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# Mutagenic Analysis of Human Immunodeficiency Virus Type 1 Vpr: Role of a Predicted N-Terminal Alpha-Helical Structure in Vpr Nuclear Localization and Virion Incorporation

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**The Vpr gene product of human immunodeficiency virus type 1 is a virion-associated protein that is important for efficient viral replication in nondividing cells such as macrophages. At the cellular level, Vpr is primarily localized in the nucleus when expressed in the absence of other viral proteins. Incorporation of Vpr into viral particles requires a determinant within the p6 domain of the Gag precursor polyprotein Pr55$^{gag}$. In the present study, we have used site-directed mutagenesis to identify a domain(s) of Vpr involved in virion incorporation and nuclear localization. Truncations of the carboxyl (C)-terminal domain, rich in basic residues, resulted in a less stable Vpr protein and in the impairment of both virion incorporation and nuclear localization. However, introduction of individual substitution mutations in this region did not impair Vpr nuclear localization and virion incorporation, suggesting that this region is necessary for the stability and/or optimal protein conformation relevant to these Vpr functions. In contrast, the substitution mutations within the amino (N)-terminal region of Vpr that is predicted to adopt an alpha-helical structure (extending from amino acids 16 to 34) impaired both virion incorporation and nuclear localization, suggesting that this structure may play a pivotal role in modulating both of these biological properties. These results are in agreement with a recent study showing that the introduction of proline residues in this predicted alpha-helical region abolished Vpr virion incorporation, presumably by disrupting this secondary structure (S. Mahalingam, S. A. Khan, R. Murali, M. A. Jabbar, C. E. Monken, R. G. Collman, and A. Srinivasan, Proc. Natl. Acad. Sci. USA 92:3794–3798, 1995). Interestingly, our results show that two Vpr mutants harboring single amino acid substitutions (L to F at position 23 [L23F] and A30F) on the hydrophobic face of the predicted helix coded for relatively stable proteins that retained their ability to translocate to the nucleus but exhibited dramatic reduction in Vpr incorporation, suggesting that this hydrophobic face might mediate protein-protein interactions required for Vpr virion incorporation but not nuclear localization. Furthermore, a single mutation (E25K) located on the hydrophilic face of this predicted alpha-helical structure affected not only virion incorporation but also nuclear localization of Vpr. The differential impairment of Vpr nuclear localization and virion incorporation by mutations in the predicted N-terminal alpha-helical region suggests that this region of Vpr plays a role in both of these biological functions of Vpr.**

The human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS, is a retrovirus that belongs to the lentiviral subfamily. Unlike simple retroviruses which generally code only for the structural and enzymatic proteins, lentiviruses code for a number of auxiliary proteins (Vif, Vpr, Tat, Rev, Vpu, and Nef, in the case of HIV-1) not found in most retroviruses. These proteins play pivotal regulatory functions which account for the tightly regulated replication of these viruses (reviewed in references 9 and 37). Both HIV-1 and HIV-2 and most simian immunodeficiency viruses code for the highly conserved open reading frame R, which lies in the central region of the genome (3, 7, 10, 13, 17, 18, 32). Vpr is a 14-kDa, 96-amino-acid protein expressed primarily from a singly spliced Rev-dependent mRNA (1, 7, 12). A recent study has characterized the Vpr protein as an oligomer (47). HIV-2 and most simian immunodeficiency viruses in addition also code for a second protein, Vpx, which shares notable sequence homology with Vpr. It has been suggested that Vpx may have arisen from Vpr by gene duplication in these viruses (40). Both of these homologous proteins are packaged efficiently in the viral particles at high copy numbers and at molar amounts comparable to those for Gag (6, 16, 31, 44). Virion localization studies place both Vpr and Vpx outside the core structure (41, 45). As Vpr and Vpx are not part of the Gag structural polyprotein, their incorporation requires an anchor to associate with the assembling capsid structures. Though the molecular mechanism is currently unknown, the C-terminal region of the Gag precursor corresponding to the p6 protein appears to constitute such an anchor, essential for the incorporation of both of these accessory proteins (4, 21, 33, 43). Recent studies suggest that the virion incorporation of Vpr involves a predicted alpha-helical structure located near the protein N terminus (29, 30).

Functionally, Vpr was first identified as a moderate and nonspecific transactivator, capable of augmenting reporter gene expression from both the HIV-1 long terminal repeat and other heterologous promoters (7). The ability to replicate in the nondividing host cell distinguishes HIV from the oncoretroviruses (27, 35). Also, Vpr was subsequently shown to induce differentiation and growth arrest in several tumor cell lines, even in the absence of any other viral proteins, indicating that the presence of Vpr by itself is sufficient to alter cellular events independent of HIV replication (25, 36). Functional studies

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have shown that Vpr accelerates HIV-1 replication in some T-lymphoid cell lines, and this augmentation of virus production is more pronounced in primary macrophages in both HIV-1 and HIV-2 systems (2, 7, 8, 15, 32, 42). The virion association of Vpr suggests that the protein may play a role early in replication. A recent study has determined that in addition to $p17^{gag}$, Vpr may also mediate the nuclear transport of HIV-1 proviral DNA in nondividing target cells (16). Interestingly, results from another study suggest that Vpr function may also require the de novo synthesis of this protein, as the virion-associated protein alone was insufficient to augment replication in cell types such as macrophages (8). Recent studies have also characterized Vpr as having transcellular activity (26). Both Vpr purified from the plasma acquired from HIV-seropositive individuals and recombinant-purified Vpr were capable of inducing latent cells into high-level viral producers when added to the culture medium at low concentrations (26). This report also suggests that Vpr can continue to act following integration events, as latent cells presumably have already progressed through these early steps. Mechanistically, it is conceivable that this transcellular activity is mediated by the same mechanisms that are operative in modifying cellular growth and differentiation. At the cellular level, Vpr has been localized mainly in the nucleus when expressed in the absence of other HIV-1 proteins (28). Even though no classical nuclear localization signal in this protein has been clearly identified, it has been suggested that Vpr may specifically interact with other nuclear proteins and may thus gain access to the nucleus (46). In this regard, two groups have identified proteins of various molecular weights that interact with Vpr (34, 46). Interestingly, one of these Vpr targets, designated Vpr-interacting protein, or RIP-1, appears to translocate to the nucleus following its interaction with Vpr and glucocorticoid receptor (34).

In this study, we have used mutational analysis to explore the region(s) of the Vpr protein involved in its nuclear localization and virion incorporation properties. Our studies indicate that the C-terminal truncation of Vpr, but not substitution mutations in this region, result in the impairment of Vpr nuclear localization and virion incorporation. These impairments are likely due to reduced protein stability and/or altered structural conformation associated with these truncated Vpr proteins. Also, we demonstrate that a region extending from amino acids 16 to 34 near the N terminus of Vpr with a predicted alpha-helical structure is involved in both Vpr nuclear localization and virion incorporation. Moreover, we identify residues located on the hydrophobic face of this predicted N-terminal alpha-helical structure capable of affecting Vpr virion incorporation without affecting the protein's ability to localize to the nucleus.

## MATERIALS AND METHODS

**HIV molecular clones.** HIV-1 provirus plasmid HxBRU used in this study is a hybrid between the two closely related proviruses, HxBc2 and BRU/LAI (24). The genotype of this molecular clone is 5′ long terminal repeat *gag*$^+$ *pol*$^+$ *vif*$^+$ *vpr*$^+$ *tat*$^+$ *rev*$^+$ *vpu env*$^+$ *nef* 3′ long terminal repeat. HxBRUR$^-$ was constructed by replacing the sequences from a *Stu*I site to a *Sal*I site (nucleotides [nt] 4989 to 5367; +1 = start of BRU initiation of transcription) of HxBRU with the corresponding sequences of ptrENVR$^-$, in which the initiation codon (ATG) of Vpr has been changed to GTG (24). HxBRUR$^-$ and HxBRU are isogenic except for the expression of Vpr. The HxBH10 provirus clone encodes a functional Vpu and a truncated Vpr (R72f) (39). This truncated R72f is due to a frameshift caused by insertion of a single T at nt 5315 (+1 = start of HxBc2 initiation of transcription) (23). HxBRUR77f was generated by digestion of HxBRU with *Sal*I located at nt 5367 and religation following blunt ending with the Klenow fragment of DNA polymerase I. These treatments created a frameshift within the Vpr open reading frame at the amino acid position 78, as described before (28).

HIV HxBRU proviral constructs harboring different Vpr substitution mutations were generated by using a two-step PCR-based method essentially as described previously (19). The 5′ primer (5′-AACACCATATGTATGTTT-3′) is located at a *Nde*I site in *vif* (nt 4705; +1 = start of BRU initiation of transcription), and the 3′ primer (5′-TGTATCATATGCTTTAGC-3′) is located at the *Nde*I site in *env* (nt 5946; +1 = start of HxBc2 initiation of transcription). Complementary oligonucleotide primers containing the desired mutations in Vpr were used to generate *Nde*I-*Nde*I PCR fragments containing the different Vpr mutations. These PCR fragments were digested with *Stu*I and *Sal*I or with *Sal*I and *Kpn*I restriction enzymes, depending on the Vpr region involved in mutagenesis. The resulting DNA fragments were subcloned into one of the intermediate vectors; one vector contained the *Apa*I-*Sal*I fragment derived from HxBRU, while the other contained the *Sal*I-*Bam*HI fragment from HxBc2. Either the *Apa*I-*Sal*I fragment or the *Sal*I-*Bam*HI fragment containing the various Vpr mutations was cloned back into the proviral construct HxBRU. The resulting HxBRU proviruses harboring the different Vpr mutations used in this study are shown in Fig. 1. Three additional Vpr mutants (EA29,30FK, IL63,64KR and LI68,70RK) were only cloned into Vpr expressor, as described later and shown in Fig. 6A. The nucleotide sequences of the sense mutagenic oligonucleotides are as follows: RE12,13PG, sense, 5′-AGGGCCACAGCCCGGGCCACACAAT-3′; L23F, sense, 5′-ACTAGAGCTCTTCGAGGAGCTT-3′; E25K, sense, 5′-GCTTTTAGAGAAGCTTAAGAA-3′; A30F, sense, 5′-TAAGAATGAATTCGTTAGACATT-3′; H33I, sense, 5′-AGCTGTTAGAATATTTCCTAGGA-3′; EA29,30FK, sense, 5′-GCTTAAGAATTTTAAAGTTAGACATT-3′; I63F, sense, 5′-ATAATAAGATTTCTGCAAC-3′; IL 63,64KR, sense, 5′-ATAATAAGAAAACGGCAACAACTG-3′; LI68,70RK, sense, 5′-CAACAACTGCGGTTTAAACATTTCAGA-3′; R73S, sense, 5′-TATCCATTTCAGCATTGGGTGTC-3′; G75N, sense, 5′-TTTCAGAATTAATTGTCGACATA-3′; SR79,80ID, sense, 5′-TGTCGACATATCGATATAGGCGTTA-3′.

**Eukaryotic Vpr expression vectors.** To construct Vpr expression vectors for the various mutations, we first amplified the Vpr sequences from the respective HxBRU proviruses by PCR, using a 5′ primer (5′-ACTTCTAGAGGATAGATGGAACAAGCC-3′) with an *Xba*I site engineered preceding the Vpr ATG and a 3′ primer (5′-CAGGAGCTCAGTCTAGGATCTACTGGC-3′) which harbors an engineered *Sac*I site following the stop codon of Vpr. These PCR fragments were digested with *Xba*I and *Sac*I restriction enzymes and then used to replace the corresponding Vpr fragment in a Vpr expression vector SVCMVER, which contains the cytomegalovirus immediate-early gene promoter (24). Four Vpr substitution mutants WEL, EAHF, HFRI, and RR90,95NN (as shown in Fig. 6A) based on the Vpr sequence derived from the HIV-1 ELI strain were also generated in the same expression vector by using an oligonucleotide-directed mutagenesis method, as described before (48). The sense mutagenic oligonucleotides used are as follows: WEL, 5′-ATACAATGAAGCAGCATTACTTCTGCAGGAGGAGCTTA-3′; EAHF, 5′-GCTTAAGAGTTTCAGAGTTAGACTGCAGCCTAGGATAT-3′; HFRI, 5′-ACTGTTTATTGGATCCGAACAGGGGTGTCAAC-3′; RR90,95NN, 5′-AGGAGAGCAAATAATGGAAGTAGTAATTCCTAGACT-3′. All expression vectors so generated were subsequently analyzed by DNA sequencing to confirm the mutations.

**Cell lines and antisera.** The transformed lymphoid cell line (MT4 cells) used in this study were maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS). This cell line has been shown to be highly sensitive to HIV-1 virus infection (14). COS-7, an African green monkey kidney cell line transformed by an origin-defective mutant of simian virus 40, was propagated in Dulbecco modified Eagle medium supplemented with 10% FCS. An HIV-1-positive human serum 162 and a rabbit anti-Vpr serum raised against an *Escherichia coli*-derived ELI Vpr protein were used in this study and have been previously described (24). Fluorescein-labeled goat anti-rabbit $F(ab')_2$ was purchased from Gibco/BRL Co.

**Transfection of cells.** MT4 cells ($5 \times 10^6$) were transfected with 10 μg of either Vpr$^+$ or mutated Vpr provirus plasmids by using the DEAE-dextran method as described before (39). The transfected cells were maintained in RPMI 1640 containing 10% FCS for 48 to 84 h. To test for Vpr expression, COS-7 cells ($2.5 \times 10^4$) cultured in shell vials were transfected with 1 μg of either wild-type or mutated VPR expressors, using the calcium-phosphate method (24). The transfected cultures were maintained in Dulbecco modified Eagle medium containing 10% FCS for 48 h.

**Immunofluorescence microscopy.** At 48 h posttransfection, COS-7 cells were fixed in acetone for 30 min at 4°C. After fixation, the cells were first incubated with the rabbit anti-Vpr serum in phosphate-buffered saline (PBS) containing 2% Carnation instant skim milk powder (Nestlé) for 12 h at 37°C and then incubated with fluorescein-labeled goat anti-rabbit antibody for 2 h. After being washed with PBS, the cells were observed at a magnification of ×100 (oil emulsion) with a Zeiss fluorescence microscope.

**Cell labeling and radioimmunoprecipitation.** At 48 to 84 h posttransfection, MT4 cells were metabolically labeled with 100 μCi of [$^{35}$S]methionine per ml and 100 μCi of [$^3$H]leucine per ml for 12 h. After labeling, virions were pelleted from supernatants by ultracentrifugation at 30,000 rpm through a 20% sucrose cushion in a Beckman SW41 rotor and lysed in radioimmunoprecipitation assay buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.25% sodium deoxycholate, and 0.2% phenylmethylsulfonyl fluoride and immunoprecipitated with a mixture of

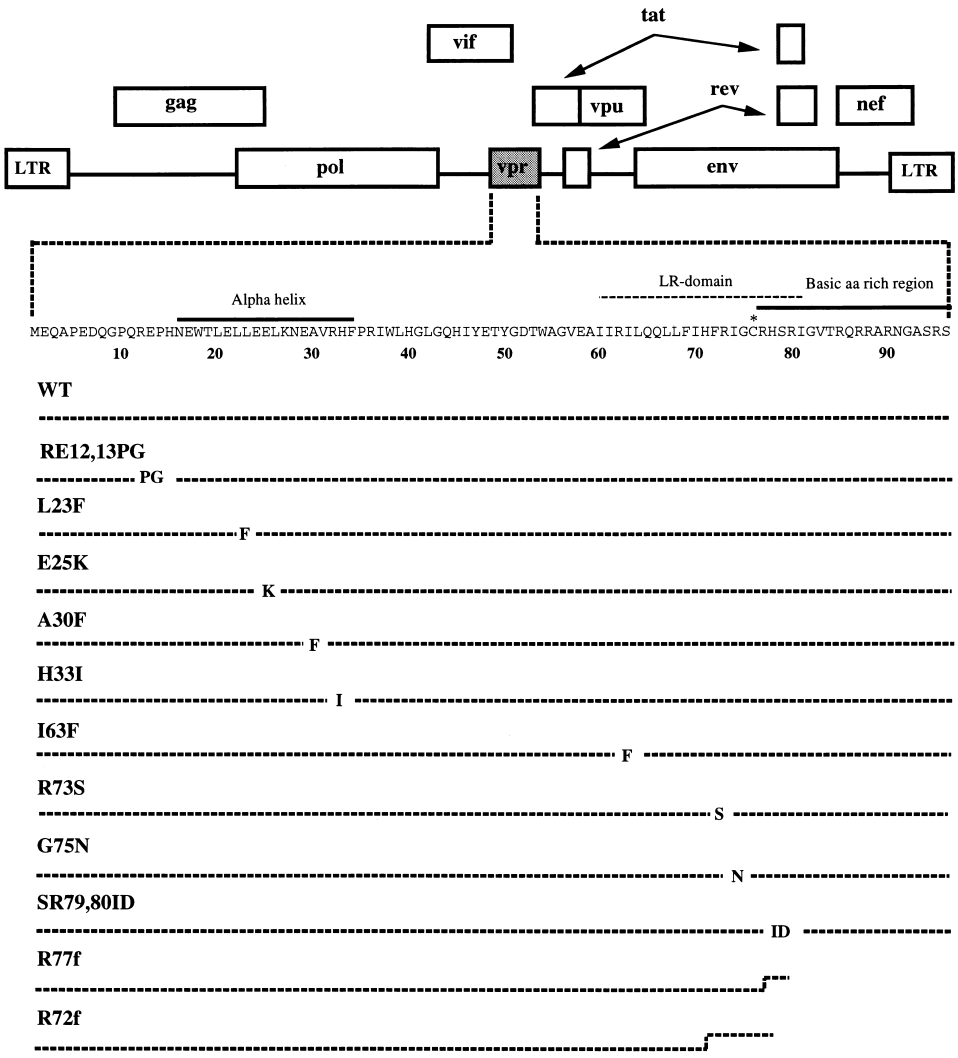



FIG. 1. Genetic organization of the HIV-1 genome and Vpr substitution and deletion mutations in proviral plasmid constructs. The schematic organization of the HIV-1 genome and the amino acid sequence of Vpr are shown at the top. The position of the predicted amphipathic alpha-helical structure extending from amino acids (aa) 16 to 34 near the N terminus of Vpr, the highly conserved cysteine residue at position 76, the previously described LR domain (46), and the basic amino-acid-rich region are indicated. The positions of the mutated amino acids of Vpr mutants are shown at the bottom. The positions of the C-terminal truncations (HxBRUR77f and HxBH10R72f) are also indicated. LTR, long terminal repeat.

HIV-1-positive human serum 162 and rabbit anti-Vpr serum, as previously described (24). Immunoprecipitates were run on an SDS–14% polyacrylamide gel and subsequently analyzed by autoradiography. In the experiment whose results are shown in Fig. 4C, the virions in the supernatants were pelleted by ultracentrifugation through a 20% sucrose cushion, lysed, directly run on SDS–14% polyacrylamide gels, and analyzed by autoradiography without additional immunoprecipitation steps.

For pulse-chase metabolic labeling, MT4 or COS-7 cells were starved 48 h posttransfection in methionine- and leucine-free RPMI 1640 medium for 15 min. The COS-7 cells were scraped off the petri dish during the starvation. Subsequently, the cells were metabolically labeled with 250 μCi of [$^{35}$S]methionine and 100 μCi of [$^{3}$H]leucine in 0.5 ml of medium for 30 min. The labeling medium was then removed, and the cells were washed once with complete RPMI 1640 containing 10% FCS. Equal aliquots of labeled cells were resuspended in 0.5 ml of complete RPMI 1640 containing 10% FCS and incubated at 37°C. At each indicated time point, the cells were lysed with radioimmunoprecipitation assay buffer and immunoprecipitated with a mixture of HIV-1-positive human serum 162 and rabbit anti-Vpr serum or with the rabbit anti-Vpr serum alone. The immunoprecipitates were analyzed as described above. Densitometric analysis of autoradiograms was performed with a Molecular Dynamics Personal densitometer, using ImageQuant software, version 3.22.

**Computer-assisted protein secondary-structure analysis.** The secondary structure of the Vpr protein was analyzed by using the MacVector sequence analysis software from International Biotechnologies Inc., New Haven, Conn. The prediction of the secondary structure of proteins by this software is based on the Robson-Garnier and Chou-Fasman methods (5, 11).

## RESULTS

**Construction of HIV-1 proviruses harboring different Vpr mutations.** In order to identify the domain(s) of Vpr involved in its capacity to be incorporated into virions, we constructed a series of infectious HIV proviruses that coded for the mutated versions of Vpr (Fig. 1). In designing these mutants, we targeted three putative structural regions in the Vpr sequence: (i) the acidic N-terminal domain containing a putative amphipathic alpha helix extending from amino acids 16 to 34 as predicted by computer-assisted analysis, (ii) the previously identified leucine-isoleucine-rich region (LR domain) (46), and (iii) the arginine-rich C-terminal domain (Fig. 1).

Substitution mutations were designed to affect specific amino acid residues found to be highly conserved among Vpr sequences from different HIV-1 isolates (40). Generally, sub-

stitutions were designed to introduce either a charge or a steric change in the amino acid sequence as described in Fig. 1. One mutant carrying substitutions of amino acids Arg-12 and Glu-13 to Pro and Gly (Fig. 1, RE12,13PG) was generated to modify the proximal N terminus of Vpr. Also, four substitution mutations were introduced in the predicted alpha-helical structure located near the N terminus of Vpr. This predicted alpha helix contains a notable acidic hydrophilic face and an aliphatic hydrophobic face, as shown in Fig. 7B. To explore the importance of each face of this predicted helical structure, two amino acids, Leu-23 and Ala-30, located at the hydrophobic face were replaced by an aromatic amino acid Phe to generate mutants L23F (an L-to-F substitution at position 23) and A30F, respectively. In two other mutants, nonconservative substitutions were made on the same helical structure, where the charged residues Glu-25 and His-33 were replaced by Lys (mutant E25K) and Ile (mutant H33I), respectively (Fig. 1). As shown in Fig. 7B, one of these residues, Glu-25, is located on the hydrophilic face of the predicted alpha-helical structure. None of these four mutants (L23F, E25K, A30F, and H33I) disrupted this putative helical structure, as predicted by computer-assisted analysis (data not shown). In addition, two Vpr frameshift mutants, R77f and R72f, which prematurely terminate the wild-type Vpr and effectively remove the last 19 and 24 amino acids, respectively, were also used in this study. Four mutants carrying substitutions of Ile-63 for Phe (I63F), Arg-73 for Ser (R73S), Gly-75 for Asn (G75N), and Ser-79 and Arg-80 for Ile and Asp (Fig. 1, SR79,80ID) were constructed to introduce changes in the LR domain and/or arginine-rich region of Vpr. Moreover, none of the Vpr mutations introduced modified other open reading frames or known splicing sites in the HIV sequence, with the exception of the mutant RE12,13PG, which changed the residues Arg-184, Gly-185, and Ser-186 in the Vif sequence to Ser, Pro, and Gly, respectively. However, this particular mutant was not restricted for replication in T-lymphoid MT4 cells.

In addition to being tested for their virion incorporation capacity, all mutated Vpr proteins were evaluated for their stability during viral replication in MT4 cells as described in the following sections. In the case of mutants that showed a reduced steady-state protein level, we performed pulse-chase experiments to further explore their stability status. Most of the Vpr mutants were also subcloned into an expression vector, as described in Materials and Methods, in order to evaluate, in parallel, Vpr stability in the absence of other viral proteins following transfection of Vpr expressors in COS-7 cells.

**Effect of C-terminal truncation on Vpr stability and virion incorporation capacity.** To determine the effect of the C-terminal region on Vpr stability, we transfected HIV provirus constructions expressing either the wild-type (HxBRU) or the C-terminal-truncated Vpr proteins (HxBRUR77f and HxBH10R 72f) in MT4 cells. At 48 h posttransfection, expression of viral proteins was analyzed by metabolic labeling and immunoprecipitation. Briefly, equal numbers of transfected MT4 cells were pulse-labeled with [$^{35}$S]methionine and [$^{3}$H]leucine for 30 min and subsequently chased at different intervals of time. The viral proteins in the cell lysates were immunoprecipitated with HIV-1-positive human serum 162 mixed with rabbit anti-Vpr serum. Immunoprecipitated proteins were analyzed by autoradiography after SDS–14% polyacrylamide gel electrophoresis (SDS–14% PAGE). The viral structural proteins were abundantly detected in cell lysates from viruses expressing both the wild-type and the truncated Vpr proteins (Fig. 2A). A protein of 14 kDa corresponding to the Vpr product was specifically immunoprecipitated from cells transfected with the wild-type Vpr expressing HxBRU (Fig. 2A, lanes 1 to 4). Different faster-migrating proteins corresponding to the two truncated Vpr proteins were evident in lysates from cells transfected with HxBRUR77f or HxBH10R72f. The molecular mass of these two truncated Vpr proteins ranged from approximately 9 to 10 kDa (Fig. 2A, lanes 5 to 8 and lanes 9 to 12, respectively). Moreover, as shown by pulse-chase labeling, these truncated Vpr species (R77f and R72f) were less stable than the wild-type Vpr. While the half-life of wild-type Vpr was more than 7 h, the half-lives of the truncated Vpr proteins (R77f and R72f) were approximately 3 and 1 h, respectively (Fig. 2B). These data clearly indicate that the removal of the last 19 or 24 amino acids from the Vpr C terminus severely impairs Vpr stability in T-lymphoid cells (MT4).

In order to evaluate the ability of these truncated Vpr proteins (R77f and R72f) to incorporate into progeny virions, MT4 cells were transfected with the different HIV proviruses (HxBRU, HxBRUR77f, and HxBH10R72f). At 48 h posttransfection, the cells were labeled with [$^{35}$S]methionine and [$^{3}$H]leucine for 12 h. The cells were then lysed, immunoprecipitated, and analyzed by SDS–14% PAGE. The results indicate that the levels of truncated Vpr mutants R77f and R72f in cell lysates were notably reduced, compared with the wild-type Vpr level (data not shown). Also, the virions in the culture supernatants were pelleted and immunoprecipitated in parallel. As truncated forms of Vpr comigrated with an unknown nonspecific protein, we performed parallel immunoprecipitations with either a mix of HIV-1-positive human serum 162 and anti-Vpr-specific antibodies or a mix of HIV-1-positive human serum 162 and normal rabbit serum. As shown in Fig. 3, Vpr proteins can be specifically immunoprecipitated from virions collected from MT4 cultures transfected with proviruses containing wild-type or truncated Vpr proteins (Fig. 3, compare lanes 1, 3, and 5 with lanes 2, 4, and 6). These results indicate that the truncated R77f and R77f are incorporated into virions even though the detected levels of these two truncated Vpr proteins in virions are much lower than that of the wild type. Such low levels of truncated Vpr proteins in the virions can be partially attributed to the instability of these mutants. These data suggest that as the truncated Vpr proteins were capable of being packaged in the virions, albeit at lower levels, the C terminus of Vpr may not be absolutely necessary for virion incorporation but may modulate this Vpr property by affecting the stability and, conceivably, the optimal protein conformation necessary for virion targeting.

**Substitution mutational analysis indicates that a predicted N-terminal alpha-helical structure is necessary for Vpr virion incorporation.** We first determined the effect of different substitution mutations on Vpr stability. MT4 cells were transfected with the proviral constructs harboring the wild-type or substitution mutations in the Vpr sequence. At 48 h after transfection, the cells were labeled with [$^{35}$S]methionine and [$^{3}$H]leucine for 12 h during the peak of replication, as determined by the appearance of HIV-induced syncytia in the cell cultures. Radiolabeling was done at 48 h posttransfection with proviruses harboring wild-type and mutated Vpr, with the exception of HxBRUL23F and HxBRUA30F (72 to 84 h), which showed a delay of viral replication. After labeling, the cells were lysed, immunoprecipitated with HIV-1-positive human serum 162 mixed with rabbit anti-Vpr serum, and analyzed by SDS–14% PAGE followed by autoradiography (Fig. 4A, B, and C, left panels). From each HIV provirus transfection, viral structural proteins were abundantly detected in cell lysates. However, the 14-kDa Vpr protein was specifically immunoprecipitated only from cultures transfected with proviruses expressing the wild-type or mutated Vpr but not in cells transfected with Vpr$^{-}$ provirus as indicated. The mobility of

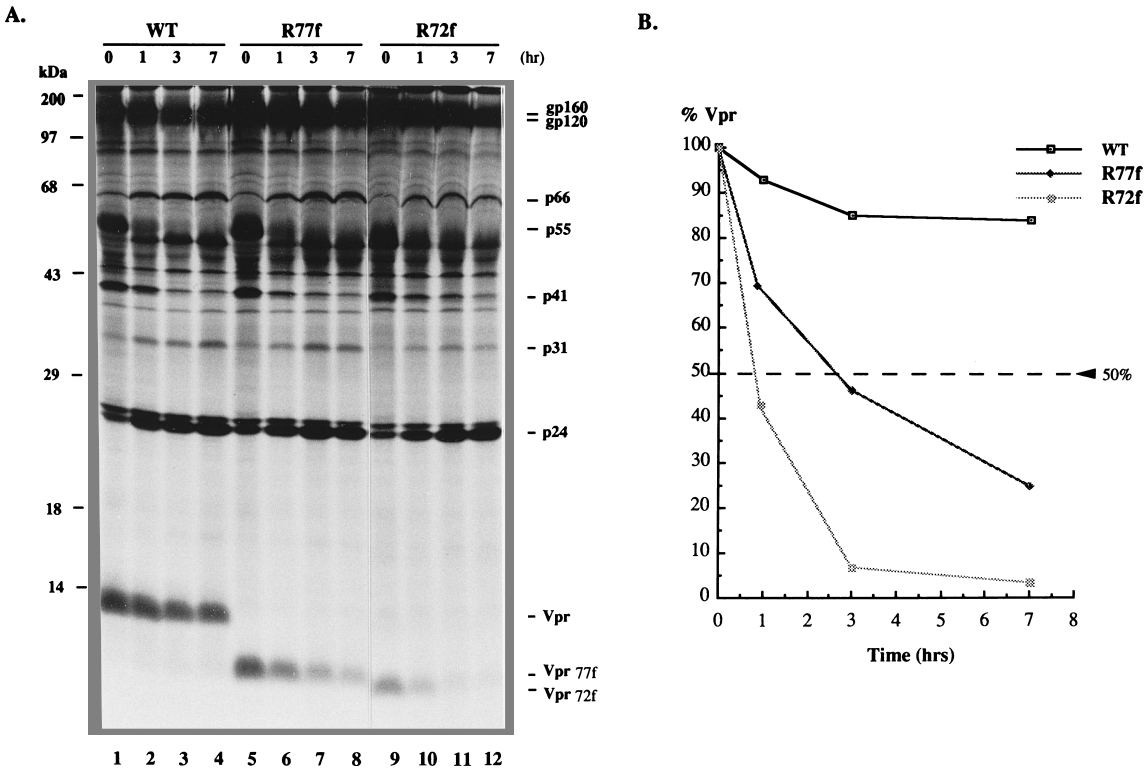



FIG. 2. Effect of C-terminal deletions on Vpr stability. (A) MT4 cells were transfected with 10 μg of HxBRUwt, HxBRUR77f, or HxBH10R72f provirus plasmid. At 48 h posttransfection, the cells were pulse-labeled with 250 μCi of [$^{35}$S]methionine and 100 μCi of [$^{3}$H]leucine for 30 min and chased at the indicated times in media containing excess unlabeled methionine and leucine. Subsequently, the cells were lysed with radioimmunoprecipitation assay buffer, and the cell lysates were immunoprecipitated with HIV-1-positive human serum 162 mixed with rabbit anti-Vpr serum. The positions of viral proteins and Vpr (wild-type [WT] and truncated forms) are indicated. The molecular mass markers are shown on the left. (B) The quantitative analysis of the wild-type (WT) and truncated Vpr bands was performed with a Molecular Dynamics Personal densitometer, using ImageQuant software, version 3.22. The percentage of Vpr proteins recovered relative to the amount present at the end of the pulse (0 min) is plotted as a function of time.

mutated Vpr (SR79,80ID) was shown to be a little faster than that of wild-type Vpr (Fig. 4B, compare lanes 4 and 2). The levels of expression and stability of all mutated Vpr proteins, with the exception of Vpr H33I, were similar to the wild-type Vpr level.

To further confirm Vpr stability results, we also performed pulse-chase immunoprecipitation analysis of some mutated Vpr proteins expressed either in a proviral context or from Vpr expression vectors in the absence of other viral proteins. Vpr expression vectors containing the substitution mutation L23F, E25K, A30F, or H33I were transfected in COS-7 cells. At 48 h posttransfection, the cells were pulse-labeled with [$^{35}$S]methionine and [$^{3}$H]leucine for 30 min and were subsequently chased at different intervals of time. The Vpr proteins in the cell lysates were immunoprecipitated with rabbit anti-Vpr serum and analyzed by autoradiography after SDS–14% PAGE. The levels of Vpr protein at different time intervals were evaluated by densitometric analysis and are presented in Fig. 5A. The half-life of the wild-type Vpr and that of the mutated Vpr (L23F, E25K, and A30F) in COS-7 cells were shown to be more than 7 h, and as expected from the preceding results, the mutated Vpr H33I was shown to be less stable, with a half-life of approximately 5.5 h. When MT4 cells were transfected with HIV proviruses expressing the same Vpr mutants, followed by pulse-chase labeling and immunoprecipitation, similar results were also obtained, indicating that the stability of these mutated Vpr proteins (L23F, E25K, and A30F) is not affected by the presence or the absence of other viral proteins (data not shown).

To determine the level of virion incorporation of the mutated Vpr proteins, virions were pelleted from the supernatants of the labeled MT4 cultures transfected with each proviral plasmid. The pelleted virions were then run on an SDS–14% polyacrylamide gel either directly after lysis (Fig. 4C, right panel) or following immunoprecipitation with the mix of the anti-Vpr and anti-HIV serum 162 (Fig. 4A and B, right panels). The results presented in Fig. 4 show that the wild-type Vpr protein was specifically immunoprecipitated from the virion lysates, indicating that wild-type Vpr can be efficiently incorporated into progeny viruses (Fig. 4A, B, and C, right panels, lanes 2). Mutated Vpr proteins were detected at levels comparable to that of the wild-type Vpr in the progeny virions obtained from proviruses harboring mutations in the proximal N-terminal region (RE12,13PG) or in the C-terminal half of the protein (I63F, R73S, G75N, or SR79,80ID) (Fig. 4A, right panel, lanes 5 and 6; B, right panel, lanes 3 and 4; C, right panel, lane 5). In contrast, low levels of Vpr products were detected from the virions obtained from proviruses harboring mutations in the region extending from amino acids 16 to 34, which is predicted to form an alpha-helical structure (L23F, E25K, A30F, or H33I) (Fig. 4A, right panel, lanes 3 and 4; Fig. 4C, right panel, lanes 3 and 4).

To determine the incorporation efficiency of the mutated Vpr proteins in the virions, the level of Vpr found in the virions

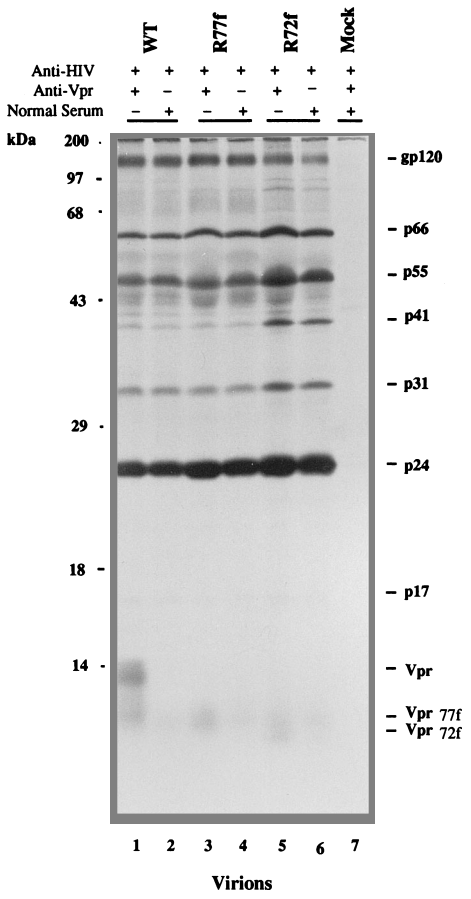



FIG. 3. Effect of C-terminal truncation on Vpr virion incorporation. MT4 cells were transfected with 10 μg of HxBRUwt, HxBRUR77f, or HxBH10R72f provirus plasmid. At 48 h posttransfection, the cells were labeled with [$^{35}$S]methionine and [$^{3}$H]leucine for 12 h. Labeled virions were collected by ultracentrifugation through a 20% sucrose cushion and immunoprecipitated with HIV-1-positive human serum 162 mixed with either rabbit anti-Vpr serum or normal rabbit serum. The positions of viral proteins and Vpr (wild-type [WT] and truncated forms) are indicated.

was evaluated by densitometric analysis of the autoradiograms presented in Fig. 4. The virion-associated Vpr level was evaluated as a ratio of virion-associated Vpr to that of the reverse transcriptase (RT) p66, which served as an internal control in each sample. The results indicate that the mutations at positions R-12 and E-13, I-63, R-73, G-75, and S-79 and R-80 allowed the efficient incorporation of mutated Vpr into virions, compared with the wild type (Fig. 5B). However, the mutated Vpr at positions L-23, E-25, A-30, and H-33 resulted in a remarkable decrease in incorporation level (Fig. 5B). In the case of H33I, the low level of Vpr incorporation could partially result from its instability (Fig. 4A, left panel, lane 4).

These results indicate that substitution mutations at residues R-12 and E-13 in the N terminus or residues I-63, R-73, G-75, and S-79 and R-80 in the C-terminal portion of Vpr affect neither its stability nor its virion incorporation. In contrast, the presence of mutations at position L-23, E-25, or A-30, all located in the putative N-terminal alpha helix, leads to the expression of stable Vpr proteins in the cells with low capacity to be incorporated in the progeny virions. From this mutational analysis, it is clear that residues L-23, E-25, and A-30 are involved in Vpr virion incorporation.

**Effect of mutations on Vpr nuclear localization.** Previous studies have shown that Vpr localizes in the nucleus when expressed in the absence of other viral proteins (28, 46). To identify the region(s) of Vpr involved in its nuclear localization, we generated a series of Vpr expressors (SVCMV-Vpr) which contain a number of substitution mutations in the different regions of Vpr as shown schematically in Fig. 6A. In addition to the BRU-derived mutants, we also analyzed substitution mutations spanning a Vpr sequence derived from another HIV isolate, ELI. The BRU mutants tested for nuclear localization by immunofluorescence analysis included RE12,13PG, located in the N terminus of Vpr, four mutants spanning the predicted alpha-helical region (L23F, E25K, A30F, and EA29,30FK), three mutants in the LR domain (I63F, IL63,64KR, and LI68,70RK), and two mutants in the C-terminal basic amino-acid-rich region (SR79,80ID and R77f) (Fig. 6A). The ELI mutants tested included the WEL and EAHF mutants, which were located in the proximal and distal regions of the N-terminal helix, respectively, while the HFRI and RR90,95NN mutants were located in the distal half of the protein (Fig. 6A).

To determine the nuclear localization of these Vpr mutants in the absence of other viral proteins, we transfected these Vpr expressors in COS-7 cells. At 48 h posttransfection, the cells were fixed, labeled with anti-Vpr serum, and analyzed by indirect immunofluorescence analysis. To evaluate the ability of various Vpr mutants to be targeted to the nucleus, the number of cells exhibiting a Vpr-specific signal from each experiment was determined in random fields. We also compared the pattern of immunofluorescence of the Vpr-positive cells to the wild-type pattern. The data presented in Fig. 6A represent the mean percentage of Vpr-positive cells with specific nuclear staining over total numbers of Vpr-positive cells from three independent experiments. As exemplified in Fig. 6B, specific nuclear staining for Vpr was clearly observed in COS-7 cells transfected with wild-type Vpr$^{+}$ expressor (Fig. 6B, panel b) but not in cells transfected with the Vpr$^{-}$ expressor (Fig. 6B, panel a). In a majority of the cells (92%) that expressed the wild-type protein, specific Vpr staining was evident predominantly in the nucleus, with occasional perinuclear staining. In contrast, as shown in Fig. 6A and exemplified in Fig. 6B, panel o, the C-terminal Vpr truncation mutant (R77f) severely impaired nuclear localization, with only 38% of Vpr-positive cells showing Vpr staining in the nucleus. Even in cells that exhibited such nuclear staining, a notable portion of the protein was still retained in the cytoplasmic compartment. This result correlates with the data obtained in previous fractionation experiments that demonstrated a severe impairment of Vpr nuclear association when the last 19 amino acids were deleted (28). Interestingly, however, neither of the substitution mutants (the ELI mutant RR90,95NN and the BRU mutant SR79,80ID) in the same region were impaired in their ability to localize to the nucleus (Fig. 6A and B, panel n). Also, several substitution mutants located in the other Vpr regions, including the BRU (RE12,13PG, L23F, EA29, 30FK, A30F, I63F, IL63,64KR, and LI68,70RK) and the ELI (HFRI) mutants, were evaluated for their capacity to translocate to the nucleus. With these mutants, approximately 70 to 93% of the cells exhibiting Vpr-specific staining had some proportion of their signal in the nucleus, suggesting that the determinant to reach the nucleus was present in these mutants. However, analysis of the immunofluorescence pattern indicated that the distribution of the protein among the cytoplasmic and nuclear compartments varied. Some mutants (RE12,13PG, A30F, EA29,30FK, and LI68,70RK), like the wild type, had predominantly nuclear staining, while other mutants had notable amounts of the pro-

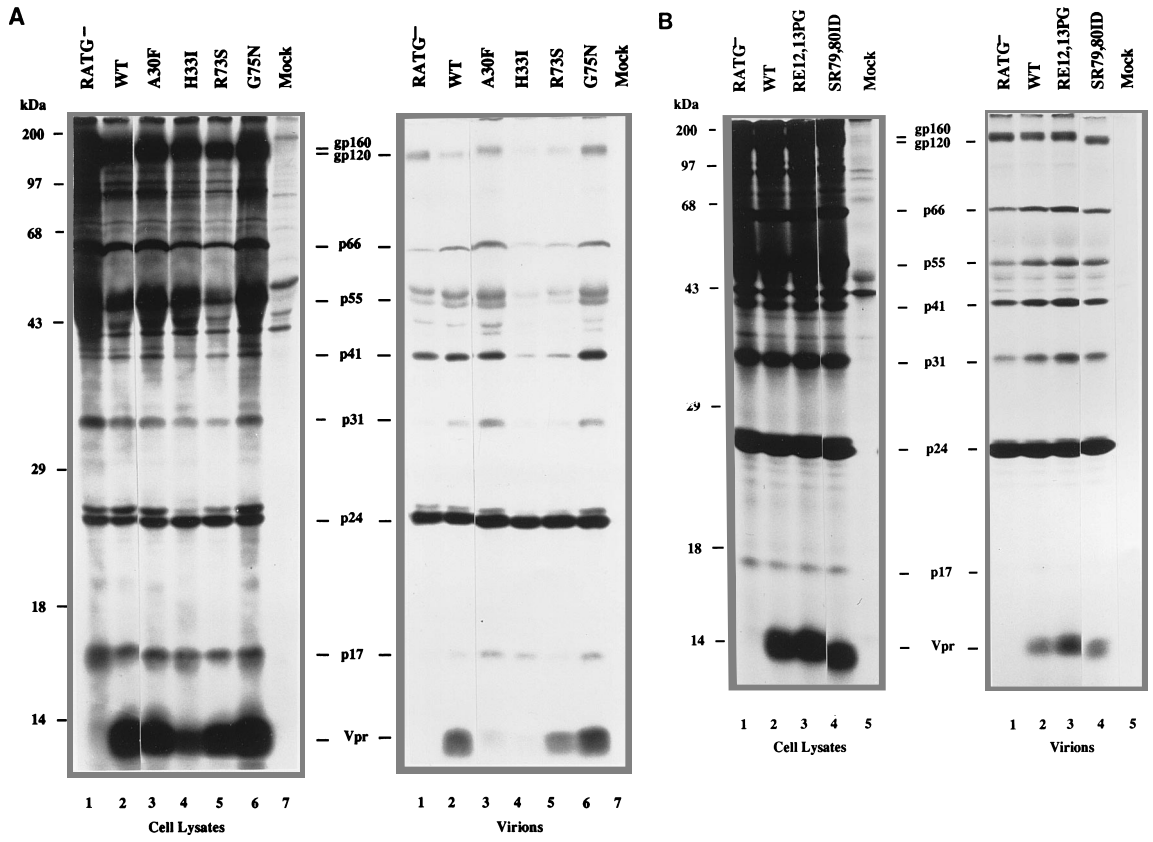



FIG. 4. Effect of substitution mutants of Vpr on virion incorporation. MT4 cells were transfected with 10 μg of proviral constructs encoding the different mutated Vpr proteins as indicated above the gels. At 48 to 84 h posttransfection, the cells were metabolically labeled with [$^{35}$S]methionine and [$^{3}$H]leucine for 12 h. The labeled cells (A, B, and C, left gels) and the virions pelleted through a sucrose cushion (A and B, right gels) were lysed and immunoprecipitated with a mix of HIV-1-positive human serum (162) and rabbit anti-Vpr serum. The immunoprecipitated proteins were analyzed after electrophoresis on an SDS–14% polyacrylamide gel; this was followed by autoradiography. (C, right gel) Pelleted virions were lysed and directly analyzed by SDS–14% PAGE. The positions of viral proteins and Vpr are indicated. RATG$^{-}$, HxBRUR$^{-}$; WT, HxBRU; Mock, uninfected.

tein in both the nucleus as well as the cytoplasm (Fig. 6A and B, panels c, e, g, h, and j to m).

Although two of the mutated Vpr proteins (L23F and IL63,64KR) were targeted to the nucleus, their intranuclear distribution was notably different from that of the wild type. Immunofluorescence staining of these two Vpr mutants did not show the wild-type diffused nuclear staining but rather the presence of condensed staining in the nucleus and cytoplasm (Fig. 6B, panels e and k). This nuclear condensed staining was also confirmed by confocal microscopic analysis (data not shown). It appears that these two mutated Vpr proteins form condensed cores in unidentified subnuclear and cytoplasmic regions. The identity of these locations is currently not known.

In contrast, the mutants from the N-terminal alpha-helical region from both ELI (WEL and EAHF) and BRU (E25K) exhibited notably different immunofluorescence patterns from that of the wild type. In these cells, Vpr staining was mainly located in the perinuclear region (Fig. 6B, panels d, f, and i). Only 4% (WEL), 31% (EAHF), and 25% (E25K) of the cells presented a Vpr-specific signal in the nucleus. Even in cells that exhibited such nuclear staining, a notable portion of the protein was still retained in the cytoplasmic compartment. These results are consistent with the notion that this putative N-terminal alpha-helical structure is essential for the preferential nuclear targeting of the Vpr protein.

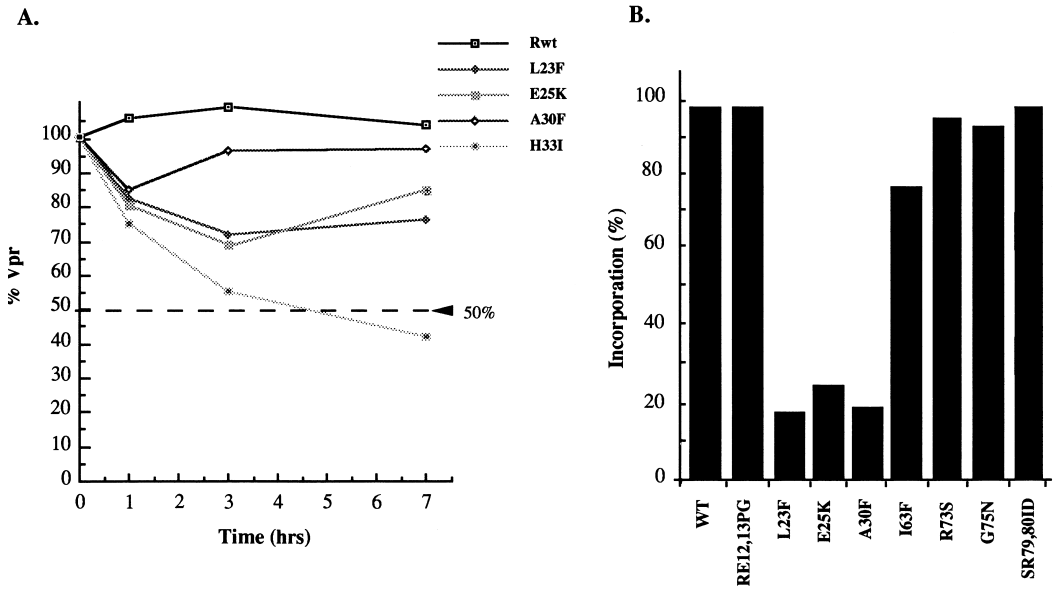



FIG. 5. Evaluation of stability and virion incorporation of different mutated Vpr proteins. (A) $10^6$ COS cells were transfected with different Vpr expressors as indicated. After 48 h, the transfected cells were pulse-labeled with [$^{35}$S]methionine and [$^{3}$H]leucine for 30 min and were subsequently chased at different intervals of time. The Vpr proteins in the cell lysates were immunoprecipitated with rabbit anti-Vpr serum and analyzed by autoradiography after SDS–14% PAGE. Expression levels at different time intervals for each of the substitution mutants were evaluated by using a Molecular Dynamics Personal densitometer. The percentage of Vpr recovered relative to the amount present at the end of the pulse (0 min) is plotted as a function of time. Rwt, wild-type Vpr. (B) Autoradiograms were scanned to determine the densitometric values of Vpr and p66 RT proteins immunoprecipitated from pelleted virions. The level of p66 RT served as an internal control in each sample. The incorporation rate of Vpr in the virions was determined by the ratio of virus-associated Vpr to RT. The wild-type (WT) Vpr/RT expression ratio was arbitrarily set at 100%.

## DISCUSSION

Of the six HIV-1 auxiliary proteins (Vif, Vpr, Tat, Vpu, Rev, and Nef), Vpr is the only gene product that is incorporated efficiently into the progeny virions (6, 38). The presence of Vpr in the virions is a strong indication that this protein may have a functional role early in viral replication. When expressed in the absence of other viral proteins, Vpr was shown to localize in the nucleus (28). These two disparate Vpr cellular localizations are likely mediated through specific protein-protein interactions during viral replication. In order to identify regions of Vpr involved in its nuclear localization and virion incorporation, we generated a number of mutations in different putative regions of the Vpr sequence: a putative N-terminal alpha-helical structure predicted by computer-assisted sequence analysis, a previously characterized region termed the LR domain toward the distal half of the protein, and a carboxyl-terminal region rich in basic residues. Our results clearly show that several individual substitution mutations in this helical region, including L23F, E25K, and A30F, resulted in relatively stable proteins that showed markedly reduced virion incorporation. One of these mutants, E25K, also impaired Vpr nuclear localization, indicating that the putative N-terminal alpha-helical region extending from amino acids 16 to 34 plays a significant role in virion incorporation as well as in the nuclear targeting of Vpr. Furthermore, the results from this study indicate that the C-terminal truncation of Vpr, but not the substitution mutations in this region, resulted in a less stable Vpr protein and in the impairment of both virion incorporation and nuclear localization. These impairments may be due to altered structural conformation and/or stability of these truncated Vpr proteins.

Amphipathic helices are commonly observed in structural motifs that involve protein-protein and protein-lipid interactions (37). One of the important structural features of Vpr includes an N-terminal domain predicted to form an amphipathic alpha helix (from amino acids 16 to 34), as shown in Fig. 7A. This putative helical structure exhibits well-demarcated hydrophilic and hydrophobic faces, as shown in Fig. 7B. Interestingly, Vpx, another virion-associated protein encoded by HIV-2 and simian immunodeficiency virus, also contains a predicted amphipathic helical structure near its N terminus (20). In addition, the C-terminal halves of the alpha-helical structures of Vpr and Vpx share notable homology in terms of the amino acid sequence and, hence, amphipathicity (Fig. 7A). Recent data indicate that, like incorporation of Vpr, Vpx incorporation also depends on the presence of a region extending from amino acids 439 to 497 at the carboxyl-terminal end of the Pr55$^{gag}$ polyprotein corresponding to the p6 region (43). These findings strongly tie the sequence homology found in these proteins with that of a common packaging mechanism for these related accessory products. Recent reports indicate that the presence of an alpha-helical structure near the N terminus of Vpr is important for its virion incorporation, since introduction of proline residues in this predicted alpha-helical region abolished Vpr virion incorporation (29, 30). Moreover, it was shown that substitution of alanine for the four leucine residues in the helical region resulted in a mutant which was not incorporated into virus-like particles, suggesting the importance of these hydrophobic amino acids in the helical region for Vpr virion incorporation (30). It is of interest to note in the present study that amino acids L-23 and A-30 are close to one another on the hydrophobic face of the predicted helix (Fig. 7B). Though substitution of L-23 or A-30 with phenylalanine conserves the hydrophobicity of this face, it notably impairs Vpr incorporation, suggesting that specific residues located on the hydrophobic face may be involved in protein-protein interactions relevant to virion targeting. Also, our

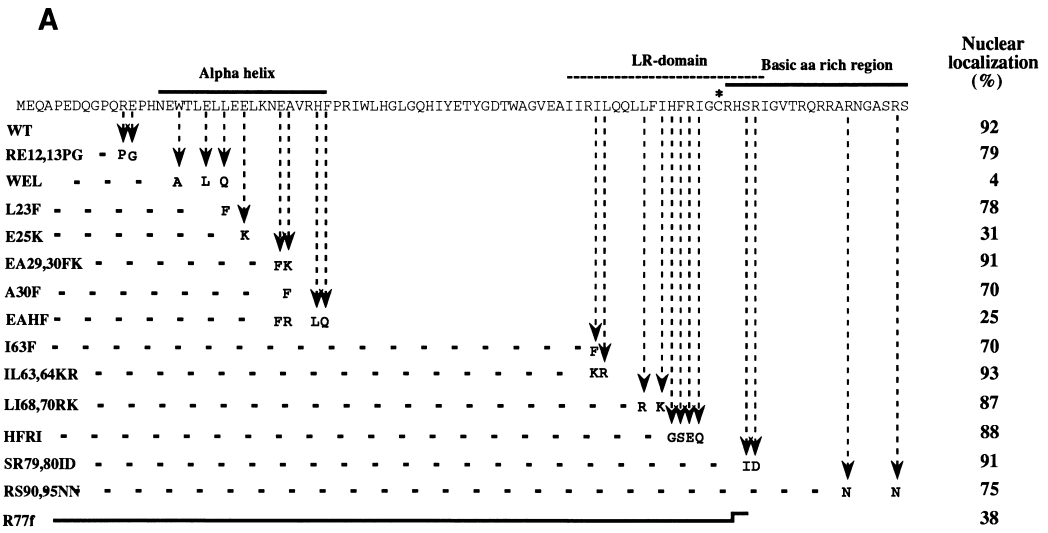


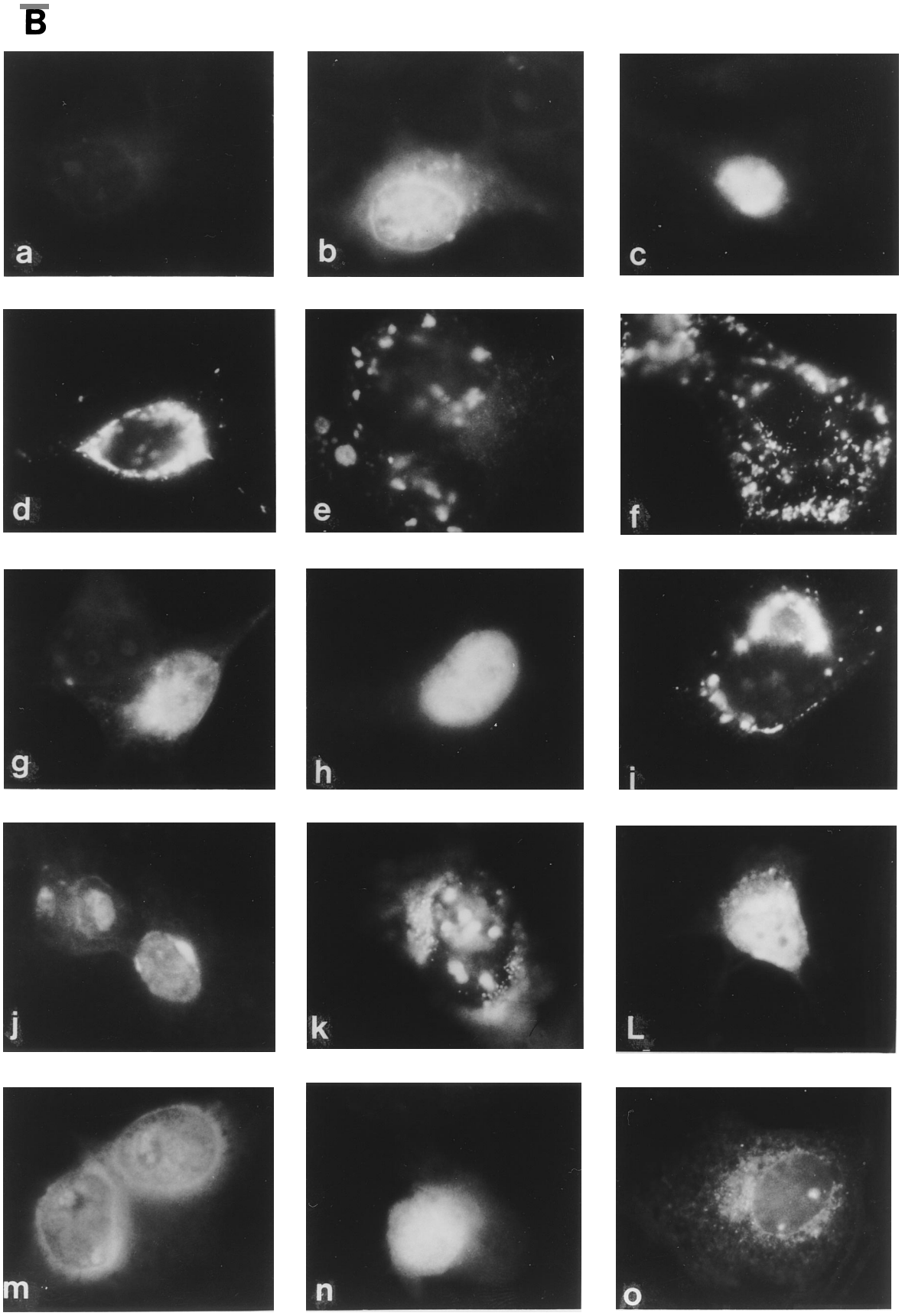

FIG. 6. Nuclear localization of different mutated Vpr proteins in COS cells. (A) The amino acid sequence of Vpr is shown at the top. The position of the predicted amphipathic alpha-helical structure, the highly conserved amino acid cysteine at position 76, the LR domain, and the basic amino-acid (aa)-rich region in the Vpr are also indicated. A series of mutated Vpr expressors (SVCMV-Vpr) were constructed (as described in Materials and Methods), and the position of each mutation on the Vpr sequence is shown by the arrow. For evaluating the nuclear localization of Vpr, COS cells cultured in shell vials were transfected with each of these Vpr expressors. After 48 h, the transfected cells were fixed, labeled with anti-Vpr antibodies, and analyzed by the indirect immunofluorescence technique (as described in Materials and Methods). The percentage of Vpr-positive cells having nuclear localization was evaluated by determining the numbers of Vpr-positive cells with specific nuclear staining over total numbers of Vpr-positive cells (shown on the right). WT, wild type. (B) Intracellular localization of wild-type and different mutated Vpr proteins in COS cells. (a) $Vpr^-$; (b) wild-type Vpr; (c) RE12,13PG; (d) WEL; (e) L23F; (f) E25K; (g) A30F; (h) EA29,30FK; (i) EAHF; (j) I63F; (k) IL63,64KR; (l) LI68,70RK; (m) HFRI; (n) SR79,80ID; (o) R77f. The localization of RR90,95NN is not shown.

results indicate that an individual substitution mutation, E25K, located at the hydrophilic side of this predicted helical region severely impairs the capacity of Vpr to be incorporated into virions. These results further extend the conclusion reached by a recent study (30), in which virion incorporation was not affected when multiple glutamic acid residues in the putative helix were replaced by a similarly charged aspartic acid residue. Interestingly, however, the authors noted a drop in incorporation when these acidic residues were replaced by an uncharged, but polar, glutamine residue. In our mutational analysis, one of the glutamic acids tested by the previous study was replaced by the oppositely charged lysine in order to disrupt the acidity associated with this hydrophilic face. Our results document a notable impairment of virion association of this mutant, which suggests the importance of the acidity rather than the identity of the amino acid residues associated with this hydrophilic face for effective virion incorporation of Vpr. Taken together, these results indicate that both sides of this secondary structure can affect Vpr incorporation.

Our results from immunofluorescence analysis clearly indicate that the predicted alpha-helical structure near the N terminus of Vpr plays an important role in Vpr nuclear localization in addition to Vpr incorporation. The importance of this predicted alpha-helical structure for nuclear targeting is also supported by the impairment of nuclear localization of a multiple substitution mutant (EAHF) which was shown to disrupt the conformation of the distal half of the helical structure, as predicted by our computer analysis (data not shown). It is of interest to note that the Vpr mutants WEL and E25K, which affect nuclear localization, carry amino acid substitutions on the hydrophilic face of the putative helix (Fig. 7B). In contrast, mutations that targeted the hydrophobic face of the Vpr N-terminal helix (L23F or A30F) resulted in mutant proteins that retained their ability to translocate to the nucleus but were impaired in their virion incorporation property.

The immunofluorescence patterns of the various Vpr mutants were also investigated. Two mutated Vpr proteins (L23F and IL63,64KR), even though clearly localized in the nucleus, still displayed different staining patterns, compared with the wild-type Vpr. Interestingly, these mutations are located either in the predicted alpha-helical region or in a previously described LR domain extending from amino acids 60 to 81 in Vpr (46) as depicted in Fig. 6A. This LR domain has been shown to be involved in the interaction with a cellular protein (RIP), and point mutations in this region (amino acids 60 to 81) abolished this interaction (46). RIP has been shown to be present in the cytoplasm and nucleus (46). So, it might be possible that the proper nuclear translocation of Vpr is related to its association with the RIP protein.

Previous studies have shown that the C-terminal portion of Vpr is involved in efficient Vpr incorporation into progeny virions (33, 41). We also tested the importance of the C-terminal portion of Vpr for its stability and biological properties. The results indicate that two premature truncation mutants that eliminate the C-terminal sequences were relatively unstable proteins, compared with the wild-type or the N-terminal mutants (Fig. 2). As the level of the virion-associated protein in these truncation mutants was lower than the level observed with the wild type, it is conceivable that the C-terminal region may also modulate the efficiency of virion association (Fig. 3). However, we cannot rule out the possibility that this is partially related to the unstable nature of the C-terminal truncation mutants. Nonetheless, the detection of these truncated Vpr proteins in the virions clearly indicates that the minimal determinant(s) required for virion incorporation is located not in the deleted C-terminal region (up to the last 24 amino acids) but in the proximal region of the protein. This is also supported by the fact that the homologous HIV-2–simian immunodeficiency virus proteins (Vpr-2 and Vpx) capable of effective virion association do not share C-terminal sequences with HIV-1 Vpr (40). Additional support suggesting the lack of specific motifs directing virion incorporation comes from our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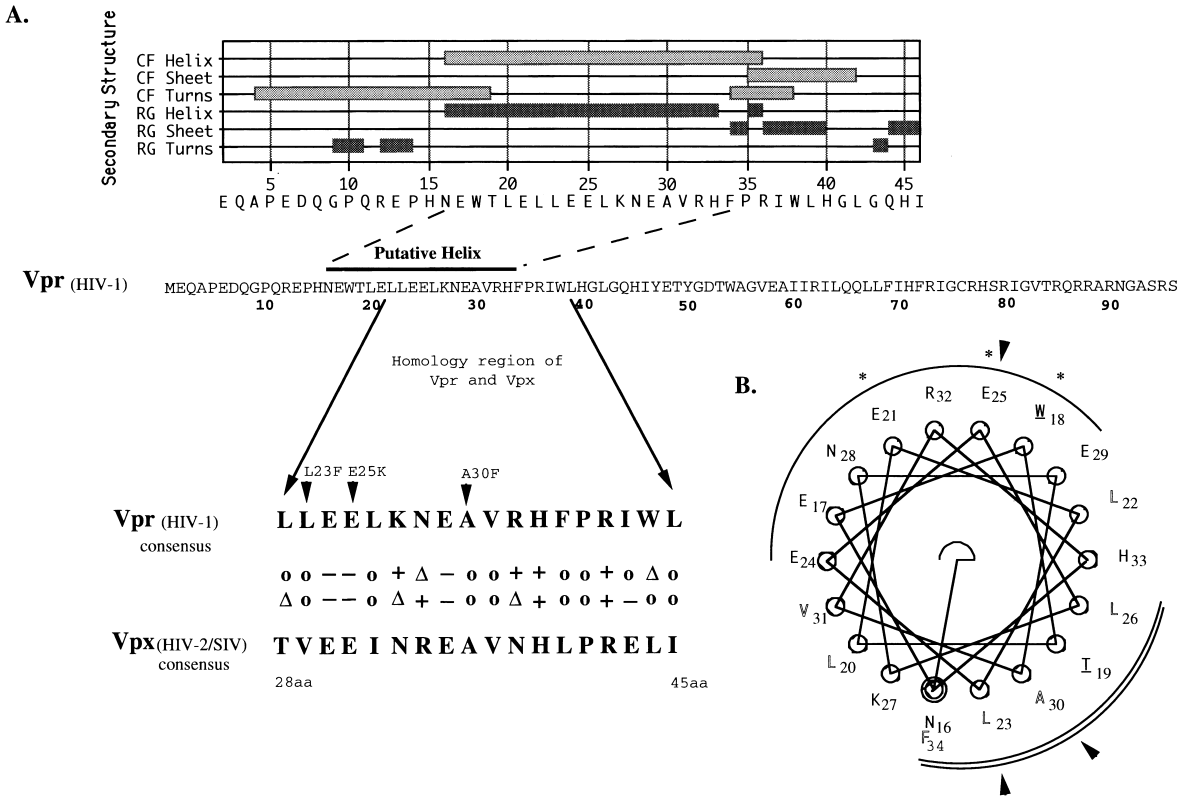


FIG. 7. The computer-predicted N-terminal alpha-helical structure in Vpr protein. (A) The amino acid sequence of Vpr and the positions of the alpha helix, β sheet, and β turn predicted by computer analysis are shown at the top. (RG, Robson-Garnier method; CF, Chou-Fasman method). A region with high sequence homology between Vpr and Vpx is shown at the bottom, and this region comprises the C-terminal half of the amphipathic helix region. The incorporation-related amino acids are indicated by arrows. ○, hydrophobic amino acids (aa); △, polar aa; —, negatively charged aa; +, positively charged aa. (B) Helical-wheel diagram of the predicted alpha-helical structure extending from amino acids 16 to 34 in the HIV-1 Vpr sequence. The hydrophilic face is indicated by a single line, and the hydrophobic face is represented by a double line. The incorporation-related amino acids are indicated by arrows, and the amino acids involved in Vpr nuclear localization are indicated by asterisks.

substitution analysis of the C-terminal region where no single residue tested directly affected the efficiency of virion incorporation (Fig. 4 and 5). Given these independent lines of evidence which suggest the lack of a minimal required determinant for virion incorporation in this region, the highly reduced levels of virion-associated truncated Vpr proteins is likely mediated by simply modulating the stability of the protein (Fig. 2) and/or by impairing the optimal conformation of the protein necessary for virion incorporation. However, the fact that some protein is clearly present in the virion excludes the possibility of a major conformational change.

Our results from immunofluorescence analysis using the same R77f mutant indicate that the essential determinant(s) for the nuclear localization of Vpr is not located within the last 19 amino acids of the Vpr C terminus. However, as is the case with virion incorporation, eliminating the last 19 amino acids resulted in a dramatic reduction in the ability of Vpr to translocate to the nucleus (Fig. 6). These results correlate with fractionation experiments that show an impairment of nuclear localization, using a similar 19-amino-acid-truncated Vpr (28). A recent study shows that the deletion of the last 9 amino acids (amino acids 87 to 96) or an internal deletion from amino acids 77 to 87 in the arginine-rich C terminus of Vpr did not abolish Vpr nuclear localization (46). Consistent with this study, our analysis of a number of substitution mutations in the C-terminal region of Vpr did not identify any specific amino acid(s) involved in Vpr nuclear localization. Taken together, these results suggest the lack of a primary determinant necessary for nuclear localization in this region. Therefore, as with virion incorporation, the impairment of nuclear localization of a large C-terminal-truncated Vpr may also be due to altered structural conformation. Though a number of mutations were included in our study, we cannot formally rule out the possibility that other single residues in this region are capable of affecting these properties.

These results strongly suggest that a predicted N-terminal alpha-helical structure is involved in protein-protein interactions which mediate virion incorporation and nuclear localization of Vpr. Our results also distinguish between the hydrophilic and hydrophobic faces of this putative secondary structure in their ability to affect nuclear localization and virion incorporation of Vpr. It is clear that the efficiency of both nuclear and virion targeting of the mutant E25K is highly impaired. However, a small, but observable, quantity of the mutant still reaches the nucleus and the virions, ruling out the possibility of a global disruption of the Vpr protein. This may suggest that a common mechanism is operative both in transporting Vpr to the nucleus as well as in incorporating it in the virion. On the other hand, proteins that show relatively high levels of nuclear localization (A30 and L23) are still incapable of effective incorporation in the virion. This clearly indicates that determinants in the hydrophobic region, in addition to those in the hydrophilic region, influence Vpr virion targeting.

These results are interesting, in light of a recent study that

suggests that Vpr may associate with a 41-kDa protein (termed Rip-1) that specifically binds the type II glucocorticoid receptor which traffics between the cytoplasm and the nucleus (34). Hence, it is conceivable that specific mutations within the Vpr sequence that affect its ability to reach the nucleus may also affect its efficient association with such partners. As the hydrophobic mutants, in spite of active nuclear localization, still affect virion incorporation, it suggests that this side of the helix either forms a direct hydrophobic interface with virion structures or, alternatively, mediates homooligomerization of the protein. In this regard, it is worth noting that Vpr oligomers have been documented previously (33, 47). We are currently exploring Vpr-Vpr and Vpr-cellular protein interactions and their ability to influence virion incorporation and nuclear localization.

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