harvested and analyzed by immunoprecipitation with an HIV-1-positive human serum. The relative positions of the HIV-1 proteins are indicated.

HIV-1 in structure (32). In this report, this system was used to study some of the parameters affecting the incorporation of Pr160$^{gag\text{-}pol}$ into virus particles. To do this, various mutations were created in the original expression plasmid pSVGAGPOL-RRE-R (see Materials and Methods), and the effect of each mutation or combinations of mutations on particle formation and Pr160$^{gag\text{-}pol}$ incorporation into particles was assayed.

Expression of Pr55^{gag} and Pr160$^{gag\text{-}pol}$ from separate plasmids. Expression plasmids were constructed which produced Pr55^{gag} and Pr160$^{gag\text{-}pol}$ individually. This was done either by creating a large deletion in pSVGAGPOL-RRE-R which removed most of the *pol* open reading frame or by creating a 5-bp deletion at the *gag-pol* boundary of the same plasmid. The 5-bp deletion introduced a permanent frameshift at the DNA level (see Materials and Methods). These plasmids were called pSVGAG-RRE-R and pSVFS5T, respectively.

We performed an experiment to determine the potential of each precursor to form virus-like particles without the presence of the other. This was done to establish how the individual precursor molecules produced in our vector system functioned and to compare our results with what was previously reported in the literature (6, 22, 25, 31, 40).

Two sets of CMT3-COS cells were transfected with either pSVGAGPOL-RRE-R, pSVGAG-RRE-R, pSVFS5T, or the control plasmid pBABY. In all cases, a second plasmid expressing *rev*, pCMVrev, was also cotransfected. At 48 h posttransfection, one set of plates was metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine for 4 h and chased for an additional 4 h with nonradioactive methionine and cysteine. The cells and medium were harvested, and an immunoprecipitation was performed with HIV-1-positive human serum (Fig. 2B and C). The second set of plates were harvested at 72 h posttransfection, and the cells were analyzed by Western blotting with the same antiserum (Fig. 2A).

Lanes which contained extracts from cells transfected with the wild-type vector, pSVGAGPOL-RRE-R, showed a series of bands which included both precursors, mature cleavage products, and products that are likely processing intermediates (Fig. 2A and B, lanes 2). Both p24 and p17 appeared to be released from these cells into the culture medium (Fig. 2C, lane 2), suggesting the release of virus-like particles. The released material was shown to be particulate both by ultracentrifugation through 20% sucrose and by precipitation with polyethylene glycol (22) (data not shown).

Lanes which contained extracts from cells transfected with pSVGAG-RRE-R showed a high level of expression of Pr55^{gag} both by Western blot (Fig. 2A, lane 3) and by immunoprecipitation (Fig. 2B, lane 3). Two virus-specific products of lower molecular weight were also observed on the Western blot. These were not analyzed further but may represent products derived by initiation at internal methionine codons or by proteolytic degradation. Analysis of the

medium from labeled cells showed the presence of uncleaved $Pr55^{gag}$ (Fig. 2C, lane 3), suggesting the release of immature virus-like particles. As before, the released material was shown to be particulate both by ultracentrifugation through 20% sucrose and by precipitation with polyethylene glycol (data not shown).

In contrast, cells transfected with pSVFS5T failed to release virus-specific proteins into the medium (Fig. 2C, lane 4) compared with cells transfected with the wild-type construct (Fig. 2C, lane 2). However, the processing of $Pr160^{gag\text{-}pol}$ intracellularly into its mature forms appeared to be similar to that of the wild-type construct pSVGAGPOL-RRE-R (compare lanes 2 and 4 in Fig. 2A and B), despite the fact that $Pr55^{gag}$ was not present. The lack of $Pr55^{gag}$ was not surprising, since the 5-bp deletion in pSVFS5T placed the *gag* and *pol* genes into the same open reading frame, bypassing the *gag* stop codon. The result of this experiment suggests that activation of the HIV protease and intracellular processing can occur when Pr160 is expressed alone, without the release of virus particles. Similar results have also been observed by others with transfected COS-1 cells (25).

Proteolytic processing is not necessary for incorporation of $Pr160^{gag\text{-}pol}$ into virus-like particles. The above experiments demonstrated that release of $Pr55^{gag}$ into the medium occurred without the participation of $Pr160^{gag\text{-}pol}$ and an active protease. Thus, sufficient information appears to reside in $Pr55^{gag}$ to direct the budding process even in the absence of proteolytic processing.

The relationship between proteolytic processing and viral particle release was also addressed in a second way. A point mutation which inactivated the viral protease was introduced into the original expression plasmid pSVGAGPOL-RRE-R to create pSVGAGPOLprotD25G (see Materials and Methods). This plasmid was then transfected into CMT3-COS cells together with a plasmid that expressed *rev*. The transfected cells were subjected to the labeling, blotting, immunoprecipitation, and gel electrophoresis protocols described above. Figure 2A, lane 5, shows that, as expected, proteolytic processing in this mutant was inhibited. The predominant HIV-specific products expressed in the transfected cells were $Pr160^{gag\text{-}pol}$ and $Pr55^{gag}$, as well as the same unidentified lower-molecular-weight products that were present when $Pr55^{gag}$ was expressed alone (Fig. 2A, lane 3). Figure 2C, lane 5, shows clearly that particle release also occurred, as both $Pr160^{gag\text{-}pol}$ and $Pr55^{gag}$ were present in the medium. The released material was shown to be particulate by both ultracentrifugation through 20% sucrose and precipitation with polyethylene glycol (data not shown). These results confirm that proteolytic processing was not necessary for virus particle release and suggest that uncleaved $Pr160^{gag\text{-}pol}$ was incorporated into the released particles.

Requirement for myristylation. Both $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ are known to contain myristic acid at their amino termini, and it has been shown that myristylation is required for virus assembly (1, 9). In these experiments, mutations were created in an infectious HIV DNA clone to produce precursors that lacked myristic acid. We introduced such a mutation into our expression plasmid to determine whether myristylation was also required for the formation of virus-like particles in our system (see Materials and Methods). The resulting plasmid was called pSVGAG-POLmyr(−). The mutation changed the conserved glycine residue in the myristic acid addition signal to a valine. Labeling experiments with [^{3}H]myristic acid confirmed that the precursors lacked myristic acid, and experiments similar to those shown in Fig. 2 confirmed that myristylation was required for the efficient release of particles from transfected cells (data not shown). As in the case of expression of $Pr160^{gag\text{-}pol}$ alone, intracellular processing of these precursors took place even in the absence of particle release. This result is different from what has been observed by others in HeLa cells (1) and may reflect different levels of protein expression.

Myristic acid is required on $Pr55^{gag}$ but not on $Pr160^{gag\text{-}pol}$ for incorporation into virus-like particles. The normal mechanism of synthesis of $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ involving ribosomal frameshifting necessitates that both precursors be synthesized with myristic acid at their amino termini. Therefore, although myristic acid was shown to be required for particle release, it was not known whether mature-particle release could still take place with myristic acid present on only one of the two precursors. To address this issue, complementation experiments were undertaken with plasmids which expressed the precursors individually.

A series of four plasmids were transfected into CMT3-COS cells in different combinations. These plasmids expressed the myristic acid-containing (Myr^+) and myristic acid-lacking (Myr^-) versions of both precursors. As before, pCMVrev was cotransfected as a source of *rev*. The labeling, extraction, immunoprecipitation, and gel electrophoresis protocols were the same as described above. The results are shown in Fig. 3.

Cells transfected with either pSVGAG-RRE-R or pSV-GAGmyr(−) expressed $Pr55^{gag}$ intracellularly (Fig. 3A and B, lanes C), but $Pr55^{gag}$ was only released into the medium when it contained myristic acid (Fig. 3A and B, lanes M). Cells transfected with either pSVFS5T or pSVFS5Tmyr(−) processed the $Pr160^{gag\text{-}pol}$ precursor intracellularly but did not release material into the medium (Fig. 3C and D). Cells cotransfected with the different combinations of Myr^+ and Myr^- plasmids processed the precursors intracellularly (Fig. 3E, F, and G). They released p24 into the medium when a myristic acid moiety was present on $Pr55^{gag}$. The released material was shown to be particulate by both ultracentrifugation through 20% sucrose and precipitation with polyethylene glycol (data not shown). The presence or absence of myristic acid on the $Pr160^{gag\text{-}pol}$ precursor did not affect this result (compare Fig. 3E and F). In contrast, cells which contained $Pr55^{gag}$ that lacked myristic acid and a $Pr160^{gag\text{-}pol}$ which contained it did not release p24 into the medium (Fig. 3G).

There were two possible interpretations of these results. First, a myristylation-deficient $Pr160^{gag\text{-}pol}$ containing a functional protease could be incorporated into virus-like particles and be released into the medium as long as $Pr55^{gag}$ contained a functional myristic acid. Presumably, a protein-protein interaction between the two precursors could facilitate the incorporation of $Pr160^{gag\text{-}pol}$ into the budding particle. The p24 seen in the medium would then be the result of proteolytic processing of the precursors by the activated protease after its incorporation into the particle. The second possibility was that the p24 seen in the medium in the cotransfections originated from cleavage of the precursors intracellularly. Since intracellular processing can occur without particle release, it was possible that p24 could have been drawn into the budding particles as $Pr55^{gag}$ budded from the membrane.

To distinguish between these possibilities, another cotransfection experiment was performed. In this experiment, the release of $Pr160^{gag\text{-}pol}$ from cells was assayed directly by expressing a $Pr160^{gag\text{-}pol}$ which lacked myristic

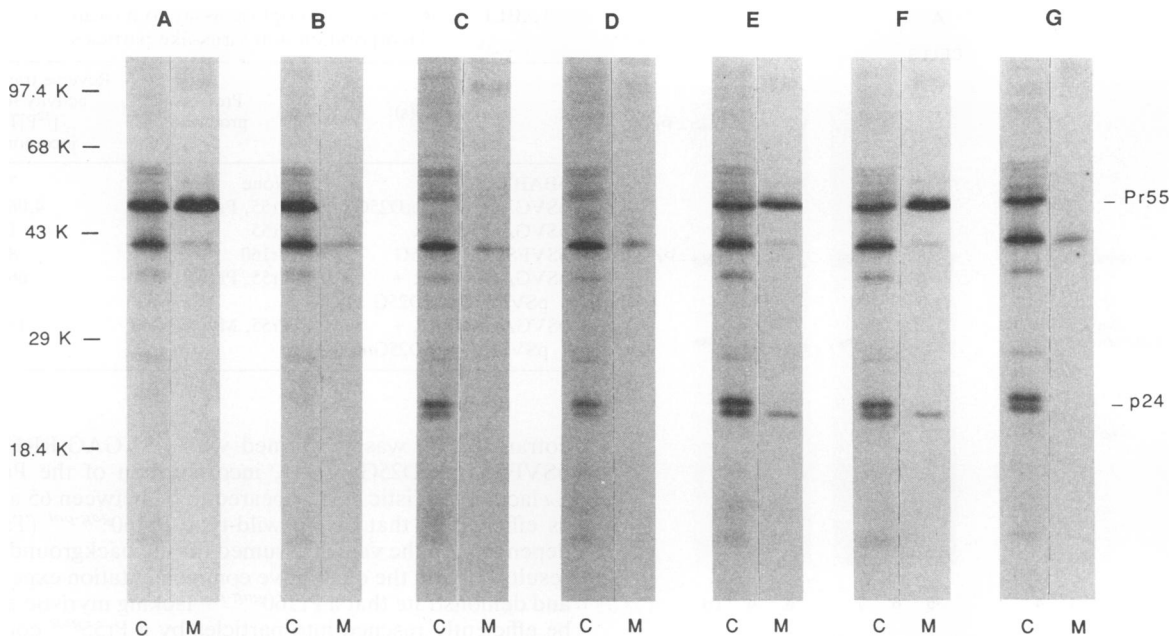

FIG. 3. Immunoprecipitation analysis of the proteins produced and released into the medium by cells transfected with various combinations of plasmids. CMT3-COS cells were transfected with the following plasmids: (A) pSVGAG-RRE-R; (B) pSVGAGmyr(−); (C) pSVFS5T; (D) pSVFS5Tmyr(−); (E) pSVGAG-RRE-R plus pSVFS5T; (F) pSVGAG-RRE-R plus pSVFS5Tmyr(−); (G) pSVGAGmyr(−) plus pSVFS5T. In all cases, cells were also transfected with pCMVrev. At 48 h posttransfection, the cells were metabolically labeled with [^{35}S]methionine. The cells (lanes C) and medium (lanes M) were then harvested and analyzed by immunoprecipitation with an HIV-1-positive human serum. The relative positions of the HIV-1 proteins and molecular weight markers (in kilodaltons) are indicated.

acid and which also contained a mutation in the viral protease. The plasmid which expressed this doubly mutated precursor was called pSVFS5TprotD25Gmyr(−).

CMT3-COS cells were transfected with either pSVGAG-RRE-R or pSVFS5TprotD25Gmyr(−) alone or with both plasmids. As controls, CMT3 cells were also transfected with either pBABY, pSVGAGPOL-RRE-R, pSVGAGPOL protD25G, or pSVGAG-RRE-R plus pSVFS5TprotD25G. In both the control and experimental transfections, pCMVrev was also transfected as a source of *rev*. At 72 h posttransfection, the cells and medium were harvested and analyzed by Western blotting with HIV-1-positive human serum followed by incubation with ^{125}I-labeled staphylococcal protein A (Fig. 4).

Control cells transfected with pSVGAGPOLprotD25G or cotransfected with pSVGAG-RRE-R plus pSVFS5Tprot D25G expressed both Pr55^{gag} and Pr160$^{gag\text{-}pol}$ intracellularly (Fig. 4A, lanes 3 and 4) and released these precursors into the medium (Fig. 4B, lanes 3 and 4). As expected, cells that were transfected with pSVGAG-RRE-R alone expressed only Pr55^{gag} (Fig. 4A, lane 5), which was also subsequently released into the medium (Fig. 4B, lane 5). Cells transfected solely with pSVFS5TprotD25Gmyr(−) expressed high levels of Pr160$^{gag\text{-}pol}$ intracellularly (Fig. 4A, lane 6) but failed to release appreciable levels into the culture medium (Fig. 4B, lane 6). However, when the Pr55^{gag}-expressing plasmid pSVGAG-RRE-R was cotransfected with pSVFS5TprotD 25Gmyr(−), significantly more Pr160$^{gag\text{-}pol}$ was released into the medium along with Pr55^{gag} (Fig. 4B, lane 7). This demonstrates directly that the release into the medium of a Pr160$^{gag\text{-}pol}$ lacking myristic acid is facilitated by the presence of a Pr55^{gag} containing myristic acid.

We also performed the reciprocal complementation experiment. In this case, the myristic acid moiety was located on the protease-deficient Pr160$^{gag\text{-}pol}$ and was lacking on Pr55^{gag}. The results are shown in Fig. 4, lanes 8 to 10. As expected, cells transfected with plasmids that expressed Pr55^{gag} lacking myristic acid [pSVGAGmyr(−)] or Pr160$^{gag\text{-}pol}$ containing a defective protease (pSVFS5TprotD25G) expressed these precursors intracellularly (Fig. 4A, lanes 8 and 9) but failed to release them into the medium (Fig. 4B, lanes 8 and 9). A high level of expression of both precursors was also observed in the cotransfection (Fig. 4A, lane 10). This result confirms that myristic acid on Pr160$^{gag\text{-}pol}$ is not sufficient to mediate the release of a nonmyristylated Pr55^{gag}.

Quantitative measurement of Pr160$^{gag\text{-}pol}$ incorporation into virus-like particles. Recent studies with protease-deficient clones of HIV-1 have demonstrated that reverse transcriptase showed significant levels of activity while still in the context of the precursor molecule (28). We took advantage of this fact to develop a quantitative measure of Pr160$^{gag\text{-}pol}$ incorporation into particles by using protease-defective versions of Pr160$^{gag\text{-}pol}$. To do this, complementation experiments similar to those described above were performed, and reverse transcriptase assays were carried out on the particles isolated from the culture medium. The results are shown in Table 1.

Cells transfected with pSVGAGPOLprotD25G alone released high levels of particulate reverse transcriptase into the medium. Significant but lower levels of reverse transcriptase were also found in the medium of cells cotransfected with pSVGAG-RRE-R and pSVFS5TprotD25G. These cells expressed the same precursors as the cells transfected with pSVGAGPOLprotD25G alone but did so from separate plasmids. Thus, incorporation of Pr160$^{gag\text{-}pol}$ into particles in the cotransfected cells appeared to be much less efficient than in the singly transfected ones. When the

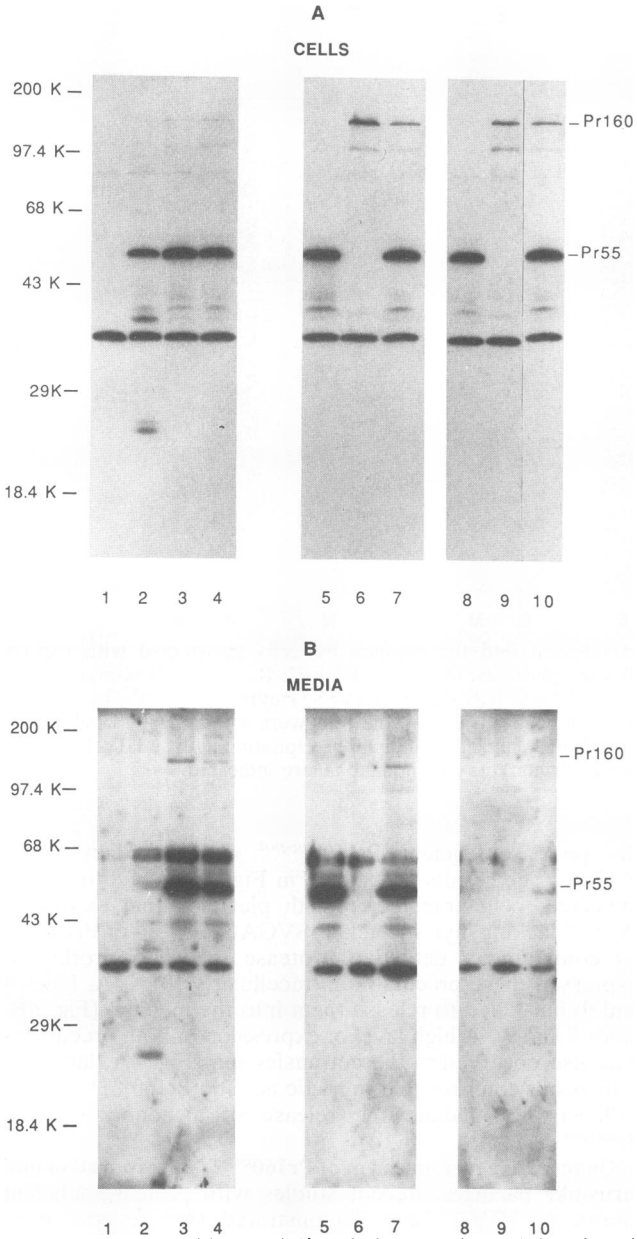

FIG. 4. Western blot analysis of the proteins produced and released into the medium by cells transfected with various combinations of plasmids. CMT3-COS cells were transfected with the following plasmids: lanes 1, pBABY; lanes 2, pSVGAGPOL-RRE-R; lanes 3, pSVGAGPOLprotD25G; lanes 4, pSVGAG-RRE-R plus pSVFS5TprotD25G; lanes 5, pSVGAG-RRE-R; lanes 6, pSVFS5TprotD25Gmyr(−); lanes 7, pSVGAG-RRE-R plus pSVFS5TprotD25Gmyr(−); lanes 8, pSVGAGmyr(−); lanes 9, pSVFS5TprotD25G; lanes 10, pSVGAGmyr(−) plus pSVFS5T protD25G. In all cases, the cells were also transfected with pCM-Vrev. (A) At 72 h posttransfection, the cells were harvested, extracted, separated on a 15% polyacrylamide gel, and transferred to an Immobilon P membrane by electroblotting. The blot was then probed with an HIV-1-positive human serum, followed by incubation with ^{125}I-staphylococcal protein A. The relative positions of the HIV-1 proteins and molecular weight markers (in thousands) are indicated. (B) At 72 h posttransfection, the medium was harvested and cleared of cellular debris, and the viral particles were precipitated with polyethylene glycol. The viral particles were then solubilized in SDS sample buffer, separated on a 15% polyacrylamide gel, and transferred to an Immobilon P membrane by electroblotting. The blot was then probed with an HIV-1-positive human serum, followed by incubation with ^{125}I-staphylococcal protein A. The relative positions of the HIV-1 proteins and molecular weight markers (in thousands) are indicated.

TABLE 1. Reverse transcriptase assay to measure Pr160$^{gag\text{-}pol}$ incorporation into virus-like particles

Plasmid(s)	Proteins produced	Reverse transcriptase activity (cpm of [^{32}P]TTP incorporated)
pBABY	None	70
pSVGAGPOLprotD25G	Pr55, Pr160	4,060
pSVGAG-RRE-R	Pr55	17
pSVFS5TprotD25G	Pr160	86
pSVGAG-RRE-R + pSVFS5TprotD25G	Pr55, Pr160	669
pSVGAG-RRE-R + pSVFS5TprotD25Gmyr(−)	Pr55, Myr$^-$ Pr160	454

cotransfection was performed with pSVGAG-RRE-R and pSVFS5TprotD25Gmyr(−), incorporation of the Pr160$^{gag\text{-}pol}$ lacking myristic acid appeared to be between 65 and 80% as efficient as that of the wild-type Pr160$^{gag\text{-}pol}$ (Table 1), depending on the values assumed for the background. These results confirm the qualitative complementation experiments and demonstrate that a Pr160$^{gag\text{-}pol}$ lacking myristic acid can be efficiently rescued into particles by a Pr55^{gag} containing myristic acid.

Electron microscopic analysis of particles produced with a normal Pr55^{gag} and Pr160$^{gag\text{-}pol}$ lacking myristic acid. To determine the nature of the virus-like particles containing the myristylation-deficient Pr160$^{gag\text{-}pol}$, an electron microscopic analysis was performed. Thin sections of cells expressing Pr55^{gag} and a protease-positive or -deficient version of Pr160$^{gag\text{-}pol}$ lacking myristic acid were examined. The photographs of these sections are shown in Fig. 5.

Cells expressing Pr55^{gag} and the myristylation-deficient Pr160$^{gag\text{-}pol}$ containing an active protease showed mature particles with characteristic condensed cores (Fig. 5A). Cells expressing Pr55^{gag} and the myristylation-deficient Pr160$^{gag\text{-}pol}$ containing an inactive protease showed only immature particles (Fig. 5B).

These results suggest that Pr160$^{gag\text{-}pol}$ lacking myristic acid was incorporated into the particles shown in Fig. 5A in a nearly normal fashion, since the mature cores appear normal and could have arisen only from the action of the viral protease. As expected, the particles shown in Fig. 5B are immature, since these cells contain an inactive protease.

DISCUSSION

The ability to express Pr55^{gag} and Pr160$^{gag\text{-}pol}$ individually from separate expression vectors allowed us to determine the potential of each molecule to form particles which bud in the absence of the other protein. Our data clearly demonstrate that Pr55^{gag} is capable of forming virus-like particles when expressed alone. These particles bud and contain the typical concentric immature core that is seen when the viral protease is inactivated. This result confirms those described previously by other workers using different expression systems and demonstrates that in this regard, Pr55^{gag} is similar to the *gag* proteins of other retroviruses (6, 22, 31, 40).

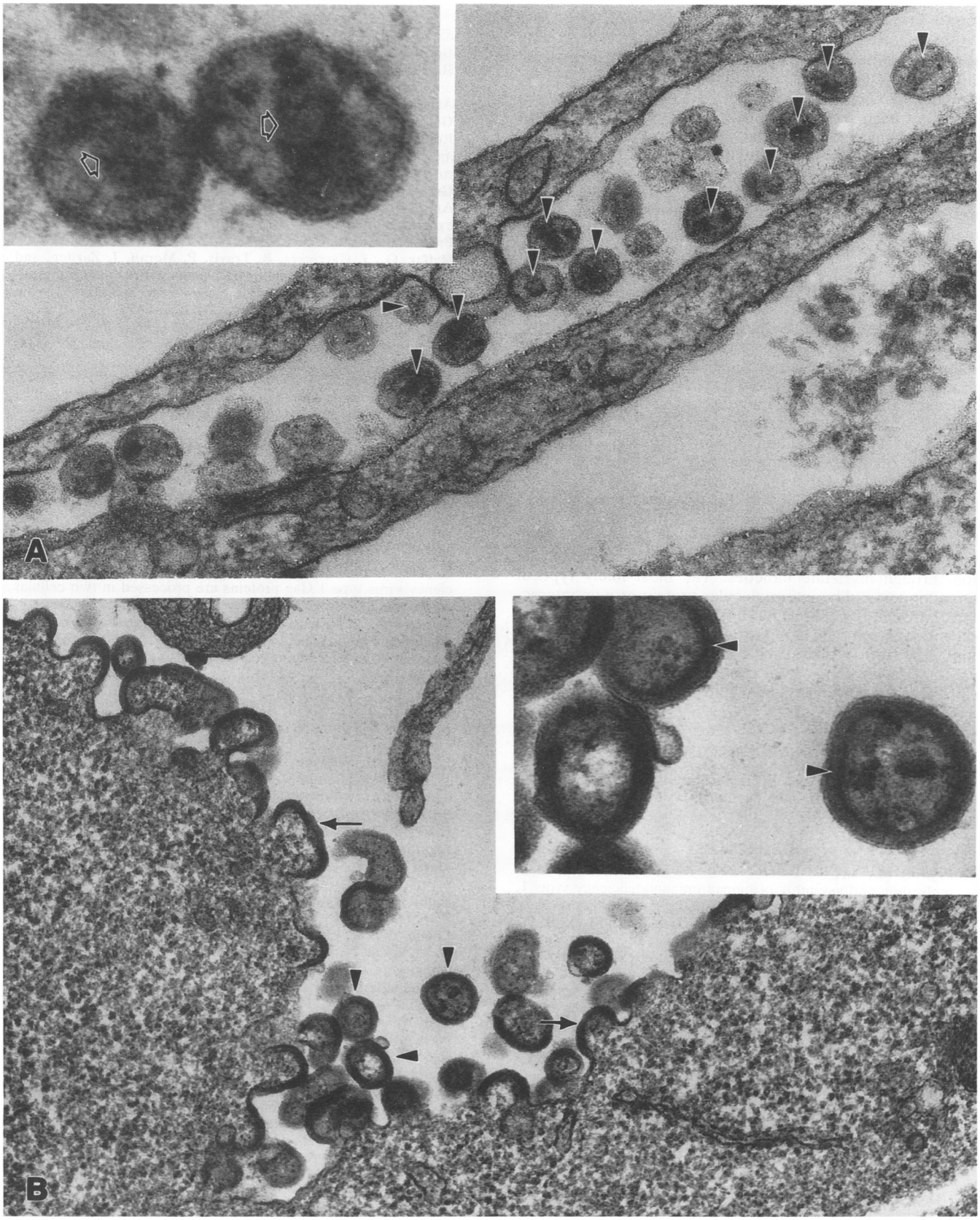

FIG. 5. Electron micrograph of cells cotransfected with plasmids expressing individual HIV-1 precursors. Thin sections of transfected CMT3-COS cells were prepared as described in Materials and Methods. (A) Cells cotransfected with pSVGAG-RRE-R and pSVFS5Tmyr(−). The arrows and arrowheads indicate the mature condensed cores. (B) Cells cotransfected with pSVGAG-RRE-R and pSVFS5TprotD25 Gmyr(−). The arrows and arrowheads indicate the immature concentric cores.

Expression of $Pr160^{gag\text{-}pol}$ by itself did not lead to viral particle formation. In some respects, this result may seem surprising, as $Pr160^{gag\text{-}pol}$ contains perfectly good p17 and p24 domains. On the other hand, $Pr160^{gag\text{-}pol}$ is lacking p6, which has been shown to be necessary for the formation of budding particles (8). It is interesting that, although particle budding did not occur, the viral protease still appeared to be activated normally. Protease activity was also observed, even in cells expressing a $Pr160^{gag\text{-}pol}$ molecule lacking myristate. Since it is well established that viral protease activation requires dimerization of the protease polypeptide, it is reasonable to assume that intermolecular association of at least two $Pr160^{gag\text{-}pol}$ molecules still occurred in these cells. Recently, cytoplasmic activation of the viral protease has been described for HIV-1-infected cells, and a role for such activation in viral pathogenesis has also been postulated (16, 30). Thus, it seems likely that the observed results reflect the presence of a novel pathway of protease activation distinct from that involved in virion morphogenesis.

Our results with the plasmid expressing both $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ containing a defective myristate addition signal confirm the notion that a functional myristate is required for particle formation (9). Using separate expression plasmids, we have also shown that the myristate on $Pr160^{gag\text{-}pol}$ is not necessary for incorporation of this precursor into virus-like particles when a functional myristate is present on $Pr55^{gag}$. This indicates that the association of $Pr160^{gag\text{-}pol}$ with $Pr55^{gag}$ probably involves mechanisms other than association through myristate moieties. The most likely type of interaction would be intermolecular protein-protein interactions between regions of the different precursor molecules. Recent data from our laboratory suggest that these associations may start to take place cotranslationally. In addition, we have shown that at least one site of association maps to a predicted α-helical region within the p24 domain of the precursors (32a).

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REFERENCES

1. **Bryant, M., and L. Ratner.** 1990. Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. Proc. Natl. Acad. Sci. USA **87**:523–527.
2. **Debouck, C., J. G. Gorniak, J. E. Strickler, T. D. Meek, B. W. Metcalf, and M. Rosenberg.** 1987. Human immunodeficiency virus protease expressed in Escherichia coli exhibits autoprocessing and specific maturation of the gag precursor. Proc. Natl. Acad. Sci. USA **84**:8903–8906.
3. **Ehrlich, L. S., B. E. Agresta, and C. A. Carter.** 1992. Assembly of recombinant human immunodeficiency virus type 1 capsid protein in vitro. J. Virol. **66**:4874–4883.
4. **Farmerie, W. G., D. D. Loeb, N. C. Casavant, C. A. Hutchison III, M. H. Edgell, and R. Swanstrom.** 1987. Expression and processing of the AIDS virus reverse transcriptase in Escherichia coli. Science **236**:305–308.
5. **Gelderblom, H. R., E. H. Hausmann, M. Ozel, G. Pauli, and M. A. Koch.** 1987. Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. Virology **156**:171–176.
6. **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.
7. **Gonda, M. A.** 1988. Molecular genetics and structure of the human immunodeficiency virus. J. Electron Microsc. Techniques **8**:17–40.
8. **Gottlinger, H. G., T. Dorfman, J. G. Sodroski, and W. A. Haseltine.** 1991. Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. Proc. Natl. Acad. Sci. USA **88**:3195–3199.
9. **Gottlinger, H. G., J. G. Sodroski, and W. A. Haseltine.** 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86**:5781–5785.
10. **Graves, M. C., J. J. Lim, E. P. Heimer, and R. A. Kramer.** 1988. An 11-kDa form of human immunodeficiency virus protease expressed in Escherichia coli is sufficient for enzymatic activity. Proc. Natl. Acad. Sci. USA **85**:2449–2453.
11. **Haffar, O., J. Garrigues, B. Travis, P. Moran, J. Zarling, and S.-L. Hu.** 1990. Human immunodeficiency virus-like, nonreplicating, *gag-env* particles assemble in a recombinant vaccinia virus expression system. J. Virol. **64**:2653–2659.
12. **Hammarskjöld, M.-L., and D. Rekosh.** 1989. The molecular biology of human immunodeficiency virus. Biochim. Biophys. Acta **989**:269–280.
13. **Hammarskjöld, M.-L., S.-C. Wang, and G. Klein.** 1986. High-level expression of the Epstein-Barr virus EBNA1 protein in CV1 cells and human lymphoid cells using a SV40 late replacement vector. Gene **43**:41–50.
14. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77**:51–59.
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. **Kaplan, A. H., and R. Swanstrom.** 1991. Human immunodeficiency virus type 1 Gag proteins are processed in two cellular compartments. Proc. Natl. Acad. Sci. USA **88**:4528–4532.
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. 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. **Lewis, N., J. Williams, D. Rekosh, and M.-L. Hammarskjöld.** 1990. Identification of a *cis*-acting element in human immunodeficiency virus type 2 (HIV-2) that is responsive to the HIV-1 *rev* and human T-cell leukemia virus types I and II *rex* proteins. J. Virol. **64**:1690–1697.
20. **Lightfoote, M. M., J. E. Coligan, T. M. Folks, A. S. Fauci, M. A. Martin, and S. Venkatesan.** 1986. Structural characterization of reverse transcriptase and endonuclease polypeptides of the acquired immunodeficiency syndrome retrovirus. J. Virol. **60**: 771–775.
21. **Lillehoj, E. P., F. H. R. Salazar, R. J. Mervis, M. G. Raum, H. W. Chan, N. Ahmad, and S. Venkatesan.** 1988. Purification and structural characterization of the putative *gag-pol* protease of human immunodeficiency virus. J. Virol. **62**:3053–3058.
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 for alternative *gag* precursors. J. Virol. **62**:3993–4002.
24. **Navia, M. A., and B. M. McKeever.** 1990. A role for the aspartyl protease from the human immunodeficiency virus type 1 (HIV-1) in the orchestration of virus assembly. Ann. N.Y. Acad. Sci. **616**:73–85.
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. **Partin, K., H. G. Krausslich, L. Ehrlich, E. Wimmer, and C. Carter.** 1990. Mutational analysis of a native substrate of the human immunodeficiency virus type 1 proteinase. J. Virol. **64**:3938–3947.
27. **Pearl, L. H., and W. R. Taylor.** 1987. A structural model for the retroviral proteases. Nature (London) **329**:351–354.
28. **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.
29. **Rekosh, D., A. Nygren, P. Flodby, M.-L. Hammarskjöld, and H. Wigzell.** 1988. Coexpression of human immunodeficiency virus envelope proteins and tat from a single simian virus 40 late replacement vector. Proc. Natl. Acad. Sci. USA **85**:334–338.
30. **Riviere, Y., V. Blank, P. Kourilsky, and A. Israel.** 1991. Processing of the precursor of NF-κB by the HIV-1 protease during acute infection. Nature (London) **350**:625–626.
31. **Royer, M., S. S. Hong, B. Gay, M. Cerutti, and P. Boulanger.** 1992. Expression and extracellular release of human immunodeficiency virus type 1 Gag precursors by recombinant baculovirus-infected cells. J. Virol. **66**:3230–3235.
32. **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.
32a. **Srinivasakumar, N.** Unpublished data.
33. **Veronese, F. D., T. D. Copeland, A. L. DeVico, R. Rahman, S. Oroszlan, R. C. Gallo, and M. G. Sarngadharan.** 1986. Characterization of highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III/LAV. Science **231**:1289–1291.
34. **Veronese, F. D., T. D. Copeland, S. Oroszlan, R. C. Gallo, and M. G. Sarngadharan.** 1988. Biochemical and immunological analysis of human immunodeficiency virus *gag* gene products p17 and p24. J. Virol. **62**:795–801.
35. **Veronese, F. D., R. Rahman, T. D. Copeland, S. Oroszlan, R. C. Gallo, and M. G. Sarngadharan.** 1987. Immunological and chemical analysis of P6, the carboxyl-terminal fragment of HIV P15. AIDS Res. Hum. Retroviruses **3**:253–264.
36. **Weber, I. T.** 1990. Comparison of the crystal structures and intersubunit interactions of human immunodeficiency and Rous sarcoma virus proteases. J. Biol. Chem. **265**:10492–10496.
37. **Weiss, R., N. Teich, H. Varmus, and J. Coffin.** 1982. Molecular biology of tumor viruses: RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Wilcox, C., J. S. Hu, and E. N. Olson.** 1987. Acylation of proteins with myristic acid occurs cotranslationally. Science **238**:1275–1278.
39. **Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theordore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. **62**:139–147.
40. **Wills, J. W., and R. C. Craven.** 1991. Form, function, and use of retroviral gag proteins (editorial). AIDS **5**:639–654.
41. **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.