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Incorporation of Vpr into Human Immunodeficiency Virus Type 1 Virions: Requirement for the p6 Region of *gag* and Mutational Analysis

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The product of the *vpr* open reading frame of human immunodeficiency virus type 1 (HIV-1) is a 15-kDa, arginine-rich protein that is present in virions in molar quantities equivalent to that of Gag. We report here the results of our investigations into the mechanism by which Vpr is incorporated into virions during assembly in infected cells. For these studies we used an expression vector encoding a Vpr molecule fused at its amino terminus to a nine-amino-acid peptide from influenza virus hemagglutinin. The tagged Vpr expression vector and a *vpr* mutant HIV-1 provirus were used to cotransfect COS cells, and the resulting virions were tested for the presence of the tagged protein on immunoblots probed with monoclonal antibody against the hemagglutinin peptide. The COS-produced virions were found to contain readily detectable amounts of tagged Vpr and smaller amounts of a putative tagged Vpr dimer. Infectivity of the particles was not altered by incorporation of tagged Vpr. Our results using this system in combination with mutant HIV-1 proviruses suggested that incorporation of Vpr into virions requires the carboxy-terminal Gag protein of HIV-1 (p6) but not gp160, Pol, or genomic viral RNA. In addition, analysis of mutated, tagged Vpr molecules suggested that amino acids near the carboxy terminus (amino acids 84 to 94) are required for incorporation of Vpr into HIV-1 virions. The single cysteine residue near the carboxy terminus was required for production of a stable protein. Arginine residues tested were not important for incorporation or stability of tagged Vpr. These results suggested a novel strategy for blocking HIV-1 replication.

Human immunodeficiency virus type 1 (HIV-1) capsids, like those of the type C retroviruses, are assembled at the cytoplasmic side of the plasma membrane from precursor polyproteins (8). The *gag*-encoded precursor, $Pr55^{gag}$, is sufficient for assembly and budding of virus-like particles, although the minimum portion of the molecule required for these events has not been clearly defined (9, 13, 28). The *gag-pol*-encoded precursor, $Pr160^{gag\text{-}pol}$, is synthesized as a result of a ribosomal frameshift near the 3′ end of *gag* (17). It is required for the cleavage of the precursor polyproteins by the *gag*-encoded protease, PR, but not for viral assembly or release (9). The mature virion proteins derived from cleavage of $Pr55^{gag}$ are (from amino to carboxy terminus) matrix (MA), capsid (CA), nucleocapsid (NC), and p6. Cleavage of $Pr160^{gag\text{-}pol}$ results in three additional *pol*-encoded proteins: PR, RT, and IN. Small spacer proteins, p2 and p1, have also been found in mature virions (15). These appear to result from closely spaced cleavages between CA and NC and between NC and p6, respectively. In the mature virion, MA is found associated with the inner side of the viral envelope (8), CA forms the cone-shape virion core (8), and NC is found associated with the viral genomic RNA (31). The role of p6 is not clear; however, it may play a role in release of virions from the cell surface, since in its absence, few virions appeared to be released from transfected cells (11). Each virion also contains two copies of viral genomic RNA (32). Incorporation of these molecules into the virion appears to be mediated by two cysteine motifs present in NC (10) and by a highly structured region of the RNA called ψ that lies between the splice donor site and the *gag* initiation codon (1, 3, 24).

vpr (34) and *vpx* (12) encode virion structural proteins of 96 and 126 amino acids, respectively. Whereas *vpr* appears to be present in the genomes of all lentiviruses, *vpx* has been found only in simian immunodeficiency virus (SIV) and HIV-2 (29). The proteins encoded by *vpr* and *vpx*, Vpr and Vpx, respectively, have been found in virions at molar amounts equivalent to that of Gag (4, 16, 35); however, unlike the other virion structural proteins, they are not synthesized as part of the *gag* or *gag-pol* precursor polyproteins. Their incorporation into virions is a property unusual for products of accessory genes: of the six known accessory gene products, Vpr and Vpx are the only ones whose products have been found at significant levels in virions (2). The mechanism by which these proteins are incorporated into virions is not well understood; a recent report suggested that incorporation of HIV-2 Vpx requires a virion structural component (19).

The role of *vpr* and *vpx* in viral replication is also not well understood. In vivo, *vpr* appears to increase pathogenicity and increase viral load in SIV-infected monkeys (23). Experimental results in vitro have been controversial; some authors have reported a critical role for *vpr* (14) and *vpx* (18, 36) in HIV-2 or SIV infection of peripheral blood lymphocytes and primary macrophages, while others have reported little effect (6, 26). Recent reports have suggested that HIV-1 *vpr* activates transcription of several cellular and viral promoters (5) and influences the differentiation state of rhabdomyosarcoma cells (25); however, the relevance of these activities to HIV-1 replication in vivo remains unclear. There is reason to believe that Vpx binds RNA: it is rich in arginine, a characteristic of many RNA binding proteins and has been shown to have affinity for an artificial RNA substrate (16).

Because of the unique role of Vpr and Vpx as accessory gene products present at high copy numbers in virions, we were

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interested in investigating the molecular basis for their incorporation into virions. To do this, we established an in vitro system in which cells were cotransfected with a *vpr* mutant HIV-1 provirus and an expression vector encoding Vpr fused to a nine-amino-acid sequence containing an epitope from the influenza virus hemagglutinin. This system allowed us to study both the viral components and the amino acids of Vpr that are important for its incorporation into virions.

MATERIALS AND METHODS

Plasmids. pcDNA-tag-Vpr was constructed by polymerase chain reaction (PCR) amplification of pNL4-3 (obtained from Malcolm Martin through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases) by using primers with sequences from the 5′ and 3′ ends of *vpr*. One primer contained a *Bam*HI cleavage site, followed by sequence encoding amino acids MYPYDVPDYA, followed by the 15 nucleotides (nt) 3′ to the *vpr* ATG initiation codon (nt 5562 to 5576) (5′-CGC GAAGCTTATGTACCCATACGATGTTCCAGATTACGC TGAACAAGCCCCAGAA-3′). The second primer contained sequence identical to the inverse complement of the sequence 3′ to *vpr* (nt 5860 to 5874) followed by a *Hin*dIII cleavage site (5′-CGCGGATCCTCCTGGATGTTCCA-3′). The amplified fragment was cleaved with *Bam*HI and *Hin*dIII and ligated to similarly cleaved pcDNAI/amp DNA (Invitrogen Corp.). The fragment was also cloned into pBS (Stratagene) for oligonucleotide-directed mutagenesis by the method of Kunkel (20). Mutated *vpr* genes were excised with *Bam*HI and *Hin*dIII and transferred to pcDNAI/amp. pcDNA-tag-Vpx was derived by amplification of *vpx* of SIVmac239 (obtained from Ronald Desrosiers through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases). Primers that hybridized to the 5′ and 3′ ends of *vpx* and contained *Hin*dIII and *Bam*HI sites, respectively, were used for ligation to pcDNAI/amp (5′-CGCGAAG CTTATGTACCCATACGATGTTCCAGATTACGCTTCA GATCCCAGGGAGAGA-3′ and 5′-CGCGGATCCTTCTTC CATTTATGCTAG-3′). Plasmids encoding amino-terminal truncations of tag-Vpr were constructed by PCR amplification as described above for pcDNA-tag-Vpr, except that the 5′ primer hybridized to nucleotides in *vpr* that were 3′ to the initiation codon.

Proviral DNAs. pNL4-3-R$^-$ was derived from pNL4-3 by cleaving with *Afl*II (nt 5633), blunting the ends with T4 DNA polymerase, and recircularizing with T4 DNA ligase. pNL4-3-R$^-$E$^-$ was derived by cleaving pNL4-3-R$^-$ at the NdeI site (nt 6400), blunting, and religating. Additional mutated proviruses were derived from pNL4-3-R$^-$E$^-$. In pNL4-3-R$^-$E$^-$-Gag$^-$, an *Acc*I site in *gag* (nt 960) was filled in and religated. In pNL4-3-R$^-$-P6$^-$, the TGG codon (nt 2101) located in the p1 spacer peptide between NC and p6 was changed to a TGA termination codon by PCR mutagenesis. This alteration also changes Ala-7 of Pol to threonine. In pNL4-3-R$^-$E$^-$-ψ^-, 32 nt (nt 754 to 785) between the splice donor and *gag* ATG were deleted by PCR amplification. In pNL4-3-R$^-$E$^-$-NC^{zn}, Cys-15 and Cys-18 (nt 1963 and 1972) of the NC first cysteine array were changed to serine by PCR mutagenesis (this mutation is identical to that previously reported by Gorelick et al. [10]). In pNL4-3-R$^-$E$^-$-NC$^-$, an AAG codon at amino acid 14 (nt 1960) was changed to a TAG termination codon by PCR mutagenesis. pNL4-3-R$^-$E$^-$NC^{zn}-ψ^- was derived by replacing the *Sph*I-*Apa*I fragment (nt 1447 to 2010) of pNL4-3-R$^-$E$^-$-ψ^- with that of pNL4-3-R$^-$E$^-$-NC^{zn}. pNL4-3-R$^-$E$^-$-Pol$^-$ was derived by removing a 23-bp *Hin*cII fragment (nt 2498 to 2521), causing a frameshift at amino acid 138 of *pol*. All mutations were verified by DNA sequencing.

Cell culture and transfections. COS cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. Cells (1.2×10^6) were cotransfected with 5 μg of provirus and 15 μg of tag-Vpr expression vector DNA (when two different expression vectors were used, 7.5 μg of each vector and 5 μg of provirus were used) by calcium phosphate precipitation as described previously (30). All plasmids were purified by double banding in cesium chloride density gradients. Cells were fed with fresh medium at 18 and 42 h posttransfection.

Infectivity assay. A301 cells (5.0×10^6) were incubated with virus (5.0 ng of $p24^{gag}$) in 2 ml of RPMI-fetal bovine serum containing 8.0 μg of Polybrene per ml for 2 h. The cells were washed twice with phosphate-buffered saline (PBS), resuspended in 5.0 ml of medium, and incubated at 37°C. Duplicate samples of culture supernatants were removed for $p24^{gag}$ analysis (Abbott Laboratories) 12 and 48 h postinfection. Culture supernatants were negative for $p24^{gag}$ (<10.0 pg/ml) at the first time point.

Preparation of virions and cell lysates. Culture supernatants were collected 76 h posttransfection and clarified by centrifugation for 10 min at 1,300 × *g*. Virions were pelleted by ultracentrifugation for 45 min at 100,000 × *g* and solubilized in 120 μl of lysis buffer (10 mM Tris [pH 7.5], 0.15 M NaCl, 2 mM EDTA, 0.5% Nonidet P-40). To prepare lysates, the cells were washed with PBS, removed from the culture dishes in 2 ml of PBS containing 0.5 mM EDTA, and pelleted by centrifugation for 5 min at 500 × *g*. The cells were then resuspended in 200 μl of ice-cold lysis buffer and incubated on ice for 4 min. Lysates were clarified by centrifugation in a microcentrifuge at 4°C for 4 min. Protein concentration was measured by using protein assay reagent (Bio-Rad). Supernatants and virions were stored at −80°C.

For density gradient analysis, virions were pelleted from six dishes of transfected COS cells. Virions were pelleted, resuspended in 500 μl of TNE (10 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA), and overlaid on an 11-ml linear sucrose gradient (20 to 60% [wt/vol] sucrose in TNE). The gradient was centrifuged for 15 h at 100,000 × *g*, and 12 fractions (0.9 ml) were collected from the bottom of the tube. Virions were pelleted and dissolved in 60 μl of lysis buffer. Half the fraction was used for immunoblot analysis, and 2 μl was used for $p24^{gag}$ enzyme-linked immunosorbent assay (ELISA).

Immunoblot analysis. Proteins from lysates (100 μg) and virions (40 μl) were separated on sodium dodecyl sulfate (SDS)–15% polyacrylamide gels in sample buffer containing 2-mercaptoethanol. The proteins were transferred onto Immobilon-P transfer membrane (Millipore Corp.) and processed as described by Landau et al. (22). Filters were probed with monoclonal antibody 12CA5 (Babco), pooled AIDS patient serum, or rabbit anti-recombinant gp120 (Genentech). Bound antibody was detected by treating with alkaline phosphatase-conjugated rabbit anti-mouse or goat anti-human immunoglobulin (Tago).

RESULTS

Incorporation of tagged Vpr into HIV-1 virions. To study the mechanism of incorporation of Vpr into virions, we constructed a Vpr expression vector (Fig. 1A, pcDNA-tag-Vpr) and an HIV-1 provirus deficient in *vpr* (Fig. 1B, pNL4-3-R$^-$). pcDNA-tag-Vpr contains an ATG codon followed by nucleotide sequence encoding the peptide YPYDVPDYA. This sequence is joined in frame to *vpr* of pNL4-3 at amino acid 2.

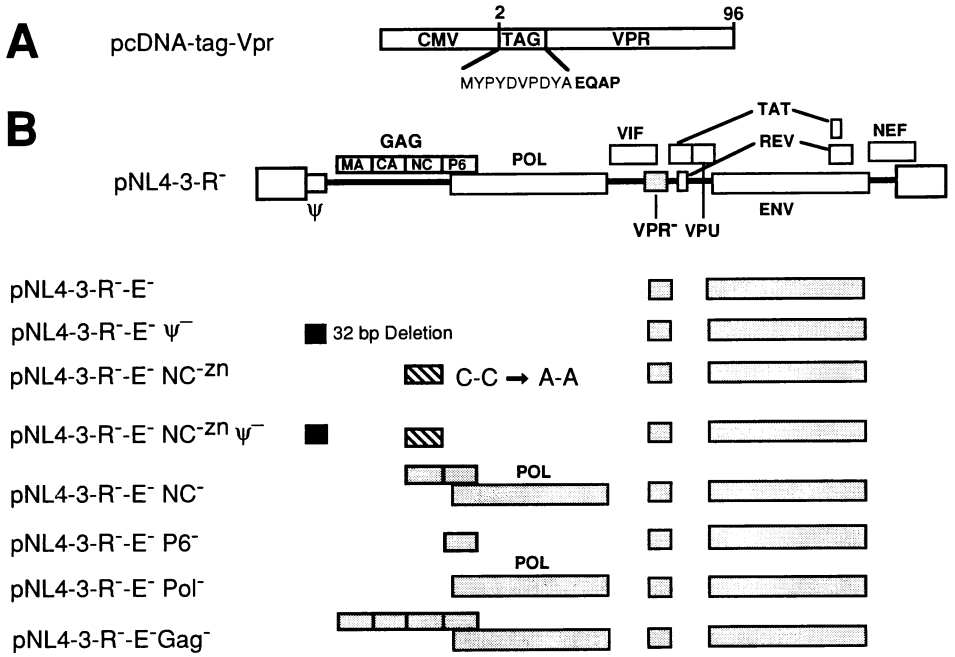

FIG. 1. Expression vectors for producing wild-type and mutant HIV-1 virions containing tagged Vpr. (A) pcDNA-tag-Vpr contains the cytomegalovirus immediate-early promoter linked to an open reading frame containing a methionine initiation codon, the nine-amino-acid influenza virus hemagglutinin epitope tag, and pNL4-3 *vpr* starting at amino acid 2. (B) Proviruses with defects in structural genes or packaging functions. pNL4-3-R$^-$ (derived from the *vpr*$^+$ HIV-1 provirus pNL4-3) encodes a *vpr* product frameshifted at amino acid 26. This frameshift does not affect the overlapping *vif* and *tat* open reading frames; pNL4-3-R$^-$E$^-$ encodes an *env* product frameshifted at amino acid 60, a location 3′ to the overlapping *vpu* reading frame (this provirus was used to generate further mutants). pNL4-3-R$^-$E$^-$-ψ$^-$ contains a 32-bp deletion of the packaging site, two bases larger than that described previously by Clavel and Orenstein (3); pNL4-3-R$^-$E$^-$-NC^{zn} has a mutation of the first cysteine array identical to that described previously by Gorelick et al. (10); pNL4-3-R$^-$E$^-$-NC^{zn}-ψ$^-$ contains both the ψ deletion and cysteine array mutation; pNL4-3-R$^-$E$^-$-NC$^-$ encodes an NC truncated at amino acid 15; pNL4-3-R$^-$E$^-$-Pol$^-$ encodes a *pol* product frameshifted at amino acid 138; pNL4-3-R$^-$E$^-$-P6$^-$ encodes a *gag* protein truncated in the p1 spacer region, nine amino acids amino-terminal to p6. This mutation also makes a conservative amino acid substitution in Pol (Ala-7 to threonine). pNL4-3-R$^-$E$^-$-Gag$^-$ encodes a *gag* protein frameshifted at amino acid 57. Affected regions are shown as boxes: gray boxes, truncations; striped boxes, the first cysteine array mutation; black boxes, ψ site deletion.

The nine-amino-acid peptide, or epitope tag, corresponds to a sequence from influenza virus hemagglutinin that is recognized by monoclonal antibody 12CA5 (7).

There were several advantages to studying the properties of tagged Vpr rather than those of the native protein. Available antipeptide antisera, as well as several batches of AIDS patient serum that we tested, did not efficiently recognize NL4-3 Vpr on immunoblots (data not shown). In addition, we could detect mutated Vpr molecules which otherwise might have been poorly recognized because of alteration of antigenic determinants. Furthermore, the epitope tag allowed us to determine whether the presence of an additional amino acid sequence at the amino terminus of Vpr would interfere with its incorporation into virions or would affect virus infectivity.

To test for incorporation of tagged Vpr into virions, we cotransfected COS cells with various combinations of pcDNA-tag-Vpr and pNL4-3-R$^-$ or pNL4-3. Supernatants were collected 48 to 72 h later, and lysates of the transfected cells were prepared. Lysates and virions pelleted from the supernatants were analyzed on immunoblots probed with monoclonal antibody 12CA5 as described in Materials and Methods. In each case, we verified that similar amounts of virus had been produced in each transfection by either hybridizing identical immunoblots with pooled anti-HIV-1 patient serum or by p24^{gag} assay.

Supernatants from COS cells cotransfected with pcDNA-tag-Vpr and either pNL4-3-R$^-$ or pNL4-3 (*vpr*$^+$) contained a particulate, 15-kDa protein recognized by 12CA5 (Fig. 2A, lanes 2 and 3). In contrast, supernatants from COS cells transfected separately with pNL4-3, pNL4-3-R$^-$, or pcDNA-tag-Vpr (Fig. 2A, lanes 4, 5, and 6) contained undetectable amounts of this protein. These observations suggested that the tagged Vpr produced in the transfected cells had been incorporated into virions. Furthermore, supernatant from COS cells transfected with pcDNA-tag-Vpr and a provirus unable to express Gag (pNL4-3-R$^-$-Gag$^-$) lacked detectable, particulate-tagged Vpr (see Fig. 5A, lanes 1 and 2). Thus, the presence of particulate-tagged Vpr in supernatants appeared to depend on the release of virions from the transfected cells. Cotransfection of COS cells with pNL4-3 (*vpr*$^+$) resulted in no significant decrease in the amount of virion-associated, tagged Vpr (Fig. 2A, lane 3), suggesting that tagged Vpr was incorporated even in the presence of provirus expressing wild-type Vpr and that tagged Vpr may be expressed at levels higher than that of the native protein expressed from the provirus. Cotransfection of pcDNA-tag-Vpr with a plasmid expressing wild-type Vpr (identical to pcDNA-tag-Vpr, except lacking the hemagglutinin peptide) resulted in a decreased amount of virion-associated, tagged Vpr (Fig. 2A, lane 1), suggesting that the tagged protein competes with wild-type protein for incorporation into virions. In this transfection, tagged Vpr was somewhat reduced in the lysate; therefore, we cannot rule out the possibility of decreased stability of tagged Vpr in this situation.

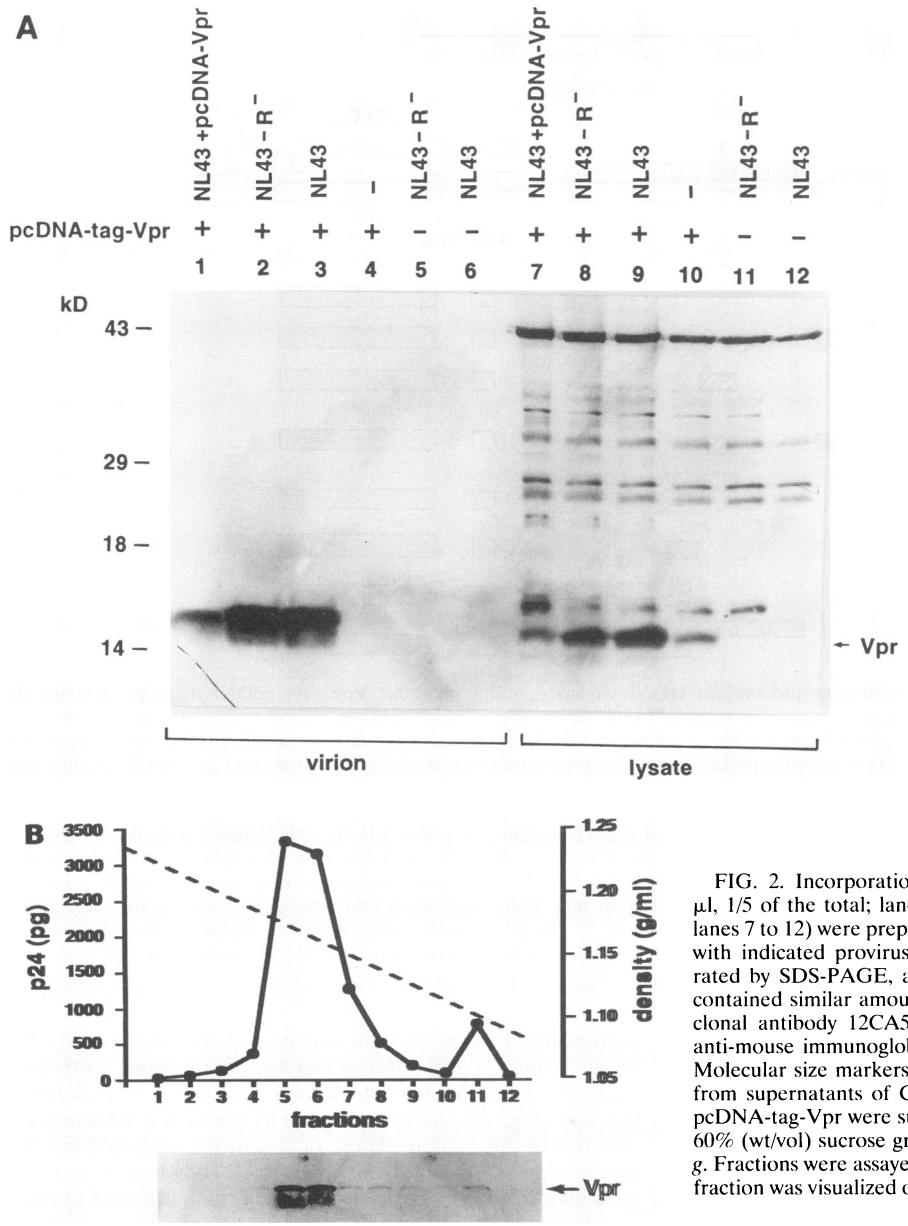

FIG. 2. Incorporation of tagged Vpr into virions. (A) Virions (40 μl, 1/5 of the total; lanes 1 to 6) and cell lysates (100 μg of protein; lanes 7 to 12) were prepared from COS cells (1.2×10^6), cotransfected with indicated provirus and with or without pcDNA-tag-Vpr, separated by SDS-PAGE, and transferred to filters. Virion preparations contained similar amounts of $p24^{gag}$. Filters were probed with monoclonal antibody 12CA5 and alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin as described in Materials and Methods. Molecular size markers are indicated to the left. (B) Pelleted virions from supernatants of COS cells cotransfected with pNL4-3-R⁻ and pcDNA-tag-Vpr were suspended in 500 μl of TNE, layered on a 20 to 60% (wt/vol) sucrose gradient, and centrifuged for 15 h at 100,000 × *g*. Fractions were assayed for $p24^{gag}$ by ELISA, and tagged Vpr in each fraction was visualized on an immunoblot probed with 12CA5 (below).

Lysates from COS cells transfected with pcDNA-tag-Vpr and pNL4-3-R⁻ generally contained more tagged Vpr than those transfected with pcDNA-tag-Vpr alone (Fig. 2A, lane 10). This effect did not appear to be due to transactivation of pcDNA-tag-Vpr by a factor encoded by the cotransfected provirus, since increased amounts of tagged Vpr were also present in lysates of COS cells transfected with vectors expressing mutant viruses that did not incorporate tagged Vpr but did express the HIV regulatory genes (as discussed below [see Fig. 5A, lanes 2, 6, and 8]). It is possible that association with virions protects Vpr from degradation by cellular proteases, thereby increasing the amount of Vpr detected in lysates of cells expressing tagged Vpr and virion components.

To test further whether the tagged Vpr secreted by the transfected COS cells was associated with HIV-1 virions, we fractionated the particles on a linear sucrose density gradient and visualized the tagged Vpr in each fraction on an immunoblot probed with 12CA5. The results showed that the particles had a density of about 1.16, consistent with that of retroviral particles (Fig. 2B). In addition, the amount of tagged Vpr in each fraction paralleled the amount of particles, further suggesting that the tagged Vpr had been incorporated into HIV-1 particles.

It was possible that virions containing tagged Vpr would be rendered noninfectious because of the presence of the amino-terminal tag. To test this, we infected A301 cells with virus (5.0 ng of $p24^{gag}$) produced by COS cells cotransfected with pNL4-3-R⁻, with or without pcDNA-tag-Vpr. Since the tagged protein is only present in the virions on the first round of viral replication, we measured $p24^{gag}$ production in the

cultures shortly after infection (48 h postinfection). The results from this assay showed that tagged Vpr had no detectable effect on viral infectivity (Vpr⁻ virus, 85.0 ± 2.8 pg/ml; tag-Vpr-containing virus, 112.1 ± 0.3 pg/ml). We cannot, however, rule out the possibility of small differences in viral infectivity that would not have been detected in this assay.

Virion components required for Vpr incorporation into HIV-1 virions. We next tested whether Vpr incorporation requires a homologous virion component. To do this, we asked whether HIV-1-tagged Vpr would be incorporated into murine leukemia virus (MLV) or human T-cell leukemia virus type II (HTLV-II) particles by using the appropriate proviral vectors (21, 22) and pcDNA-tag-Vpr. We also constructed a tagged SIVmac239 Vpx expression vector and tested whether the protein expressed would be incorporated into HIV-1 particles. In both cases, incorporation showed specificity for the homologous virion: tagged Vpr was not detectably incorporated into MLV or HTLV-2 particles, nor was tagged Vpx incorporated into HIV-1 particles (data not shown). These results suggested that incorporation into HIV-1 particles requires a specific interaction of Vpr with one or more HIV-1 virion components.

Virion structural components that we considered as possibly being required for incorporation of Vpr into HIV-1 virions included Gag, Pol, Env, and viral genomic RNA. To determine which of these might be important for Vpr incorporation, we prepared mutated pNL4-3-R⁻ proviruses, each deficient in *vpr* and one or more virion structural genes (Fig. 1B). Virions produced from COS cells cotransfected with *env* mutant provirus (pNL4-3-R⁻E⁻) and pcDNA-tag-Vpr lacked detectable gp160 (Fig. 3, bottom panel) but contained wild-type levels of tagged Vpr. This result suggested that gp160 is not required for Vpr incorporation. Because of this observation, we used pNL4-3-R⁻E⁻ to make proviruses with additional mutations.

We next prepared virions from proviruses mutated in RNA packaging functions (Fig. 2B, pNL4-3-R⁻E⁻-ψ⁻, pNL4-3-R⁻E⁻-NC^{zn}, and pNL4-3-R⁻E⁻-NC^{zn}-ψ⁻). The ψ deletion and the NC first cysteine array mutation have been previously reported to reduce virion viral RNA content by 5- to 10-fold (3) and 50-fold (10), respectively, without affecting virion release. RNase protection analysis and p24^{gag} assay of these mutant virions showed decreased amounts of viral RNA and similar amounts of virions, consistent with these reports (data not shown). Virions produced from cells transfected with these proviruses and pcDNA-tag-Vpr contained wild-type amounts of tagged Vpr (Fig. 4). These results suggested that viral RNA encapsidation is not required for incorporation of tagged Vpr.

We next prepared virions from proviruses containing termination or frameshift mutations in Gag or Pol (Fig. 1B; pNL4-3-R⁻E⁻-NC⁻, pNL4-3-R⁻E⁻-P6⁻, and pNL4-3-R⁻E⁻-Pol⁻). Virions produced by transfection of pNL4-3-R⁻E⁻-Pol⁻ contained a protein corresponding to Pr55^{gag}, a smaller amount of a 60-kDa protein corresponding to a truncated Pr160$^{gag\text{-}pol}$, and unidentified heterogeneous proteins of high molecular weight (Fig. 5C, lanes 3 and 4). Because of the truncation of PR in this provirus, no processed *gag* proteins were observed. These virions appeared to incorporate wild-type amounts of tagged Vpr (Fig. 5A, lane 4). Thus, the region of Pol carboxy terminal to amino acid 138 does not appear to be required for tagged Vpr incorporation into virions. These results also suggested that incorporation of tagged Vpr does not require proteolytic processing of the capsid precursor polyproteins.

pNL4-3-R⁻E⁻-NC⁻ is deficient in NC, p6, and Pol. Virions produced by COS cells transfected with this provirus contained a protein of 40 kDa corresponding to a truncated, unprocessed Gag precursor. They appeared to be secreted less efficiently

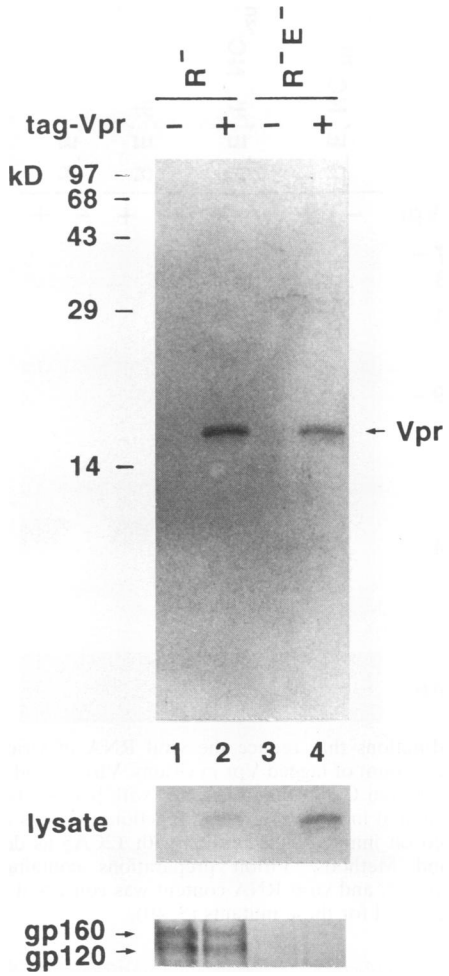

FIG. 3. gp160 does not influence the amount of tagged Vpr in virions. Virions (top panel) and cell lysates (middle and bottom panels) were prepared from COS cells transfected with pcDNA-tag-Vpr and pNL4-3-R⁻ or pNL4-3-R⁻E⁻. Virions and lysates were analyzed on immunoblots probed with 12CA5 (top and middle panel) or rabbit anti-recombinant gp120 (bottom panel) as described in Materials and Methods. Virion preparations contained similar amounts of p24^{gag}.

than *pol* mutant virions (Fig. 5C, lanes 5 and 6); however, we were not able to quantitate p24^{gag} in these virions to confirm this observation, since the commercial ELISA was inaccurate for measuring unprocessed Gag (data not shown). pNL4-3-R⁻E⁻-P6⁻ contains a termination codon in the p1 spacer, 9 amino acids before the amino terminus of p6. Because of the overlap in *gag* and *pol* open reading frames, this mutation also changed a single amino acid in Pol (Thr-7 to alanine). Upon transfection of COS cells with this provirus, virions were efficiently produced. They contained a truncated Gag precursor of 44 kDa, p24^{gag}, and proteins with molecular weights consistent with Gag processing intermediates containing MA-CA and CA-NC (39 and 32 kDa, respectively). Processing appeared to be somewhat less efficient in these virions; they contained more unprocessed and partially processed Gag precursors than did the wild-type particles. Virions produced by transfection of COS cells with either the NC⁻ or p6⁻ provirus lacked detectable tagged Vpr. Taken together, these results suggested that the region of Gag corresponding to p6 is critical for Vpr incorporation into HIV-1 virions.

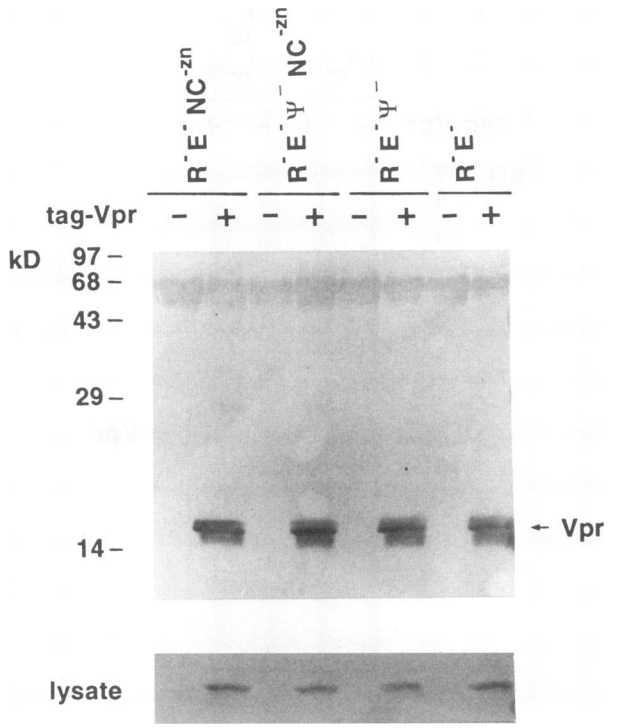

FIG. 4. Mutations that reduce the viral RNA of virions do not influence the amount of tagged Vpr in virions. Virions and cell lysates were prepared from COS cells transfected with pcDNA-tag-Vpr and proviruses mutated in RNA packaging functions. Virions and lysates were analyzed on immunoblots probed with 12CA5 as described in Materials and Methods. Virion preparations contained similar amounts of $p24^{gag}$, and viral RNA content was consistent with those previously reported for these mutants (3, 10).

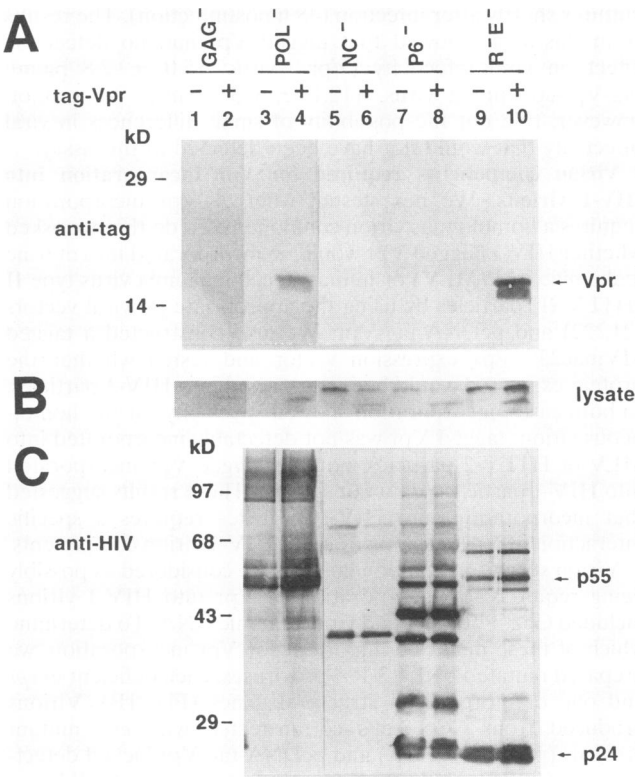

FIG. 5. Tagged Vpr incorporation requires the carboxy-terminal region of Gag corresponding to p6. Virions (A and C) and lysates (B) were prepared from COS cells transfected with pcDNA-tag-Vpr and the indicated mutated proviruses. Virions and lysates were separated by SDS-PAGE on 15% (A and B) or 10% (C) gels. Proteins were transferred to filters and probed with 12CA5 as described in Materials and Methods. (C) $Pr55^{gag}$ and $p24^{gag}$ are indicated by arrows. Bands at 40 kDa in lanes 5 and 6 represent truncated Gag protein; bands at the 54 and 58 kDa (lanes 7 and 8) are likely to be processing intermediates of $Pr160^{gag\text{-}pol}$. Lanes 1 and 2, 3 and 4, and 5 to 10 were from independent experiments and therefore show some differences in band intensities due to experimental variability.

Amino acids of Vpr critical for incorporation into HIV-1 virions. To determine which regions of Vpr are important for its incorporation into virions, we prepared a panel of mutated pcDNA-tag-Vpr expression vectors and tested them in the cotransfection assay. Because Vpr is rich in arginine, we mutated several of these residues to determine whether they are critical for virion incorporation. Mutation of Arg-32 and Arg-62 to Ala had no effect on stability or incorporation of tagged Vpr; neither did deletion of Arg-95 in the stop-94 truncation (Fig. 6B, lanes 2, 3, 10, and 11). In addition, mutation of two other arginine residues (Arg-73 and Arg-88 to alanine) had no effect on incorporation or stability of tagged Vpr (not shown). Mutation of Cys-76 to alanine appeared to destabilize the protein, since no tagged protein could be detected in virions or lysates. Cys-76 is conserved in all of the sequenced HIV-1 and SIV isolates and is the only cysteine residue in Vpr (27). These results suggested that the arginine residues tested are not critical for incorporation and that Cys-76 plays a crucial structural role.

A second, less abundant protein of 28 kDa was often visible on immunoblots when large amounts of tagged Vpr were present (Fig. 6, lanes 2, 3, and 7). This protein was of appropriate size for a Vpr dimer. It was possible that the 28-kDa protein represented a dimer linked by interchain disulfide bonding of Cys-76; however, the ratio of putative dimer to monomer was not increased on nonreducing SDS-polyacrylamide gel electrophoresis (PAGE) (not shown), as would have been expected for a disulfide-linked dimer. This protein could still represent a highly stable, non-disulfide-linked Vpr dimer.

Truncation of two amino acids from the carboxy terminus (stop-94) had no effect on tagged Vpr incorporation or stability (Fig. 6, lane 5); however, it appeared to decrease the amount of tagged Vpr detected in the lysate (Fig. 6, lane 13). This could be due either to an enhancement in its ability to be incorporated into virions or to decreased stability in lysates, although we have not studied this further. Further truncation of the carboxy-terminal 13 amino acids (stop-84) resulted in a relatively stable protein that was detected in cells but did not appear to be efficiently incorporated into virions (Fig. 6, lane 4). This result suggested that the region of Vpr spanning amino acids 84 to 93 is critical for incorporation into virions. This region was not sufficient to direct the protein into virions, however, since a tagged protein encoding this region alone (residues 84 to 96) was not incorporated into virions (not shown). The stop-84 protein was present in the cell lysates at levels lower than those of full-length tagged Vpr. However, this is not likely to account for its low level in virions, since stop-94 was present at even lower level in lysates but was readily

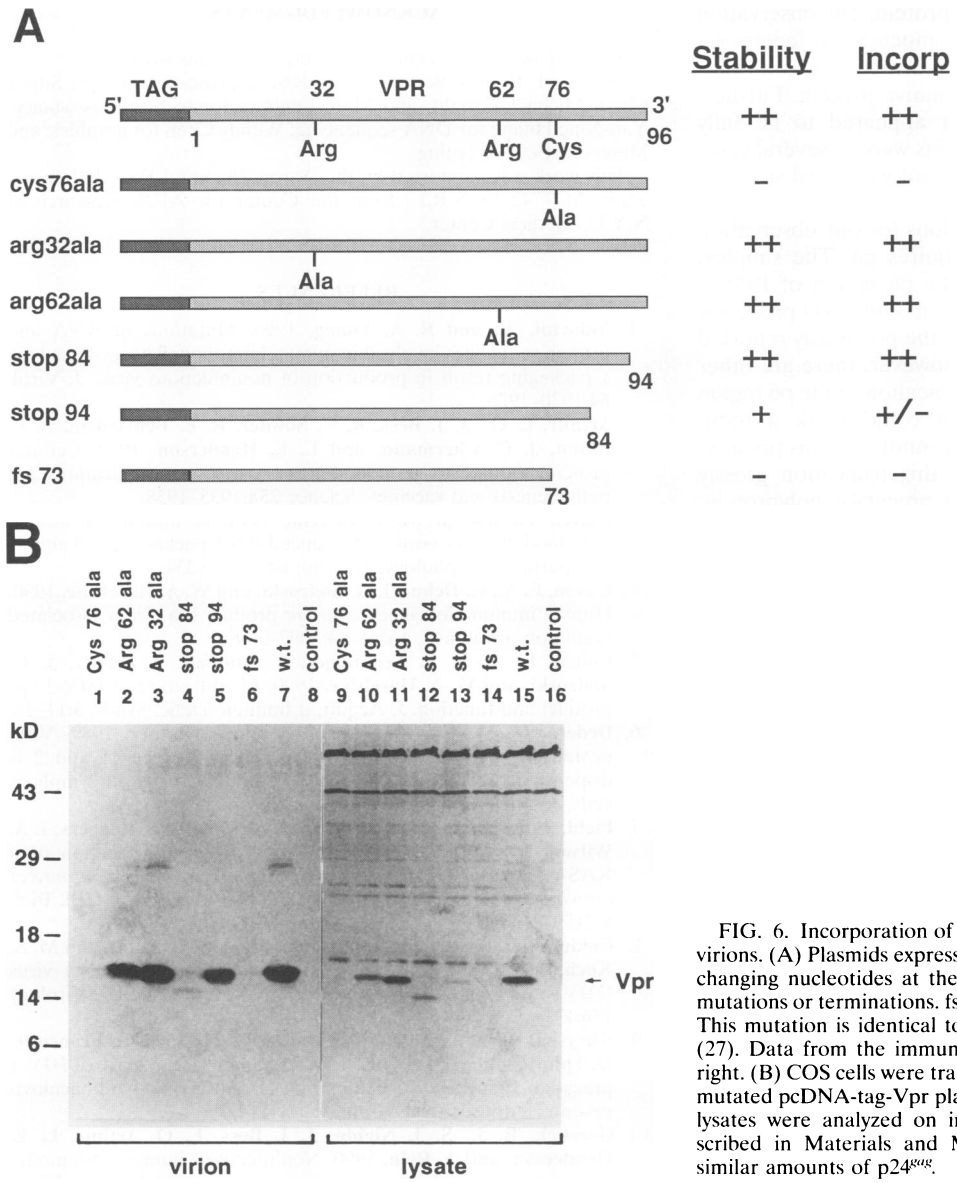

FIG. 6. Incorporation of mutant tagged Vpr molecules into HIV-1 virions. (A) Plasmids expressing mutant tagged Vpr were generated by changing nucleotides at the indicated codons to introduce missense mutations or terminations. fs-73 contains an insertion of a T at nt 5774. This mutation is identical to that of *vpr* from HIV-1 provirus HXB2 (27). Data from the immunoblot in panel B are summarized to the right. (B) COS cells were transfected with pNL4-3-R$^-$E$^-$ (5.0 μg) and mutated pcDNA-tag-Vpr plasmids (15.0 μg) as indicated. Virions and lysates were analyzed on immunoblots probed with 12CA5 as described in Materials and Methods. Virion preparations contained similar amounts of p24^{gag}.

detected in virions. Furthermore, in our experiments, tagged Vpr is detected with higher sensitivity in virions than in cell lysates; for example, in some experiments, we observed only a faint band in lysates, but a strong signal from the virions (for example, Fig. 5A and B, lanes 10).

Further truncation of tagged Vpr at codon 73 of Vpr (fs-73) failed to yield detectable protein in virions or lysates. This may be due, at least in part, to the absence of Cys-76, a residue that appears to be critical for stable production of protein. The protein encoded by fs-73 is similar to that of HIV-1 provirus HXB2. HXB2 *vpr*, like fs-73, is frameshifted by insertion of a single T nucleotide (nt 5774). In both cases, a protein of 78 amino acids that contains the same sequence of five frameshifted amino acids at positions 74 to 78 (the only difference in this region is a substitution of Arg-73 to glutamine) is produced. Because the protein encoded by fs-73 was not incorporated into virions, it is likely that HXB2 virions do not contain Vpr.

DISCUSSION

We present here evidence that HIV-1 Vpr which has been fused to a nine-amino-acid peptide corresponding to an epitope from influenza virus hemagglutinin can be incorporated during the assembly of HIV-1 virions. Incorporation of tagged Vpr into HIV-1 virions appeared to be a specific process; Vpr was not incorporated into MLV or HTLV-II particles, nor was a Vpr-related protein, SIV Vpx, incorporated into HIV-1 virions. These results suggested that Vpr incorporation into virions results from specific interactions between Vpr and a virion component(s) during assembly. Further investigation supported this hypothesis; HIV-1 virions lacking p6 failed to incorporate detectable amounts of tagged Vpr, while those deficient in Pol, Env, or genomic RNA were unaffected.

Although our studies examined the properties of tagged Vpr, there are several reasons to believe that our observations

largely reflect properties of the native protein. The observation that tagged Vpr was both stable and competent for incorporation into virions suggested that its conformation was at least not grossly different from that of the native protein. Furthermore, virions containing tagged Vpr appeared to be fully infectious. Mutated or truncated proteins were in several cases either not incorporated into virions or not expressed stably in the transfected cells.

There are several possible explanations for our observation that incorporation of tagged Vpr requires p6. The simplest model is that Vpr binds directly to the p6 region of $Pr55^{gag}$ during virion assembly. Direct interaction of the Gag precursor polyprotein with Vpr is consistent with the previously reported equimolar ratio of the two proteins; however, there are other possible explanations. For example, truncation of the p6 region from the Gag precursor polyprotein could mask a more amino-terminal Vpr binding site. We cannot rule this possibility out; however, it is unlikely that this truncation grossly distorts the conformation of the Gag precursor polyprotein, since it was appropriately cleaved by the viral protease and was efficiently assembled into virions which were released from the cells. Another possibility is that deletion of p6 alters assembly such that Vpr is not accessible to the assembling virion. This could be due to a difference in cellular localization or timing of the events in virion assembly.

It is interesting to speculate on the virion localization of Vpr on the basis of our results. The proteins that result from proteolytic cleavage of $Pr55^{gag}$ are organized such that their order in the polyprotein follows their arrangement in the mature virion (amino to carboxy terminal, MA-CA-NC). In accordance with this, Pol-derived proteins are carboxy terminal to Gag in $Pr160^{gag\text{-}pol}$ and are located within the capsid. If it is bound to the carboxy-terminal region of $Pr55^{gag}$, Vpr should also be localized in the mature virion within the capsid. This localization would bring Vpr into the proximity of the viral genomic RNA, possibly allowing the two molecules to associate. It will also be of interest to test whether Vpr exists as a dimer or multimer, as was suggested by the presence of a small amount of highly stable putative dimer apparent on the immunoblots.

Because several of the structural components of HIV-1 are dispensable for virion assembly and release, as is the case for the other retroviruses that have been examined (33), we were able to assess the ability of Vpr to be incorporated into particles deficient in various components. It is well established that RNA, gp160, and Pol are dispensable for virion assembly and release (32). MA and CA appear to be critical for HIV-1 assembly and release (32, 33); however, the role of NC and p6 in these events has not been extensively studied. In our studies, p6 and NC appeared to be unnecessary for virion production. Our results differed from those of a previous report which showed that particles deficient in p6 were inefficiently released from cells (11). This discrepancy may be due in part to differences in the mutations introduced to truncate $Pr55^{gag}$ or to the differences in the strain of HIV-1 used in that study.

Our observation that tagged Vpr is incorporated into virions suggests a possible method for loading peptide sequences of interest into HIV-1 particles. Such peptide sequences could have, for example, nucleolytic or proteolytic activity. Expression in target cells of such Vpr fusion proteins might result in release of defective particles. Although expression of exogenous genes in vivo is still difficult, it is conceivable that such an approach could have therapeutic value. It remains to be determined what size peptide can be fused to Vpr without interfering with its ability to be incorporated into virions.

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