Requirement of the Pr55\textsuperscript{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 consist of two molecules of genomic RNA as well as molecules originating from \textit{gag}, \textit{pol}, and \textit{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\textsuperscript{−} 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\textsuperscript{gag} polypeptide precursor. Incorporation of Vpr requires the expression but not the processing of \textit{gag} products and is independent of \textit{pol} and \textit{env} expression. Direct interaction of Vpr with the Pr55\textsuperscript{gag} precursor protein was demonstrated by coprecipitation experiments with \textit{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 \textit{gag}, \textit{pol}, and \textit{env} genes, which are found in all known replication-competent retroviruses, HIV-1 encodes at least six nonstructural viral proteins (\textit{vif}, \textit{vpr}, \textit{tat}, \textit{rev}, \textit{vpu}, and \textit{nef}), called auxiliary genes, that regulate virus production and infectivity (10, 22, 37). \textit{tat} and \textit{rev} encode regulatory gene products essential for HIV-1 replication in cell culture. \textit{vif}, \textit{vpr}, \textit{vpu}, and \textit{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\textsuperscript{+} 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 \textit{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 \textit{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 \textit{vpr} and \textit{vpx} gene products showed regions of similarity, suggesting that \textit{vpx} in the HIV-SIV group may have arisen by duplication of the \textit{vpr} gene (36).

Functional studies have shown that \textit{vpr} accelerates virus replication and cytopathic effects in CD4\textsuperscript{+} T-cell lines (8, 31). Recently Westervelt et al. have shown that mutation of HIV-1 \textit{vpr} resulted in attenuation of virus replication in primary monocytes (39). This notion was supported by experiments in which antisense phosphorothioate oligodeoxynucleotides targeted to \textit{vpr} inhibited viral replication in primary human macrophages (5). In addition, in vivo experiments indicate that \textit{vpr} plays an important role in establishing a persistent high-level infection in SIV\textsubscript{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 \textit{gag} and \textit{pol} genes are incorporated into virions in the form of their polyprotein precursors, namely Pr55\textsuperscript{gag}, NH\textsubscript{2}-p17(\textbf{MA})-p24(\textbf{CA})-p7(\textbf{NC})-p6-COOH, and Pr160\textsuperscript{pol} : NH\textsubscript{2}-p17(\textbf{MA})-p24(\textbf{CA})-p7(\textbf{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 nucleo-protein 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-
Incorporation of Vpr product into HIV-1

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 S' long terminal repeat (LTR) gag pol rev vif vpr tat 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 (Apal site at nucleotide 1555, where +1 is the start of the HxBc2 initiation of transcription) and the C-terminal portion of vpr (SalI site at nucleotide 5331) of HxBc2 by the corresponding sequences of BRU/LAI that encode a full-length functional vpr gene (7, 8).

The HxBc2ΔEABCAT proviral construct is derived from the pHxBΔenvCAT plasmid, which contains a 580-bp long deletion (BglII 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 EcoRI sites at nucleotides 4193 and 5288 was introduced into pHxBΔenvCAT to generate HxBc2ΔEABCAT. This provirus does not encode Int, Vif, Vpr, or Nef but does express a truncated form of gp120. The envelope-deficient provirus HxBH10-env' is characterized by a mutation of the env initiation codon as well as a frameshift mutation at a KpnI site at nucleotide 5893, which prevents the synthesis of the env glycoprotein precursor (41). The RNA-packaging mutant provirus HxBRU-C2849S 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 NcoI (nucleotide 5220) and SalI (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).
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 10⁶ COS-7 cells with 10 μg of HxBc2 (Vpr-) proviral DNA and 8 μg of pSVMVER. As a negative control, HxBc2 was transfected alone or cotransfected with 8 μg of pSVMVERstop, 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 [³⁵S]methionine and [¹⁴C]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 pSVMVER (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 pSVMVERstop (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 pSVMVER or the negative control pSVMVERstop. 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. 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. pHxBc2ΔΔCAT contains 1,095 (ΔEcoRI; nucleotides 4193 to 5288) and 580 (ΔBglII; 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 Δα contains a mutation in the initiation codon of env and a frameshift at a Kpnl site (nucleotide 5983) 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. prENV 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. prENV encodes Vif, Vpr, Tat, Rev, Vpu, and gp41. The pH1gCAR plasmid encodes Pr55⁰⁰⁰ and the protease domain of the pol gene and contains the Rev-responsive element (RRE/CAR) sequence.
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 pSVMVER (lane 4) or pSVMVERstop (lane 5). At 48 h posttransfection, [35S]methionine- and [3H]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 pSVMVERstop (lane 1) or pSVMVER (lanes 2). [35S]methionine- and [3H]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 efficiency of trans- versus cis-incorporation of Vpr into virus particles was evaluated by densitometric analysis. Autoradiograms 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 protein 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 expression 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 expression 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 pSVMVERstop (Fig. 3B, lane 4). The
incorporation of Vpr in the absence of env gene product was also confirmed by cotransfecting pSVMVER with HxBH10-env⁻* (Fig. 3, lanes 2), a Vpr⁻⁷ 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 viruses 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 HxBc2ΔΔΔΔΔΔΔΔCAT 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 pSVMVER 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 Pr55prov (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 Pr55prov 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 Pr55prov 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.
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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. [35S]methionine- and [3H]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.

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 Pr55gag 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 Pr55gag 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 p55gag precursor. The results obtained with the deleted proviruses indicate that Vpr incorporation requires only the expression of the Gag polyprotein Pr55gag 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 Pr55gag to be incorporated led us to use a system in which large quantities of Pr55gag 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
cells cotransfected with pHlgagCAR and ptrENV− (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 polypeptide 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 polypeptide 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 Pr55gag 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 Pr55gag was shown by coprecipitation experiments in which the 14-kDa Vpr protein and the Pr55gag product were precipitated from virus-like particles pellets from supernatant fluid of COS cells cotransfected with a Pr55gag expressor, pHHagCAR, and a plasmid expressor, ptgENV, encoding Vpr in addition to Rev. The coprecipitation of Vpr with Pr55gag 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 Pr55gag 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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