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Mutations of RNA and Protein Sequences Involved in Human Immunodeficiency Virus Type 1 Packaging Result in Production of Noninfectious Virus

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To identify RNA and protein sequences involved in packaging of human immunodeficiency virus type 1 (HIV-1), various mutations were introduced into the viral genome. Portions of the human immunodeficiency virus type 1 genome between the first splice donor site and the *gag* initiation codon were deleted to investigate the RNA packaging site (Ψ). Point mutations that alter cysteine residues in one or both zinc finger motifs of p7, a cleavage product of the *gag* precursor, were created to study the role of the *gag* zinc fingers in packaging. The Ψ site mutants and the *gag* mutants exhibited similar phenotypes. Cells transfected with the mutant genomes, while expressing normal levels of human immunodeficiency virus type 1 RNA and proteins, produced viral particles that were normal in protein content but lacked detectable viral RNA. These mutant virions were unable to productively infect cells. The combination of human immunodeficiency virus type 1 packaging mutations should minimize fortuitous assembly of infectious virus and may provide a means to produce noninfectious particles for candidate vaccines.

Packaging, the process by which viral proteins and RNA get together to form an infectious particle, is an essential step in the retrovirus life cycle. Unspliced genomic RNA is an indispensable component of the viral particle, and the retrovirus packaging process can discriminate between unspliced viral RNA and spliced viral or cellular mRNAs. This discrimination is very likely the result of interaction between specific viral RNA sequences and viral proteins (34, 38). RNA sequences essential for packaging have been mapped to a site near the 5′ end of the viral genome in avian and murine retroviruses (16a, 17, 21–23, 33, 37). This site occurs within intervening sequences that are removed during splicing, producing an unpackageable RNA.

One or more proteins encoded by sequences at the 3′ end of the *gag* gene are thought to be involved in the recognition of viral RNA (6, 15), because deletion mutations at this locus appear to produce particles lacking viral RNA (25, 26). A zinc finger motif of the form $CysX_2CysX_4HisX_4Cys$ is a common feature of the carboxyl terminus of the *gag* precursor in all retroviruses (4). This motif occurs once in the murine retroviruses and twice in most other retroviruses studied thus far. Because nucleic acid-binding properties have been attributed to proteins containing zinc finger motifs (9), a direct role for the zinc finger in RNA binding has been investigated for Rous sarcoma and Moloney leukemia viruses (16). In Rous sarcoma virus, deletion of the first of the two zinc finger motifs abolishes RNA packaging and infectivity. Deletion of the second motif reduces viral infectivity approximately 100-fold. Both zinc fingers are important for 70S RNA dimer formation (25, 26). Similar results have been observed for Moloney leukemia virus, in which the lack of packaging of genomic RNA seems to correlate with nonspecific packaging of spliced viral RNA and cellular mRNAs (13, 24, 29). One other function has been attributed to the *gag* zinc fingers; they are necessary for the correct positioning of the tRNA primer on the replication initiation site at the 5′ end of the RNA genome (29).

We report here an investigation of the RNA and protein sequences involved in human immunodeficiency virus type 1 (HIV-1) packaging. Mutations have been constructed both in HIV-1 RNA sequences suspected to be essential for packaging and in nucleotides that encode the *gag* zinc fingers. Viral particles produced by transfection of cells with these mutants are normal in protein content but are missing genomic RNA and are not infectious.

MATERIALS AND METHODS

Cell lines and viruses. The simian virus 40-transformed African green monkey cell line cos-1 (12) was obtained from G. Khoury (National Institutes of Health) and maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. The H9 and H9 IIIB T-lymphoid cell lines were maintained as described previously (28). Viral infections (28) and reverse transcriptase assays (7) were carried out as described previously after filtration of culture supernatants through a 0.45-μm-pore-size filter (Millipore Corp., Bedford, Mass.).

Site-directed mutagenesis and plasmid construction. The parent DNA used for these studies is the biologically active clone pHXB2gtp (11). The *Sac*I-*Sal*I (base pairs 39 to 5366) (31) 5.3-kilobase-pair (kb) fragment was cloned in M13mp18, and oligo-mediated site-directed mutagenesis was performed on the single-stranded DNA by the protocol of Kunkel et al. (18). The following oligomers were used for the mutagenesis: A3, TGACGCTCTCGCACCCATCTCTCACCAGTCGCCG CCCCTC, deleting nucleotides 293 to 331 (31); A4, CTCTCT CCTTCTAGCCTCCGCTCACCAGTCGCCGCCCCTC, deleting nucleotides 293 to 313; A14, TGCCCTTCTTTGCCAT AATTGAAATACTTAACAATCTTTC, changing guanines at positions 1508 and 1517 to adenines; A15, TGTCCTTCC TTTCGATATTTCCAATAGCCCTTTTTCCTAG, changing the guanosines at positions 1571 and 1580 to adenines. The introduction of the mutations was verified by sequencing

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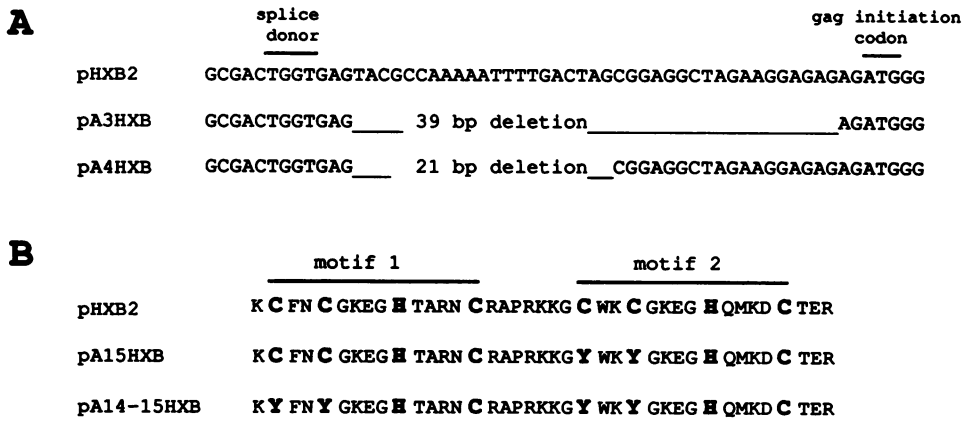

FIG. 1. HIV-1 mutations. (A) Locations and sizes of deletions that affect the HIV-1 Ψ site. (B) Amino acid changes in the zinc fingers of HIV-1 *gag* produced by various point mutations. The guanine nucleotides in the second position of codons coding for cysteine residues were changed to adenine nucleotides, producing codons coding for tyrosine.

different M13mp18 recombinant DNAs by using two specific oligomers: A5 (CCATCGATCTAATTCTC) and A16 (GGC CAGATCTTCCCTAA) (3). A *Bss*hII-*Bal*I 1.9-kb DNA fragment from the mutated M13mp18 recombinant clones was used to transfer all of the different mutations to the wild-type plasmid pHXB2gpt. The full-length HIV-1 mutant clones were designated pA3HXB, pA4HXB, pA15HXB, and pA14-15HXB, according to the oligonucleotides used in the mutagenesis. The entire region that had been transferred from the M13mp18 plasmids was sequenced by using double-stranded DNA sequencing methods (3) to confirm the presence of the desired mutations and the absence of any other alterations. The plasmid pHXB2Bam p3 is described elsewhere (10). All DNA manipulations were according to standard procedures (3).

Eucaryotic cell transfections. cos-1 cells were seeded at a density of 10^6 per 100-mm plate, 24 h before transfection, in DME medium supplemented with 10% fetal calf serum and incubated at 37°C in 5% CO_2. Transfection was generally carried out by using 10 μg of plasmid DNA per 100-mm plate as described by Chen and Okayama (5). When viral particles were destined for RNA analysis, transfections in cos-1 cells were performed by the method of Seldon et al. (32) to avoid the heavy DNA contamination of samples that was observed when calcium phosphate transformation was performed.

Nucleic acid analysis. RNA was extracted from cos-1 cells by using the hot phenol method of Queen and Baltimore (30). Viral RNA was extracted from supernatants containing equal amounts of p24 and was carried out in presence of equal amounts of added tRNA, which was used to monitor the final recovery of RNA. RNA samples were resuspended in H_2O to obtain identical concentrations of tRNA (1 μg/μl). Northern (RNA) blot analysis and RNA slot blot analysis were performed as previously described (3). A 9-kb HIV-1 DNA probe from pHXB2gpt was labeled with [^{32}P]dATP by random priming and was used in the Northern blot hybridization. A similarly labeled 3.8-kb *Cla*I-*Eco*RI *gag-pol* fragment from pHXB2gpt was used as a probe for the slot blot. All filters were washed extensively with 0.5× SSC (1× SSC is 0.15 NaCl and 0.015 M sodium citrate, pH 7.0)–0.1% sodium dodecyl sulfate at 65°C. Signals were detected by autoradiography with Kodak XAR film with a Du Pont Quanta III screen.

Analysis of viral protein. cos-1 cells transfected 48 h earlier with 10 μg were metabolically labeled with 500 μCi of [^{35}S]methionine for 4 h. Cell lysates were prepared and immunoprecipitations were performed as described previously (36). The HIV-1-positive human serum used in these experiments had demonstrated reactivity with all known viral structural proteins (10). Immunoprecipitated proteins were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (19). Virus was pelleted by centrifugation for 3 h at 27,000 rpm in an SW27 rotor. The pellet was suspended in dissociation buffer (0.01 M Tris hydrochloride [pH 7.3], 0.2% Triton X-100, 0.001 M EDTA, 0.005 M dithiothreitol, 0.006 M KCl) when reverse transcriptase activity was to be measured (7) or in 0.2% Triton X-100 and Laemli buffer (19) when protein analysis was to be performed. Western blot analysis and radioimmunoprecipitation followed the procedure of Veronese et al. (36). P24 analysis on tissue culture supernatants or on pelleted virus was performed by using a Du Pont P24 kit as recommended by the manufacturer.

RESULTS

Construction of viral mutants. To investigate the RNA and protein sequences involved in HIV-1 packaging, we have introduced a variety of mutations into the viral genome. Using the defined Ψ sites of murine leukemia, spleen necrosis, and avian sarcoma viruses as a guide, two deletion mutations were constructed in homologous sequences in the HIV-1 genome to investigate whether this region acts as a packaging signal for the human virus. By using oligo-mediated mutagenesis, nucleotides 293 through 331 (31) were deleted to create the mutant pA3HXB and nucleotides 293 through 313 were deleted to produce pA4HXB (Fig. 1A). These deletions occurred between the first splice donor site and the *gag* initiation codon, leaving the consensus sequence for the first splicing donor site intact.

To investigate the role of the *gag* zinc fingers in virus assembly, the p7 protein-coding sequence was mutagenized (Fig. 1B). The p7 and p6 proteins of HIV-1 are adjacent products of the *gag* precursor (14, 35), analogous to the p12 protein of Rous sarcoma virus and the p10 protein of Moloney leukemia virus. Oligo-mediated mutagenesis was employed to substitute tyrosines for the first two cysteines of the second zinc finger motif, producing the mutant pA15HXB. A second mutant, pA14-15HXB, in which both of the zinc finger motifs were altered, was constructed by

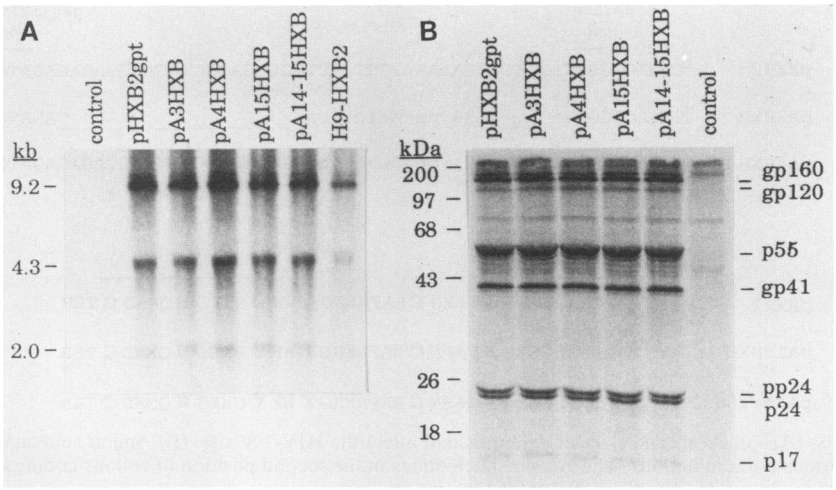

FIG. 2. Transcription and translation of HIV-1 mutants in cos-1 cells. (A) Northern blot analysis comparing viral transcripts in RNA extracted from transfected cos-1 cells. A 10-μg sample of DNase I-treated total cellular RNA was loaded in each lane. RNA from mock-transfected cos-1 cells was used as a negative control, and 5 μg of RNA from H9 cells chronically infected with HXB2 virus was used as a positive control. The HIV-1-specific probe was a ^{32}P-labeled full-length viral DNA. (B) HIV-1-specific proteins in transfected cos-1 cells. Lysates derived from cos-1 cells transfected with each plasmid indicated were immunoprecipitated with an HIV-1-positive human serum (10). As a negative control, a lysate from cells transfected with the plasmid pHXB2Bam p3, which does not produce virus due to a posttranscriptional defect (10), was used. Immunoprecipitated proteins were resolved by using a 10% sodium dodecyl sulfate–polyacrylamide gel (19).

further mutating pA15HXB to substitute tyrosines for the first two cysteines of the first zinc finger motif.

Analysis of HIV-1 mutants in cos-1 cells. The ability of the HIV-1 mutants to express RNA and proteins was examined in the African green monkey kidney cell line cos-1. Transfection of cos-1 cells with plasmids carrying biologically active HIV-1 DNA and a simian virus 40 origin of replication results in the recovery of infectious HIV-1 virus. Viral transcription, translation, assembly, and budding occur normally in these cells, and the recovered virus is capable of infecting susceptible cells (10). cos-1 cells were transfected with the wild-type pHXB2gpt plasmid and the four mutated molecular clones of HIV-1 to investigate viral gene expression. The patterns of RNA transcripts in cells transfected with the mutant plasmids were indistinguishable from that obtained from cells transfected with the wild-type plasmid (Fig. 2A). In each case, all three classes of HIV-1 mRNA were present: the 9.2-kb genomic mRNA, a 4.3-kb spliced mRNA encoding the *env* and the *vif* genes (1), and the heterogeneous collection of messages in the range of 2 kb which includes *tat-III*, *rev*, and *nef* mRNA (2). Thus, these HIV-1 mutations do not appear to affect the expression of viral RNA.

The expression of HIV-1 structural proteins was investigated by metabolic labeling of transfected cos-1 cells, followed by immunoprecipitation of viral proteins with human serum antibodies with demonstrated specificity for HIV-1 antigens (Fig. 2B). All of the major structural proteins of the virus were present in cells transfected with either the HIV-1 wild-type or mutant plasmids. The presence of gp160, gp120, p55, gp41, p24, and p17 in all of these transfected cells indicates that the HIV-1 mutations do not substantially alter the synthesis and processing of *gag* and envelope precursors.

The relative expression of one of the regulatory proteins, *tat*, was also investigated for these mutants. Cotransfection of wild-type or mutant HIV-1 plasmids with the plasmid pC15, in which a chloramphenicol acetyltransferase gene is under the control of the HIV-1 long terminal repeat (10), indicated that each of the mutants produce normal levels of *tat* (data not shown). We conclude that the four HIV-1 mutants under study do not exhibit substantial alterations in the expression of viral RNAs and proteins.

Biological activity of viral mutants. To assess the biological activity of the HIV-1 particles produced by transfection of cos-1 cells with pHXB2gpt, pA3HXB, pA4HXB, pA15 HXB, and pA14-15HXB2, culture supernatants were harvested, filtered, and examined for the presence of reverse transcriptase activity and the *gag* protein p24. All of the supernatants contained substantial, although varied, levels of reverse transcriptase and p24. Supernatants adjusted to contain comparable levels of p24 (15 ng) were used to infect H9 cells, which are susceptible to infection and permissive for HIV-1 replication (28). In H9 cells infected with supernatant from cos-1 cells transfected with pHXB2gpt, both the percentage of HIV-1 antigen-positive cells and the reverse transcriptase activity increased with time (Table 1). In contrast, H9 cultures exposed to supernatants derived from cos-1 cells transfected with mutant viral genomes were negative in cellular p24 immunofluorescence, reverse transcriptase activity, or particle-associated p24 assays. These assays continued to be negative for 30 days after the H9 cells were exposed to mutant viral particles. These data indicate that the viral particles produced by all four HIV-1 mutants in cos-1 cells lack infectivity when transferred to a susceptible cell line.

Analysis of viral particles. The protein and nucleic acid composition of mutant viral particles from cos-1 cell transfections was studied to determine whether the lack of productive infection of H9 cells was due to defective virions. Three different assays indicated that the amounts of HIV-1 protein present in supernatants of cos-1 cells transfected with the mutant HIV-1 plasmids were similar to that obtained with the wild-type plasmid. Viral particles in the cos-1 supernatants were pelleted by centrifugation and a quantitative analysis of the amounts of p24 present in the pellet was

TABLE 1. Infectivity of HIV-1 genomes in H9 cells[a]

Day	Immunofluorescence (% of control)	p24 (ng/ml)	Reverse transcriptase activity (cpm of [^{32}P]dGTP per ml per min) × 10^3
3	0.5	>20	ND
6	5	>20	32.0
9	30	>20	91.4
12	65	>20	156.6
16	90	>20	56.7
30	90	>20	56.6

[a] H9 cells were infected by filtered (0.45 μm (Millipore) supernatants from cos-1 cells that had been transfected 48 h earlier with the indicated plasmids. Immunofluorescence assays were performed with murine monoclonal antibody specific for the p24gag protein (36) and the H9-HIV IIIB cell line as a positive control. p24 assays were performed with a p24 enzyme-linked immunosorbent assay kit (Du Pont Co.). Reverse transcriptase assays (7) were carried out after filtration of culture supernatants. Results are reported for pHXB2gpt. Negative results were obtained for the following plasmid constructs: pA3HXB, pA4HXB, pA15HXB, and pA14-15HXB. ND, Not determined.

performed by using a p24 enzyme-linked immunosorbent assay. p24 values ranged from 15 to 20 ng in each sample (Table 2). A reverse transcriptase assay performed on the pelleted viral particles revealed a twofold reduction in mutant levels relative to the wild type (Table 2). Finally, Western blot analysis showed that the wild type and each of the mutants produced particles containing comparable amounts of *gag* and *env* proteins (Fig. 3). To determine whether the transfection frequency was similar among the wild-type and mutant transfections of the cos-1 cells, an immunofluorescence assay was performed on fixed cos-1 cells 48 h after transfection by using an anti-p24-specific mouse monoclonal antibody (36). The fraction of cells producing positive signals was nearly identical among the different transfections, ranging between 17 and 20%. Together, these data indicated that the number and protein composition of viral particles produced by the HIV-1 mutants were similar to those of the wild type.

The amount of viral RNA present in these virions was investigated by extracting RNA directly from virions present in the supernatant, immobilizing the nucleic acid on nitrocellulose slot blots, and probing with a labeled *Cla*I-*Eco*RI 3.8-kb *gag-pol* fragment (Fig. 4). At least a 100-fold reduction in the RNA content was observed for both Ψ and cysteine mutants relative to that of the wild type. Thus, for both Ψ and *gag* zinc finger mutants, HIV-1 RNA is not packaged efficiently in the viral particles.

Wild-type and mutant HIV-1 particle morphology was

TABLE 2. Quantitative analysis of HIV-1 viral proteins in supernatants of transfected cells[a]

Plasmid construct	Virus-associated p24 (ng/ml)	Reverse transcriptase activity (cpm of [^{32}P]dGTP per ml per min)
pHXB2gpt	1.90	5,060
pA3HXB	1.70	3,133
pA4HXB	1.60	2,718
pA15HXB	1.75	3,436
pA14-15HXB	1.65	2,949
pHXB2 Bam p3	0.00	740

[a] Each transfection was overlaid with 10 ml of medium, and protein assays were carried out 48 h later. A Du Pont enzyme-linked immunosorbent assay kit was used to measure p24 levels in three different dilutions of each supernatant. Reverse transcriptase activity is given as the mean of three experiments.

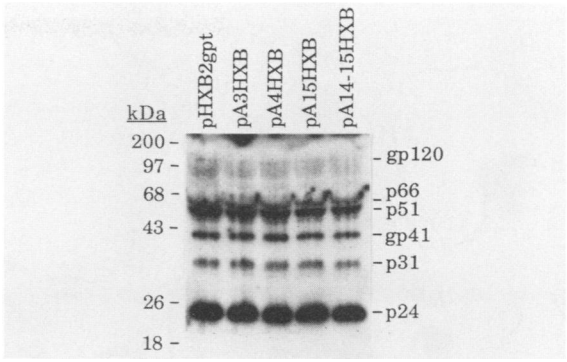

FIG. 3. Analysis of the protein content of HIV-1 mutant particles by Western blotting. Virus present in supernatants from cos-1 cells transfected with wild-type and mutant plasmids was concentrated by centrifugation at 27,000 rpm for 3 h in an SW28 rotor. Each pellet was suspended in 0.2% Triton X-100, and the concentration of p24 was determined by p24 enzyme-linked immunosorbent assay. Viral protein lysates, normalized to contain 75 ng of p24 each, were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with HIV-1-positive human serum (10). kDa, Kilodaltons.

examined by electron microscopy. Careful scoring of the sections indicated that the majority of the mutant particles were less electron dense than wild-type viral particles (Fig. 5). The appearance of empty mutant virions is consistent with the RNA and protein analyses, which indicated that the mutant virions lacked genomic RNA.

DISCUSSION

The experiments described here identify a site in the HIV-1 genome that is required for the encapsidation of viral genomic RNA and reveal that HIV-1 packaging site mutants and *gag* p7 zinc finger mutants exhibit a similar packaging-defective phenotype. These data are consistent with the idea that retroviral RNA packaging requires a specific interaction between particular viral RNA sequences and proteins.

Our data indicate that the HIV-1 Ψ site is located in the 5′ leader region of the genomic viral RNA, and deletions located immediately 3′ to the splice donor site as small as 21 base pairs can produce a defect in packaging. These results indicate that HIV-1 Ψ site mutants exhibit normal patterns of gene expression in transfected cells and that the viral particles produced by these mutants, although lacking detectable RNA, appear normal in protein composition. A recent report

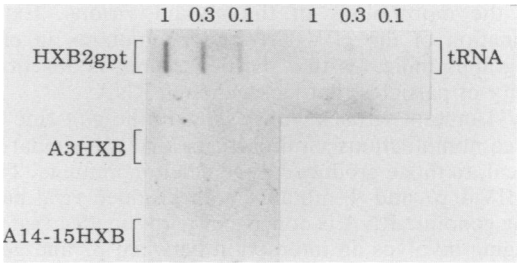

FIG. 4. Nucleic acid content of viral particles. Samples of 1, 0.3, and 0.1 eq of RNA were loaded on nitrocellulose, where 1 eq represents the amount of RNA obtained from cos-1 supernatants containing 18 ng of p24. A labeled 3.8-kb *Cla*I-*Eco*RI *gag-pol* fragment from pHXB2gpt was used as a probe.

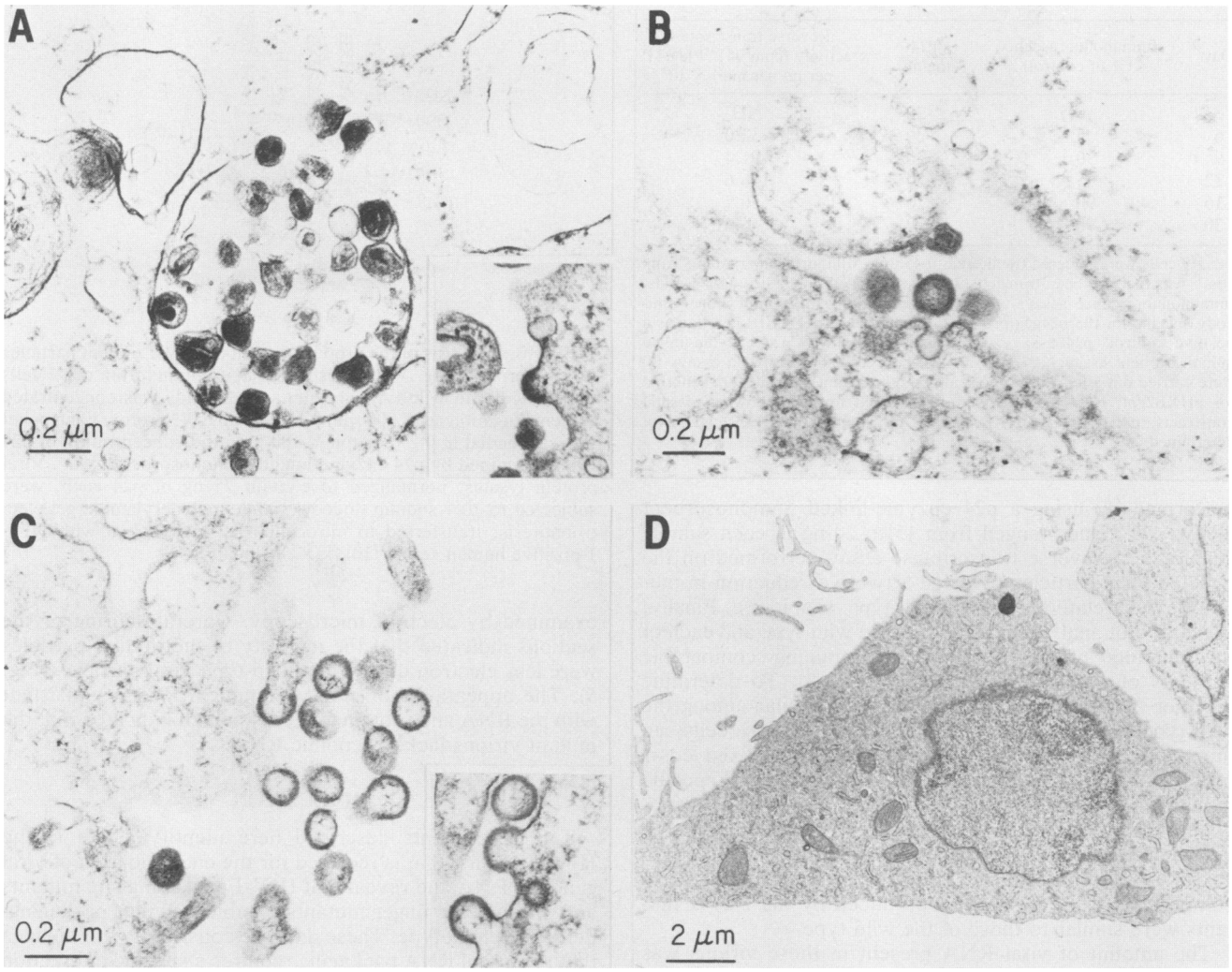

FIG. 5. Electron micrographs of transfected cos-1 cells: A, pHXB2gpt; B, pA3HXB; C, pA14-15HXB; D, pHXB2Bam p3 (negative control). The fields shown in panels A, B, and C were selected to show viral morphology, and the insets were selected to show viral budding. Pictures were taken at a magnification of ×38,500 for panels A, B, and C, and ×4,500 for the negative control in panel D.

describing another deletion mutation that overlaps this region also concluded that the HIV-1 Ψ site occurs at this position but did not further characterize the protein content of the viral particles, perhaps because these particles appeared to have normal morphology in electron micrographs (20). In contrast, electron micrographs of the HIV-1 Ψ mutants described here indicate that the lack of RNA does affect the morphology of the mutant virions. Extensive examination of the HIV-1 Ψ and p7 mutants in electron micrographs indicates that the dense core is absent in the majority of particles that lack genomic RNA.

HIV-1 mutant viruses with lesions in the *gag* zinc fingers produce noninfectious viral particles that are similar, if not identical, to those produced by Ψ deletion mutants. The fact that HIV-1 p7 and Ψ mutants both produce viral particles lacking genomic RNA is consistent with the idea that HIV-1 packaging involves an interaction between p7 and genomic RNA. If p7 interacts with genomic RNA, the zinc fingers could play a direct role in binding. It is also possible that the *gag* zinc fingers interact with other nucleic acids, i.e., the tRNA primer (29), or with other proteins that are important for proper RNA packaging.

It is clear from the analysis of the packaging mutants that assembly of viral capsids occurs in the absence of RNA packaging. A particle whose protein composition is similar to that of wild-type virions is assembled even when the genomic RNA cannot be packaged. Mutations in the zinc fingers of p7, a protein that is probably directly involved in RNA packaging, do not affect capsid assembly, indicating that the zinc finger domain of p7 does not play a role in assembly.

The extent to which retroviral packaging mutations reduce viral infectivity is not yet clear. Results obtained with Molony and spleen necrosis viruses with packaging defects indicate that these mutants lose 2 to 4 log units of infectivity relative to that of wild-type viruses (22, 23, 37). When infectious virus is recovered from cells that contain packaging mutants, the recovered virus is probably the result of recombination between the mutant virus and endogenous retroviral sequences (23). If recombination is responsible for the leakiness of murine retroviral packaging mutations, the lack of endogenous retroviruses with sequences similar to that of HIV-1 in human cells makes this mechanism of reversion unlikely for HIV-1 packaging mutants.

Recent results indicate that whole inactivated simian immunodeficiency virus type 1 can provide some protection from simian immunodeficiency virus type 1 infection in rhesus monkeys (8, 27). The HIV-1 packaging mutations described here may provide a means to produce noninfectious antigenic particles useful for induction of anti-HIV-1 immune responses. Viral particles of this type may better retain immunogenic characteristics than virus inactivated by chemical or thermal treatments. A cell line with a stably integrated mutant provirus is being constructed to provide a source of such noninfectious particles, and its properties are under investigation. Potential reversion of point mutations in the provirus should be minimized by the simultaneous presence of the Ψ site deletion and the four *gag* cysteine mutations present in A14-15HXB. Although we cannot eliminate the possibility that a mutant viral genome might occasionally be packaged, inadvertant packaging of an HIV-1 genome may not lead to a productive infection, both because of the packaging defect and because of the role of the *gag* zinc fingers in correctly positioning the tRNA primer necessary for reverse transcription (29). Finally, the HIV-1 genome containing packaging mutations can be divided into two independent constructs stably integrated in a cell line, probably eliminating fortuitous production of infectious virus.

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