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# Targeting Foreign Proteins to Human Immunodeficiency Virus Particles via Fusion with Vpr and Vpx

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**The human immunodeficiency virus type 1 (HIV-1) and HIV-2 Vpr and Vpx proteins are packaged into virions through virus type-specific interactions with the Gag polyprotein precursor. To examine whether HIV-1 Vpr (Vpr1) and HIV-2 Vpx (Vpx2) could be used to target foreign proteins to the HIV particle, their open reading frames were fused in frame with genes encoding the bacterial staphylococcal nuclease (SN), an enzymatically inactive mutant of SN (SN*), and chloramphenicol acetyltransferase (CAT). Transient expression in a T7-based vaccinia virus system demonstrated the synthesis of appropriately sized Vpr1-SN/SN* and Vpx2-SN/SN* fusion proteins which, when coexpressed with their cognate $p55^{Gag}$ protein, were efficiently incorporated into virus-like particles. Packaging of the fusion proteins was dependent on virus type-specific determinants, as previously seen with wild-type Vpr and Vpx proteins. Particle-associated Vpr1-SN and Vpx2-SN fusion proteins were enzymatically active, as determined by in vitro digestion of lambda phage DNA. To determine whether functional Vpr1 and Vpx2 fusion proteins could be targeted to HIV particles, the gene fusions were cloned into an HIV-2 long terminal repeat/Rev response element-regulated expression vector and cotransfected with wild-type HIV-1 and HIV-2 proviruses. Western blot (immunoblot) analysis of sucrose gradient-purified virions revealed that both Vpr1 and Vpx2 fusion proteins were efficiently packaged regardless of whether SN, SN*, or CAT was used as the C-terminal fusion partner. Moreover, the fusion proteins remained enzymatically active and were packaged in the presence of wild-type Vpr and Vpx proteins. Interestingly, virions also contained smaller proteins that reacted with antibodies specific for the accessory proteins as well as SN and CAT fusion partners. Since similar proteins were absent from Gag-derived virus-like particles and from virions propagated in the presence of an HIV protease inhibitor, they must represent cleavage products produced by the viral protease. Taken together, these results demonstrate that Vpr and Vpx can be used to target functional proteins, including potentially deleterious enzymes, to the human or simian immunodeficiency virus particle. These properties may be exploitable for studies of HIV particle assembly and maturation and for the development of novel antiviral strategies.**

Unlike simple retroviruses, human and simian immunodeficiency viruses (HIV and SIV) encode proteins in addition to Gag, Pol, and Env that are packaged into virus particles. These include the Vpr protein, present in all primate lentiviruses, and the Vpx protein, which is unique to the HIV type 2 (HIV-2)/$SIV_{SM}$/$SIV_{MAC}$ group of viruses (for review, see references 32 and 35). Since Vpr and Vpx are present in infectious virions, they have long been thought to play important roles early in the virus life cycle (2, 13, 17, 40). Indeed, recent studies of HIV-1 Vpr have shown that this protein has nucleophilic properties and that it facilitates, together with the matrix protein, nuclear transport of the viral preintegration complex in nondividing cells, such as the macrophage (11). Similarly, Vpx-deficient HIV-2 has been shown to exhibit delayed replication kinetics and to require 2 to 3 orders of magnitude more virus to produce and maintain a productive infection in peripheral blood mononuclear cells (10, 16). Thus, both accessory proteins appear to be important for efficient replication and spread of HIV/SIV in primary target cells and may thus represent useful targets for rational drug design.

Vpr and Vpx packaging is mediated by the Gag precursor and thus must play an important role in HIV assembly processes (21, 24, 30, 40). To test whether Vpr and Vpx could also be used as vehicles to target foreign proteins to HIV/SIV virions, we constructed HIV-1 *vpr* (*vpr1*) and HIV-2 *vpx* (*vpx2*) gene fusions with the bacterial staphylococcal nuclease (*SN*) and chloramphenicol acetyltransferase (*CAT*) genes. Incorporation of foreign proteins into retrovirus particles has previously been reported by fusion with *gag* (15, 36, 38). Using the yeast retrotransposon Ty1 as a retrovirus assembly model, Natsoulis and Boeke (28) tested this approach as a novel means to interfere with viral replication. More recently, the expression of a murine retrovirus capsid-SN fusion protein was found to inhibit murine leukemia virus replication in tissue culture cells (29). Since the HIV accessory proteins, Vpr and Vpx, can be packaged in quantities similar to those of the major structural proteins, we reasoned that they may be exploited for the same purpose; unlike Gag or Pol proteins, Vpr and Vpx are dispensable for viral replication in immortalized T-cell lines. Thus, structural alteration of these accessory proteins may be more readily tolerated than similar changes in Gag or Gag/Pol. Fusion proteins containing a Vpx or Vpr moiety should be pack-

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aged into HIV particles by expression in *trans*, since their incorporation should be mediated by the same interactions with Gag that facilitates wild-type Vpr and Vpx protein packaging.

To evaluate the feasibility of this strategy, we have constructed Vpr1 and Vpx2 fusion proteins and analyzed their abilities to package into HIV particles. For fusion partners, we have selected SN because of its potential to degrade viral nucleic acid upon packaging and CAT because of its utility as a functional marker. To control for cytotoxicity, we have also used an enzymatically inactive nuclease mutant (SN*), derived from SN by site-directed mutagenesis. This SN* mutant differs from wild-type SN by two amino acid substitutions; Glu was changed to Ser (position 43), and Arg was changed to Gly (position 87). SN* folds normally but has a specific activity that is $10^6$-fold lower than that of wild-type SN (37). Using transient expression systems and in *trans* complementation approaches, we have addressed questions of fusion protein stability, function, and packaging requirements. Our results show that Vpr1 and Vpx2 fusion proteins can be expressed in mammalian cells and are incorporated into HIV particles even in the presence of wild-type Vpr and/or Vpx proteins. Most importantly, however, our results show that virion-incorporated Vpr and Vpx fusions remain enzymatically active. Thus, targeting heterologous Vpr and Vpx fusion proteins, including deleterious enzymes, to virions may represent a promising new avenue toward anti-HIV drug discovery.

## MATERIALS AND METHODS

**Cells and viruses.** HeLa, HeLa-tat (HLtat), and CV-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HLtat cells constitutively express the first exon of HIV-1 *tat* (5) and were kindly provided by B. Felber and G. Pavlakis. A recombinant vaccinia virus (rVT7) containing the bacteriophage T7 RNA polymerase gene (7) was used to facilitate expression of viral genes placed under the control of a T7 promoter (4). Stocks of rVT7 were prepared and titrated in CV-1 cells as described previously (39). HIV-$1_{YU2}$ (23), HIV-1 pNL 4-3-R$^-$ and pNL 4-3 (30), HIV-$1_{HXB2D}$ (31), HIV-$1_{SG3}$ (8a), HIV-$2_{ST}$, (20), and HIV-$2_{7312A}$ (unpublished data) proviral clones were used for the construction of recombinant expression plasmids and the generation of transfection-derived viruses.

**Antibodies.** To generate HIV-1 Vpr-specific antibodies, the HIV-$1_{YU2}$ (23) *vpr* open reading frame was amplified by PCR using primers (sense [5′-GCCACCT TTGTCGACTGTTAAAAAACT-3′] and antisense [5′-GTCCTAGGCAAGC TTCCTGGATGC-3′]) containing *Sal*I and *Hin*dIII sites and ligated into the prokaryotic expression vector pGEX (9), generating pGEX-vpr1. This construct allowed expression of Vpr1 as a C-terminal fusion protein with glutathione *S*-transferase (GST), thus allowing protein purification by affinity chromatography. *Escherichia coli* DH5α was transformed with pGEX-vpr1, and protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG). Expression of the GST-Vpr1 fusion protein was confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Soluble GST-Vpr1 protein was purified, and Vpr1 was released by thrombin cleavage, using previously described procedures (34). New Zealand White rabbits were immunized with 0.4 mg of purified Vpr1 protein emulsified 1:1 in Freund's complete adjuvant, boosted three times at 2-week intervals with 0.25 mg of Vpr1 mixed 1:1 in Freund's incomplete adjuvant, and bled 8 and 10 weeks after the first immunization to collect antisera (27a).

Additional antibodies used in these studies included monoclonal antibodies to HIV-1 Gag (ATC1 [40]) and HIV-2 Gag (RIC7 [26, 40]), polyclonal rabbit antibodies raised against the HIV-2 Vpx protein (17, 18), and anti-SN antiserum raised against purified bacterially expressed SN protein.

**Construction of T7-based expression plasmids.** A DNA fragment encompassing HIV-$1_{HXB2D}$ *gag* (nucleotides 335 to 1837) was amplified by PCR using primers (sense [5′-AAGGAGAGCCATGGGTGCGAGAGCG-3′] and antisense [5′-GGGGATCCCTTTATTGTGACGAGGGG-3′]) containing *Nco*I and *Bam*HI restriction sites (underlined). The PCR product was digested with *Nco*I and *Bam*HI, purified, and ligated into the polylinker of the pTM1 vector (27), generating pTM-gag1. Similarly, a DNA fragment containing the *gag* coding region of HIV-$2_{ST}$ (nucleotides 547 to 2113) was amplified by PCR using sense and antisense primers 5′-ATTGTGGGCCATGGGCGCGAGAAAC-3′ and 5′-GGGGGGCCCCTACTGGTCTTTTCC-3′, respectively. The reaction product was cut with *Nco*I and *Sma*I (underlined), purified, and ligated into the polylinker of pTM1, generating pTM-gag2.

For expression of Vpr1 under the control of the T7 promoter, a DNA fragment containing the HIV-$1_{YU2}$ *vpr* coding region (nucleotides 5107 to 5400) was amplified by PCR using primers (sense [5′-GAAGATCTACCATGGAAGCCC CAGAAGA-3′] and antisense [5′-CGCGGATCCGTTAACATCTACTGGCTC CATTTCTTGCTC-3′]) containing *Nco*I and *Hpa*I-*Bam*HI sites, respectively (underlined). The reaction product was cut with *Nco*I and *Bam*HI and ligated into pTM1, generating pTM-vpr1 (Fig. 1A). To fuse SN and SN* in frame with *vpr1*, their coding regions were excised from pGN1561.1 and pGN1709.3, respectively (27a), and, through a series of subcloning steps, ligated into the *Sma*I-*Xho*I sites of pTM-vpr1, generating pTM-vpr1SN and pTM-vpr1SN*. This approach changed the translational stop codon of Vpr1 to a Trp codon and the C-terminal Ser residue to a Cys. The resulting junctions between *vpr1* and *SN*/*SN** are depicted in Fig. 1C.

For expression of Vpx2 under T7 control, a DNA fragment containing the HIV-$2_{ST}$ *vpx* coding sequence (nucleotides 5343 to 5691) was amplified by PCR using primers (sense [5′-GTGCAACACCATGGCAGGCCCCAGA-3′] and antisense [5′-TGCACTGCAGGAAGATCTTAGACCTGGAGGGGGAGGA GG-3′]) containing *Nco*I and *Bgl*II sites, respectively (underlined). After cleavage with *Bgl*II and Klenow fill-in, the PCR product was cleaved with *Nco*I, purified, and ligated into the *Nco*I and *Sma*I sites of pTM1, generating pTM-vpx2 (Fig. 1B). To construct in-frame fusions with *vpx2*, *Bam*HI-*Xho*I SN- and

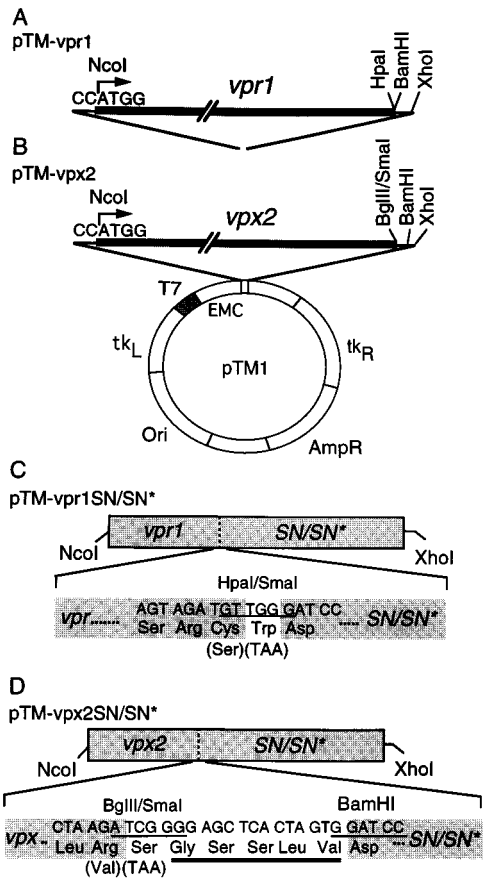


FIG. 1. Construction of *vpr1*, *vpr1SN*/*SN**, *vpx2*, and *vpx2SN*/*SN** expression plasmids. (A) Illustration of the pTM-vpr1 expression plasmid. The HIV-$1_{YU2}$ *vpr* coding region was amplified by PCR and ligated into pTM1 at the *Nco*I and *Bam*HI restriction sites. (B) Illustration of the pTM-vpx2 expression plasmid. The HIV-$2_{ST}$ *vpx* coding region was amplified by PCR and ligated into pTM1 at the *Nco*I and *Bgl*II-*Sma*I sites. (C) Illustration of the fusion junctions of the pTM-vpr1SN/SN* expression plasmids. *Sma*I-*Xho*I DNA fragments containing *SN* and *SN** were ligated into *Hpa*I-*Xho*I-cut pTM-vpr1. Blunt-end ligation at *Hpa*I and *Sma*I sites changed the *vpr* translational stop codon (TAA) to Trp and substituted the C-terminal Ser with a Cys residue. (D) Illustration of the fusion junctions of the pTM-vpx2SN/SN* expression plasmids. *Bam*HI-*Xho*I DNA fragments containing *SN* and *SN** were ligated into *Bam*HI-*Xho*I-cut pTM-vpx2. In the construction of these plasmids, the Vpx C-terminal Arg codon was changed to a Val codon and a Ser residue was introduced in place of the Vpx translational stop codon (TAA). Fusion of *vpx* and *SN*/*SN** at the *Bam*HI sites left a short amino acid sequence of the pTM1 polylinker (double underlined) between the two coding regions.

SN*-containing DNA fragments were excised from pTM-vpr1SN and pTM-vpr1SN* and ligated into pTM-vpx2, generating pTM-vpx2SN and pTM-vpx2SN*, respectively. This approach introduced one amino acid substitution at the C terminus of Vpx (Val to Arg), changed the translational stop codon of *vpx* to Ser, and left five amino acids residues of the pTM1 plasmid polylinker. The resulting junctions between *vpx2* and *SN*/*SN** are depicted in Fig. 1D.

**Construction of HIV LTR-based expression plasmids.** For efficient expression of Vpr and Vpx fusion proteins in the presence HIV, we constructed a eukaryotic expression vector (termed pLR2P) which contains both an HIV-2 long terminal repeat (LTR) (HIV-$2_{ST}$, coordinates −544 to 466) and an HIV-2 Rev response element (RRE) (HIV-$2_{ROD}$, coordinates 7320 to 7972) (see Fig. 7A). The HIV-2 LTR and RRE were chosen because they respond to both HIV-1 and HIV-2 Tat and Rev proteins (6, 22, 25). The *vpr1*, *vpr1SN*, *vpx2*, and *vpx2SN* coding regions were excised from their respective pTM expression plasmids (Fig. 1) with *Nco*I and *Xho*I restriction enzymes and ligated into pLR2P, generating pLR2P-vpr1, pLR2P-vpr1SN, pLR2P-vpx2, and pLR2P-vpx2SN, respectively (see Fig. 7A). For construction and expression of *vpr*- and *vpx-CAT* gene fusions, the *SN*-containing regions (*Bam*HI-*Xho*I fragments) of pLR2P-vpr1SN and pLR2P-vpx2SN were removed and substituted with a PCR-amplified *Bgl*II-*Xho*I DNA fragment containing *CAT* (1), generating pLR2P-vpr1CAT and pLR2P-vpx2CAT, respectively (see Fig. 10A).

**Transfections.** Transfections of proviral clones were performed in HLtat cells, using calcium phosphate DNA precipitation methods as described by the manufacturer (Stratagene). T7-based (pTM1) expression constructs were transfected by using Lipofectin (Bio-Rad) into rVT7-infected HeLa cells as described previously (40).

**Western blot (immunoblot) analysis.** Virions and virus-like particles (VLPs) were concentrated from the supernatants of transfected or infected cells by ultracentrifugation through 20% cushions of sucrose (125,000 × *g*, 2 h, 4°C). Pellets and infected or transfected cells were solubilized in loading buffer (62.5 mM Tris-HCl [pH 6.8], 0.2% SDS, 5% 2-mercaptoethanol, 10% glycerol), boiled, and separated on 12.5% polyacrylamide gels containing SDS. Following electrophoresis, proteins were transferred to nitrocellulose (0.2-μm pore size; Schleicher & Schuell) by electroblotting and incubated for 1 h at room temperature in blocking buffer (5% nonfat dry milk in phosphate-buffered saline [PBS]) and then for 2 h with the appropriate antibodies diluted in blocking buffer. Protein bound antibodies were detected with horseradish peroxidase-conjugated specific secondary antibodies, using enhanced chemiluminescence methods as instructed by the manufacturer (Amersham).

**SN nuclease activity assay.** Cells and viral pellets were resuspended in nuclease lysis buffer (40 mM Tris-HCl [pH 6.8], 100 mM NaCl, 0.1% SDS, 1% Triton X-100) and clarified by low-speed centrifugation (1,000 × *g*, 10 min). Tenfold dilutions were made in nuclease reaction cocktail buffer (100 mM Tris-HCl [pH 8.8], 10 mM $CaCl_2$, 0.1% Nonidet P-40) and boiled for 1 min. Five microliters of each dilution was added to 14 μl of reaction cocktail buffer containing 500 ng of lambda phage DNA (*Hin*dIII fragments) and incubated at 37°C for 2 h. Reaction products were electrophoresed on 0.8% agarose gels, and DNA was visualized by ethidium bromide staining.

## RESULTS

**Expression of Vpr1-SN, Vpr1-SN*, Vpx2-SN, and Vpx2-SN* fusion proteins in mammalian cells.** Expression of Vpr1- and Vpx2-SN/SN* fusion proteins in mammalian cells was assessed using the rVT7 system. HeLa cells were grown to 75 to 80% confluency and transfected with the recombinant plasmids pTM-vpr1, pTM-vpx2, pTM-vpr1SN/SN*, and pTM-vpx2SN/SN* (Fig. 1). Twenty-four hours after transfection, cells were washed twice with PBS and lysed. Soluble proteins were separated by SDS-PAGE and subjected to immunoblot blot analysis. The results are shown in Fig. 2. Transfection of pTM-vpr1SN and pTM-vpr1SN* resulted in the expression of a 34-kDa fusion protein that was detectable with both anti-Vpr and anti-SN antibodies (Fig. 2A). Similarly, transfection of pTM-vpx2SN and pTM-vpx2SN* resulted in the expression of a 35-kDa fusion protein which was detected with anti-Vpx and anti-SN antibodies (Fig. 2B). Both fusion proteins were found to migrate slightly slower than expected, as judged from the combined molecular masses of Vpr1 (14.5 kDa) and SN (16 kDa) and of Vpx2 (15 kDa) and SN, respectively. Transfection of pTM-vpr1 and pTM-vpx2 alone yielded appropriately sized wild-type Vpr and Vpx proteins. Anti-Vpr, anti-Vpx, and anti-SN antibodies were not reactive with lysates of pTM1-transfected cells included as controls. These results demonstrate

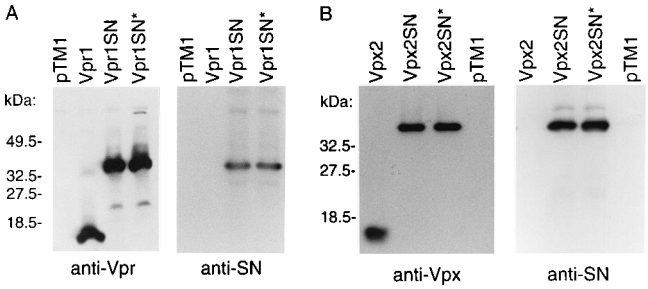


FIG. 2. Expression of Vpr1-SN and -SN* and Vpx2-SN and -SN* fusion proteins in mammalian cells. (A) pTM1, pTM-vpr1, pTM-vpr1SN, and pTM-vpr1SN* were transfected into HeLa cells 1 h after infection with rVT7 (multiplicity of infection = 10). Twenty-four hours later, cell lysates were prepared and examined by immunoblot analysis. Replica blots were probed with anti-Vpr1 (left) and anti-SN (right) antibodies. (B) Replica blots, prepared from rVT7-infected HeLa cells transfected with pTM1, pTM-vpx2, pTM-vpx2SN, and pTM-vpx2SN*, were probed with anti-Vpx2 (left) and anti-SN (right) antibodies. Bound antibodies were detected by enhanced chemiluminescence (Amersham) methods as described by the manufacturer.

both SN and SN* fusion proteins can be expressed in mammalian cells.

**Incorporation of Vpr1- and Vpx2-SN/SN* fusion proteins into VLPs.** In vaccinia virus and baculovirus systems, the expression of HIV Gag is sufficient for assembly and extracellular release of VLPs (3, 8, 19, 33). Our earlier data and those of others demonstrated that Vpr1 and Vpx2 can be efficiently incorporated into Gag particles without the expression of other viral gene products (21, 24, 30, 40). To test whether the Vpr1 and Vpx2 fusion proteins could be similarly packaged into VLPs, recombinant plasmids were coexpressed with HIV-1 and HIV-2 Gag proteins in the rVT7 system. pTM-vpr1, pTM-vpr1SN, and pTM-vpr1SN* were transfected into HeLa cells alone and in combination with the HIV-1 Gag expression plasmid, pTM-gag1. Twenty-four hours after transfection, cell and VLP extracts were prepared and analyzed by immunoblot analysis (Fig. 3A). Anti-Vpr antibody detected Vpr1, Vpr1SN, and Vpr1SN* in cell lysates (top panel) and in pelleted VLPs derived by coexpression with pTM-gag1 (middle panel). In the absence of HIV-1 Gag expression, Vpr1 and Vpr1SN were not detected in pellets of culture supernatants (middle panel). As expected, VLPs also contained p55 Gag (bottom panel). Thus, Vpr1-SN/SN* fusion proteins were successfully packaged into VLPs.

To analyze whether Vpx2SN was similarly capable of packaging into HIV-2 VLPs, pTM-vpx2, pTM-vpx2SN, and pTM-vpx2SN* were transfected into HeLa cells alone and in combination with the HIV-2 Gag expression plasmid, pTM-gag2. Western blots were prepared with lysates of cells and VLPs concentrated from culture supernatants by ultracentrifugation (Fig. 3B). Anti-Vpx antibody detected Vpx2, Vpx2-SN, and Vpx2-SN* in cell lysates (top panel) and in VLPs derived by coexpression with pTM-gag2 (middle panel). Anti-Gag antibody detected p55 Gag in VLP pellets (bottom panel). Comparison of the relative protein signal intensities suggested that the Vpr1-SN and -SN* and Vpx2-SN and -SN* fusion proteins were packaged into VLPs in amounts similar to those of wild-type Vpr1 and Vpx2 proteins. Sucrose gradient analysis of VLPs containing Vpr1-SN and Vpx2-SN demonstrated cosedimentation of these fusion proteins with VLPs (data not shown).

Earlier studies demonstrated that the Gag C-terminal region is required for incorporation of Vpr1 and Vpx2 into virions (21, 24, 30, 40). However, packaging was found to be virus type

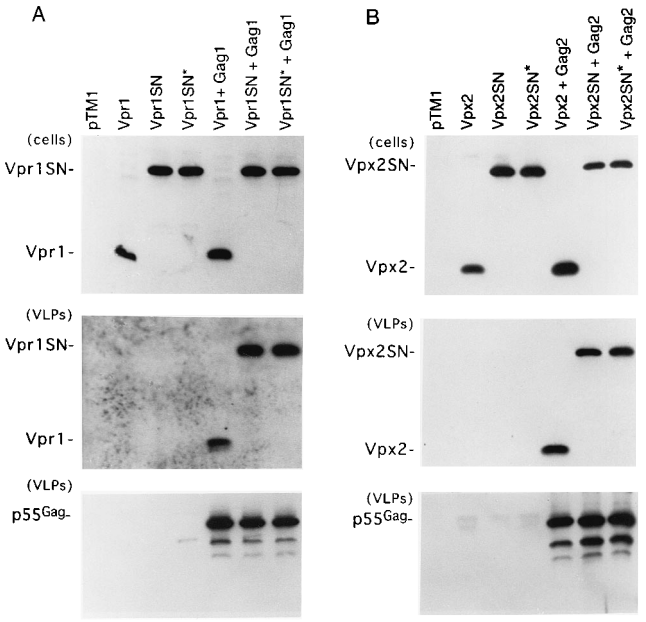


FIG. 3. Incorporation of Vpr1-SN and -SN* and Vpx2-SN and -SN* fusion proteins into VLPs. (A) T7-expressing (rVT7-infected) HeLa cells were transfected with pTM-vpr1, pTM-vpr1SN, and pTM-vpr1SN* alone and in combination with pTM-gag1. pTM1 was also transfected for control. Culture supernatants were collected 24 h after transfection, clarified by centrifugation (1,000 × *g*, 10 min), and ultracentrifuged (125,000 × *g*, 2 h) over cushions of 20% sucrose. Pellets (VLPs; middle and bottom panels) and cells (top panel) were solubilized in loading buffer and examined by immunoblot analysis using anti-Vpr1 (top and middle) and anti-Gag (bottom) antibodies as probes. (B) T7-expressing HeLa cells were transfected with pTM-vpx2, pTM-vpx2SN, and pTM-vpx2SN* alone and in combination with pTM-gag2. Pellets (VLPs; middle and bottom panels) and cells (top panel) were lysed, and proteins were separated by SDS-PAGE and electroblotted to nitrocellulose as described above. Replica blots were probed with anti-Vpx2 (top and middle panels) and anti-Gag (bottom panel) antibodies. Bound antibodies were detected by enhanced chemiluminescence methods.

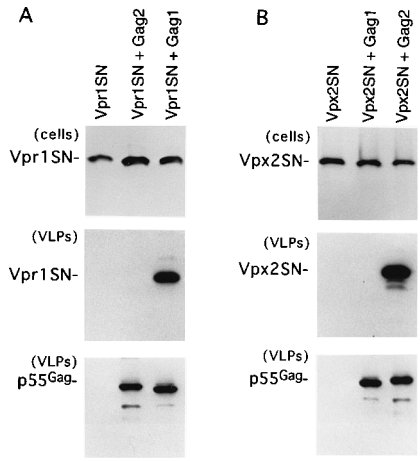


FIG. 4. Virus type-specific signals mediate incorporation of Vpr- and Vpx-SN into VLPs. (A) HIV-1 Gag mediates packaging of Vpr1-SN. rVT7-infected (T7-expressing) HeLa cells were transfected with pTM-vpr1SN alone and in combination with pTM-gag2 and pTM-gag1. Pellets (VLPs; middle and bottom panels) and cells (top panel) were prepared 24 h after transfection and examined by immunoblot analysis using anti-Vpr1 (top and middle) and anti-Gag (bottom) antibodies for probes. (B) HIV-2 Gag mediates packaging of Vpx2-SN. T7-expressing HeLa cells were transfected with pTM-vpx2SN alone and in combination with pTM-gag1 and pTM-gag2. Pellets (VLPs; middle and bottom panels) and cells (top panel) were prepared 24 h after transfection and examined by immunoblot analysis using anti-Vpx2 (top and middle) and anti-Gag (bottom) antibodies for probes.

specific; that is, when expressed in *trans*, Vpx2 was efficiently incorporated only into HIV-2 virions (18) and HIV-2 VLPs (40). Similarly, HIV-1 Vpr required interaction with the HIV-1 Gag precursor for incorporation into HIV-1 VLPs (24, 40). To confirm that the association of Vpr1-SN and Vpx2-SN with VLPs was not mediated by the SN moiety but was due to Vpr- and Vpx-specific packaging signals, pTM-vpr1SN and pTM-vpx2SN were cotransfected individually with either pTM-gag1 or pTM-gag2. For control, pTM-vpr1 and pTM-vpx2 were also transfected alone. Twenty-four hours later, lysates of cells and pelleted VLPs were examined by immunoblotting (Fig. 4). Vpr1-SN was expressed in all cells (Fig. 4A, top panel) but was associated only with VLPs derived from cells transfected with pTM-gag1 (middle panel). Similarly, Vpx2-SN was detected in all pTM-vpx2-transfected cells (Fig. 4B, top panel) but was associated only with VLPs derived by cotransfection with pTM-gag2 (middle panel). HIV-1 and HIV-2 Gag monoclonal antibodies confirmed the presence of Gag precursor protein in each VLP pellet (bottom panel). These data indicate that incorporation of Vpr1-SN and Vpx2-SN into VLPs requires interaction with the cognate Gag precursor protein, just as for native Vpr1 and Vpx2.

While Vpr1-SN and Vpx2-SN fusion proteins clearly associated with VLPs (Fig. 3), the question remained as to whether they would continue to do so in the presence of the native accessory proteins. We therefore compared the efficiency of Vpr1-SN and Vpx2-SN packaging by competition analysis (Fig. 5). pTM-vpr1SN and pTM-vpx2SN were cotransfected with pTM-gag1/pTM-vpr1 and pTMgag2/pTM-vpx2, respectively, using ratios that ranged from 1:4 to 4:1 (Fig. 5A and B, left panels). For comparison, pTM-vpr1SN and pTM-vpr1 were transfected individually with pTM-gag1 (Fig. 5A, middle and

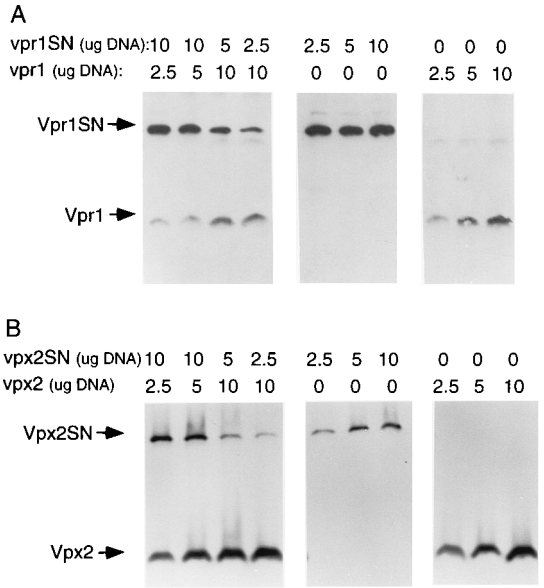


FIG. 5. Competition analysis of Vpr1-SN and Vpx2-SN for incorporation into VLPs. (A) T7-expressing HeLa cells were transfected with different amounts of pTM-vpr1 (2.5, 5, and 10 μg) and pTM-vpr1SN (2.5, 5, and 10 μg), either individually or together in combination with pTM-gag1 (10 μg). (B) HeLa cells were transfected with different amounts of pTM-vpx2 (2.5, 5, and 10 μg) and pTM-vpx2SN (2.5, 5, and 10 μg), either individually or together with pTM-gag2 (10 μg). Twenty hours after transfection, particles were concentrated by ultracentrifugation through sucrose cushions and analyzed by immunoblotting using anti-Vpr1 (A) or anti-Vpx2 (B) antibodies.

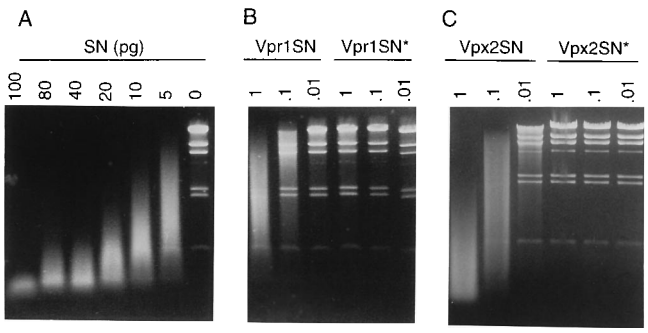


FIG. 6. Nuclease activity of VLP-associated Vpr1-SN and Vpx2-SN proteins. VLPs were concentrated from culture supernatants of T7-expressing HeLa cells cotransfected with pTM-gag1/pTM-vpr1SN, pTM-gag1/pTM-vpr1SN*, pTM-gag2/pTM-vpx2SN, and pTM-gag2/pTM-vpx2SN* by ultracentrifugation (125,000 × *g*, 2 h) through 20% cushions of sucrose. Pellets containing Vpr1-SN and -SN* (B) and Vpx2-SN and -SN* (C) were resuspended in PBS. Tenfold dilutions were made in nuclease reaction cocktail buffer (100 mM Tris-HCl [pH 8.8], 10 mM $CaCl_2$, 0.1% Nonidet P-40) and boiled for 1 min. Five microliters of each dilution was added to 14 μl of reaction cocktail buffer containing 500 ng of lambda phage DNA (*Hin*dIII fragments) and incubated at 37°C for 2 h. Reaction products were electrophoresed on 0.8% agarose gels, and DNA was visualized by ethidium bromide staining. Standards (A) were prepared by dilution of purified SN (kindly provided by A. Mildvan) into cocktail buffer and assayed as described above.

right panels, respectively), and pTM-vpx2SN and pTM-vpx2 were transfected with pTM-gag2 (Fig. 5B, middle and right panels, respectively). VLPs were pelleted through sucrose cushions, lysed, separated by PAGE, blotted onto nitrocellulose, and probed with the anti-SN antibody. The results revealed the presence of both Vpr1 and Vpr1-SN in VLPs when cotransfected into the same cells (Fig. 5A, left panel). Similarly, coexpressed Vpx2 and Vpx2-SN were also copackaged (Fig. 5B, left panel). Comparison of the relative amounts of VLP-associated Vpr1-SN and Vpx2-SN when expressed in the presence and absence of the native protein indicated that there were no significant packaging differences. These results indicate that Vpr1 and Vpx2 fusion proteins can efficiently compete with wild-type proteins for virion incorporation.

**Vpr1-SN and Vpx2-SN fusion proteins possess nuclease activity.** To test whether virion-associated SN fusion proteins were enzymatically active, VLPs concentrated by ultracentrifugation from culture supernatants of HeLa cells transfected with pTM-gag1/pTM-vpr1SN and pTM-gag2/pTM-vpx2SN were analyzed for nuclease activity in an in vitro DNA digestion assay. Prior to this analysis, immunoblotting confirmed the association of Vpr1-SN and Vpx2-SN with VLPs (data not shown). Figure 6 shows lambda phage DNA fragments in 0.8% agarose gels after incubation with dilutions of VLPs lysates that contained Vpr1- or Vpx2-SN fusion proteins. VLPs containing Vpr1-SN* and Vpx2-SN* were included as negative controls, and dilutions of purified SN served as reference standards (Fig. 6A). Both virion-associated Vpr1-SN (Fig. 6B) and Vpx2-SN (Fig. 6C) fusion proteins exhibited nuclease activity, as demonstrated by degradation of lambda phage DNA. Cell-associated Vpr1-SN and Vpx2-SN fusion proteins also possessed nuclease activity when analyzed in this system (data not shown). To control for SN specificity, this analysis was also conducted in buffers devoid of $Ca^{2+}$, and under these conditions no SN activity was detected (data not shown). These results indicate that SN remains enzymatically active when expressed as a fusion protein and packaged into VLPs.

**Incorporation of Vpx2-SN fusion protein into HIV-2 virions.** Our earlier studies indicated that Vpx is incorporated into HIV-2 virions when expressed in *trans* (18). To test whether Vpx2 fusion proteins were similarly capable of packaging into wild-type HIV-2 virions, we constructed an expression plasmid (pLR2P) placing the *vpx2SN* and *vpx2SN** coding regions under control of the HIV-2 LTR and RRE. The HIV-2 RRE was positioned downstream of the fusion genes to ensure mRNA stability and efficient translation (Fig. 7A). To examine whether the fusion proteins could package when expressed in *trans*, HIV-$2_{ST}$ proviral DNA (pSXB1) was transfected alone and in combination with pLR2P-vpx2SN and pLR2P-vpx2SN*. Forty-eight hours later, extracellular virus was pelleted from culture supernatants by ultracentrifugation through cushions of 20% sucrose and examined by immunoblot analysis (Fig. 7B). Duplicate blots were probed with anti-Vpx, anti-SN, and anti-Gag antibodies. The anti-Vpx antibody detected the 15-kDa Vpx2 protein in all viral pellets. In virions derived by cotransfection of HIV-$2_{ST}$ with pLR2P-vpx2SN and pLR2P-vpx2SN*, additional proteins of approximately 35 and 32 kDa were clearly visible. The same two proteins were also apparent on a duplicate blot probed with anti-SN antibodies, indicating that they represented Vpx2-SN fusion proteins (Fig. 7B, middle panel). The predicted molecular mass of full-length Vpx2-SN fusion protein is 33 kDa. As native Vpx and SN run slightly more slowly than predicted, it is likely that the 35-kDa species represents the full-length Vpx2-SN fusion protein. Anti-SN antibodies detected additional proteins of approximately 21 and 17 kDa (these proteins were more apparent after longer exposure). Since only the 35-kDa protein was detected in Gag-derived VLPs, which lack Pol proteins (Fig. 2), we suspected that the smaller proteins represented cleavage products of Vpx2-SN and Vpx2-SN* generated by the viral protease. Anti-Gag antibodies confirmed the presence of approximately equivalent amounts of virions from each transfection.

To confirm packaging of Vpx2-SN into HIV-2 virions, sucrose gradient analysis was performed. Extracellular virus collected from culture supernatants of HLtat cells 48 h after cotransfection with pLR2P-vpx2SN and HIV-$2_{ST}$ was pelleted through cushions of 20% sucrose. Pellets were resuspended in PBS and then centrifuged for 18 h over linear gradients of 20 to 60% sucrose. Fractions were collected and analyzed by immunoblotting (Fig. 7C). Duplicate blots were probed separately with anti-SN and anti-Gag antibodies. Peak concentrations of both Vpx2-SN and Gag were detected in fractions 8 to 11, demonstrating direct association and packaging of Vpx2SN into HIV-2 virions. These same sucrose fractions were found to have densities of between 1.16 and 1.17 g/ml, as determined by refractometric analysis (data not shown). Again, both the 35- and 32-kDa forms of Vpx2-SN were detected, providing further evidence for protease cleavage following packaging into virus particles.

Since HIV-$2_{ST}$ is defective in *vpr* (20), we asked whether this affected the packaging of the Vpx2-SN fusion protein. We therefore analyzed a second strain of HIV-2, termed HIV-$2_{7312A}$, which was cloned from short-term peripheral blood mononuclear cell culture and contains open reading frames for all genes, including intact *vpr* and *vpx* genes (unpublished data). A plasmid clone of HIV-$2_{7312A}$ proviral DNA (pJK) was transfected alone and in combination with pLR2P-vpx2SN into HLtat cells. For comparison, HIV-$2_{ST}$ was also cotransfected with pLR2P-vpx2SN. Progeny virus was concentrated by ultracentrifugation through sucrose cushions and examined by immunoblot analysis (Fig. 7D). Duplicate blots were probed with anti-Vpx and anti-Gag antibodies. The results revealed comparable levels of Vpx2-SN incorporation into *vpr*-competent virus (HIV-$2_{7312A}$) compared with *vpr*-defective virus (HIV-$2_{ST}$). Moreover, the 35- and 32-kDa proteins were again detected in HIV-$2_{7312A}$ virions. These results confirmed efficient

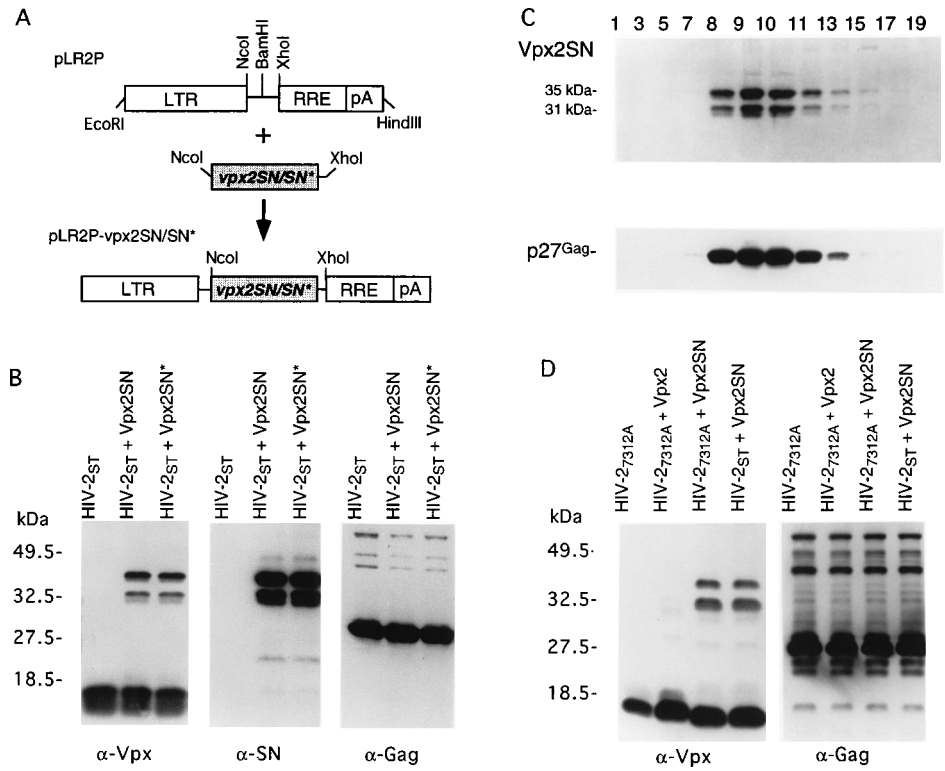


FIG. 7. Incorporation of Vpx2SN into HIV-2 by *trans* complementation. (A) Construction of the pLR2P-vpx2SN/SN* expression plasmids. To facilitate efficient expression of HIV genes, the HIV-2 LTR and RRE were engineered into the polylinker of pTZ19U, generating pLR2P. The organization of these elements within the pTZ19U polylinker is illustrated. *Nco*I-*Xho*I *vpx2SN* and *vpx2SN** (vpx2SN/SN*)-containing DNA fragments were ligated into pLR2P, generating pLR2P-vpx2SN and pLR2P-vpx2SN* (pLR2P-vpx2SN/SN*). (B) Association of Vpx2-SN with HIV-2 virions. Monolayer cultures of HLtat cells were transfected with HIV-$2_{ST}$ proviral DNA (pSXB1) and cotransfected with pSXB1/pLR2P-vpx2SN and pSXB1/pLR2P-vpx2SN*. Extracellular virus was concentrated from culture supernatants 48 h after transfection by ultracentrifugation (125,000 × *g*, 2 h) through cushions of 20% sucrose. Duplicate Western blots of viral pellets were prepared and probed independently with anti-Vpx2 (left), anti-SN (middle), and anti-Gag (right) antibodies. (C) Sucrose gradient analysis. Pellets of supernatant-virus prepared from pSXB1/pLR2P-vpx2SN-cotransfected HLtat cells were resuspended in PBS, layered over a 20 to 60% linear gradient of sucrose, and centrifuged for 18 h at 125,000 × *g*. Fractions (0.5 ml) were collected from the bottom of the tube, diluted 1:3 in PBS, reprecipitated, and solubilized in electrophoresis buffer for immunoblot analysis. Replica blots were probed with anti-SN (top) and anti-Gag (bottom) antibodies. Fraction 1 represents the first collection from the bottom of the gradient, and fraction 19 represents the last collection. Only alternate fractions are shown, except at the peak of protein detection. (D) Incorporation of Vpx2-SN into HIV-$2_{7312A}$ Vpr- and Vpx-competent virus. Virus concentrated from supernatants of HLtat cells transfected with HIV-$2_{7312A}$ proviral DNA (pJK) or cotransfected with pJK/pLR2P-vpx2SN or pJK/pLR2P-vpx2 was prepared for immunoblot analysis as described above. Included for control were virions derived by pSXB1/pLR2P-vpx2SN cotransfection. Duplicate blots were probed with anti-Vpx (left) and anti-Gag (right) antibodies.

incorporation of the Vpx2-SN protein into replication-competent wild-type HIV-2, even in the presence of native Vpr and Vpx proteins.

**Incorporation of Vpr1-SN into HIV-1 virions.** Using the same LTR/RRE-based expression plasmid, we also tested whether Vpr1-SN could package into HIV-1 virions by coexpression with HIV-1 provirus (as discussed above, the HIV-2 LTR can be transactivated by HIV-1 Tat and the HIV-2 RRE is sensitive to the HIV-1 Rev protein). Virions released into the culture medium 48 h after transfection of HLtat cells with pNL4-3 (HIV-1) and pNL4-3-$R^-$ (HIV-1-$R^-$) (30) alone and in combination with pLR2P-vpr1SN were concentrated by ultracentrifugation and examined by immunoblot analysis (Fig. 8). As observed in cotransfection experiments with HIV-2, anti-SN antibodies identified two major Vpr1-SN fusion proteins of approximately 34 and 31 kDa. These proteins were not detected in virions produced by transfection of pNL4-3 and pNL4-3-$R^-$ alone. From expression in the rVT7 system, the full-length Vpr1-SN fusion protein was expected to migrate at 34 kDa. Therefore, the 31-kDa protein likely represents a cleavage product. Anti-SN antibodies also detected a protein migrating at 17 kDa. Anti-Vpr antibody detected the 34- and 31-kDa proteins in virions derived from cotransfected cells. It

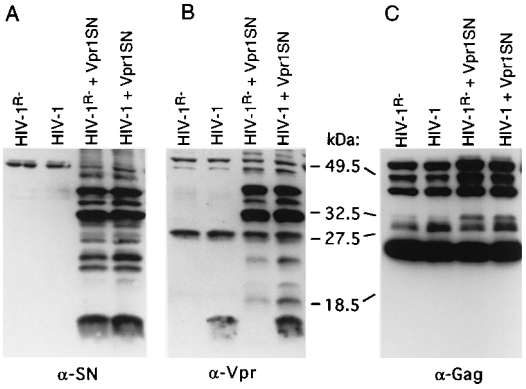


FIG. 8. Incorporation of Vpr1-SN into HIV-1 virions by *trans* complementation. Culture supernatant virus from HLtat cells transfected with pNL4-3 (HIV-1) and pNL4-3$R^-$ (HIV-1 *vpr* mutant) or cotransfected with pNL4-3/pLR2P-vpr1SN and pNL4-3$R^-$/pLR2P-vpr1SN was prepared for immunoblot analysis as described above. Blots were probed with anti-SN (A), anti-Vpr (B), and anti-Gag (C) antibodies.

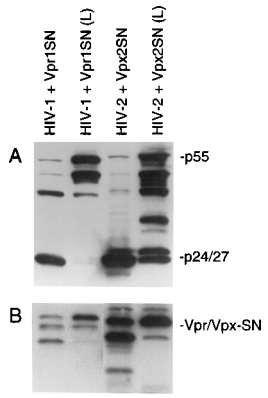


FIG. 9. Inhibition of Vpr1/Vpx2-SN processing by an HIV protease inhibitor. HIV-1 (pSG3) and HIV-2 (pSXB1) proviral DNAs were cotransfected separately into replica cultures of HLtat cells with pLR2P-vpr1SN and pLR2P-vpx2SN, respectively. One culture of each transfection contained medium supplemented with 1 μM the HIV protease inhibitor L-699,502. Virions were concentrated from culture supernatants by ultracentrifugation through cushions of 20% sucrose and examined by immunoblot analysis using anti-Gag (A) and anti-SN (B) antibodies.

is noteworthy that both the anti-Vpr and anti-SN antibodies detected the 31-kDa protein most strongly and that anti-Vpr antibody did not detect the 17-kDa protein recognized by anti-SN antibody. These results also show that even in virions in which native Vpr protein was packaged, Vpr1-SN was incorporated in abundance. The Gag monoclonal antibody detected similar amounts of Gag protein in all viral pellets and demonstrated processing of the $p55^{gag}$ precursor (Fig. 8C).

To demonstrate more directly that cleavage of the Vpr1- and Vpx2-SN fusion proteins was mediated by the HIV protease, virus was concentrated from pNL4-3-$R^-$/pLR2P-vpr1SN- and pSXB1/pLR2P-vpx2SN-transfected cells that were cultured in the presence of 1 μM the HIV protease inhibitor L-689,502 (kindly provided by E. Emini, Merck & Co., Inc.). As expected, immunoblot analysis of virions demonstrated substantially less processing of $p55^{gag}$ (Fig. 9A). Similarly, virions produced in the presence of L-689,502 also contained greater amounts of the uncleaved species of Vpr1-SN and Vpx2-SN fusion proteins (Fig. 9B). Taken together, these results confirm that Vpr1- and Vpx2-SN fusion proteins are subject to protease cleavage during or subsequent to virus assembly.

**Incorporation of Vpr1- and Vpx2-CAT fusion proteins into HIV virions.** To determine whether Vpx2 and Vpr1 could target additional proteins to the HIV particle, the entire 740-bp *CAT* gene was substituted for *SN* in the pLR2P-vpx2SN and pLR2P-vpr1SN vectors, generating pLR2P-vpr1CAT and pLR2P-vpx2CAT (Fig. 10A). pNL4-3/pLR2P-vpr1CAT, pNL4-3-$R^-$/pLR2P-vpr1CAT, and pSXB1/pLR2P-vpx2CAT were cotransfected into HLtat cells. As controls, pNL4-3, pNL4-3-$R^-$, and pSXB1 were transfected alone. Progeny virions, concentrated from culture supernatants, were analyzed by immunoblotting (Fig. 10B and C). With anti-Vpr antibodies, 40-kDa fusion proteins were detected in viral pellets derived by cotransfection of pRL2P-vpr1CAT with both pNL4-3 and pNL4-3-$R^-$ (Fig. 10B). This size is consistent with the predicted molecular mass of the full-length Vpr1-CAT fusion protein. In addition, anti-Vpr antibodies also detected a 17-kDa protein which did not correspond to the molecular mass of native Vpr1 protein (14.5 kDa in virions derived from cells transfected with pNL4-3). The same protein was recognized weakly with anti-CAT antibodies (data not shown), suggesting a fusion protein cleavage product containing mostly Vpr sequence. Very similar

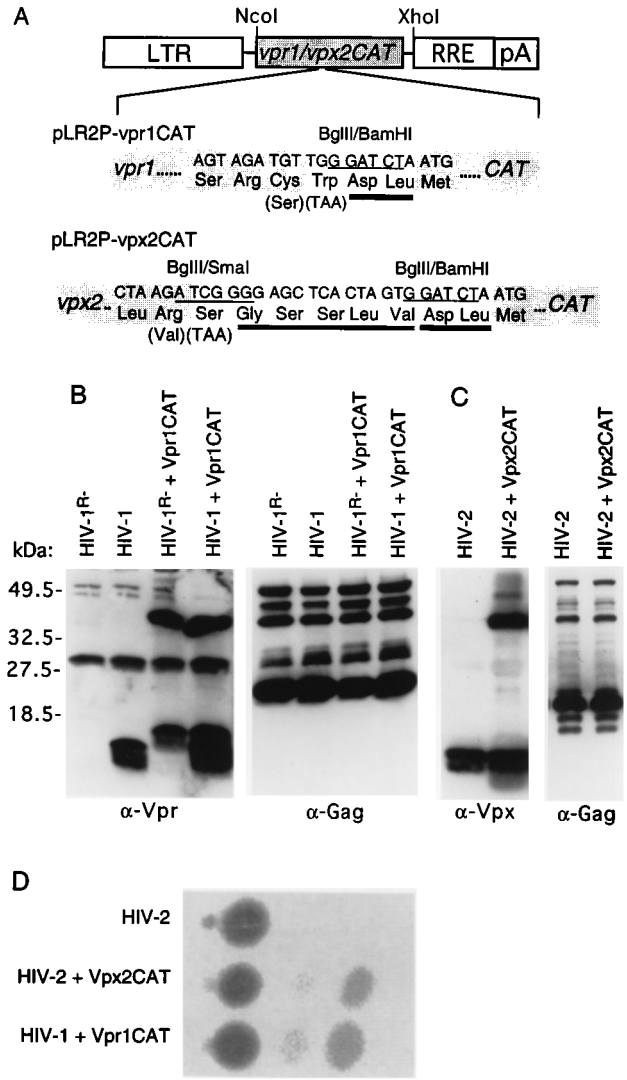


FIG. 10. Incorporation of enzymatically active Vpr1- and Vpx2-CAT fusion proteins into HIV virions. (A) Illustration of the fusion junctions of the pLR2P-vpr1CAT and pLR2P-vpx2CAT expression plasmids. PCR-amplified *Bam*HI-*Xho*I DNA fragments containing *CAT* were ligated into *Bgl*II-*Xho*I-cut pLR2P-vpr1SN and pLR2P-vpx2SN, replacing *SN* (see Fig. 1). This construction introduced two additional amino acid residues (Asp and Leu, above the blackened bar) between the *vpr1/vpx2CAT* coding regions. (B) Incorporation of Vpr1-CAT into HIV-1 virions. Virus produced from HLtat cells transfected with pNL4-3 (HIV-1) and pNL4-3$R^-$ (HIV-1-$R^-$) or cotransfected with pNL4-3/pLR2P-vpr1CAT and pNL4-3$R^-$/pLR2P-vpr1CAT was prepared as described above and examined by immunoblot analysis. Replica blots were probed with anti-Vpr1 (left) and anti-Gag (right) antibodies. (C) Incorporation of Vpx2-CAT into HIV-2 virions. Virus produced from HLtat cells transfected with pSXB1 (HIV-2) or cotransfected with pSXB1/pLR2P-vpx2CAT was prepared as described above and examined by immunoblot analysis. Replica blots were probed with anti-Vpx2 (left) and anti-Gag (right) antibodies. (D) Virion-incorporated Vpr1- and Vpx2-CAT fusion proteins possess enzymatic activity. Viruses pelleted from HLtat cells transfected with pSXB1 (HIV-2) or cotransfected with pSXB1/pLR2P-vpx2CAT and pNL4-3/pLR2P-vpr1CAT were lysed and analyzed for CAT activity by using previously described methods (12). HIV-2 was included as a negative control.

results were obtained with virions derived from HLtat cells cotransfected with HIV-$2_{ST}$ and pRL2P-vpx2CAT, in which the anti-Vpx antibody detected 41- and 15-kDa proteins (Fig. 10C). These results demonstrate that Vpr1-CAT and Vpx2-CAT fusion proteins are packaged into virions. However, like in the case of SN fusion proteins, CAT fusion proteins were

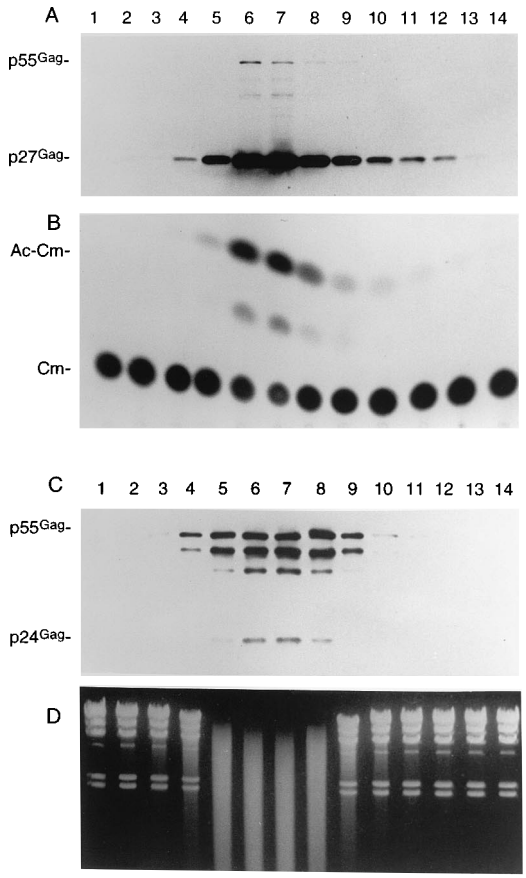


FIG. 11. Virion association of enzymatically active CAT and SN fusion proteins. (A) HIV-2 virions collected from the culture supernatant of HLtat cells cotransfected with pSXB1 and pLR2P-vpx2CAT were sedimented in linear gradients of 20 to 60% sucrose. Fractions of 0.7 ml were collected and analyzed by immunoblot analysis using Gag monoclonal antibodies as a probe. (B) CAT enzyme activity was determined in each fraction by standard methods. The positions of nonacetylated [$^{14}$C]chloramphenicol (Cm) and acetylated chloramphenicol (Ac-Cm) are indicated. (C) HIV-1 virions derived from HLtat cells cotransfected with pSG3 and pLR2P-vpr1SN and cultured in the presence of L-689,502 were sedimented in linear gradients of 20 to 60% sucrose. Fractions were collected and analyzed for virus content by immunoblot analysis using Gag monoclonal antibodies. (D) SN activity was determined in each fraction as described for Fig. 6.

cleaved by the HIV protease (the Vpx2-CAT cleavage product is not visible because of comigration with the native Vpx protein). CAT cleavage appeared less extensive, as judged from the intensity of the full-length CAT fusion protein on immunoblots.

Lysates of HIV-1 and HIV-2 viral particles were diluted 1:50 in 20 mM Tris base and analyzed for CAT activity by previously described methods (1). Figure 10D indicates that virions which contained Vpr1-CAT and Vpx2-CAT proteins possessed CAT activity. These results suggest the packaging of active Vpr1- and Vpx2-CAT fusion proteins.

**Virion-incorporated SN and CAT fusion proteins are enzymatically active.** The ability of Vpr1 and Vpx2 to deliver functionally active proteins to the virus particle was further confirmed by sucrose gradient analysis. Virions derived from HLtat cells cotransfected with HIV-$2_{ST}$ and pLR2P-vpx2CAT were sedimented in linear gradients of 20 to 60% sucrose as described above. Fractions were collected and analyzed for viral Gag protein (Fig. 11A) and corresponding CAT activity (Fig. 11B). Peak amounts of Gag protein were detected in fractions 6 and 7 (densities of 1.16 and 1.17 g/ml, respectively). Similarly, peak amounts of acetylated chloramphenicol were also detected in fractions 6 and 7.

We also tested whether virion-associated SN fusion protein retained nuclease activity, HIV-$1_{SG3}$ virions containing Vpr1-SN were analyzed after sedimentation in linear gradients of sucrose (Fig. 11). Since preliminary studies demonstrated that protease cleavage of SN fusion proteins (Fig. 7 to 9) markedly reduced Vpr1-SN nuclease activity (data not shown), these experiments were performed by culturing pSG3/pLR2P-vpr1SN cotransfected cells in the presence of L-689,502 as described above. Immunoblot analysis of sedimented virions revealed peak concentrations of Gag in fractions 6 and 7 and substantially reduced p55 processing (Fig. 11C). Peak SN activity was associated with the fractions that contained the highest concentrations of virus (Fig. 11D). These results thus document that virion incorporation per se does not abrogate the enzymatic activity of Vpr and Vpx fusion proteins, although cleavage by the viral protease may inactivate the fusion partners.

## DISCUSSION

In this report, we demonstrated the capability of HIV-1 Vpr and HIV-2 Vpx to direct the packaging of foreign proteins into HIV virions when expressed as heterologous fusion molecules. Coexpression of Vpr1 and Vpx2 fusion proteins with HIV Gag proteins resulted in their incorporation into VLPs, as has been shown for the wild-type Vpr and Vpx proteins (14, 18, 40). The *trans*-complementation experiments with HIV proviral DNA revealed that Vpr1 and Vpx2 fusion proteins were also incorporated into replication-competent viruses. Moreover, packaging of the fusion proteins in the presence of wild-type Vpx and/or Vpr proteins (Fig. 5, 7, and 8) indicated that the viral signals mediating their packaging were not obstructed by the foreign components of the fusion molecules. Likewise, virion-associated SN and CAT fusion proteins remained enzymatically active.

Data from our immunoblot analysis of VLPs and virions indicated that both virion-associated CAT and SN/SN* are susceptible to cleavage by the viral protease. There appears to be at least one cleavage site in CAT and two cleavage sites in the SN and SN* proteins. From calculated molecular weights of the major SN/SN* cleavage products, it appears that SN and SN* are cleaved once near their C termini and once near the fusion protein junctions. Since the fusion protein junctions of Vpr1-SN and Vpx2-SN are not identical, it is also possible that these regions differ with respect to susceptibility to the viral protease. Although Vpx2-SN/SN* are processed to a lesser extent than Vpr1-SN (Fig. 7 and 8), the major cleavage sites appear to be conserved. From the present data, we cannot determine the precise positions of the protease cleavage sites in Vpr1-SN and Vpx2-SN. However, there is no doubt that both the HIV-1 and HIV-2 proteases recognize processing sites in the fusion partners and that there is sufficient physical contact to enable cleavage. This is evidenced both by the reduction of cleavage product intensities on immunoblots as well as by an increased enzymatic activity in the presence of an HIV protease inhibitor. In a recent study with Moloney murine leukemia virus Gag-SN fusion proteins, the production of a 17-kDa form of an anti-SN-immunoreactive species that was dependent on coassembly of the fusion protein into virions indicated that SN was also cleaved by the Moloney murine leukemia virus protease (29). Taken together, these results

indicate that SN and CAT are susceptible to cleavage by retroviral proteases.

The demonstration that Vpr1 and Vpx2 fusion proteins are capable of associating with both VLPs and virions will facilitate various future studies on these accessory proteins and on HIV assembly in general. The approach of generating deletion mutants to study protein structure-function relationships is often of limited value since this can reduce protein stability or change the three-dimensional structure of the protein. In the case of Vpr, a single amino acid substitution at residue 76 has been shown to destabilize its expression in infected cells (30). Studies in our laboratory have indicated that deletion mutations in *vpr* and *vpx* result in premature degradation of the proteins following expression (unpublished data). Fusions of Vpr and Vpx mutant proteins with SN or CAT increase stability and hence may facilitate deletional studies aimed at identifying their functional domains. Future application of such an approach may include mapping the determinants that mediate interaction with $p6^{Gag}$ (40) or those responsible for the nucleophilic properties of Vpr and its association with the preintegration nucleoprotein complex (11).

The successful packaging of Vpr1/Vpx2-SN fusion proteins into virions indicates their potential use for accessory protein targeted viral inactivation (28). The notion of interfering with virus replication by targeting Gag-SN fusion proteins to viral particles was first demonstrated by using the yeast Ty1 retrotransposon, in which targeting of capsid-nuclease fusion proteins to particles resulted in a 98 to 99% reduction in the efficiency of Ty1 transposition in vivo, presumably by degradation of the RNA genome (28). This idea, termed capsid-targeted viral inactivation, was extended recently to show that Gag-SN fusion proteins of Moloney murine leukemia virus were targeted to virions and inhibited virus multiplication (29). Our data support the concept that Vpr and Vpx may also serve as vehicles for specific targeting of virus-inhibitory molecules, including SN. In contrast to HIV Gag, Vpr and Vpx are small proteins that can be manipulated relatively easily without altering virus replication and thus may represent vehicles with considerable versatility for application to such an antiviral strategy.

In this report, we have demonstrated that Vpr and Vpx can serve as vehicles to deliver functionally active enzymes to the HIV virion, including those that may exert an antiviral activity such as SN. Unfortunately, we do not know whether the incorporation of a Vpr/Vpx-SN fusion protein degrades HIV nucleic acid so as to exert an antiviral effect. This is primarily due to the processing and thus inactivation of the SN fusion partner by the viral protease. In addition, we still do not know whether HIV particles contain a high enough $Ca^{2+}$ concentration to activate SN (millimolar concentrations required) and whether Vpr- and Vpx-mediated incorporation places the enzyme in sufficient close contact to allow hydrolysis of the viral RNA. Regardless of these questions, we have demonstrated that in principle, the concept of accessory protein-targeted virus inactivation is feasible. As the processes that regulate HIV replication, viral assembly, maturation, and infectivity become better understood, we anticipate application of this concept to a variety of other potential fusion partners and ultimately the design of a viable antiviral strategy.

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