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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

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Human immunodeficiency virus type 1 (HIV-1) *gag* and *pol* genes were expressed by using fragments of the BH10 clone of HIV inserted into a simian virus 40 late replacement vector. An initial construct containing the entire coding regions of *gag*, *pol*, and *vif* produced only minute amounts of the *gag* precursor, Pr55^{gag}. However, high-level expression was obtained when an additional sequence from the *env* gene (the *rev*-responsive element) was inserted 3′ of *vif* in the correct orientation, and *rev* was provided in *trans* from a second vector. Western immunoblot analysis of transfected cells showed the presence of large amounts of both Pr55^{gag} and Pr160$^{gag\text{-}pol}$ as well as all of the expected cleavage products. Electron microscopy of thin sections of transfected cells showed a multitude of viruslike particles. Both immature particles in the process of budding and particles containing the condensed core characteristic of HIV were observed. Analysis of the released viruslike particles showed the presence of active reverse transcriptase. Sucrose gradient analysis of particles produced from [^{3}H]uridine-labeled cells indicated a peak of radioactivity which cosedimented with a peak of p24, suggesting that the particles contained RNA.

The human immunodeficiency virus (HIV), like other retroviruses, assembles its nucleocapsid from two precursor polypeptides encoded by the viral genome (for reviews, see references 18a, 20, and 49). During assembly, the nucleocapsid precursors, together with the two strands of viral RNA to be packaged into particles, are believed to aggregate under the plasma membrane at regions which contain the viral envelope proteins. Virus particles are then formed by the exocytotic process known as budding (49). Electron microscopic studies indicate that budding virus particles contain an immature core which appears as an electron-dense ring structure closely apposed to the lipid bilayer (10, 14). After budding, it is believed that proteolytic cleavages of the precursors by the viral protease cause the core to rearrange and mature into its characteristic condensed structure. Although there have been many morphological studies which visualize the budding process in detail (10, 14), little is known about the molecular interactions which drive the formation of these particles or of the targeting mechanisms that bring core and envelope proteins together. For HIV-1, there is some data which indicate that an additional protein, vpu, may also be needed to promote efficient assembly (42, 45). However, vpu is not present in mature virus particles (2, 32, 43).

The core precursor made in the largest amounts in HIV-1-infected cells is Pr55^{gag} (39, 47). This polypeptide is the product of the *gag* open reading frame, and it is myristylated at its amino terminus (33, 47). Upon assembly, proteolytic cleavage of Pr55^{gag} gives rise to the myristylated matrix polypeptide p17, the major core polypeptide p24 (33, 47), and the proteins believed to be associated with the viral RNA, p9 and p6 (48). It has been known for some time that in both avian and murine retrovirus systems, expression of a *gag* precursor by itself can lead to the formation of viruslike particles, even in the absence of expression of viral envelope glycoproteins, reverse transcriptase, or viral RNA (for a review, see reference 49). Myristylation of the precursor is required for efficient particle formation in mammalian but not in avian cells (15, 50). The second precursor (p160$^{gag\text{-}pol}$) is made in much smaller amounts, approximately 5 to 10% of the level of the first precursor. It is a fusion product of the *gag* and *pol* open reading frames and is the result of a ribosomal frameshift mechanism that shifts reading frames from *gag* to *pol* shortly before the stop codon at the 3′ end of *gag* (21, 51). This precursor is also myristylated and is cleaved upon assembly to yield the products of *gag* as well as the products of *pol*. The proteins encoded within *pol* are the viral protease (4, 16, 26, 30), reverse transcriptase (7, 29, 46), and integrase proteins.

Expression of the HIV-1 structural genes in virus-infected cells requires coexpression of the *rev* gene (8, 41). Cytoplasmic RNAs capable of producing *gag*, *gag-pol*, or *env* are not found in the absence of a functional *rev* gene product (8). More recent studies have shown that *rev* is also required for structural protein expression from eucaryotic expression vectors which undergo nuclear transcription (3, 6, 9, 17, 18, 25). In these systems, *rev* appears to be necessary for the transport of mRNAs encoding the structural proteins from the nucleus to the cytoplasm.

Viruslike particle formation by Pr55^{gag} has recently been shown from a baculovirus vector in insect cells (12). Expression in this system did not require *rev* coexpression. In these studies, the particles contained a core structure which closely resembled the cores of immature particles seen in HIV-infected cells. These particles presumably contained only uncleaved Pr55^{gag} since Pr160$^{gag\text{-}pol}$ containing the viral protease was not expressed in this system. The same group of workers also reported similar results with the simian immunodeficiency virus *gag* precursor (5).

In the present study, we report the use of a simian virus 40 (SV40) late replacement vector to produce large amounts of the HIV-1 *gag* and *gag-pol* precursors. Expression is totally

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dependent on *rev* coexpression and the *cis*-acting *rev*-responsive element. Our results show that in this system, viruslike particles which contain reverse transcriptase and RNA are formed, and that the budding and maturation process is independent of envelope protein coexpression. In addition, proteolytic processing of the precursors appears to take place normally, and the released virus particles have mature core structures.

MATERIALS AND METHODS

Cells and transfections. CMT3 COS cells (11) were transfected by using DEAE-dextran as previously described (19). The cells were harvested at 60 to 72 h posttransfection.

Western immunoblot analysis. Western blots were performed by using Immobilon P membranes (Millipore Corp., Bedford, Mass.) as previously described (18, 19). The blots were developed with either a human serum from a HIV-positive individual, which was a kind gift from Tun Ho Lee (Harvard University, Boston, Mass.), or a monoclonal antibody directed against p24 obtained from Epitope, Inc. (Beaverton, Oreg.).

Ultrastructural studies. Cells on plates were transfected with the appropriate vectors. At 65 h posttransfection, they were washed twice with phosphate-buffered saline and fixed on the plates for 10 minutes with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. The cells were then scraped from the plates into the same solution and centrifuged at 2,000 rpm for 5 min in a benchtop IEC centrifuge. The cell pellets were then fixed for an additional 2 h in the same solution. After being washed in the same buffer, the pellets were then postfixed with 1% osmium tetraoxide for 1.5 h in s-collidine buffer pH 7.2. En bloc staining was then carried out for 1 h in 1% uranyl acetate in 0.1 M maleate buffer, pH 6.0. After being washed in the same buffer, the samples were dehydrated in a graduated series of cold ethanols and propylene oxide before infiltration with PolyBed 812. They were then placed in flat embedding molds containing a fresh mixture of PolyBed 812. Polymerization was accomplished at 60°C for 48 h. Ultrathin sections (90 nm in thickness) were cut on an Ultracut-E equipped with a diamond knife. The sections were then doubly stained with saturated uranyl acetate in 50% ethanol and lead citrate. The stained sections were then examined and photographed with a Hitachi T-600 electron microscope.

p24 antigen assays. These assays were performed by using either a p24 radioimmune assay system (catalog no. NEK-040; Dupont, NEN Research Products, Boston, Mass.) or a p24 antigen enzyme-linked immunosorbent assay kit (catalog no. 0801002; Cellular Products Inc. Buffalo, N.Y.) according to the directions provided by the manufacturers. In each case, a standard curve was generated by using a series of serial dilutions of a standard provided by the manufacturer. The test dilution was always determined to be within the linear range of the assay. In the experiments which involved measuring the distribution of p24 present in the cells and medium, proportional amounts were assayed so that a direct comparison of the distribution could be made.

Reverse transcriptase (RT) assays. Particle-associated RT was assayed by standard procedures (44). Briefly, the culture medium from a dish of transfected cells (3×10^6 cells and 10 ml per dish) was centrifuged at 1,500 rpm for 3 min in a benchtop IEC centrifuge to remove the cells. One milliliter was then filtered (pore size, 0.45 μm) to remove cellular debris and centrifuged again at 15,000 rpm (Eppendorf centrifuge) for 2 h to pellet the viruslike particles. The pellet was then assayed for RT activity. RT activity was measured directly by adding 80 μl of RT reaction mixture to the tube, incubating it for 1 h at 37°C, and precipitating the product with 10% trichloroacetic acid containing 0.01 M sodium pyrophosphate. The precipitate was then collected on glass fiber filters and counted in a scintillation counter. Each reaction mix contained 0.05 M Tris hydrochloride (pH 8.0), 0.01 M $MgCl_2$, 0.06 M KCl, 0.12 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), 0.36 mM glutathione, 0.002 M dithiothreitol, 0.08% Triton X-100, 4 μg of poly(rA)-poly (dT)template, and 20 μCi of [^{3}H]TTP.

Expression plasmids. Several different plasmids were used to express the HIV-1 *rev* gene. pSVrev1 contains the *rev*-coding sequences from a HIV-1 cDNA clone (pCV1) (1) under the control of the SV40 late promoter. This plasmid was constructed by inserting a Bsu96I fragment from pCV1 into the vector pBABY (36) at the unique *Xho*I cloning site after repair of both fragment and vector with T4 DNA polymerase.

pRev1 contains the same cDNA fragment under the control of the promoter-enhancer region from the simian cytomegalovirus (CMV) IE94 gene (−650 to +30) (22). This plasmid was constructed by inserting the Bsu96I fragment from pCV1 into the vector pCMV. In pCMV, the SV40 sequences contained within pBABY have been exchanged for the CMV promoter region (28).

The plasmid pSVSX3Δ3 contains a genomic copy of the *rev* gene. This plasmid is similar to the previously described pSVSX1Δ3, except that it is missing most of the first coding exon of *tat* (18). It consists of a *Sal*I-*Xho*I fragment of HIV-1 proviral DNA (HXB2 clone nucleotides 5496 to 8896) which was removed from the vector pIII-env3-1 (13) and inserted into the *Xho*I site of pBABY. This places the HIV sequences under the control of the SV40 late promoter. A deletion was made in the *env* open reading frame (HXB2 nucleotides 7040 to 7620) so that the vector produces *rev* and a truncated form of gp120 (gp70).

The plasmid used to express HIV-1 envelope proteins was pSVSX3. This vector is identical to pSVSX3Δ3 described above, except that it contains an intact *env* gene. It produces *rev*, gp160, gp120, and gp41.

pGAGPOL contains a DNA fragment from the proviral BH10 clone of HIV-1 (corresponding to HXB2 nucleotides 679 to 5785) inserted into the *Xho*I site of pBABY. This fragment includes the open reading frames for *gag*, *pol*, and *vif*. It was isolated from a plasmid which contained the entire BH10 proviral clone as a *Sac*I fragment in the vector pSP64 (Promega Biotec, Madison, Wis.). The HIV-1 sequences were excised as a *Sal*I fragment between the *Sal*I site in the pSP64 polylinker and the *Sal*I site at 5785 in the HIV-1 DNA. After cloning into pBABY, a small deletion (*Ava*I-*Xba*I) was made in the remaining pSP64 polylinker by restriction enzyme digestion, T4 DNA polymerase repair, and religation. This deletion removed a *Bam*HI site which was present in the polylinker. A second *Bam*HI site at the boundary of the SV40 and pBR322 DNA sequences was also removed by restriction enzyme digestion and repair. These manipulations were performed to facilitate the construction of pGAGPOL-RRE-r.

pGAGPOL-RRE-r and pGAGPOL-RRE-w were created by inserting the *Bgl*II-*Bam*HI fragment from the HIV-1 BH10 clone (corresponding to HXB2 nucleotides 7620 to 8474) into the unique *Bam*HI site present in pGAGPOL. This fragment contains the RRE. To make pGAGPOL-RRE-r, this fragment was inserted in the same orientation with

respect to *gag*, *pol*, and *vif* as in the viral genome. To make pGAGPOL-RRE-w, the fragment was inserted in the reverse orientation.

Sedimentation analysis. Fifteen milliliters of medium from transfected cells was first cleared of cellular debris by centrifugation at 2,500 rpm for 5 min in a benchtop IEC centrifuge. It was then layered onto a sucrose step gradient consisting of 5 ml of 60% sucrose and 15 ml of 20% sucrose in a buffer containing 0.025M Tris hydrochloride, pH 7.5, 0.140 M NaCl, 0.005 M KCl, and 0.007 M $NaHPO_4$. The gradient was then subjected to ultracentrifugation at 28,000 rpm for 4 h in an SW28 rotor. Twenty fractions (1 ml) were collected from the bottom of the tube. Aliquots (50 μl) of each fraction were assayed for radioactivity by precipitation with 10% trichloroacetic acid. The precipitate was collected on glass fiber filters and counted in a scintillation counter. Peak fractions were also assayed for p24 antigen by using an enzyme-linked immunosorbent assay kit as described above.

RESULTS

***gag* and *gag-pol* expression from a SV40 vector requires *rev* and the *rev*-responsive element (RRE).** Previously, we demonstrated that a SV40 late replacement vector (pBABY) could be used to express large amounts of properly processed HIV-1 envelope proteins and *tat* in transfected mammalian cells (36). This system mimicked the situation in HIV-1-infected cells in that production of envelope protein was completely dependent on the coexpression of a functional *rev* gene (18). We also showed that envelope protein expression required the presence of a *cis*-acting sequence within the envelope gene which was initially mapped to an 854-base-pair *Bgl*II-*Bam*HI restriction enzyme fragment. Subsequent studies by us and others have mapped this RRE to a smaller 268-base-pair fragment (28, 31). It seemed likely that expression of *gag* and *gag-pol* from pBABY would also require *rev* and the RRE, since *rev* had been shown to regulate *gag* and *gag-pol* expression as well as *env* expression in HIV-1-infected cells. In addition, others had shown that expression of *gag* alone from a vector containing the HIV-1 long terminal repeat was *rev* dependent (3, 17). To test this notion, three pBABY-(HIV-1 DNA) recombinant plasmids were constructed. These plasmids as well as the original pBABY vector are shown in Fig. 1.

Each of the three plasmids contains the *Sac*I-*Sal*I restriction enzyme fragment from the BH10 proviral DNA clone of HIV-1 which encodes the *gag*, *pol*, and *vif* genes. To construct these vectors, a small polylinker containing a *Sal*I site was added to the *Sac*I end of the HIV-1 fragment (see Materials and Methods). The fragment was then cloned as a *Sal*I-*Sal*I fragment into the *Xho*I site of pBABY, creating the plasmid pGAGPOL. The orientation of the fragment was such that the SV40 late promoter was immediately 5′ of the *gag* open reading frame. Rabbit β-globin sequences 3′ of the HIV DNA provided splice and polyadenylation signals. To create the plasmids pGAGPOL-RRE-r and pGAGPOL-RRE-w, the 854-base-pair *Bgl*II-*Bam*HI fragment containing the RRE was cloned into the *Bam*HI site of pGAGPOL in either the right or the wrong orientation.

Each of the *gag-pol*-containing plasmids was then transfected into CMT3 COS cells with or without an additional plasmid that could supply *rev* in *trans*. In this experiment the plasmids used to supply *rev* were pSVrev (c-*rev*) which contained *rev* as a cDNA or pSVSX1Δ3 (g-*rev*) which contained *rev* in a fragment of proviral DNA. At 70 h posttransfection, the cells were harvested, and extracts were

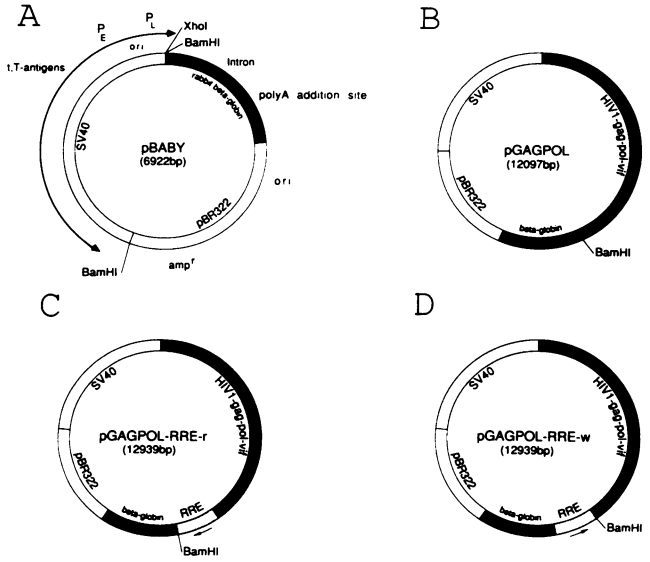

FIG. 1. Diagrams of various plasmids used in this study. The plasmids shown in panels B, C, and D were constructed from the SV40 late replacement vector pBABY shown in panel A. (See Materials and Methods for details.)

analyzed by Western blotting with a serum from an HIV-1-infected individual. The blot is shown in Fig. 2A. In each of the lanes containing either pGAGPOL or pGAGPOL-RRE-w with or without *rev* coexpression (lanes 1 to 6), only a series of weakly staining bands were detected. These weak bands were also present in extracts of CMT3 COS cells which were transfected with pBABY alone (data not shown). By contrast, in the lanes transfected with pGAGPOL-RRE-r and either of the *rev*-expressing plasmids (lanes 7 and 8), a series of about 10 prominent bands was visible. These bands were absent in the lane transfected with pGAGPOL-RRE-r alone (lane 9). From the position and relative intensity of these bands on the Western blot, it seemed likely that they represented the products of the *gag* and *gag-pol* reading frames. The band migrating with the position of highest molecular weight was of the size expected for the gag-pol precursor ($p160^{gag\text{-}pol}$). There was also a series of somewhat weaker bands in the range of 80 to 120 kilodaltons which correspond in size to expected cleavage intermediates derived from this precursor. There was a clear band at about 66 kilodaltons which corresponded in size to the reverse transcriptase (46). The strongest bands corresponded in size to $Pr55^{gag}$, the *gag* precursor; p41, a known cleavage intermediate (33, 47); and p24, the major core antigen. Although not visible on this gel, a band corresponding to p17 was also observed in other experiments (data not shown).

To provide further evidence that the various proteins expressed from pGAGPOL-RRE-r were authentic products of *gag*, another experiment was performed. In this case, pGAGPOL-RRE-r was cotransfected with pRev1, a plasmid containing a *rev* cDNA under the control of a CMV promoter (see Materials and Methods). At 70 h posttransfection, cell extracts were made and analyzed on a Western blot by using a monoclonal antibody known to be directed against p24. As a control, extracts from cells transfected with the parent vector pBABY were also analyzed. The blot is shown in Fig. 2b. As expected, in the lane containing the extract from cells transfected with pGAGPOL-RRE-r and pRev1 (Fig. 2b, lane 1), several bands were visualized. These bands corre-

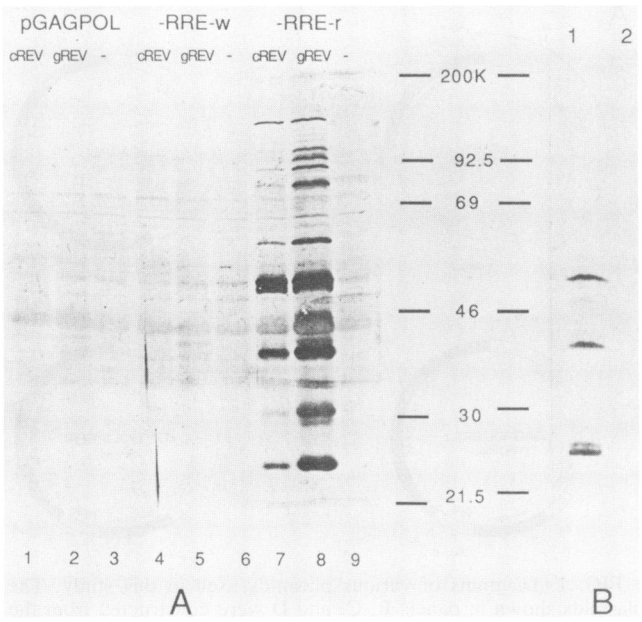

FIG. 2. Western blot analysis of proteins from cells transfected with various plasmids. Proteins from transfected cells were separated on a 12% sodium dodecyl sulfate–polyacrylamide gel and transferred to an Immobilon P membrane by electroblotting. (A) Western blot developed with serum from an HIV-positive individual. Lanes 1 to 3 were derived from cells transfected with gGAGPOL, lanes 4 to 6 were derived from cells transfected with gGAGPOL-RRE-w, and lanes 7 to 9 were derived from cells transfected with pGAGPOL-RRE-r. Where indicated, these vectors were also cotransfected with vectors that supplied *rev* in *trans*. cREV refers to pSVrev, which expresses a cDNA copy of the *rev* gene. gREV refers to pSVSX3Δ3, which expresses a genomic copy of *rev*. (B) Western blot developed with a monoclonal antibody directed against p24. Lane 1 is derived from cells transfected with pGAGPOL-RRE-r and pRev1. pRev1 expresses a cDNA copy of the *rev* gene from the simian CMV immediate early promoter. Lane 2 is derived from cells transfected with pBABY. The positions of molecular weight markers run in parallel lanes are indicated.

sponded to some of the bands detected with human HIV-positive serum and migrated to the positions expected for $Pr55^{gag}$, p41, and p24.

p24 is released into the medium from transfected cells. Our previous work with the HIV-1 *env* expressed from the same parent vector as that used to express *gag* and *pol* suggested a level of expression corresponding to about 5 μg of envelope protein per transfected 100-mm dish (approximately 3×10^6 to 5×10^6 cells) (36). To determine whether the levels of *gag* and *gag-pol* products produced in this system were comparable with the levels achieved with the *env* vector and to assess the distribution of product between cells and medium, a quantitative p24 antigen assay was performed. To do this, cells were transfected with pGAGPOL-RRE-r alone, pGAGPOL-RRE-r plus pRev1, or pGAGPOL-RRE-r plus pSVSX3, a plasmid which expresses both *rev* and functional HIV-1 envelope proteins. Parallel cultures were harvested at various times posttransfection, and samples of both media and cells were assayed for p24 (Table 1). Consistent with the Western blot data of Fig. 2, p24 expression was again totally dependent on *rev* coexpression. In contrast, coexpression of HIV-1 envelope proteins appeared to have no significant influence either on the total levels of p24 or on its distribution. Except for the earliest time points, the medium contained the majority of detectable p24. There was a dramatic increase in the overall amount of p24 accumulated between 30 and 44 h posttransfection, as would be expected for a product expressed from an SV40 late replacement vector. The high levels of p24 in the medium suggested that p24 was being released from the cells and raised the possibility that viruslike particles were being made.

TABLE 1. p24 antigen assay of cells and media from cells transfected or cotransfected with various plasmids

Vector and time of harvest (h posttransfection)[a]	p24 present in:	
	Cell lysate[b] (ng of p24/0.1 dish)	Medium (ng of p24/ml)[c]
pGAGPOL-RRE-r		
30	0	0
44	0	0
58	0	0
72	0	0
pGAGPOL-RRE-r+pRev1		
30	52	40
44	200	240
58	180	280
72	200	300
pGAGPOL-RRE-r+pSVSX3		
30	35	8
44	230	200
58	195	230
72	190	250

[a] Four dishes of CMT3 COS cells were transfected or cotransfected with each of the indicated vectors, and at the times indicated the medium and cells were harvested separately from one dish.
[b] Cell pellets were suspended in 1 ml of Tris-buffered saline, pH 7.2. Each dish contained approximately 3×10^5 cells.
[c] Each dish of cells contained 10 ml.

Viruslike particles are produced in transfected cells. To examine this possibility directly, cells were again transfected with either pGAGPOL-RRE-r plus pRev1 or pGAGPOL-RRE-r plus pSVSX3. pRev1 expresses only *rev*, while pSVSX3 expresses both *rev* and functional envelope proteins (see Materials and Methods). At 65 h posttransfection, the cells were fixed, harvested, cut into thin sections, stained, and examined in the electron microscope. A composite photograph of what was observed is shown in Fig. 3. In cells transfected with either combination of vectors, viruslike particles which bore a remarkable resemblance to either the immature or mature particles observed in HIV-1-infected cells were visualized. Budding immature particles contained an electron-dense ring just under their membranes (Fig. 3C, D, and E). Surface projections, resembling envelope protein spikes, were observed in some of the preparations derived from cells which were cotransfected with the vector expressing the envelope proteins (Fig. 3C). Fully matured particles contained the condensed core structure characteristic of HIV-1 (Fig. 3A, B, and F). Particles with this condensed core were visible even in both transfections.

Viruslike particles from transfected cells contain RT and RNA. Since the particles released from transfected cells contained mature condensed cores that were indistinguishable from that of HIV-1, it seemed a likely possibility that they would contain active RT and RNA. To examine the particles for RT, cells were transfected with pGAGPOL-RRE-r alone, pGAGPOL-RRE-r plus pSVrev, pGAGPOL-RRE-r plus pRev1, or pGAGPOL-RRE-r plus pSVSX3. After 72 h, the medium was harvested, filtered, and sedi-

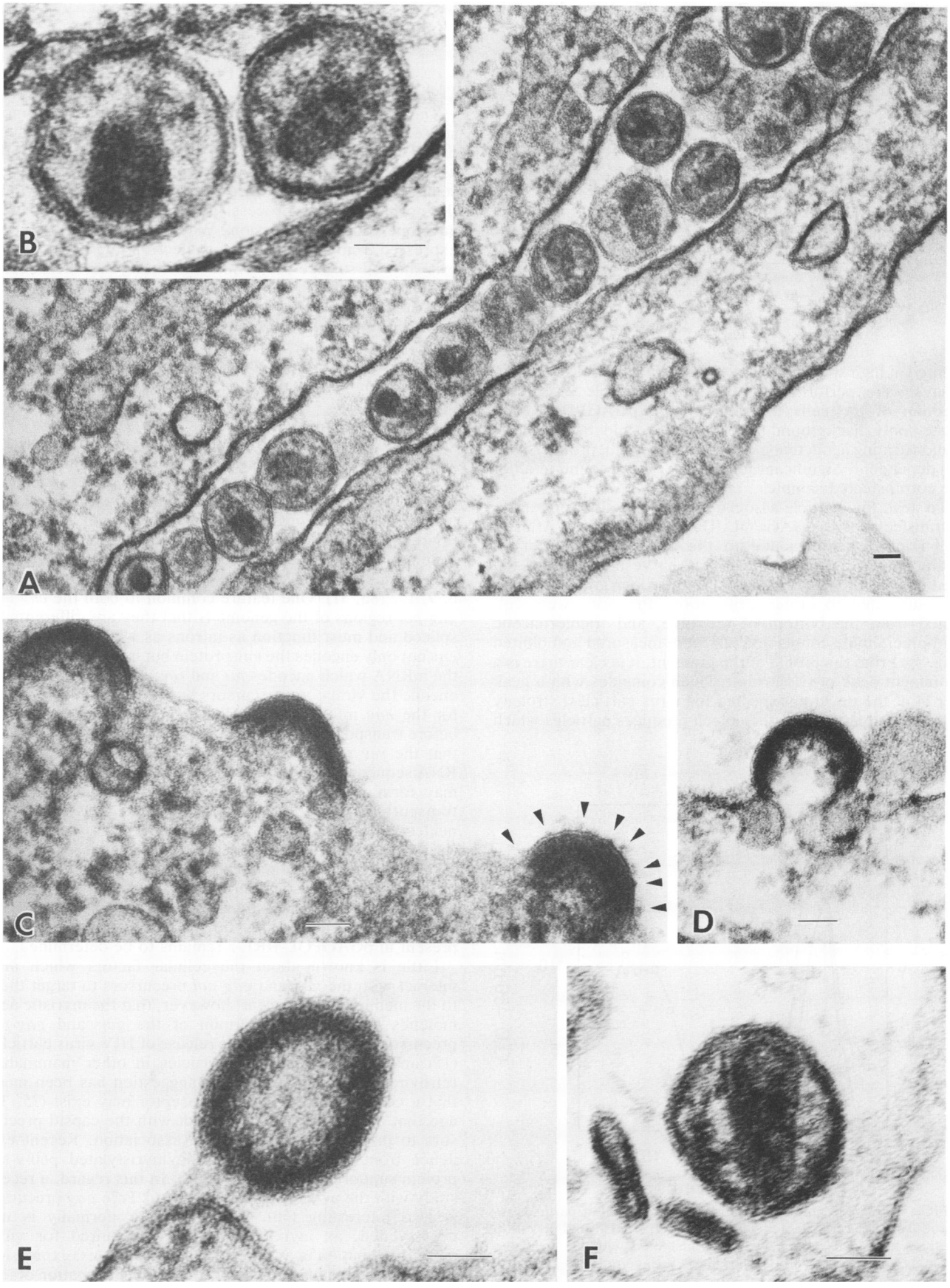

FIG. 3. Electron micrographs of cells transfected with various plasmids. Thin sections of transfected cells were prepared as described in Materials and Methods. Panels A, B, and C are from cells transfected with pGAGPOL-RRE-r and pSVSX3. Panels D, E, and F are from cells transfected with pGAGPOL-RRE-r and pRev1. The bar in each micrograph represents 60 nm.

TABLE 2. RT assay of particles released from cells transfected or cotransfected with various plasmids[a]

Vector	RT activity ([^{3}H]TTP cpm) in:	
	Expt 1	Expt 2
pGAGPOL-RRE-r	120	870
pGAGPOL-RRE-r+pSVrev1	16,580	ND[b]
pGAGPOL-RRE-r+pRev1	ND	332,160
pGAGPOL-RRE-r+pSVSX3	43,200	17,280

[a] The high-speed pellet from 1 ml of filtered medium was assayed for RT activity as described in Materials and Methods.
[b] ND, Not determined.

mented at high speed to pellet the viruslike particles, and RT assays were performed on the pellets (Table 2). In the medium of the cells transfected with pGAGPOL-RRE-r alone, only background levels of RT could be detected, demonstrating again that expression of *gag-pol* products was *rev* dependent. Significant levels of RT were found in all of the cotransfected samples.

To test for particle-associated RNA, cells were again cotransfected with pGAGPOL-RRE-r and pSVSX3. After 24 h, [^{3}H]uridine was added to the medium, and after an additional 48 h, the medium was harvested. Part of the medium was then layered onto a sucrose step gradient which was subjected to ultracentrifugation. Fractions were collected from the bottom of the tube, and trichloroacetic acid-precipitable counts and p24 were measured and plotted (Fig. 4). From the profile of the gradient, it is clear there is a prominent peak of [^{3}H]uridine which coincides with a peak of p24 at the position expected for virus particles, strongly suggesting that the transfected cell produces particles which contain RNA.

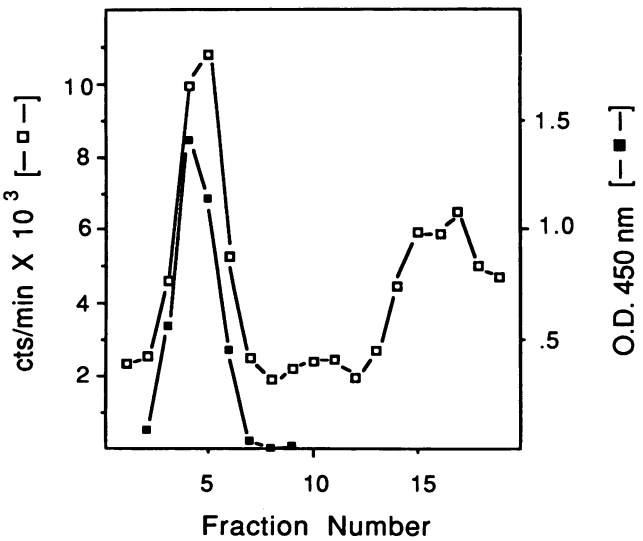

FIG. 4. Sucrose gradient sedimentation analysis of particles released from cells transfected with pGAGPOL-RRE-r and pSVSX3. Medium from cells which were cotransfected with the indicated plasmids and labeled with [^{3}H]uridine was layered onto a sucrose step gradient and subjected to ultracentrifugation as described in Materials and Methods. Fractions were collected and counted for trichloroacetic acid-precipitable ^{3}H. p24 assays were then performed on the peak fractions. O.D., Optical density.

DISCUSSION

The experiments reported in this study clearly demonstrate that expression of the *gag* and *pol* open reading frames of HIV-1 in transfected CMT3 COS cells leads to the assembly of particles which contain cores that closely resemble infectious virus. These particles appeared to form by the normal budding process, even in the absence of viral envelope protein expression. When envelope protein was expressed from a second vector, electron microscopic analysis showed surface projections indicating that the budding particles contained a normal viral envelope. Our results also indicated that the particles contained RNA.

After this paper was submitted for publication, two different groups have reported HIV viruslike particle formation by using vaccinia virus vectors (17a, 23). In one case, mature particles containing both *gag* and envelope proteins were observed (17a).

Efficient expression of *gag* and *gag-pol* from the SV40 vector was also shown to require coexpression of the *rev* gene and the *cis*-acting RRE, in a manner similar to that previously described by us for *env* (18). This result is consistent with findings reported for other expression systems (3, 17) and indicates that sequences which convey the *rev*-requiring phenotype must reside throughout the HIV-1 genome. In the absence of *rev*, mRNA transcribed from these genes has been shown to accumulate in the nucleus (3, 6, 9, 17, 18a, 31). One feature common to both the *env* and *gag-pol* regions of the genome is that they are differentially spliced and must function as introns as well as exons (i.e., *env* not only encodes the *env* protein but is also the intron for the mRNA which encodes *tat* and *rev*; *gag* and *pol* not only encode the viral core precursors but form part of the intron for the *env* mRNA). Since introns are normally removed before transport from the nucleus, it is tempting to speculate that the *rev* requirement stems from features of the HIV RNA sequences that mark them as introns. *rev* and the RRE may form part of a mechanism that has arisen to allow transport of intron containing *gag* and *env* mRNA from nucleus to cytoplasm. Recent experiments in our laboratories have demonstrated that stable cytoplasmic *env* mRNA formation requires the presence of a 5′ splice site upstream of the *env* sequences and an interaction of this site with U1 snRNA (X. Lu et al., unpublished data). Whether expression of *gag* and *gag-pol* requires the 5′ upstream splice site that is present in pGAGPOL-RRE-r remains to be determined.

Little is known about the cellular factors which may interact with the *gag* and *gag-pol* precursors to target them to the membrane. It is clear, however, that the myristic acid moieties on the amino termini of the *gag* and *gag-pol* precursors are essential for the release of HIV virus particles (15) and for the release of particles in other mammalian retroviral systems (35, 38). The suggestion has been made that a cellular myristyl-protein receptor may exist (35, 38) and that this protein could interact with the capsid precursors to promote their membrane association. Recent evidence from experiments with the myristylated p60-v-src protein support this hypothesis (37). In this regard, a recent study with the avian rous sarcoma virus Pr76 *gag* precursor is also interesting (50). This precursor normally is not myristylated, as myristylation is not required for virus particle formation in avian cells. Because of this, expression of Pr76 in mammalian cells only leads to the formation of low levels of extracellular particles. However, a fivefold enhancement of particle release was observed when the normal amino terminus of the precursor was exchanged for the

sequence from p60 v-src which was known to be sufficient for myristylation.

The finding that retrovirus core precursors can target themselves to the plasma membrane and drive budding, even in the absence of viral envelope protein expression, presents an interesting dilemma, since it has been known for some time that viral envelope proteins themselves possess signals which target them to specific membrane surfaces (24, 34, 40). Also, in infected cells, virus budding is believed to take place only in regions which contain viral envelope proteins. What then restricts the evagination process to these regions? Are core precursors and envelope proteins targeted to adjoining sites by independent mechanisms, or is there a common cellular factor involved? Does expression of the viral envelope protein in cells expressing the core precursors alter the sites of budding? Answers to these questions must await further experimentation.

Our data also suggests that RNA is incorporated into the viruslike particles, although we have not yet identified its nature. Recently, a sequence of 19 nucleotides which appears to be required for efficient packaging of viral RNA has been identified by using a mutated proviral clone (27). This element is positioned just 5′ from the start of the *gag* open reading frame and is contained within the plasmid pGAGPOL-RRE-r. Therefore, the possibility exists that specific RNA transcribed from this plasmid is packaged into the viruslike particles. The system described in the present study may thus be useful for further examining all aspects of the assembly process, including the mechanisms underlying RNA packaging.

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