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A virion-specific inhibitory molecule with therapeutic potential for human immunodeficiency virus type 1

(AIDS/antiretroviral therapy/Vpx protein)

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ABSTRACT **A potential new approach for gene therapy against human immunodeficiency virus type 1 (HIV-1) infection is the design of a nonstructural gene-based virion-specific inhibitory molecule that is packaged with virus to destroy its infectivity. We tested this approach for HIV-1 by using Vpx, a virion-associated protein of HIV-2 and simian immunodeficiency virus. Vpx was incorporated into HIV-1 virions and the resulting cell-free virus lost infectivity in CD4+ human T cells. This demonstrates the therapeutic potential of an accessory gene-based virion-specific inhibitory molecule. Vpx and its derivatives can be regarded as a new class of anti-HIV-1 molecule.**

A major goal of AIDS research is to identify a class of molecule that can specifically interfere with replication of human immunodeficiency virus type 1 (HIV-1). One class of potential anti-HIV-1 molecule is an altered HIV-1 protein produced by a mutation designed to adversely affect viral structure or function. Modified structural genes produce representative molecules whose potential for gene therapy has been reported (1–3). These molecules are directed to the target virion by inclusion of structural gene motifs of the original viruses. Therapeutic effects are introduced either by transdominant mutations of the structural genes themselves (1, 2) or by the fusion of an enzyme to the structural gene motif (3). An inherent disadvantage of structural gene derivatives is the occurrence of homologous recombination during reverse transcription. In addition, approaches that exploit the fusion of a nuclease or protease to a structural gene require strict regulation of the enzyme activity to avoid toxicity caused by nonspecific destruction of host macromolecules.

As an alternative to structural gene-based approaches, we sought to identify a protein based on a nonstructural gene that could be specifically targeted to HIV-1 virions to destroy infectivity. We expected a candidate molecule to have a specific virion-association motif and inhibitory activity. In addition to these two essential requirements, we set three criteria for the selection of an appropriate molecule. (*i*) The protein should be foreign to HIV-1 and not encoded by the HIV-1 genome. Although this might impose structural or functional constraints on the virion once it was incorporated, it should decrease homologous recombination and resulting loss of effectiveness. (*ii*) The protein should be small enough to facilitate future modification and analysis. (*iii*) The protein molecule should not have general irrelevant enzymatic activity that requires regulation.

Based on these criteria, we tested Vpx, a nonstructural virion-associated accessory protein of HIV-2 and simian immunodeficiency virus (SIV) (4–7). We chose the Vpx protein for the following reasons. First, there is significant homology between Vpx and Vpr^{HIV-1} (refs. 8 and 9; Fig. 1), a virion-associated protein of HIV-1 (10, 11), suggesting the presence of a common virion-association motif. Second, despite its overall homology with Vpr^{HIV-1}, Vpx is foreign to HIV-1 and has some distinct characteristics that could potentially serve in an inhibitory manner. In this study we used Vpx of SIVmac as a representative Vpx (12). Vpx^{SIVmac}, with 112 amino acid residues, is slightly larger than Vpr^{HIV-1}, which has 96 residues (Fig. 1). In addition, seven consecutive proline residues near the carboxyl terminus of Vpx^{SIVmac} are absent in Vpr^{HIV-1} (Fig. 1). Thus, Vpx^{SIVmac} satisfied our initial requirements for a virion-association motif of nonstructural gene origin with potential for inhibitory activity. Furthermore, although the exact function of Vpx in the viral life cycle is yet to be determined (13–17), no general enzymatic activity such as a protease or nuclease is known for Vpx^{SIVmac}.

Vpx is a small protein with an affinity for nucleic acids (4). This affinity may help disturb the molecular organization of the HIV-1 virion when Vpx is present in HIV-1 virions. Its small size should facilitate engineering of Vpx derivatives that have inhibitory effects for HIV-1. In this study, we show that Vpx^{SIVmac}, expressed in the context of an HIV-1 genome, can be incorporated into an HIV-1 virion with inhibitory effects on HIV-1 replication.

MATERIALS AND METHODS

Reagents. [α-[^{35}S]thio]dATP (37.0–55.5 TBq/mmol) for DNA sequencing and [*methyl*,1′,2′-^{3}H]dTTP (3.33–4.44 TBq/mmol) for the reverse transcriptase (RT) assay were obtained from New England Nuclear. The sequencing gel mix for DNA sequencing was obtained from National Diagnostics (Atlanta). Biotinylated anti-human immunoglobulin, sheep/goat immunoglobulin antibodies, and the streptavidin–biotinylated horseradish peroxidase complex used for immunoblot analysis were purchased from Amersham.

Cells. Reagents for cell culture were obtained from GIBCO. COS-7 cells (ATCC CRL1651), which constitutively express simian virus 40 (SV40) large T antigen, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (DMEM complete medium). The CD4+ human T lymphoid cell line SupT1 was maintained at 37°C in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum and antibiotics. Cells were kept in T75 flasks (Corning) under 4.5% CO_2 at 37°C.

Plasmid Construction. Procedures for DNA manipulation were standard techniques (18). The parental infectious HIV-1 molecular clone was pSP65HXB2gpt (19). It contains the

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Abbreviations: HIV, human immunodeficiency virus; RT, reverse transcriptase; SIV, simian immunodeficiency virus; SV40, simian virus 40; VSIM, virion-specific inhibitory molecule.

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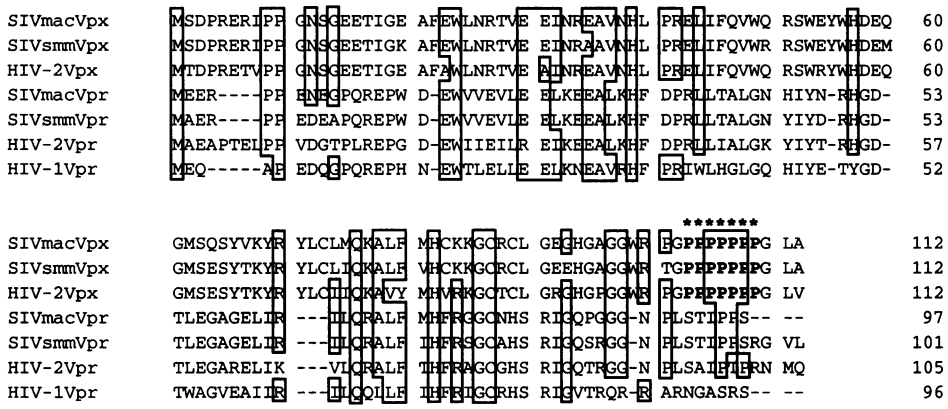

FIG. 1. Comparison of the primary structures of several Vpr and Vpx proteins from primate lentiviruses. The primary structure was compared by a multiple sequence alignment program (see *Materials and Methods*). The following isolates were analyzed: HIV-1_{HXB2R}, HIV-2_{ROD}, $SIVsmm_{H4}$, and $SIVmac_{MM251}$. Amino acids conserved between Vpr and Vpx are boxed. Dashes represent gaps in the sequences. Consecutive prolines uniquely found among various Vpx molecules are marked in bold with stars above them. The single-letter code for amino acids is used.

HIV-1 provirus HXB2 with an SV40 origin and the *Escherichia coli gpt* gene as a selective marker in a pSP65 background (Promega). Mutagenesis was performed according to Kunkel (20). Oligonucleotides for mutagenesis and PCR were synthesized with the PCR Mate 391 (Applied Biosystems). The phagemid used to make a uracil-containing single-stranded DNA template for mutagenesis was pGEM-3Zf(−)ΔpolyNdeIB2. It contains a 1.3-kb *Nde* I–*Nde* I fragment of HXB2 at the *Nde* I site. Multiple cloning sites of this phagemid were previously removed by *Hin*dIII and *Eco*RI digestion followed by filling with the Klenow fragment of DNA polymerase I and self-ligation. A *Cla* I site was introduced at the beginning of the *vpr* gene with a mutagenic oligonucleotide: 5′-GGG-CTT-GTT-CCA-TCG-ATT-CTC-TGT-CAG-TTT-C-3′ (restriction site underlined). The initiation codon for *vpu* was reinstalled by PCR-mediated mutagenesis that changed ACG to ATG (unpublished result). Each mutation was verified by a modified Sanger DNA sequencing method using Sequenase (United States Biochemical) and double-stranded DNA as the template. To replace *vpr* with *vpx*, the DNA fragment containing the *vpx* gene of pBK28 was prepared by PCR. PCR was performed with two primers: 5′-TAA-AAG-TAG-TAA-TCG-ATG-TCA-GAT-CCC-AGG-GAG-3′ and 5′-GCG-GGG-GTC-GAC-TTA-TGC-TAG-TCC-TGG-AGG-GGG-3′. Each contained a restriction site (*Cla* I and *Sal* I, respectively) that was used for cloning. The PCR condition was suggested by the manufacturer (Perkin–Elmer). The PCR product, ≈340 bp in length, was cloned into the modified pGEM-3Zf(−)ΔpolyNdeIB2 as a *Cla* I–*Sal* I fragment. The 1.3-kb *Nde* I fragment was excised and cloned back into pSP65HXB2gpt. Since *vpr* partially overlapped with *vif*, two oligonucleotides were used to reconstruct the *vif* gene truncated by this procedure: 5′-CGC-TGG-AAC-AAG-CCG-CAG-AAG-ACG-AAG-GGC-CAT-CGC-GGC-AGC-CAC-ACG-ATC-AAC-GGA-CAC-TAG-TCA-CCA-T-3′ and 5′-CGA-TGG-TGA-CTA-GTG-TCC-GTT-GAT-CGT-GTG-GCT-GCC-GCG-ATG-GCC-CTT-CGT-CTT-CTG-CGG-CTT-GTT-CCA-G-3′. These oligonucleotides were synthesized, annealed, and cloned into the *Cla* I site. The resulting clone, HXB2UX, was verified by DNA sequencing.

As a control, a DNA fragment containing *vpx* with a premature stop codon after the first 20 amino acid residues was also prepared by PCR with the same primer set as the intact *vpx* but with pBK28ΔX as an alternative template. The resulting PCR product was cloned into HXB2UX as a *Cla* I–*Sal* I fragment. This clone was called HXB2UΔX. pBK28 and pBK28ΔX have been described (5).

Transfection and Preparation of Viruses for Infection Study and Sucrose Gradient Purification. DNA was introduced into COS-7 cells by DEAE-dextran-mediated transfection (5). The concentration of cells was 3–4 × 10^6 cells per ml, and the concentration of DNA during transfection was 5 μg/ml. After 60–72 hr, the culture supernatant was collected, precleared of cell debris by centrifugation (3000 rpm, 4°C, Sorvall H1000B, RT-6000B centrifuge), and filtered through a 0.22-μm Millipore filter. For the infection study, the RT activity of the prepared viral stock was determined, and the virus was used to infect SupT1 cells without further preparation. SupT1 cells (2.4 × 10^6) were incubated overnight with the viruses (1.9 × 10^6 ^{3}H cpm of RT activity) at 37°C, washed, and resuspended in fresh complete RPMI-1640 medium. For sucrose gradient purification, the virus was precipitated on a 20% sucrose cushion (Beckman SW28 rotor, 20,000 rpm, 4°C). The viral pellet was suspended in a small volume of DMEM and stored overnight at 4°C. The resuspended viral pellet was applied to a discontinuous sucrose gradient (20–60%, 2.5% step) and fractions of about 2 ml were collected as described (11). Fractions of peak RT activity were combined and the virus was collected by centrifugation (Beckman SW28, 20,000 rpm for 2 hr at 4°C) to be used for immunoblot analysis. For the cotransfection study, SupT1 cells were transfected with 1 μg of HXB2RU (*vpr*$^+$, *vpu*$^+$) together with 5 μg of HXB2UX or HXB2UΔX or irrelevant plasmid DNA [pGEM-7Zf(+); Promega].

RT Assay. The RT activity of each sample was determined by incubating the sample with 100 μl of RT cocktail [40 mM Tris·HCl, pH 7.8/8 mM dithiothreitol/10 mM $MgCl_2$ containing 0.05 A_{260} unit of poly(rA)·$(dT)_{15}$ (Boehringer Mannheim) and 2.5 μCi (92.5 kBq) of [*methyl*,1′,2′-^{3}H]dTTP] at 37°C for 2 hr. Radioactivity of the acid-insoluble fraction was determined by precipitating the reaction product with 3 ml of chilled 10% (wt/vol) trichloroacetic acid with tRNA as a carrier. The reaction mixture was filtered through Whatman GF/C glass microfiber filters and washed four times with 5% trichloroacetic acid to remove unincorporated [*methyl*,1′,2′-^{3}H]dTTP. Radioactivity was quantified by a Beckman LS 1800 scintillation counter.

The RT activity of each fraction of the sucrose gradient was determined by incubating 45-μl aliquots of each fraction after the addition of Triton X-100 (Sigma) to a final concentration of 1% (vol/vol). The RT activity of the viral stock for the infection study and the samples collected during the infection study were determined by concentrating the virus from 1 ml of precleared culture supernatant by incubating it with 0.5 ml of 30% (wt/vol) PEG/1.6 M NaCl solution at 4°C for a minimum of 15 hr and then centrifuging the mixture at 3000 rpm for 30 min at 4°C (Beckman GH 3.7 rotor, CPKR centrifuge). The viral pellets were resuspended in 100 μl of 20

mM Tris·HCl, pH 7.5/5 mM dithiothreitol/0.15 mM EDTA/0.5 M KCl/20% (wt/vol) glycerol/1% (vol/vol) Triton X-100, and 10-μl aliquots were mixed with 100 μl of RT cocktail.

Immunoblot Analysis. The purified viral pellets were resuspended in sample buffer (80 mM Tris·HCl, pH 6.8/100 mM dithiothreitol/2% SDS/10% glycerol/0.2% bromophenol blue), boiled for 2 min, and loaded onto an SDS/15% polyacrylamide gel (Laemmli) in a Bio-Rad Mini-Protean II gel system. After electrophoresis, the separated proteins were transferred to two sheets of nitrocellulose paper (pore size, 0.45 μm; Schleicher & Schuell) by passive transfer and antigens were detected by immunoblot with a representative serum from an HIV-1-infected individual or the specific goat anti-Vpx serum as described (21).

Protein Sequence Alignment. The primary structures of several Vpr and Vpx proteins of primate lentiviruses were compared by using a multiple sequence alignment program (GeneWorks; IntelliGenetics). The sequences used were obtained from the Human Retroviruses and AIDS Database (22), except for the HIV-1 sequence (5).

RESULTS

To test whether Vpx^{SIVmac} could be packaged into HIV-1 virions, HXB2UX was generated (*Materials and Methods*, Fig. 2). HXB2UX is a derivative of HXB2, an infectious molecular clone of HIV-1. HXB2UX has vpx^{SIVmac} in place of an original nonfunctional *vpr*. The rationale for the replacement of *vpr* with *vpx* is that *vpr* and *vpx* are related both in their sequence and in their position in the genome (8, 9). As a control, HXB2UΔX, an isogenic clone of HXB2UX, was prepared with a premature stop codon in vpx^{SIVmac} after the first 20 amino acid residues (*Materials and Methods*; Fig. 2). This control helps to rule out potential cis effects of the HXB2UX construct, since HXB2UΔX differs from HXB2UX only in the presence of a premature stop codon in the *vpx* gene.

Both constructs were transfected into COS-7 cells. The amounts of virus produced from the transfected COS-7 cells were comparable for HXB2UX and HXB2UΔX as monitored by released virion-associated RT activity (data not shown). The released virus was purified through a sucrose gradient and analyzed by immunoblot with a reference serum from an HIV-1-infected individual and a goat serum specific for Vpx (Fig. 3). Human serum revealed an almost identical protein profile for both virions. The *gag* products, p17 and p24, and the *pol* products, p51 and p66, were readily detected (Fig. 3, lanes 1 and 2). When probed with the goat anti-Vpx serum, a protein of $M_r \approx 12{,}000$ was detected in HXB2UX virions (Fig. 3, lane 4), but not in HXB2UΔX virions (lane 3). The size of the detected protein in HXB2UX corresponded with that of the Vpx described previously (5). These data show that Vpx^{SIVmac}, expressed in the context of an HIV-1 genome, was packaged into cell-free HIV-1 virions. The expression of Vpx apparently did not affect the expression or processing of the HIV-1 proteins. This protein profile sug-

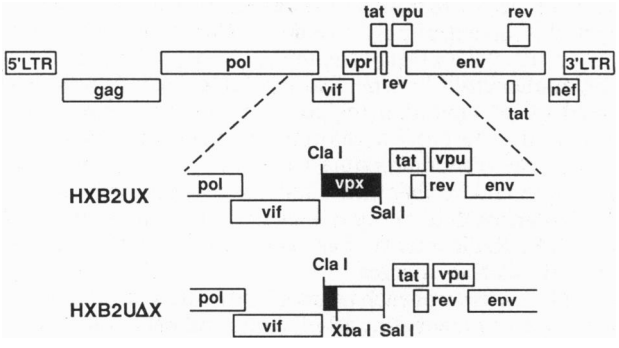

FIG. 2. Genomic organization of HXB2UX and HXB2UΔX. A representative genomic organization of the HIV-1 and part of the HXB2UX and HXB2UΔX are shown. The construction of both clones is described in *Materials and Methods*. HXB2UX contains *vpx* derived from SIVmac (BK28) (8). HXB2UΔX has *vpx* with a premature stop codon introduced by an *Xba* I linker after the first 20 amino acids of Vpx (5). Both clones have an intact *vpu* gene. The restriction enzymes used for the cloning are shown. LTR, long terminal repeat.

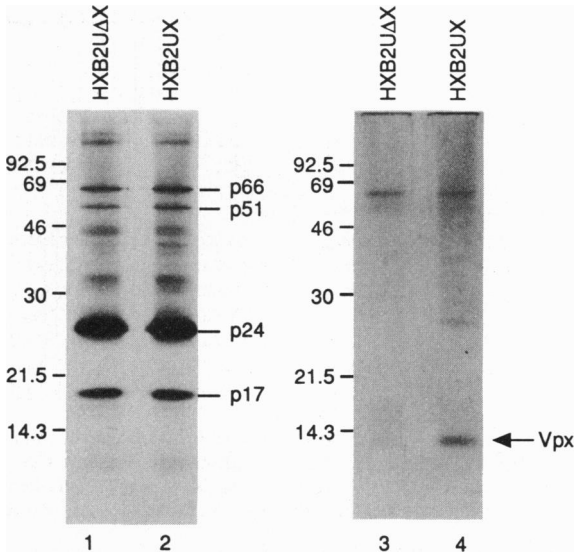

FIG. 3. Immunoblot analysis of the sucrose gradient-purified HXB2UX and HXB2UΔX virions. Proviral DNAs HXB2UΔX and HXB2UX were transfected into COS-7 cells by the DEAE-dextran method. Seventy-two hours posttransfection, viruses were collected, and purified by sucrose density gradient centrifugation and subjected to immunoblot analysis. HXB2UΔX (lane 1) and HXB2UX (lane 2) antigens were probed with a representative serum from an HIV-1-infected individual. Positions of the *gag* products p17 and p24 and the *pol* products p66 and p51 are indicated. The results of the immunoblot probed with the goat anti-Vpx serum are shown in lane 3 (HXB2UΔX) and lane 4 (HXB2UX). The position of Vpx is indicated by an arrow. Standard relative molecular mass markers ($M_r \times 10^{-3}$) for proteins are indicated at left.

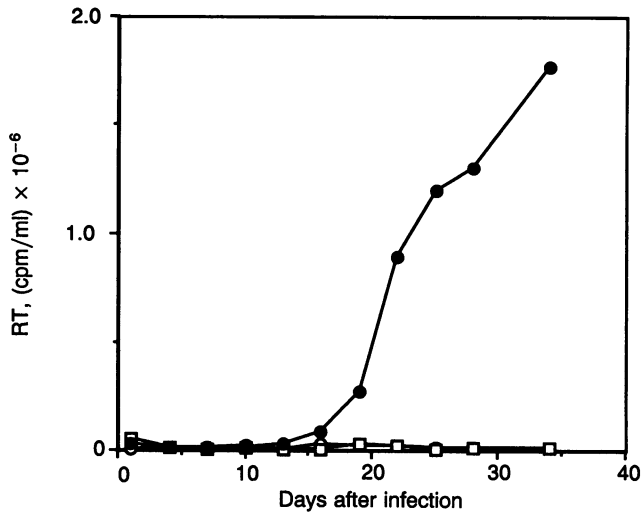

FIG. 4. Representative infectivity study on a human $CD4^+$ T-cell line, SupT1, with HXB2UX and HXB2UΔX viruses. The viruses produced from the transfected COS-7 cells were used to infect SupT1 cells. The RT activity released into the culture supernatant by HXB2UX (□), HXB2UΔX (●), and mock (○)-infected SupT1 cells was determined at the times indicated.

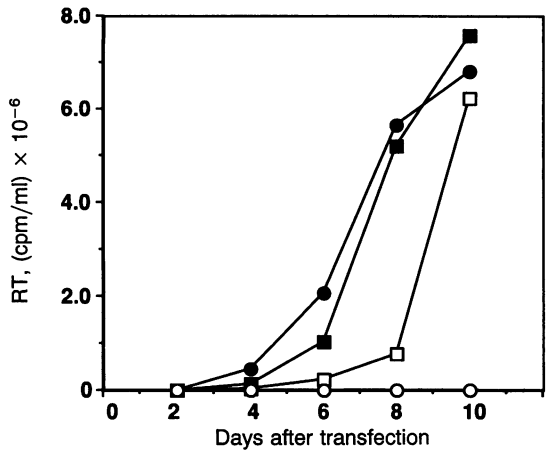

FIG. 5. Representative result of the cotransfection study. A wild-type proviral DNA, HXB2RU (1 μg), was cotransfected together with 5 μg of HXB2UX (□), HXB2UΔX (●), or an irrelevant plasmid DNA pGEM-7Zf(+) (■) into SupT1 cells by the DEAE-dextran method. The culture was monitored for RT activity released into the medium. ○, Mock transfection.

gests that *rev* regulation is intact in the constructs HXB2UX and HXB2UΔX. The comparable RT activity in the transfected COS-7 cell supernatant suggests that HXB2UX has no significant defect in the release step.

To examine the effects of the incorporated Vpx on viral infectivity, HXB2UX and HXB2UΔX viruses derived from the transfected COS-7 cells were adjusted in amount by RT activity and used to infect SupT1 cells, which are susceptible to HIV-1 infection. The infection was monitored by RT activity of the culture supernatant. A representative result is shown in Fig. 4. RT activity was detected in medium from HXB2UΔX-infected SupT1 cells at day 15 and continued to increase thereafter. In contrast, the RT activity of HXB2UX-infected SupT1 cultures was similar to that of an uninfected control throughout the observation period of 34 days. Consistent with these data, cytopathic effects were readily detected in HXB2UΔX-infected cells whereas no obvious cytopathic effects were observed in HXB2UX-infected cells (data not shown). Similar results were observed by direct transfection of HXB2UX and HXB2UΔX DNA into SupT1 cells (data not shown).

To further confirm the trans effect of Vpx, we cotransfected HXB2UX, HXB2UΔX, or irrelevant plasmid DNA together with HXB2RU into SupT1 cells. HXB2RU was used as a wild-type virus. HXB2RU is a derivative of HXB2 and has the capacity to encode a full-length functional Vpr and Vpu (ref. 11; Z.M. and X.Y., unpublished result). A representative result is shown in Fig. 5. Although the degree of inhibition varied with each experiment, the replication of HXB2RU was reproducibly retarded at least 3–4 days when it was cotransfected with HXB2UX compared with HXB2UΔX or irrelevant DNA. On several occasions, the inhibition was more prominent than the result shown in Fig. 5. Similar levels of inhibition were observed when a *vpr*$^-$ clone was used for the cotransfection as a wild type (data not shown). These results show that Vpx expressed in trans can interfere with wild-type HIV-1 replication. This negative effect does not seem to be affected by the presence of *vpr*.

DISCUSSION

In this study we tested the anti-HIV-1 therapeutic potential of a virion-specific inhibitory molecule (VSIM) of nonstructural gene origin. Here we use the term VSIM to represent an entity that can be specifically targeted to a virion to destroy the structural or functional integrity of the virus. We used Vpx to demonstrate the feasibility of the VSIM approach. Vpx expressed in the context of an HIV-1 genome was incorporated into HIV-1 virions and the viruses were not infectious in a CD4$^+$ T-cell line. Although we replaced *vpr* with *vpx*, the observed inhibitory effect was not due to the loss of *vpr* function. (*i*) *vpr* of HXB2 was originally in a truncated form and not functional. (*ii*) The effect of the *vpr* mutation should be negligible in an established T-cell line such as SupT1, especially when a high multiplicity of infection is employed as in our study (23). (*iii*) There was no inhibitory effect in HXB2UΔX, which is isogenic to HXB2UX except for an introduced premature stop codon in *vpx*. This clearly supports the idea that the negative effects were due to the expression of the Vpx protein and not due to the nucleotide change introduced by the construction of HXB2UX. The cotransfection experiment (Fig. 5) indicates that the observed inhibition was the result of a trans effect of Vpx. Furthermore, the observed inhibition of the *vpr*$^+$ HIV-1 clone during cotransfection (Fig. 5) suggests that *vpx* or a derivative of *vpx* would work in the presence of the intact *vpr* in HIV-1.

The exact mechanism of the observed inhibition of HIV-1 replication by Vpx remains to be elucidated. It is possible that the targeting of Vpx, which is foreign to HIV-1 virions, may affect the assembly and maturation of HIV-1. Alternatively, Vpx may affect other steps of the HIV-1 life cycle. Effects of Vpx on transcription or translation are less likely, however, as there were no significant differences in the major viral components between HXB2UX and HXB2UΔX virions produced from COS-7 cells (Fig. 3, lanes 1 and 2). Furthermore, the absence of a significant difference in RT activity in the transfected COS-7 cell supernatants seems to rule out a defect in the release step. Regardless of the mechanism, the inhibitory effects of Vpx are likely to involve regions of Vpx that are not shared by Vpr^{HIV-1} (Fig. 1). One such region may include the carboxyl terminus of Vpx, which has seven consecutive prolines. No such succession of proline residues is present in Vpr^{HIV-1}. The middle region of Vpx (amino acids 43–75 of Vpx^{SIVmac}; Fig. 1), which is flanked by two regions that are highly conserved between Vpx and Vpr^{HIV-1}, is also a candidate for an inhibitory effect. This region is distinct between Vpx and Vpr^{HIV-1} and is longer in Vpx than in Vpr^{HIV-1}. The relative hydrophilicity of this region suggests that it may be located on the surface of the protein. This region may be an interaction site for Vpx or Vpr with unidentified component(s) of the wild-type virion. Differences in this region may reflect the way each molecule interacts with other proteins in the virion, including the introduction of structural constraints in HIV-1 virions that contain Vpx.

Based on a comparison of the primary sequences, Tristem *et al.* (8, 9) proposed that *vpx* arose from *vpr* by gene duplication. Since Vpx and Vpr are both virion-associated proteins of primate lentiviruses (4–7, 10, 11), the incorporation of Vpx into HIV-1 virions may suggest the presence of a conserved common virion-association motif between Vpx and Vpr. A comparison of the primary sequences of Vpr and Vpx reveals two highly conserved regions. In Vpx^{SIVmac} and Vpr^{HIV-1}, the conserved regions are (*i*) amino acids 30–42 of Vpx with amino acids 24–36 of Vpr and (*ii*) amino acids 76–87 of Vpx with amino acids 65–76 of Vpr (Fig. 1). Although sequences in this region may be conserved for proper folding or some other function, these regions may be involved in the formation of a common virion-association motif. The identification of a common virion-association motif may be important for an understanding of the mechanism of how such a protein is incorporated into lentiviral virions. Furthermore, this motif may be useful as a vehicle to transduce therapeutic molecules into HIV-1 virions.

It is likely that a successful antiviral approach for HIV-1 will involve multiple complementary approaches. Although issues regarding delivery efficiency and targeting are major concerns, gene therapy has great potential for controlling HIV-1 infection. To achieve synergistic therapeutic effects, the use of multiple gene products with different action points may be necessary. From this point of view, a Vpx derivative seems advantageous, since its virion-association motif is of nonstructural gene origin and it may work synergistically when combined with other, structural gene-based transdominant mutants. Vpx or its derivatives belong to a different category from previously described transdominant negative mutants (1, 2, 24, 25) or other, structural gene derivatives (3), because *vpx* has no counterpart in the target virus HIV-1. We propose the term VSIM to represent a new class of molecule such as Vpx. A VSIM in its broad meaning can include any molecule that is specifically targeted to virions and inhibits infectivity. Therefore some transdominant structural gene products (1, 2) or their derivatives (3) can also be regarded as VSIMs.

In this study, we used the HXB2UX construct to demonstrate the principle of VSIMs. For practical purposes, other vector systems should be utilized to achieve better delivery, since HXB2UX cannot replicate in a T-cell line (Fig. 4). Additionally, Vpx^{SIVmac} may be considered a model for more precise protein engineering to achieve a final formulation of a VSIM. Further delineation of the exact virion-association motif will lead to targeting of therapeutic proteins into the virions. Analysis of the inhibitory domain of Vpx should facilitate the refinement of this promising VSIM. Refined Vpx-derived VSIMs may prove useful in a gene-therapy setting.

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