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A Novel Gene of HIV-1, *vpu*, and Its 16-Kilodalton Product

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A 16-kilodalton protein expressed in cells producing the human immunodeficiency virus (HIV-1) was identified as the gene product of the *vpu* open reading frame. When expressed in vitro, the 81–amino acid *vpu* protein reacted with about one-third of the serum samples from AIDS patients that were tested, indicating that the *vpu* open reading frame is expressed in vivo as well. Introduction of a frame-shift mutation into the *vpu* open reading frame did not significantly interfere with expression of the major viral proteins in a transient expression system. However, a five- to tenfold reduction in progeny virions was observed after the infection of T lymphocytes with the mutant virus. These data suggest that the *vpu* gene product is required for efficient virus replication and may have a role in assembly or maturation of progeny virions.

LIKE OTHER MEMBERS OF THE LENtivirus subfamily, HIV-1 contains several open reading frames (ORFs) in addition to the *gag*, *pol*, and *env* ORFs present in other retroviruses. Some of these genes, such as *tat*, *rev* (formerly *art/trs*), and *nef* (*3'-orf*, B), are thought to regulate viral gene expression. Another ORF, *vpr* (R), has been reported to be expressed in vivo even though replication of HIV-1 in T lymphocyte cell lines is not affected by the presence or absence of a functional *vpr* gene (*1*). In this study, we show that another small (81 codons) HIV-1 ORF, designated *vpu* ["U," see (*2*)] encodes a 16-kD protein that is expressed in vitro and in vivo. Mutagenesis of *vpu* in an infectious molecular clone of HIV-1 results in the synthesis of particles with altered replicative capacity.

The *vpu* gene product was initially studied by translating, in vitro, SP6-directed RNA transcripts of subcloned segments of HIV-1 proviral DNA. Two plasmids were constructed for that purpose (Fig. 1A). The first, pSP-6, could potentially encode the first exons of *tat* (72 amino acids) and *rev* (27 amino acids), the initial 756 codons of the *env* gene, and the 81-residue *vpu* gene product. The second, pSP-9 could specify only the *vpu* protein and a truncated (292–amino acid) *env* protein. The coding capacities of both plasmids can be further reduced by restriction enzyme digestion prior to in vitro transcription. Linearization of both pSP-6 and pSP-9 DNAs at the Kpn I site interrupts the *env* gene at codon 42 but leaves the *tat*, *rev*, and *vpu* ORFs intact in pSP-6. Cleavage of pSP-6 DNA with Hind III eliminates *env* and *vpu* coding sequences but does not affect the first exons of *tat* and *rev*.

Run-off RNA transcripts from linearized pSP-6 and pSP-9 plasmids were synthesized and translated in rabbit reticulocyte lysates. Proteins were metabolically labeled with [^{35}S]methionine and reacted with serum from either a seronegative individual or an AIDS patient and analyzed by SDS–polyacrylamide gel electrophoresis. As shown in Fig. 1B, pSP-6 RNA "A," which contained *tat*, *vpu*, and *env* coding sequences, elicited the synthesis of 14-, 16- and 80-kD proteins that immunoreacted with the AIDS patient serum. The shorter pSP-6 RNA "B," which has the capacity to code for *tat* and *vpu* products as well as a truncated (42-residue)

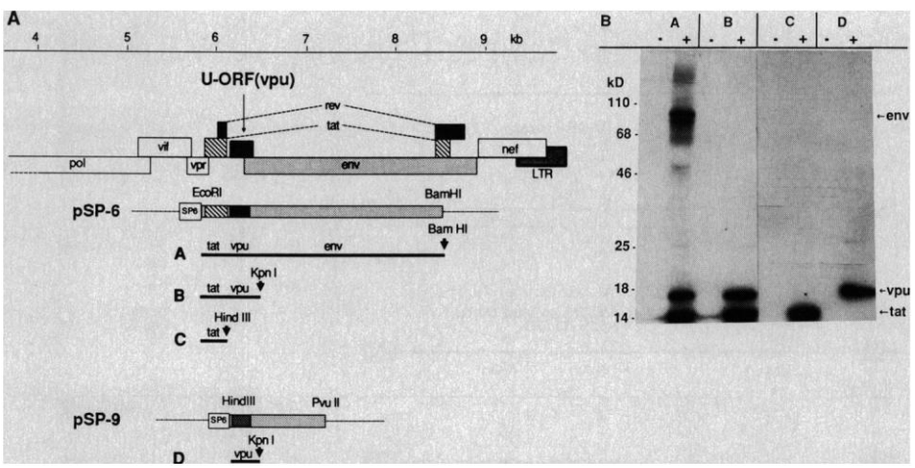

Fig. 1. Expression of *vpu* in vitro and immunoprecipitation of HIV-1–specific proteins with AIDS patient serum. (**A**) Two plasmids were constructed for the in vitro transcription of HIV-1–specific sequences: pSP-6 contains a 2.75-kb Eco RI–Bam HI fragment (nucleotides 5779 to 8522) from the infectious molecular clone pNL432 (*3*) cloned into pSP65 (*4*). Similarly, pSP-9 contains a 1.1-kb Hind III–Pvu II restriction fragment (nucleotides 6022 to 7130) cloned into the same vector. In both constructs initiation of translation of the *vpu* protein starts at internal sites: The start codons for *vpu* correspond to the third and second AUG codons on the pSP-6 and pSP-9 transcripts, respectively. pSP-6 DNA was linearized with Bam HI (A), Kpn I (B), or Hind III (C). pSP-9 DNA was linearized with Kpn I (D). Run-off transcripts (shown by solid bars) were synthesized in a reaction containing 40 m*M* tris-HCl, *p*H 7.5, 6 m*M* $MgCl_2$, 2 m*M* spermidine, 10 m*M* DTT, 1 U/μl of RNasin (Promega), 500 μ*M* rNTP's, linearized DNA (100 μg/ml), and SP6 RNA polymerase (1 U/μg DNA) for 1 hour at 40°C (*4*). Arrows show the sites used for linearization of the DNA templates. The individual genes are designated according to the new nomenclature for HIV-1 (*13*). (**B**) Aliquots of the transcripts "A" to "D" corresponding to 0.5 μg of DNA template were used for in vitro translation in a reaction containing 40 μl of rabbit reticulocyte lysate, 10 μl of [^{35}S]methionine (10 μCi), and 5 μl of RNA. The translation reaction was performed at 30°C for 1 hour. HIV-1–specific proteins were immunoprecipitated with an AIDS patient serum (+) or a negative control serum (−) and separated on a 10 to 20% SDS-polyacrylamide gel prior to autoradiography. The proteins immunoprecipitated with the AIDS patient serum are indicated on the right.

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env polypeptide, directed the translation of the 14- and 16-kD proteins. As expected, the 80-kD *env* protein, specified by pSP-6 RNA "A," was not synthesized in reticulocyte lysates incubated with pSP-6 RNA "B." When pSP-6 DNA was linearized with Hind III, which interrupted the *vpu* ORF, the resulting 0.3-kb RNA "C" directed the synthesis of only the 14-kD protein species (Fig. 1B).

Taken together, these results are compatible with the *tat* and *env* ORFs of the pSP-6 plasmid encoding 14- and 80-kD proteins, respectively, and *vpu* directing the synthesis of the 16-kD protein present in lanes A and B of Fig. 1B. This, in fact, was confirmed when pSP-9 was digested with Kpn I prior to in vitro transcription. The resulting RNA "D," capable of encoding the *vpu* polypeptide and 42 residues of the *env* gene product, was translated as a single 16-kD protein (Fig. 1B, lane D).

In vitro mutagenesis of *vpu* was carried out to delineate its function in virus-producing cells. A frame-shift mutation was constructed by introducing an 8-bp Xho I linker fragment (CCTCGAGG) at the Ssp I site (position 6189) within the 2.7-kb Eco RI–Bam HI segment of the infectious molecular clone (Fig. 2A). Insertion of the Xho I linker at this position would generate a translational frame shift and result in premature termination 35 codons from the NH_2-terminus of the *vpu* protein. The Eco RI–Bam HI fragment, which contained the mutated *vpu* gene (confirmed by nucleotide sequencing), was then inserted into a previously described (*5*) HIV-1 LTR-driven expression vector, pNL-A1, which lacks *gag* and *pol* sequences and directs the synthesis of HIV-1 *tat*, *rev*, *env*, and *vif* proteins (*6*). The resulting plasmid, pNL-A1/U_{35} (see Fig. 2A), as well as the parental pNL-A1 plasmid, were transfected into SW480 cells (*3*) and 20-hour lysates were examined by immunoblotting. As can be seen in the overexposed portion of the gel (Fig. 2B, lane 1), pNL-A1 elicited the synthesis of a 16-kD protein that was not detectable in cells transfected with pNL-Al/U_{35} containing the *vpu* mutation (lane 2). When the Eco RI–Bam HI fragment harboring the *vpu* frame-shift mutation was inserted into the pSP-6 plasmid and its expression monitored in vitro, no 16-kD *vpu* protein was produced (data not shown).

The *vpu* gene mutation was also introduced into the infectious HIV-1 proviral DNA clone pNL432 (*3*) to give rise to a plasmid designated pNL-U_{35}. SW480 cells were transfected with pNL432 and pNL-U_{35} DNAs and the resulting viral proteins were analyzed by immunoblotting. As can be seen in Fig. 2B (lanes 3 and 4), equivalent amounts of viral proteins were synthesized under conditions (transient expression in $CD4^-$ cells) where virus spread is precluded. When the tissue culture medium was examined for secreted progeny virions, similar amounts [as measured by reverse transcriptase (RT) activity] were found. The synthesis of substantial amounts of p17 *gag* obscures any 16-kD *vpu* protein migrating in the same region of the gel.

The role of the HIV-1 *vpu* gene product during virus replication was assessed by infecting A3.01 cells with equivalent amounts (RT activity) of progeny virions present in the cell-free filtrates of SW480 cells, previously transfected with pNL432 or pNL-U_{35} DNAs. Supernatant fluids from infected cultures were collected at 2-day intervals and analyzed for RT activity. As shown in Fig. 3A, the medium collected from cells infected with wild-type virus contained about six times more RT activity than the supernatant fluid harvested from cultures infected with the HIV-1 *vpu* mutant. The kinetics of infection were indistinguishable; in both instances the peak of RT activity occurred on day 8. In other experiments of this type, one-fourth to one-tenth the number of progeny virions were observed repeatedly in cultures infected with the *vpu* mutant. Furthermore, extensive and similar cytopathicity, including syncytia formation and cell death, was observed in cultures infected with wild-type and mutant virus.

In addition to the AIDS patient serum used in the in vitro translation and transient expression assays (see Figs. 1 and 2), sera from 14 randomly selected seropositive individuals were also tested for immunoreactivity with the HIV-1 *vpu* protein. This was done by immunoprecipitating the products (*tat*, *vpu*, and *env* proteins) of an in vitro translation of pSP-6 RNA "A" (see lane A, Fig. 1B). Four of the 14 sera, all of which reacted with the *env* protein, also immunoprecipitated the *vpu* and *tat* proteins. In summary, all of the 15 AIDS patient sera used in this study were positive for the HIV-1 *env* protein, five recognized *vpu* and ten showed reaction with *tat*.

An unusual feature of the *vpu* mutant is that the reduction in secreted progeny virions is not associated with either diminished cytopathicity or slower kinetics of replication (Fig. 3A). Furthermore, analyses of viral proteins synthesized in cultures of T lymphocytes prior to the peak of RT pro-

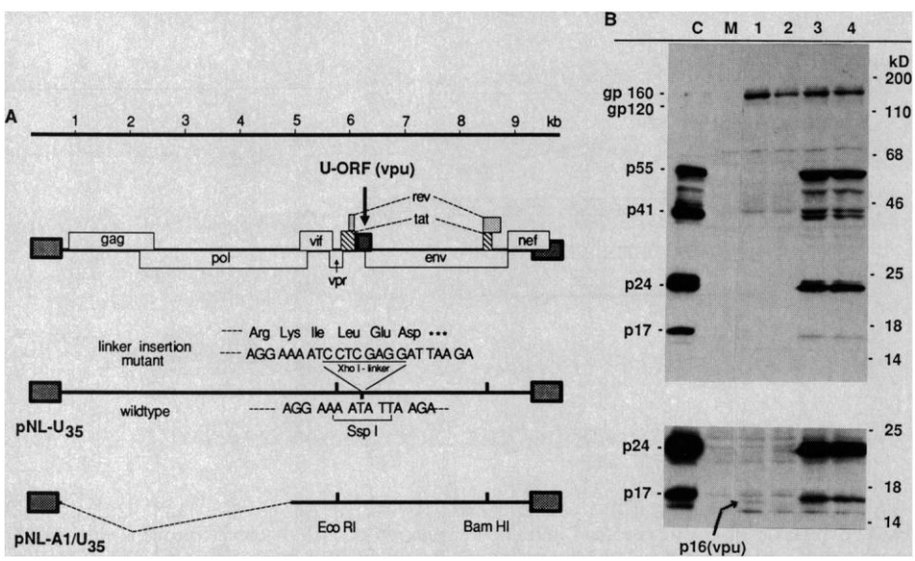

Fig. 2. Introduction of a frame-shift mutation into the *vpu* gene and analysis of the effect on expression of HIV-1 genes in SW480 cells. (**A**) A frame-shift mutant (pNL-U_{35}) was created by introducing an 8-bp Xho I linker fragment into the Ssp I site (nucleotide position 6189) within the *vpu* ORF of the infectious molecular clone pNL432. The nucleotide sequences of the wild-type and the mutant construct around the Ssp I site are shown. In addition, the predicted amino acid sequence of the COOH-terminus of the mutated *vpu* protein is shown above the nucleotide sequence starting at amino acid 30 (Arg) of the *vpu*-gene. pNL-A1/U_{35} has been constructed by introducing the Eco RI–Bam HI fragment from pNL-U_{35} into pNL-A1 (*5*). (**B**) Protein immunoblot analysis of lysates from SW480 cells transfected with pNL-A1 (lane 1), pNL-A1/U_{35} (lane 2), pNL432 (lane 3), pNL-U_{35} (lane 4), or mock transfected cells (M), respectively. (**C**) Control lysate from LAV-infected A3.01 cells. The plasmid DNAs (15 μg of each) were transfected into subconfluent SW480 cells using the standard calcium-phosphate precipitation technique (*14*). Cells were lysed in 50 m*M* tris-HCl, *p*H 8.0, 5 m*M* EDTA, 100 m*M* NaCl, 0.5% Chaps, and 0.2% deoxycholate 20 hours after the transfection and equal portions of the lysates were separated on a 10 to 20% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and reacted with AIDS patient serum followed by incubation with ^{125}I-labeled Protein A and subjected to autoradiography. In the top part of the figure exposure was for 13 hours; the bottom part is an overexposure (96 hours) of the same blot.

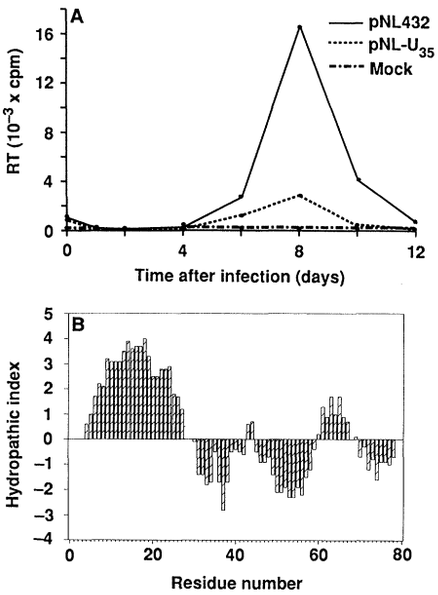

Fig. 3. (**A**) Infection of A3.01 cells with virus defective in *vpu*. SW480 cells, cultivated in DMEM + 10% fetal calf serum (FCS), were transfected with pNL-U_{35} DNA or with the wild-type plasmid DNA pNL432 using the standard calcium phosphate precipitation technique (*14*). After the transfection, SW480 cells were cultivated in RPMI 1640 containing 10% FCS. Supernatants were collected 20 hours after the transfection and filtered (0.22 μm). The RT activity of the supernatants was subsequently determined and equal RT-units, corresponding to 2.0 to 2.8 ml of the supernatant, were used to infect 2×10^6 A3.01 cells in a total volume of 5 ml of RPMI 1640. RT activity in the supernatants was determined at the indicated time points in a standard RT assay (*15*). The values given represent enzyme activity present in 1.5 μl of culture supernatant. (**B**) Hydrophobicity profile of the predicted amino acid sequence encoded by the HIV-1 *vpu* gene. The relative hydrophobicity of the amino acid residues is shown as a function of the amino acid position in the predicted *vpu* protein of our LAV isolate, HIVBru (*16*). Values were calculated by the method of Kyte and Doolittle (*17*).

duction indicate that the cells infected with the *vpu* mutant contain up to five times more *gag*, *pol*, and *env* proteins than those producing wild-type particles (*7*). This intracellular accumulation of viral proteins coupled with decreased virion synthesis suggests that *vpu* has a role in virus assembly or maturation.

As shown in Fig. 3B, the NH_2-terminus of *vpu* contains a 27–amino acid stretch of hydrophobic residues, which is characteristic of membrane-associated proteins. Adjacent to this hydrophobic domain is a run of 32 residues that includes 15 positively or negatively charged amino acids. This hydrophilic region of the HIV-1 *vpu* protein is highly conserved among several HIV isolates. The *vpu* protein thus possesses some of the structural features of the small, membrane-associated proteins present in ortho- and paramyxovirus infected cells. For example, the 15-kD membrane-bound M2 protein of influenza virus contains a charged cytoplasmic tail and is thought to be involved in virus assembly (*8*).

Although it is possible that the functional *vpu* protein is expressed from several exons in addition to the *vpu* ORF, this seems unlikely since its size (16 kD) in cells transfected with pNL-A1 was indistinguishable from that observed after in vitro translation. A consensus splice acceptor is located within the first translated exons of *tat* and *rev*, 85 nucleotides upstream from the initiation codon of the *vpu* gene (position 6012); this AUG codon is the first that precedes an ORF.

It is of interest that a number of the sequenced HIV-1 proviruses lack a functional *vpu* gene. The published sequence for HXB2, BH8, Ma1, and Z6 proviral DNAs do not contain the initiator AUG for the *vpu* ORF (*9*); the cloned ARV-2 proviral DNA contains a 1-bp deletion within *vpu* leading to a frame shift and termination after amino acid 38 (*10*). Since a functional *vpu* gene is not absolutely essential for the production of HIV-1, each of these isolates would give rise to progeny virions with altered replicative potential. In fact, variations in replicative rates, levels of progeny virion production, and cytopathicity have been described for HIV-1 isolates obtained at different points in disease (*12*) which may be relevant for understanding viral persistence.

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Platelet-Derived Growth Factor A Chain Is Maternally Encoded in *Xenopus* Embryos

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Transcription of zygotic genes does not occur in early *Xenopus* embryos until the mid-blastula transition, 6 to 7 hours after fertilization. Before this time, development is directed by maternal proteins and messenger RNAs stored within the egg. Two different forms of the A chain of platelet-derived growth factor (PDGF) are shown here to be encoded by maternal messenger RNAs. The two forms closely resemble human PDGF; however, the long form contains a hydrophobic region near the carboxyl terminus. The presence of PDGF messenger RNA in the embryo supports the idea that endogenous growth factors act at the earliest stages of embryogenesis.

CONDITIONED CULTURE MEDIUM from murine embryonal carcinoma cells contains a PDGF-like activity (*1*). These cells resemble derivatives of the inner cell mass of the mouse blastocyst, thus PDGF may be involved in early mammalian development. Because of the difficulties in manipulating the mammalian embryo, we wished to determine if PDGF participates in the early development of the experimentally more accessible *Xenopus* embryo.

In humans, two peptide chains encoded by separate genes form three biologically active isoforms of PDGF, the homodimers A:A and B:B and the heterodimer A:B (*2*). We used human A chain cDNA (*3*) and simian sarcoma virus v-*sis* (B chain) clones (*4*) as hybridization probes for the DNA blot analysis of *Xenopus laevis* genomic

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