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Requirement of the Pr55^{gag} Precursor for Incorporation of the Vpr Product into Human Immunodeficiency Virus Type 1 Viral Particles

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The human immunodeficiency virus type 1 (HIV-1) particles consists of two molecules of genomic RNA as well as molecules originating from *gag*, *pol*, and *env* products, all synthesized as precursor proteins. The 96-amino-acid Vpr protein, the only virion-associated HIV-1 regulatory protein, is not part of the virus polyprotein precursors, and its incorporation into virus particles must occur by way of an interaction with a component normally found in virions. To investigate the mechanism of incorporation of Vpr into the HIV-1 virion, Vpr$^-$ proviral DNA constructs harboring mutations or deletions in specific virion-associated gene products were cotransfected with Vpr expressor plasmids in COS cells. Virus released from the transfected cells was tested for the presence of Vpr by immunoprecipitation with Vpr-specific antibodies. The results of these experiments show that Vpr is *trans*-incorporated into virions but at a lower efficiency than when Vpr is expressed from a proviral construct. The minimal viral genetic information necessary for Vpr incorporation was a deleted provirus encoding only the pr55^{gag} polyprotein precursor. Incorporation of Vpr requires the expression but not the processing of *gag* products and is independent of *pol* and *env* expression. Direct interaction of Vpr with the Pr55^{gag} precursor protein was demonstrated by coprecipitation experiments with *gag* product-specific antibodies. Overall, these results indicate that HIV-1 Vpr is incorporated into the nascent virion through an interaction with the Gag precursor polyprotein and demonstrate a novel mechanism by which viral protein can be incorporated into virus particles.

The human immunodeficiency virus type 1 (HIV-1) displays a high level of genetic complexity, which accounts for its tightly regulated replication. In addition to *gag*, *pol*, and *env* genes, which are found in all known replication-competent retroviruses, HIV-1 encodes at least six nonstructural viral proteins (*vif*, *vpr*, *tat*, *rev*, *vpu*, and *nef*), called auxiliary genes, that regulate virus production and infectivity (10, 22, 37). *tat* and *rev* encode regulatory gene products essential for HIV-1 replication in cell culture. *vif*, *vpr*, *vpu*, and *nef* have been termed accessory genes because many of them are defective in HIV strains that are extensively passaged in tissue culture and their deletion does not abrogate viral replication in CD4$^+$ T-cell lines. However, the conservation of these accessory genes in natural isolates and in distinct lentiviruses of different primate species argues for a vital role in vivo pathogenesis.

The *vpr* gene encodes a 96-amino-acid protein that has an apparent molecular mass of approximately 14 kDa in sodium dodecyl sulfate (SDS)-polyacrylamide gels (8, 30). The *vpr* open reading frame is also present in HIV-2 isolates and in most but not all isolates of simian immunodeficiency virus (SIV) (6, 14, 20, 25). Recent amino acid comparison between HIV-2 *vpr* and *vpx* gene products showed regions of similarity, suggesting that *vpx* in the HIV-SIV group may have arisen by duplication of the *vpr* gene (36).

Functional studies have shown that *vpr* accelerates virus replication and cytopathic effects in CD4$^+$ T-cell lines (8, 31). Recently Westervelt et al. have shown that mutation of HIV-1 *vpr* resulted in attenuation of virus replication in primary monocytes (39). This notion was supported by experiments in which antisense phosphorothioate oligodeoxynucleotides targeted to *vpr* inhibited viral replication in primary human macrophages (5). In addition, in vivo experiments indicate that *vpr* plays an important role in establishing a persistent high-level infection in SIV_{MAC}-infected rhesus monkeys (27). The exact function and mechanism of action of Vpr at the molecular and cellular levels are yet to be established.

The Vpr and Vpx proteins of HIV and SIV have been shown to be present in multiple copies in mature virus particles (7, 24, 42, 43, 46). Interestingly, of the six auxiliary gene products found in the HIV-1 genome, only Vpr is virion associated. The presence of Vpr in the virus particle suggests that this protein, like all other virus-specified enzymes (viral protease, RNA-dependent DNA polymerase, RNase H, integrase) that are packaged in the virus particle, has a role in the early stages of infection before the transcription of the proviral DNA can be initiated.

The assembly and maturation of HIV-1 virus particles are complex processes in which the products of *gag* and *pol* genes are incorporated into virions in the form of their polyprotein precursors, namely Pr55^{gag}, NH_2-p17(MA)-p24(CA)-p7(NC)-p6-COOH, and Pr160$^{gag\text{-}pol}$, NH_2-p17(MA)-p24(CA)-p7(NC)-p10(PR)-p66(RT)-p32(IN)-COOH, respectively, and subsequently proteolytically cleaved by the virus-encoded protease during or after budding to form the core of the virus (22, 37, 38). Packaging of viral RNA involves the interaction of *cis*-acting packaging signal in the RNA genome and the nucleoprotein domain of the Gag and Gag-Pol precursors that contain a zinc finger motif (28). The incorporation of viral envelope glycoproteins gp120 and gp41 into the virions, syn-

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thesized initially as a precursor gp160 that is cleaved intracellularly, is believed to be mediated by an interaction between the p17(MA) domain of the Gag precursor and the Env glycoprotein complex (45). The observation that expression of Gag precursors from expression vectors leads to the budding of virus-like particles clearly indicates that the *pol* and *env* gene products are dispensable for the production of retroviral particles containing viral RNA (16, 26, 35).

Vpr is made from a singly spliced *rev*-dependent mRNA species that accumulates late in infection and is distinct from the unspliced genomic RNA and the large, singly spliced mRNA species encoding *gag*, *pol*, and *env* gene products (4, 15). Since Vpr is not encapsidated as part of a structural polyprotein precursor, an interaction of Vpr with a virion component is likely to constitute the mechanism by which Vpr is incorporated into virus particles.

The present study investigates the mechanism of Vpr incorporation in HIV-1 and the possible involvement of *gag*, *pol*, and *env* gene products in this process.

MATERIALS AND METHODS

Cell lines and molecular clones. COS-7, an African green monkey kidney cell line transformed by an origin-defective mutant of simian virus 40 (21), was propagated in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% fetal bovine serum (Flow Laboratories Inc.).

The pHxB-SV plasmid contains the infectious DNA molecular clone HxBc2 that originates from the HIV-1 IIIB isolate (11, 34). The genotype of this molecular clone is 5′ long terminal repeat (LTR) *gag*$^+$ *pol*$^+$ *vif*$^+$ *vpr tat*$^+$ *rev*$^+$ *vpu env*$^+$ *nef* 3′ LTR. pHxBRU, a hybrid between the two closely related HxBc2 and BRU/LAI proviruses, was constructed by replacing the sequences between the C terminal of *gag* (*Apa*I site at nucleotide 1555, where +1 is the start of the HxBc2 initiation of transcription) and the C-terminal portion of *vpr* (*Sal*I site at nucleotide 5331) of HxBc2 by the corresponding sequences of BRU/LAI that encode a full-length functional *vpr* gene (7, 8). The HxBc2ΔEΔBCAT proviral construct is derived from the pHxBΔenvCAT plasmid, which contains a 580-bp deletion (*Bgl*II sites at nucleotides 6583 and 7163) in the *env* gene and a chloramphenicol acetyltransferase (CAT) gene replacing the *nef* gene (23). An internal deletion located between two *Eco*RI sites at nucleotides 4193 and 5288 was introduced into pHxBΔenvCAT to generate HxBc2ΔEΔBCAT. This provirus does not encode Int, Vif, Vpr, or Nef but does express a truncated form of gp120. The envelope-deficient provirus HxBH10-env$^-$fs is characterized by a mutation of the *env* initiation codon as well as a frameshift mutation at a *Kpn*I site (nucleotide 5893), which prevents the synthesis of the *env* glycoprotein precursor (41). The RNA-packaging mutant provirus HxBRU-C28/49S is characterized by mutations within the p7(NC) region of *gag* where Cys residues 28 and 49 were changed to Ser (13). This provirus was modified to a *vpr*$^+$ genotype by replacing the sequence between *Nco*I (nucleotide 5220) and *Sal*I (nucleotide 5331) by the corresponding sequences from BRU/LAI strain that encode a functional *vpr* gene. The SVC-C1 and SVC-P1 molecular clones are characterized by a modification of the cleavage site at the junction of p17-p24 of *gag* and a substitution of Asp residue 25 for Arg in the active site of the viral protease, respectively. These proviruses show a deficient processing of the Gag intermediate p41 (p17-p24) and the Gag precursor p55, respectively (19). ptrENV, kindly provided by Lautaro G. Perez, is derived from the BRU/LAI molecular clone and is characterized by large deletions in *gag*, *pol*, and *env* genes (nucleotides 989 to 4098 and 5925 to 7219, where +1 is the site of transcription initiation) (33). This proviral construct expresses Vif, Vpr, Tat, Rev, Vpu, and gp41. ptrENVR$^-$ was derived from ptrENV in that a point mutation in the initiation codon of *vpr*, replacing ATG with GTG, was introduced by oligonucleotide-directed mutagenesis (47). The genetic maps of the proviruses used in this study are summarized in Fig. 1.

Plasmids. pIIIgagCAR, obtained from Andrew I. Dayton, is an expressor of *gag* and protease genes driven by the HIV-1 LTR (Fig. 1) (12). pSVCMVER is a simian virus 40 origin-based expression vector of *vpr* derived from the HIV-1 ELI strain (8). pSVCMVERstop contains a premature termination codon after Vpr residue 35, resulting in the expression of a nonfunctional Vpr truncated protein (8).

Antisera. HIV-1-positive human serum 38.2 contains antibodies that recognize all HIV-1 structural proteins as well as the *vpr* gene product, whereas human sera 162 and 243 react only with HIV-1 structural proteins. Rabbit antisera raised against a peptide encompassing amino acids 1 to 19 of Vpr (8) and an *Escherichia coli*-derived Vpr_{ELI} protein were also used in this study. For the preparation of the latter, the *vpr* gene was amplified by PCR from a molecular clone that originates from the ELI strain of HIV-1 (2) and cloned downstream of the *malE* gene in the procaryotic expression vector pMALcRI (New England Biolabs). Induction of bacteria carrying this plasmid with isopropyl-β-D-thiogalactopyranoside (IPTG) resulted in the expression of a fusion protein that was purified and cleaved with factor Xa protease. The Vpr moiety was purified, and polyclonal serum against Vpr was raised by immunizing rabbits (27a).

Transfection of cells. COS-7 cells (10^6) were transfected by the calcium phosphate method (9). The transfected cultures were maintained 48 to 60 h in Dulbecco modified Eagle medium supplemented with 10% newborn calf serum.

Cell labeling and radioimmunoprecipitation. Transfected COS-7 cells were metabolically labeled 48 h posttransfection with 50 μCi of [^{35}S]methionine per ml and 50 μCi of [^{3}H]leucine per ml for 16 h. Following centrifugation of the cell cultures at 3,290 × *g* for 30 min, labeled cells and virion-containing supernatants were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl [pH 7.2], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) at 0°C and immunoprecipitated as previously described (17). Immunoprecipitates were then collected by centrifugation, washed three times with RIPA buffer without sodium deoxycholate, and analyzed on a 13 to 18% gradient SDS-polyacrylamide gel and autoradiography. For the coprecipitation experiments, cell-free labeled supernatants were first ultracentrifuged at 35,000 rpm through a 20% sucrose cushion for 3 h in a Beckman SW41 rotor to pellet virus-like particles. Pellets were then lysed with either a mild RIPA buffer (140 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS) or alternatively with an NP-40-based buffer (50 mM Tris-HCl [pH 7.2], 150 mM NaCl, 1% Nonidet P-40) at 0°C for 30 min and immunoprecipitated with HIV-positive human sera. Immunocomplexes were analyzed on a 12.5% gradient SDS-polyacrylamide gel and autoradiography. Densitometric analysis of autoradiograms was performed with a Molecular Dynamics Personal densitometer and Image Quant software version 3.22.

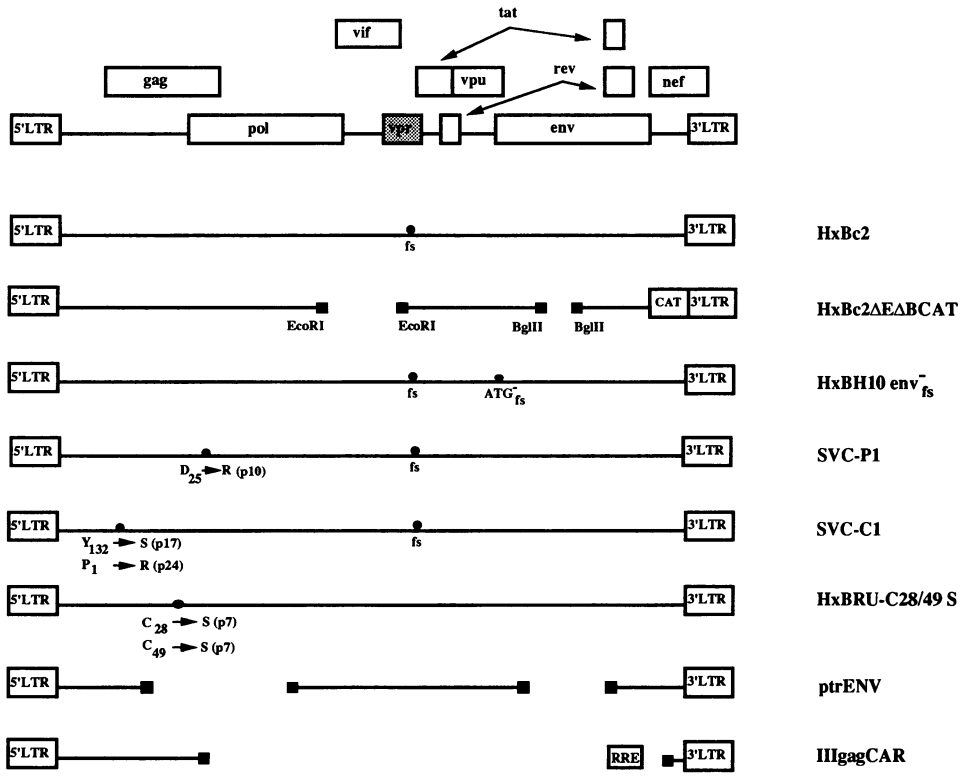

FIG. 1. Genetic organization of the HIV-1 genome and structure of the proviral plasmid constructs. Deletions are shown as empty space between black squares. Locations of the mutations are marked by black dots, under which the mutated amino acids and their positions in the respective viral proteins are indicated. The one-letter amino acid code is used. The HxBc2 molecular clone of HIV contains an insertion of an additional thymidine between nucleotides 5351 and 5352 (+1 is the site of initiation of transcription), causing a frameshift in the *vpr* reading frame after amino acid 72. pHxBc2ΔEΔBCAT contains 1,095 (Δ*Eco*RI; nucleotides 4193 to 5288) and 580 (Δ*Bgl*II; nucleotides 4193 to 5288) base pair deletions affecting the INT domain of *pol*, *vif*, *vpr*, and the gp120 domain. In addition, *nef* is replaced by the CAT reporter gene. The pHxBH10env$^{-}_{fs}$ contains a mutation in the initiation codon of *env* and a frameshift at a *Kpn*I site (nucleotide 5893) located in the same gene. SVC-P1 encodes an inactive form of the protease as a result of a substitution of an Asp residue at position 25 for an Arg in the enzyme catalytic site. The p17-p24 cleavage site mutant SVC-C1 contains a first substitution of a Tyr residue for a Ser at position 132 in p17 and a second substitution of a Pro residue for an Arg at position 1 in p24. The RNA-packaging-defective HxBRU-C28/49S provirus contains substitutions of Cys residues 28 and 49 for Ser in the p7 domain of Gag. ptrENV contains 3,109 (nucleotides 989 to 4098) and 1,294 (nucleotides 5925 to 7219) base pair deletions affecting respectively *gag*, *pol*, and the gp120 domain of *env* genes. ptrENV encodes Vif, Vpr, Tat, Rev, Vpu, and gp41. The pIIIgagCAR plasmid encodes Pr55^{gag} and the protease domain of the *pol* gene and contains the Rev-responsive element (RRE/CAR) sequence.

RESULTS

Incorporation of Vpr in *trans*. To determine whether the Vpr protein could be incorporated in *trans* into virus particles, we tested the ability of an expressor plasmid to target Vpr into virions produced from a cotransfected Vpr^{-} proviral plasmid. For this purpose, we cotransfected 10^6 COS-7 cells with 10 μg of HxBc2 (Vpr^{-}) proviral DNA and 8 μg of pSVCMVER. As a negative control, HxBc2 was transfected alone or cotransfected with 8 μg of pSVCMVERstop, a plasmid that encodes a truncated *vpr* product of 35 amino acids that was described previously to be nonfunctional (8). As a positive control, the HxBRU plasmid that expresses the *vpr* gene in the context of an infectious clone of HIV (in *cis*) was used. At 48 h posttransfection, cells were labeled with [^{35}S]methionine and [^{3}H]leucine for 16 h, and viral proteins in the cell lysate or cell-free supernatant were immunoprecipitated with the 38.2 HIV-1-positive human serum that recognizes HIV-1 structural proteins as well as Vpr. All immunoprecipitated proteins were analyzed by electrophoresis on an SDS-polyacrylamide gel followed by autoradiography. As shown in Fig. 2A, a protein of 14 kDa corresponding to the *vpr* product was specifically precipitated by the HIV-1-positive human serum 38.2 from the cell lysate and virus-containing clarified supernatant fluid of the culture transfected with the Vpr^{+} HxBRU (lane 3) or cotransfected with HxBc2 and pSVCMVER (lane 4). In contrast, the *vpr* product was not detected in the cell lysate and supernatant fluid of COS cells transfected with only HxBc2 (lane 2) or cotransfected with HxBc2 and pSVCMVERstop (lane 5). As indicated in Fig. 2A, all HIV-1 structural proteins were also immunoprecipitated with the HIV-1-positive human serum 38.2 in cell and supernatant fluid of cultures transfected with proviral DNA (lanes 2 to 5) and were absent from the mock-transfected COS cell samples (lane 1).

To eliminate the possibility that the 14-kDa *vpr* product detected in the supernatant of cells cotransfected with the Vpr expressor plasmid was the result of cell lysis or possibly secretion, COS cells were transfected only with the Vpr expressor pSVCMVER or the negative control pSVCMVERstop. At 48 h posttransfection, the transfected-cell cultures were labeled and the cell lysate and cell-free supernatant were immunoprecipitated with the HIV-1-positive human serum 38.2. As shown in Fig. 2B, the *vpr* product was not detected in

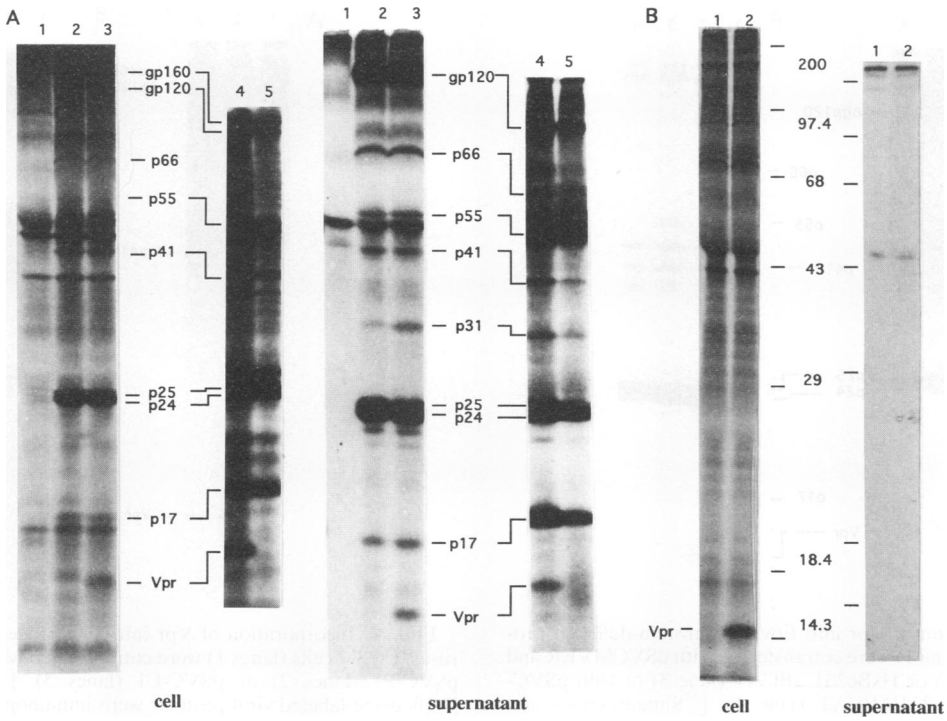

FIG. 2. *trans* incorporation of Vpr into virions. (A) COS-7 cells (lane 1) were transfected with pHxBc2 (Vpr^-) (lane 2) and HxBRU (Vpr^+) proviruses (lane 3) or cotransfected with pHxBc2 and pSVCMVER (lane 4) or pSVCMVERstop (lane 5). At 48 h posttransfection, [^{35}S]methionine- and [^{3}H]leucine-labeled viral proteins were immunoprecipitated from the cell lysates (cell) or the cell-free supernatant fluid (supernatant) with the HIV-1-positive human serum 38.2 and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). (B) COS-7 cells were transfected with pSVCMVERstop (lane 1) or pSVCMVER (lanes 2). [^{35}S]methionine- and [^{3}H]leucine-labeled viral proteins were immunoprecipitated from cell lysates (cell) or cell-free supernatant (supernatant) with the HIV-1-positive human serum 38.2 (lanes 1 and 2). Immunoprecipitated proteins were resolved by SDS-PAGE and autoradiography.

the pSVCMVER-transfected cell supernatant sample treated with the 38.2 serum (lane 2, right panel) although it was precipitated in the cell lysate sample with the same antiserum (lane 2, left panel). The truncated product of *vpr* encoded by pSVCMVERstop was not detected in both the cell lysate and the supernatant, suggesting that it was unstable or did not contain the epitope(s) recognized by the antiserum (lane 1).

The efficiency of *trans*- versus *cis*-incorporation of Vpr into virus particles was evaluated by densitometric analysis. Autoradiographs from several experiments were scanned, and the intensity of p14 (Vpr) and p66 reverse transcriptase (RT) bands associated with virion-containing supernatant fluid were quantified by using a Molecular Dynamics densitometer. The level of Vpr incorporation into virus particles was determined by calculating the ratio of the Vpr band intensity over the p66 (RT) band intensity. Comparison of the Vpr/RT ratios obtained in two independent experiments in which Vpr was expressed in *cis* and *trans* consistently showed that the value for *cis* incorporation was at least threefold higher than the value for *trans* incorporation (data not shown). These results clearly demonstrate that the Vpr product can be incorporated in *trans* into HIV-1 virus particles, although at a lower efficiency than when it is expressed in the context of a complete provirus.

Determination of HIV-1 components involved in Vpr incorporation into virions. To assess the possible role of viral structural proteins in the incorporation of Vpr into HIV-1 particles, we tested proviruses harboring deletions or mutations in specific virion-associated gene products. Since these modified proviral clones cannot replicate in $CD4^+$ cells, an experimental system that does not depend on virus replication is required. COS African green monkey kidney cells were transiently transfected with simian virus 40-based HIV-1 molecular clones encoding these mutations. As COS cells express high levels of simian virus 40 T antigen, the plasmids containing the replication-deficient proviral constructs replicate, providing large amounts of template for expression. The proviruses that did not encode a functional *vpr* gene were cotransfected with a Vpr expressor plasmid since Vpr can be incorporated in *trans*. At 48 h posttransfection, cells were metabolically labeled and lysates from cells and virion-containing supernatants were immunoprecipitated with antibodies against HIV-1 structural viral proteins and Vpr to determine whether Vpr was incorporated into virus particle. Quantification of Vpr incorporation was determined by calculating the virion-associated Vpr/RT ratio.

To determine whether *env* gene products are required for incorporation of Vpr into HIV-1 particles, we used the HxBc2ΔEΔBCAT provirus (Fig. 1). As shown in Fig. 3, this Vpr^- proviral construct expresses a truncated form of gp160 that, after processing, yields a truncated gp120 (Δgp120) that is not incorporated into virus particles as demonstrated by the presence of a truncated gp120 in the cell lysate and its absence in the supernatant fluid (lanes 3 and 4). When the Vpr expressor was cotransfected with this provirus into COS-7 cells, the 14-kDa *vpr* gene product was detected in the virion-containing supernatant fluid (Fig. 3B, lane 3). However, Vpr was not detected in the supernatant when HxBc2ΔEΔBCAT was cotransfected with pSVCMVERstop (Fig. 3B, lane 4). The

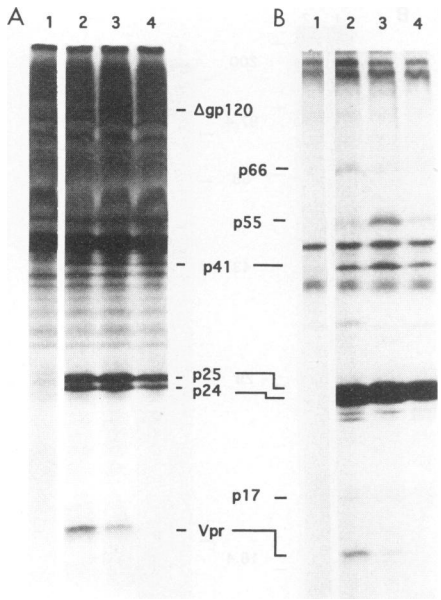

FIG. 3. Incorporation of Vpr into Env glycoprotein-deficient proviruses. COS-7 cells (lane 1) were cotransfected with pSVCMVER and HxBH10env-fs (lane 2) or HxBc2ΔEΔBCAT (lane 3) or with pSVCMVERstop and HxBc2ΔEΔBCAT (lane 4). [^{35}S]methionine- and [^{3}H]leucine-labeled viral protein were immunoprecipitated from cell lysates (A) or cell-free supernatant (B) with the HIV-1-positive human serum 162 mixed to a rabbit antiserum raised against recombinant Vpr (1:1 ratio) and analyzed by SDS-PAGE.

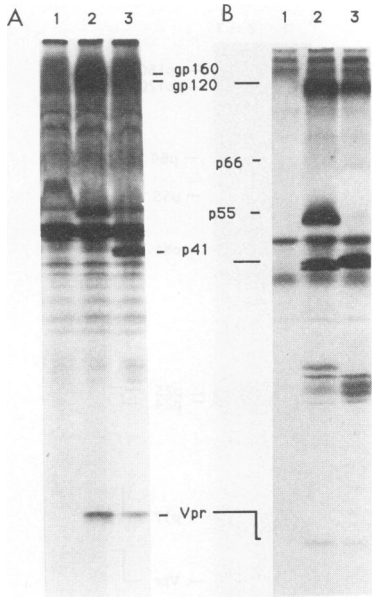

FIG. 4. Incorporation of Vpr into Gag processing-deficient proviruses. COS-7 cells (lanes 1) were cotransfected with pSVCMVER and pSVC-P1 (lanes 2) or pSVC-C1 (lanes 3). [^{35}S]methionine- and [^{3}H]leucine-labeled viral proteins were immunoprecipitated from cell lysates (A) or cell-free supernatant (B) with the HIV-1-positive human serum 162 mixed with a rabbit antiserum raised against recombinant Vpr (1:1 ratio) and analyzed by SDS-PAGE.

incorporation of Vpr in the absence of *env* gene product was also confirmed by cotransfecting pSVCMVER with HxBH10-env^{-fs} (Fig. 3, lanes 2), a Vpr$^-$ provirus that cannot express gp160 owing to a mutation in the *env* initiation codon and a frameshift mutation in the signal peptide sequence (Fig. 1). Immunoprecipitation of the cell lysate and supernatant fluid with the HIV-1-positive human serum 162 mixed with the rabbit polyclonal anti-Vpr serum (1:1 ratio) clearly demonstrates the absence of *env* products in cells and supernatants, but Vpr can still be detected in the supernatant fluid (Fig. 3, lanes 2). Quantitative comparison of Vpr incorporation in *env* glycoprotein-deficient and wild-type virions as measured by virion-associated Vpr/RT ratio did not indicate significant differences (data not shown). From these results, we can conclude that *env* glycoprotein expression is not required for Vpr incorporation into HIV-1 particles. Moreover, since HxBc2ΔEΔBCAT contains a deletion encompassing the 3′ portion of *pol* (Int$^-$), *vif*, and the 5′ portion of *vpr* and cannot express *nef*, it can also be deduced that integrase, *vif*, and *nef* products are not required for Vpr incorporation into virions.

To determine whether incorporation of Vpr into virus particles was dependent on proteolytic maturation, we cotransfected pSVCMVER in COS cells with two Vpr$^-$ molecular clones, SVC-P1 and SVC-C1 (Fig. 1), which harbor mutations that inactivate the viral protease or alter the cleavage site between p17 and p24, respectively, as described previously (19). As shown in Fig. 4, SVC-P1 and SVC-C1 expression resulted in the accumulation of Gag precursor Pr55^{gag} (Fig. 4A, lane 2) and *gag* intermediate product p41 (Fig. 4A, lane 3), respectively, in cell lysate as well as in the production of immature virus particles in supernatant samples as indicated by the presence of Pr55^{gag} and p41 and the absence of p24/p25 (Fig. 4B, lanes 2 and 3). The 14-kDa Vpr protein was detectable in the supernatant fraction of cells transfected with SVC-P1 or SVC-C1 (Fig. 4B, lanes 2 and 3). The efficiency of Vpr incorporation in SVC-P1 or SVC-C1 particles, as determined by measurement of the virion-associated Vpr/RT ratio, was comparable to that of the control HxBc2, which has the capacity to fully process the Pr55^{gag} precursor, suggesting that processing of Gag precursor protein is not required for the incorporation of Vpr into HIV-1 virions (data not shown).

The genomic RNA molecules constitute another virion component by which Vpr could be incorporated into virus particles. RNA-packaging mutant proviruses were therefore used to examine whether genomic RNA played a role in Vpr incorporation. Mutations in different retroviruses, including HIV-1, that affect any of the conserved Cys and His residues in the nucleocapsid domain of *gag* yield particles that are defective for RNA encapsidation (1, 13, 18). HxBRU-C28/49S, a Vpr$^+$ provirus that contains mutations that substitute Cys residues 28 and 49 in p7(NC) for Ser (Fig. 1), was recently shown to be severely attenuated for viral RNA packaging (13). Evaluation of the viral RNA content of HxBRU-C28/49S viral particles was shown to represent less than 5% of the RNA content normally found in wild-type virions (13). The HxBRU-C28/49S provirus and a positive control provirus, HxBRU, were transfected in COS cells. Following metabolic labeling, cell-free supernatants were centrifuged onto a 20% sucrose cushion to pellet viral particles. As shown in Fig. 5, patterns of precipitated viral proteins were similar for the positive control HxBRU (lane 2) and HxBRU-C28/49S (lane 3) in the cell and pelleted virus samples. Similar to the findings of Dorfman et al. (13), we observed a marked defect in viral particle production when Cys residues 28 and 49 of p7(NC) were substituted (compare lanes 2 and 3, right panel). The 14-kDa *vpr* gene product was detected in the virion-containing supernatant fluid

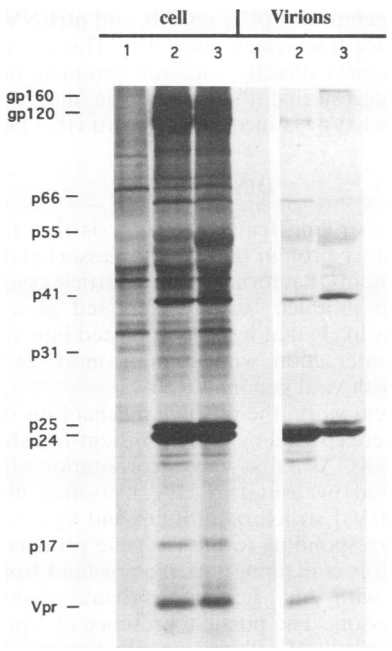

FIG. 5. Incorporation of Vpr into RNA-packaging-deficient proviruses. COS-7 cells (lane 1) were transfected with HxBRU (lane 2) or HxBRU-C28/49S (lane 3) proviral DNA. [^{35}S]methionine- and [^{3}H]leucine-labeled viral proteins were immunoprecipitated from cell lysates (cell) or pelleted virions (virions) with the HIV-1-positive human serum 162 mixed with a rabbit antiserum raised against recombinant Vpr (1:1 ratio) and analyzed by SDS-PAGE.

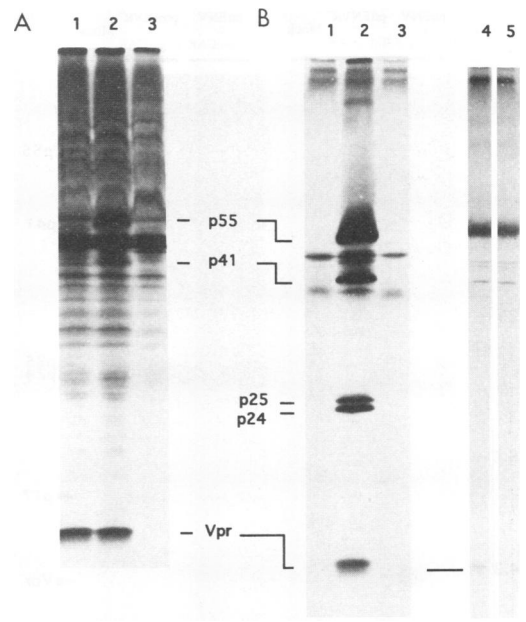

FIG. 6. Incorporation of Vpr into virus-like particles expressed from a Gag plasmid expressor. COS-7 cells (lane 3) were transfected with ptrENV (lane 1) or cotransfected with ptrENV and pIIIgagCAR (lanes 2, 4, and 5). [^{35}S]methionine- and [^{3}H]leucine-labeled viral proteins were immunoprecipitated from cell lysates (A) and cell-free supernatants (B) with the HIV-1-positive human serum 162 mixed with a rabbit antiserum raised against recombinant Vpr (1:1 ratio) (lanes 1 to 3). The viral proteins were also immunoprecipitated from pelleted virus by using the rabbit anti-Vpr peptide serum (lane 4) or the anti-Vpr peptide serum which was preincubated with the Vpr peptide antigen (lane 5). Precipitated proteins were analyzed by SDS-PAGE and autoradiography.

of cells transfected with either HxBRU (lane 2, right panel) or HxBRU-C28/49S (lane 3, right panel). Similar results were also obtained with different packaging-mutant proviruses harboring mutations in the packaging signal or His-23 in p7(NC) (data not shown). Quantification of the amount of Vpr incorporated in the wild-type or packaging-defective virus particle did not reveal significant differences, suggesting that the incorporation of Vpr into HIV-1 does not involve an interaction with genomic RNA.

All the data accumulated so far point toward an interaction of Vpr with Gag precursors to explain its incorporation into virus particles. A basic system to investigate the incorporation of Vpr into virus particles resulting from *gag* expression only was therefore developed. In this system two expressor plasmids were used: pIIIgagCAR, a *rev*-dependent Gag expressor plasmid that encodes Pr55^{gag} and protease (p10) (11, 12), and ptrENV, a gp41 expressor that expresses all HIV-1 regulatory genes, including *rev* and *vpr*, and a small portion of *gag* (p17 and part of p24) but not the structural *pol* and gp120 *env* genes (33). The data in Fig. 6 show that upon transfection of COS cells with ptrENV, the 14-kDa *vpr* product can be immunoprecipitated in the cell lysate (Fig. 6A, lane 1) but not in the supernatant fluid (Fig. 6B, lane 1), because no core particles are produced from such cells. Cotransfection of pIIIgagCAR with ptrENV results in the production of core particles in the supernatant fluid because *rev* encoded by the ptrENV expressor can act in *trans* and permits expression of Pr55^{gag} encoded by pIIIgagCAR. Immunoprecipitation of cell lysate (Fig. 6A, lane 2) with HIV human serum 162 mixed with rabbit anti-Vpr polyclonal serum clearly demonstrates the expression of p55 and p41 as well as the presence of the 14-kDa Vpr protein. Analysis of the virus-containing supernatant fluid with the same set of antisera clearly shows the presence of the 14-kDa Vpr protein in addition to HIV structural proteins (Fig. 6B, lane 2). The detection of *gag* cleavage products, p41 and p24/25, in the supernatant fluid indicates that the protease encoded by pIIIgagCAR was functional. Moreover, *pol* was not expressed, as indicated by the absence of the p66 (RT) product in the cell lysate and supernatant fluid. To demonstrate that the 14-kDa Vpr protein detected in the supernatant fluid was incorporated into the core particles, we centrifuged the supernatant through a 20% sucrose cushion. Immunoprecipitation of the pelleted viral particles with anti-Vpr peptide serum (Fig. 6B, lane 4) or anti-Vpr peptide serum incubated with the peptide antigen prior to the immunoprecipitation (Fig. 6B, lane 5) demonstrates that Vpr is *trans* incorporated into viral particles.

Interaction of Vpr with p55^{gag} precursor. The results obtained with the deleted proviruses indicate that Vpr incorporation requires only the expression of the Gag polyprotein Pr55^{gag} and is not dependent on the expression of *env* and *pol* products or packaging of genomic RNA. The possibility that Vpr interacts only with unprocessed Pr55^{gag} to be incorporated led us to use a system in which large quantities of Pr55^{gag} as well as other processed or partially processed *gag* products are associated with virus particles. Our previous results have shown that such conditions are obtained when ptrENV and pIIIgagCAR plasmids are used together (Fig. 6, lanes 2). To investigate a possible direct interaction of Vpr with *gag* products, we performed coprecipitation experiments with HIV-1-positive human sera recognizing *gag* products but not Vpr. For

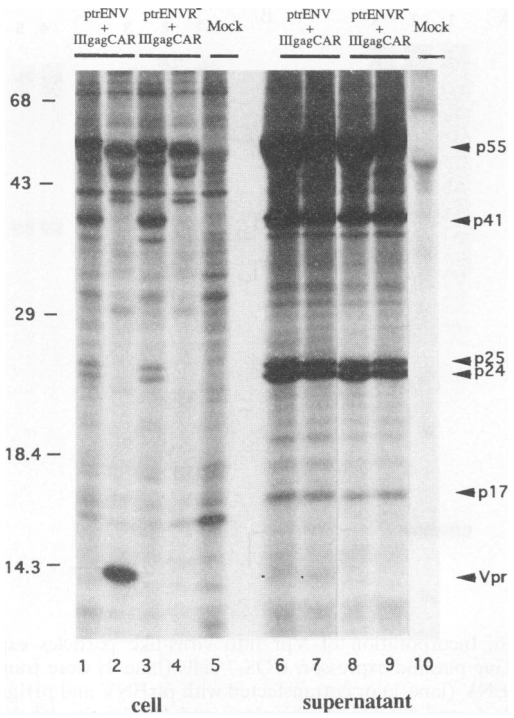

FIG. 7. Interaction between Vpr and Gag proteins. COS-7 cells (lanes 5 and 10) were cotransfected with pIIIgagCAR and ptrENV (lanes 1, 2, 6, and 7) or ptrENVR$^-$ (lanes 3, 4, 8, and 9). [^{35}S]methionine- and [^{3}H]leucine-labeled proteins were immunoprecipitated from cell lysates (lanes 1 to 5) or pelleted virions (lanes 6 to 10) with a rabbit anti-recombinant Vpr serum (lanes 2 and 4) or a pool of HIV-1-positive human sera 162 and 243 (lanes 1 and 3) or HIV-positive human serum 162 (lanes 6 and 8) or 243 (lanes 7 and 9). Precipitated proteins were analyzed by SDS-PAGE and autoradiography.

this purpose, COS cells were cotransfected with both ptrENV and pIIIgagCAR and metabolically labeled with [^{35}S]methionine and [^{3}H]leucine. To optimize the coprecipitation conditions, we tested two sets of lysis and radioimmunoprecipitation buffer conditions. Under one set of conditions a buffer similar to the RIPA buffer described in Materials and Methods was used. The second type of buffer contained only Nonidet P-40 and was used in the experiment whose results are shown in Fig. 7 since coprecipitation of Vpr with *gag* products was more efficient under these conditions. Lysed cells and sucrose cushion-pelleted viruses were immunoprecipitated separately with two distinct HIV-1-positive human sera, 162 and 243. As negative controls, mock-transfected COS cells as well as cells transfected with pIIIgagCAR and ptrENVR$^-$, a plasmid isogenic to ptrENV except for the expression of *vpr*, were used and processed similarly. Data in Fig. 7 clearly show that the rabbit anti-Vpr polyclonal serum immunoprecipitates Vpr in lysate from cells cotransfected with ptrENV (lane 2) but not in lysate from cells cotransfected with ptrENVR$^-$ (lane 4). Under similar conditions, a pool of human patient sera 162 and 243 precipitated pr55^{gag}, p41, and p24/25 but did not detect Vpr (lanes 1 and 3). In contrast, when pelleted virus from the supernatant fluid of cells cotransfected with pIIIgagCAR and ptrENV were reacted with the 162 or 243 human antiserum, a protein of 14 kDa corresponding to Vpr was coprecipitated with the *gag* products (lanes 6 and 7). This 14-kDa protein was not coprecipitated with *gag* products in the supernatant fluid of cells cotransfected with pIIIgagCAR and ptrENVR$^-$ (lanes 8 and 9) or mock transfected (lane 10). These results indicate that Vpr interacts directly with *gag* products in the HIV-1 virion and suggest that this interaction may constitute the means by which Vpr is incorporated into virus particles.

DISCUSSION

One of the striking features of Vpr is that it is the only HIV-1 regulatory protein that is virion associated. Since Vpr, unlike the proteins that form the virus particles such as *gag*, *pol*, and *env* gene products, is not expressed as a polyprotein precursor, it is likely that it is incorporated into virus particles through an interaction with one or more of these gene products or with viral genomic RNA.

In the present work, the possible mechanisms of incorporation of Vpr were studied by using transient-transfection assays with COS-7 cells. As a first step, incorporation of Vpr in *trans* was determined by using an HIV-1-positive human serum recognizing HIV-1 structural proteins and Vpr. A polypeptide of 14 kDa corresponding to the *vpr* gene product was precipitated from virus-containing supernatant fluid from COS cells cotransfected with Vpr$^-$ infectious proviral plasmids and Vpr plasmid expressors. The possible presence of Vpr in supernatant fluid as a result of cell lysis or secretion was ruled out on the basis of the detection of the protein in virus particles pelleted by ultracentrifugation through a 20% sucrose cushion and the absence of the 14-kDa *vpr* product from the supernatant fluid of COS cells transfected with Vpr plasmid expressor only. Incorporation of Vpr in *trans* was shown to be approximately threefold less efficient than incorporation of Vpr in *cis*, suggesting that the coordinate expression of the various genes encoding HIV-1 virion-associated protein is critical.

To study the possible involvement of HIV-1 proteins in the mechanism of Vpr incorporation, proviruses harboring different deletions or mutations in genes encoding virion-associated components were transfected or cotransfected in COS cells with Vpr expressor plasmids. The absence of *pol* and *env* gene products did not prevent incorporation of Vpr, indicating that expression of these gene products is not required for Vpr incorporation. Unprocessed or partially processed Gag precursor did not prevent or affect the incorporation of Vpr into immature virus particles, as demonstrated by the incorporation of Vpr into virions in which the protease function was deficient or the cleavage site between p17 and p24 was mutated. This observation is in agreement with the current model of HIV virion assembly and formation in which the virus is assembled as an immature particle in the form of polyprotein precursors that are then processed by the viral protease during or after release to generate a mature virion with a condensed core (38, 40).

The role of viral genomic RNA in Vpr incorporation was evaluated by comparing wild-type and partially deficient RNA-packaging proviruses. The data in Fig. 5 clearly show that a provirus which was shown to encapsidate viral RNA at a level representing less than 5% of that in the parental virus (13) incorporated Vpr as efficiently as a wild-type virus did. Although these results indicate that incorporation of Vpr occurs independently of viral RNA, a role of genomic RNA cannot be completely ruled out in light of recent data by Aronoff et al. (3). These authors reported that avian retroviral mutants deleted for both nucleocapsid Cys-His boxes retained the capacity to encapsidate RNA-containing appropriate packaging sequences, although this RNA was unstable and hence difficult to detect in mature particles. However, viral RNA could be readily detected in mutant virions that had been

treated with proteinase K to inactivate nucleases prior to RNA extraction. Our demonstration of a direct interaction of Vpr with *gag* products, as well as the recent localization of HIV-2 Vpx, a protein closely related to Vpr, primarily outside the core particle (44), renders this possibility unlikely.

Our results also demonstrate that the native *vpr* product is incorporated into virus-like particles produced by a $Pr55^{gag}$ expressor plasmid. This finding suggests that Vpr associates directly or indirectly with a portion of the Gag precursor protein during virion assembly. A direct interaction between Vpr and $Pr55^{gag}$ was shown by coprecipitation experiments in which the 14-kDa Vpr protein and the $Pr55^{gag}$ product were precipitated from virus-like particles pelleted from supernatant fluid of COS cells cotransfected with a $Pr55^{gag}$ expressor, pIIIgagCAR, and a plasmid expressor, ptrENV, encoding Vpr in addition to Rev. The coprecipitation of Vpr with $Pr55^{gag}$ was detected by using two distinct HIV-1-positive human sera that do not recognize Vpr. As demonstrated by the data in Fig. 7, coprecipitation of Vpr was not very efficient. This observation may be due in part to the weakness of the interaction or to the coprecipitation conditions that are still not optimal. Another possibility is that the processing of the Gag precursor releases Vpr and thus affects coprecipitation. Surprisingly, all attempts to detect a coprecipitation of Vpr with *gag* products in the cell lysate by using the same antisera were unsuccessful. At present the reason for this discrepancy remains unclear.

The domain of $Pr55^{gag}$ that is involved in the interaction with Vpr remains to be determined. An interaction with the p6 domain of the Gag precursor appears to be a fair possibility because both Vpr and the p6 portion of Gag are unique to HIV-1 and HIV-2 as well as to SIV and are not found in other animal retroviruses. The transient-transfection assay system used in this study should be useful to identify the domain(s) of Vpr and Gag implicated in this interaction by site-directed mutagenesis.

Overall, these results demonstrate a novel mechanism by which a viral protein can be incorporated into viral particles independently of the polyprotein precursors encoding the various protein components of the virus. Identification of a specific virion association motif(s) in the Vpr protein may permit the development of chimeric molecules that can be specifically targeted into the mature HIV-1 virion to affect its structural organization or functional integrity.

We note that shortly after the original submission of this report, Paxton et al. (32) and Lu et al. (29), using approaches that overlap with those described in this paper, demonstrated that incorporation of Vpr into virions requires the carboxy-terminal Gag protein of HIV(p6).

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