JOURNAL OF VIROLOGY, September 1997, p. 6339–6347 Vol. 71, No. 9
0022-538X/97/$04.00+0

Nuclear Import, Virion Incorporation, and Cell Cycle Arrest/ Differentiation Are Mediated by Distinct Functional Domains of Human Immunodeficiency Virus Type 1 Vpr

SUNDARASAMY MAHALINGAM, VELPANDI AYYAVOO, MAMATA PATEL, THOMAS KIEBER-EMMONS, AND DAVID B. WEINER*

Department of Pathology and Laboratory Medicine, School of Medicine, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104

Received 13 February 1997/Accepted 23 May 1997

The *vpr* gene product of human immunodeficiency virus type 1 (HIV-1) is a virion-associated protein that is essential for efficient viral replication in monocytes/macrophages. Vpr is primarily localized in the nucleus when expressed in the absence of other viral proteins. Vpr is packaged efficiently into viral particles through interactions with the p6 domain of the Gag precursor polyprotein $p55^{gag}$. We developed a panel of expression vectors encoding Vpr molecules mutated in the amino-terminal helical domain, leucine-isoleucine (LR) domain, and carboxy-terminal domain to map the different functional domains and to define the interrelationships between virion incorporation, nuclear localization, cell cycle arrest, and differentiation functions of Vpr. We observed that substitution mutations in the N-terminal domain of Vpr impaired both nuclear localization and virion packaging, suggesting that the helical structure may play a vital role in modulating both of these biological properties. The LR domain was found to be involved in the nuclear localization of Vpr. In contrast, cell cycle arrest appears to be largely controlled by the C-terminal domain of Vpr. The LR and C-terminal domains do not appear to be essential for virion incorporation of Vpr. Interestingly, we found that two Vpr mutants harboring single amino acid substitutions (A30L and G75A) retained the ability to translocate to the nucleus but were impaired in the cell cycle arrest function. In contrast, mutation of Leu68 to Ser resulted in a protein that localizes in the cytoplasm while retaining the ability to arrest host cell proliferation. We speculate that the nuclear localization and cell cycle arrest functions of Vpr are not interrelated and that these functions are mediated by separable putative functional domains of Vpr.

The human immunodeficiency virus type 1 (HIV-1) accessory gene *vpr*, while dispensable for viral replication in T-cell lines and activated primary peripheral blood mononuclear cells (1, 2, 8, 13, 43), is required for efficient replication in primary monocytes/macrophages. The role of Vpr in AIDS pathogenesis is not well understood. In one study, macaques infected with simian immunodeficiency virus (SIV) isolate SIVmac239 containing a mutation in the Vpr initiation codon methionine progressed to AIDS more slowly than those infected with wild-type virus (27). In another, a *vpr* mutant of SIVmac239 retained full pathogenicity but, in conjunction with a mutation in *vpx*, replicated at low levels and was nonpathogenic when inoculated into macaques (20). HIV-1 *vpr* encodes a 14-kDa, 96-amino-acid protein expressed primarily from a singly spliced Rev-dependent mRNA (3, 9, 12, 19, 49).

A recent study has characterized the Vpr protein as an oligomer (59). HIV-2 and SIV code for a second protein, Vpx, which has considerable sequence homology with Vpr (40, 51). Both proteins are packaged efficiently in HIV and SIV particles (9, 39, 55, 57). Virion localization studies place both Vpr and Vpx outside the core structure (51, 56). Although Vpr and Vpx are not part of the Gag structural polyprotein, their incorporation requires an anchor to associate with the assembling capsid structures. The C-terminal portion of the Gag precursor corresponding to the p6 protein appears to constitute such an anchor through an unknown mechanism. In addition, p6 is essential for the incorporation of both Vpr and Vpx into virus particles (26, 28, 44, 53). A predicted putative α-helical domain near the amino terminus plays an important role in the packaging of Vpr into virions and in maintaining protein stability (35, 36).

Several possible roles have been suggested for Vpr in HIV-1 replication. Vpr can modestly transactivate the HIV-1 long terminal repeat (8) and thus may upregulate viral gene expression in newly infected cells before the appearance of Tat. It has been found to enhance the nuclear migration of the preintegration complex in newly infected nondividing cells (22). Significantly, Vpr induces cellular differentiation which includes the activation of specific host cell gene transcription and growth arrest in several tumor cell lines, even in the absence of any other viral proteins (29, 38). This finding suggests that Vpr, itself, may be sufficient to alter cellular functions. Rogel et al. (47) recently reported that Vpr blocks cell cycling in the G_2/M phase of the cell cycle. This finding has been associated with a change in the phosphorylation state of CDC2 kinase (21, 24, 45). Furthermore, Vpr expression appears to inhibit the establishment of chronic infection (32, 41, 47). Macreadie et al. (34) reported that Vpr causes growth arrest and structural defects in yeast. Functional studies have shown that Vpr accelerates HIV-1 replication in some T-lymphoid cell lines and in primary macrophages where the effects of Vpr are more pronounced (4, 5, 10).

It has also been reported that Vpr has transcellular activity (31). Both Vpr purified from plasma of HIV-1-seropositive individuals and purified recombinant Vpr were capable of inducing latent cells into high-level viral producers when added to culture media at low concentrations (30). Mechanistically, it

* Corresponding author. Mailing address: Department of Pathology, Stellar-Chance Labs, Rm. 505, University of Pennsylvania, 422 Curie Blvd., Philadelphia, PA 19104. Phone: (215) 349-8365. Fax: (215) 573-9436.

is conceivable that this transcellular activity is mediated by the same mechanisms that modify cellular growth and differentiation. It has been reported that Vpr is primarily localized in the nucleus when expressed in the absence of other HIV-1 proteins (14, 33, 37, 54). Although no classical nuclear localization signal (NLS) has been clearly identified for Vpr, it has been suggested that Vpr may gain access to the nucleus by specific interactions with nuclear proteins (46, 58). In this regard, proteins that interact with Vpr in host cells have been reported but not molecularly cloned. Interestingly, one of these Vpr targets, designated Vpr-interacting protein, or RIP-1, appears to be translocated to the nucleus following its interaction with Vpr or triggering by glucocorticoid receptor (GR) ligands, supporting a possible role for the glucocorticoid receptor pathway in Vpr function (46). The molecular relationship between these different functions of Vpr has not yet been defined.

To define the interrelationship between the different functions of Vpr, we created a panel of expression vectors encoding mutant Vpr molecules. We tested a panel of Vpr mutants for the ability to arrest the cell cycle, to localize to the nucleus, to affect cellular differentiation, and to be packaged into virus-like particles. Our studies indicate that mutations in the amino-terminal acidic domain of Vpr with a predicted α-helical domain reduces virion packaging of Vpr and alters its nuclear localization. We postulate that these impairments are likely due to reduced protein stability and/or structural conformation of the mutant Vpr proteins. Moreover, we also identified amino acid residues located in the leucine-isoleucine (LR) motif which appear to control the nuclear localization. Finally, the C-terminal domain appears to control the cell cycle arrest activity of Vpr. Importantly, these studies demonstrate that nuclear localization and cell cycle arrest appear to be separable functions of Vpr.

MATERIALS AND METHODS

Cells, virus, and expression plasmids. The recombinant vaccinia virus vTF7-3, which synthesizes T7 RNA polymerase in infected cells, was used for expression studies (18). The genes encoding HIV-1 Gag polyprotein and Vpr were cloned downstream of the T7 promoter in pCDNA3 (Invitrogen) to generate expression plasmids pCDGag and pCDVpr, respectively (35). HeLa and human embryonal rhabdomyosarcoma (RD) cells were used for transfection experiments and were maintained as monolayer cultures in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS).

PCR-mediated mutagenesis. Overlap extension PCR (23) was used to introduce the site-specific mutations into the HIV-1 *vpr* gene. Briefly, two PCRs were performed with HIV-1 proviral DNA as a template. The first-round amplification products overlap at the mutation site. A second round of amplification was then carried out with the outer primer pair and a mixture of the first two reaction products as a template to generate a product containing the desired amino acid substitution mutation. The PCR products were digested with *Hin*dIII and *Xho*I (sites for which recognition sequences were incorporated into the outer primer pairs), cloned into pCDNA3, and sequenced to verify mutations and ensure the integrity of the *vpr* gene.

Infection and transfection. HeLa cells (10^6) grown in 35-mm-diameter dishes were infected with vTF7-3 at a multiplicity of infection of 10 for 1 h in 5% CO_2 at 37°C with rocking every 15 min. At the end of the incubation period, the virus inoculum was removed and the cells were washed once with phosphate-buffered saline (PBS) before transfection. Three to 6 μg of plasmid DNA and 10 μl of Lipofectin (Bethesda Research Laboratories) were added to separate polystyrene tubes containing 0.1 ml of serum-free Opti-MEM and then combined. The mixture was incubated at room temperature for 15 min, supplemented with 0.6 ml of fresh Opti-MEM, and layered onto infected cells for 3 h. At 3 h posttransfection, 0.8 ml of DMEM containing 10% heat-inactivated FBS was added to the cultures, and the incubation was continued for an additional 12 h.

Transfection of RD cells with wild-type and mutant expression plasmids and examination of cell cycle distribution were performed as described previously (38). Briefly, RD cells were cotransfected with wild-type and different mutant Vpr expression plasmids and pBabepuro (a vector that expresses puromycin resistance). Two days later, puromycin (2 μg/ml) was added to eliminate the untransfected cells; 7 to 10 days posttransfection, the RD cell nuclei were stained with propidium iodide for analysis of DNA content by flow cytometry.

Metabolic labeling and immunoprecipitation. Transfected HeLa cells were washed twice with PBS, starved for 1 h in DMEM lacking serum, methionine, and cysteine, and then labeled with ^{35}S protein labeling mix (200 μCi/ml; 1,200 Ci/mmol; NEN/Dupont). Labeled cells were lysed in 0.5 ml of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl [pH 7.6], 150 mM NaCl, 0.2% Triton X-100, 0.2% deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride) on ice and then clarified by centrifugation at 15,000 rpm for 10 min in a Sorvall RC5B rotor. The clarified lysates were incubated with anti-Vpr antibody or anti-HIV-1 antibody for 90 min on ice. Protein A-Sepharose was added to antigen-antibody complexes and mixed by shaking at 4°C for 90 min. The protein pellet was resuspended in 50 μl of 1× sample buffer and heated at 100°C for 3 to 5 min after extensive washing in buffers containing high salt and bovine serum albumin. A fraction of the protein sample was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (12% gel). For fluorography, gels were soaked in 1 M sodium salicylate containing 10% glycerol for 15 min, dried, and autoradiographed, using Kodak X-Omat AR film.

Analysis of Vpr incorporation into virus-like particles directed by HIV-1 *gag*. Recombinant vaccinia virus-infected HeLa cells were transfected with a HIV-1 Gag expression vector (pCDGag) together with wild-type or mutant Vpr expression plasmids as described above. After overnight incubation, cells were labeled with ^{35}S protein labeling mix (200 μCi/ml). The culture medium was collected and clarified by centrifugation at 15,000 rpm for 15 min in a Sorvall RC5B rotor after 5 h of continuous labeling. The clarified medium was loaded into Centricon-30 concentrators (Amicon), which have a 30,000-molecular-weight size exclusion barrier, and centrifuged at 3,000 rpm for 25 min in a Sorvall RC5B rotor. The virus-like particles attached to the filter were resuspended with 0.5 ml of RIPA buffer. Immunoprecipitation was performed with anti-Vpr serum alone, anti-HIV-1 antibody alone, or both antibodies to determine the presence of Vpr in the virus-like particles.

Immunofluorescence assay. HeLa cells were maintained in DMEM containing 10% FBS and seeded onto poly-L-lysine-coated glass coverslips at a density of 10^6 cells per dish (35-mm diameter). Cells were infected with vTF7-3 and transfected as described above after 24 h. Sixteen to 24 h after transfection, the cells were washed with PBS and fixed with methanol at room temperature for 30 min. The cells were then washed with PBS and incubated for 90 min with primary antiserum (1:50). After washing with PBS, the coverslips were incubated for 90 min with fluorescein isothiocyanate-conjugated affinity-purified $F(ab')_2$ fragment of goat anti-rabbit immunoglobulin G (ICN Biochemicals, Irvine, Calif.) and washed six times with PBS. Coverslips were then counterstained for 5 min with Evans blue (0.02% in PBS; Sigma, St. Louis, Mo.) and rewashed prior to mounting on glass slides, using a fade-resistant mounting medium (Citifluor, London, England). All incubations were carried out at 37°C in a humidification chamber.

RESULTS

Construction of Vpr mutants. To identify the domain(s) of Vpr involved in virion incorporation, nuclear localization, differentiation, and cell cycle arrest functions, we constructed a series of mutated versions of Vpr molecules (Fig. 1B). In designing these mutants, we targeted three putative structural regions in the Vpr sequence: (i) the N-terminal acidic domain containing a putative α helix (amino acids 17 to 34), (ii) the LR domain, and (iii) the carboxy-terminal domain. Substitution mutations designed to affect specific amino acid residues were found to be highly conserved among *vpr* sequences from different HIV-1 isolates (Fig. 1A). The amino-terminal domain contains five negatively charged residues (amino acid positions 17, 21, 24, 25, and 29) which are highly conserved. Structural analysis of the amino acid sequences in this region strongly predicts an amphipathic α helix. Similar structures have been shown to be involved in protein-protein interactions and virion incorporation of viral proteins in other viruses (48, 50). We used site-specific mutations of these residues which would disrupt the predicted structure. Two of these residues (glutamic acids 21 and 24) were replaced with proline, which has low potential for supporting an α-helical structure (Fig. 1B).

To explore the importance of the highly conserved alanine (Ala30), we replaced this nonpolar alanine with a bulky polar residue leucine and a hydroxyl amino acid serine to generate mutants A30L and A30S, respectively. In addition, another helical Vpr mutant was generated by replacing the four hydrophobic polar leucines (amino acid positions 20, 22, 23, and 26) with small nonpolar alanines (αL-A). To investigate the role of a second helix, we generated Vpr mutant A59P by changing alanine at amino acid position 59 to proline. Three Vpr mutants carrying substitutions of leucine to serine at amino acid

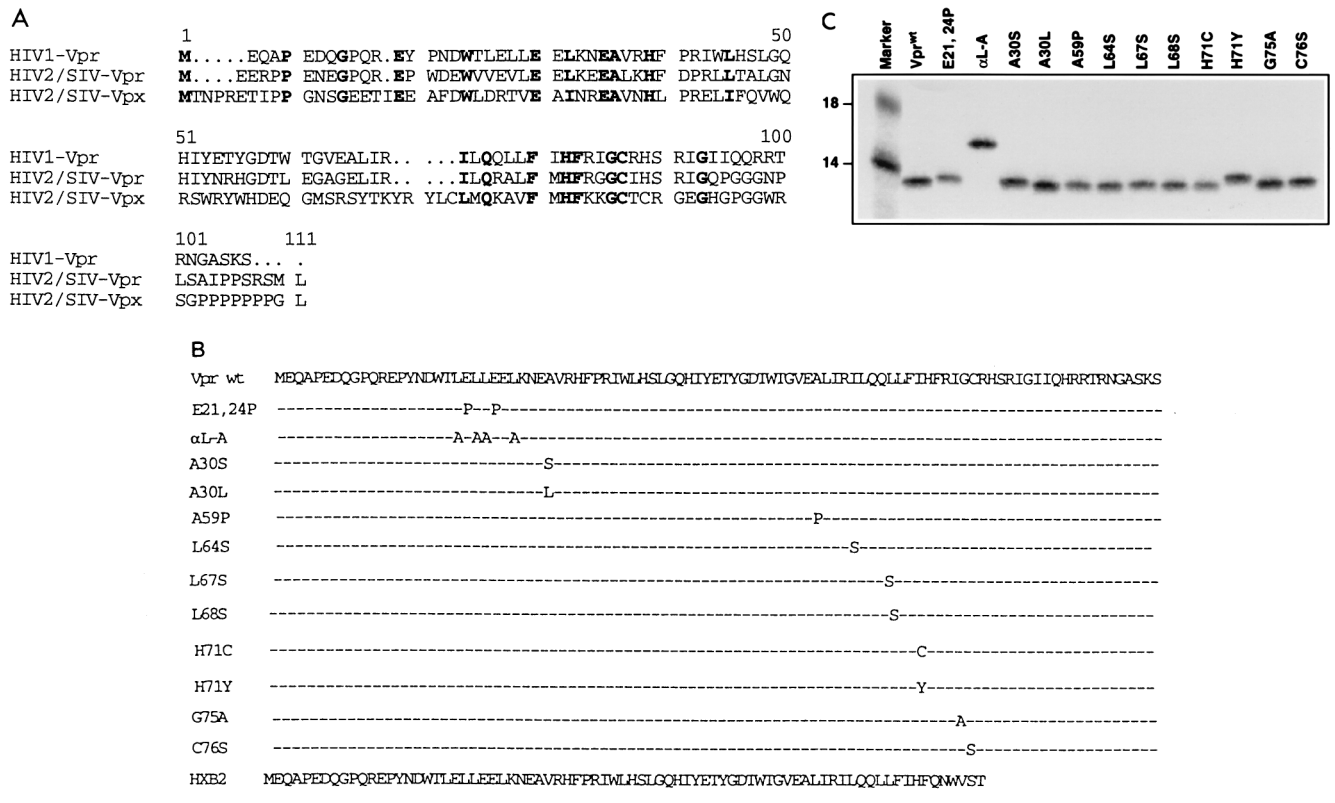

FIG. 1. Construction and expression of mutant Vpr molecules. (A) Amino acid sequence comparison of Vpr of HIV-1 and HIV-2/SIV and Vpx of HIV-2/SIV. Numbers denote positions of amino acid residues. (B) Expression plasmids for the synthesis of mutant Vpr molecules were generated by overlap PCR at the indicated codons. PCR-amplified mutant *vpr* gene fragments were digested with *Hin*dIII and *Xho*I and ligated to the pCDNA3 vector to produce Vpr mutant expression plasmids. (C) Recombinant vaccinia virus vTF7-3-infected HeLa cells were transfected with wild-type and mutant *vpr* expression plasmids. Transfected cells were labeled with ^{35}S protein labeling mix for 2 h, and the cell-associated Vpr proteins were immunoprecipitated with anti-Vpr antiserum as described in Materials and Methods. Immunoprecipitates were analyzed by SDS-PAGE (12% gel). Designations of the Vpr plasmids are indicated at the top. Vpr^{wt}, wild-type Vpr. Sizes are indicated in kilodaltons). (D) The secondary structure of Vpr was calculated by using nnpredict (25), a program that predicts the secondary structure type for each residue in an amino acid sequence based on the prediction of a two-layer, feed-forward neural network. H, helical; E, extended; −, undefined. αL-A, L64S, and H71C display the same secondary profile as wild-type Vpr, suggesting the importance of the Leu residues on the hydrophobic face and His in the C terminus. Introduction of a proline residue in E21,24 and A59 disrupts the respective helical domains.

positions 64, 67, and 68 (L64S, L67S, and L68S) were constructed to introduce mutations in the LR domain (Fig. 1B).

Histidine 71, glycine 75, and cystine 76 in the C terminus of Vpr are highly conserved among different HIV-1 isolates and in both Vpr and Vpx of HIV-2/SIV. Cystine has been shown to play a major role in protein stabilization and in protein-protein interactions (6, 11, 16). We targeted His, Gly, and Cys residues for mutagenesis to evaluate the role of this motif in the expression, virion packaging, subcellular localization, and cell cycle arrest functions of HIV-1 Vpr (Fig. 1B). All the substitution Vpr mutants were generated by the overlap PCR method and subcloned into pCDNA3 mammalian expression vector as described in Materials and Methods. The resulting constructs were verified by DNA sequence analysis.

Effects of mutations on the expression and virion packaging of Vpr. We used a vaccinia virus T7-RNA polymerase expression system (vTF7-3) to study the effect of mutations on expression of Vpr in cells and incorporation into virus-like particles directed by the HIV-1 *gag* gene. vTF7-3-infected HeLa cells were transfected with wild-type or mutant Vpr expression plasmids by the Lipofectin method. Cells were labeled for 2 h with [^{35}S]methionine, lysed, immunoprecipitated with anti-Vpr antiserum, and analyzed by SDS-PAGE (12% gel). As expected, the cells transfected with Vpr expression plasmids pro-

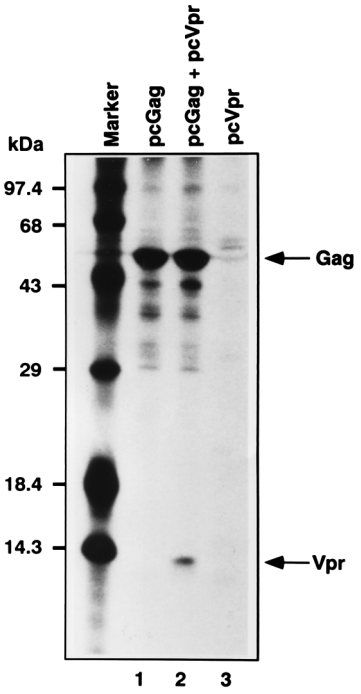

FIG. 2. Incorporation of Vpr into virus-like particles directed by HIV-1 Gag. Cotransfection of pCDGag with wild-type or mutant Vpr expression plasmids was carried out by using vTF7-3-infected HeLa cells as described in Materials and Methods. Transfected cells were labeled with ^{35}S protein labeling mix for 5 h, the culture medium was cleared by centrifugation and concentrated in Centricon-30 concentrators, and virus-like particles were resuspended with RIPA buffer. Immunoprecipitation was carried out with anti-HIV and anti-Vpr antiserum and analyzed by SDS-PAGE (12% gel). The electrophoretic positions of Gag and Vpr are shown at the right, and molecular mass markers are shown at the left.

duced a 14-kDa protein (Fig. 1C). Transfection with each of the mutants resulted in detectable levels of Vpr in the cell lysate. Interestingly, slower migration of Vpr was noticed in cells transfected with the Vpr αL-A expression plasmid. Vpr mutants E2124P and H71Y also exhibited migration slightly slower than that of wild-type Vpr (Fig. 1C). This difference in electrophoretic migration may have resulted from the altered conformation of mutant Vpr proteins relative to the wild-type polypeptide or changes in the hydrophobic face (αL-A) of the first helical domain (Fig. 1D).

To define the amino acid residues of Vpr that are required for virion incorporation, we tested several Vpr mutant molecules for the ability to be packaged into virus particles. We used a transient packaging system which is generated by the HIV-1 *gag* gene as we described previously (35, 36). In this assay, mutant Vpr expression plasmids are cotransfected with HIV-1 Gag expression vectors into vTF7-3-infected HeLa cells. After overnight transfection, the cells were labeled with [^{35}S]methionine, and the virus-like particles that had been secreted into the culture medium were collected and concentrated in a Centricon-30 concentrator. The amount of Vpr present in the virion, and cell-associated Vpr, was then detected by immunoprecipitation. Expression of Gag resulted in a 55-kDa protein product in the cell culture supernatant, and cotransfection of Gag and wild-type Vpr expression vectors resulted in the efficient packaging of Vpr into the virus-like particles as expected (Fig. 2). In contrast, no Vpr could be detected in the virus-like particles from the cells cotransfected with Gag and Vpr mutants E21,24P, αL-A, and A59P, despite

TABLE 1. Effects of mutagenesis on Vpr virion incorporation, subcellular localization, and cell cycle arrest/differentiation functions

Clone designation[a]	Incorporation into virus-like particles	Subcellular localization[b]	Cell cycle arrest	Differentiation
Vpr (wild type)	+	Nuc	+	+
E21,24P	−	Cyto	−	−
αL-A	−	Cyto	+	+
A30S	+	Cyto > nuc	−	−
A30L	+	Nuc	−	−
A59P	−	Cyto	−	−
L64S	+	Cyto > nuc	+/−[c]	+/−
L67S	+	Cyto	−	−
L68S	+	Cyto	+	+
H71C	+	Cyto > nuc	−	−
H71Y	+/−	Cyto + nuc	−	−
G75A	+	Nuc	+/−	+/−
C76S	+	Cyto + nuc	+/−	+/−
Vpr (HXB2)	+	Cyto + nuc	−	ND[d]

[a] All clones were positive for expression.
[b] Nuc, nuclear; cyto, cytoplasmic.
[c] +/−, intermediate.
[d] ND, not done.

detectable levels of expression in the cells (Fig. 1C and Table 1). Vpr molecules with mutations at alanine 30 (A30S and A30L) and His71 showed reduced levels of Vpr incorporation into virus-like particles (Table 1). These results indicate that mutations at amino acid positions 64, 67, and 68 (L64S, L67S, and L68S), Gly75, and Cys76 allow efficient packaging of Vpr into virus-like particles. Taken together with results of other studies, these results support the importance of a putative helical domain with these additional mutants in the packaging of Vpr into HIV-1 particles.

Substitution mutational analysis reveals that putative helical and LR domains are necessary for nuclear transport of Vpr. Previously it has been shown that Vpr localizes in the nuclei of infected and transfected cells in the absence of other viral proteins despite the lack of a canonical NLS (14, 33, 37, 54). We analyzed our series of Vpr expression plasmids to define the amino acid residues required for nuclear localization as shown schematically in Fig. 1B. We transfected the mutant plasmids into vTF7-3-infected HeLa cells to determine the subcellular localization of the Vpr mutant molecules. After overnight transfection, the cells were fixed with methanol, labeled with anti-Vpr antiserum, and analyzed by an indirect immunofluorescence assay to evaluate the abilities of various Vpr mutants to be targeted to the nucleus. As expected, the wild-type Vpr localized primarily in the nuclei of the transfected cells, and no signal was observed in the vector-transfected cells or in wild-type Vpr-transfected cells stained with preimmune serum (Fig. 3). In contrast, Vpr mutants E21,24P, αL-A, A59P, L67S, and L68S severely impaired the nuclear localization of Vpr, as shown in Fig. 3. The majority of cells expressing this mutant Vpr protein showed localization in the cytoplasm. Interestingly, A30L and G75A mutant Vpr molecules retained the ability to localize in the nucleus (Fig. 3). Mutants A30S, L64S, H71C, H71Y, and C76S had notable amounts of protein in both the nucleus and cytoplasm (Fig. 3). Mutants from the putative helical and LR domains exhibited immunofluorescence patterns notably different from that of wild-type Vpr. These results suggest that both N-terminal α-helix and LR domains are essential for the transport of Vpr into the nucleus.

Amino acid residues of Vpr are required for cell cycle arrest at the G_2/M phase of the cell cycle. Previous studies have

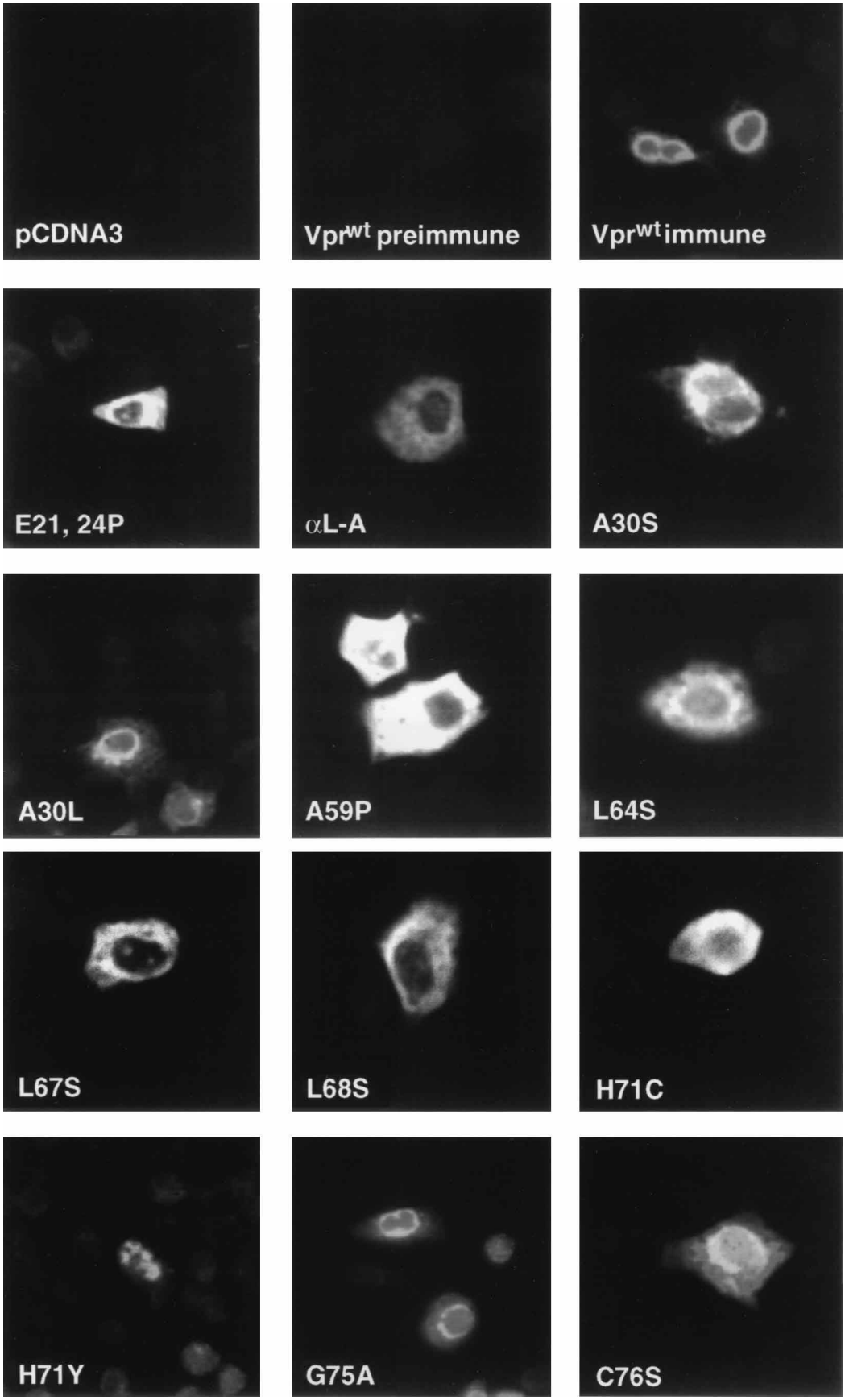

FIG. 3. Subcellular localization of wild-type Vpr (Vpr^{wt}) and effects of substitutions at the different structural domains of Vpr. HeLa cells were infected with recombinant vaccinia virus vTF7-3 and transfected with Vpr expression plasmids. After overnight transfection, the cells were fixed and stained with anti-Vpr serum followed by affinity-purified fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G.

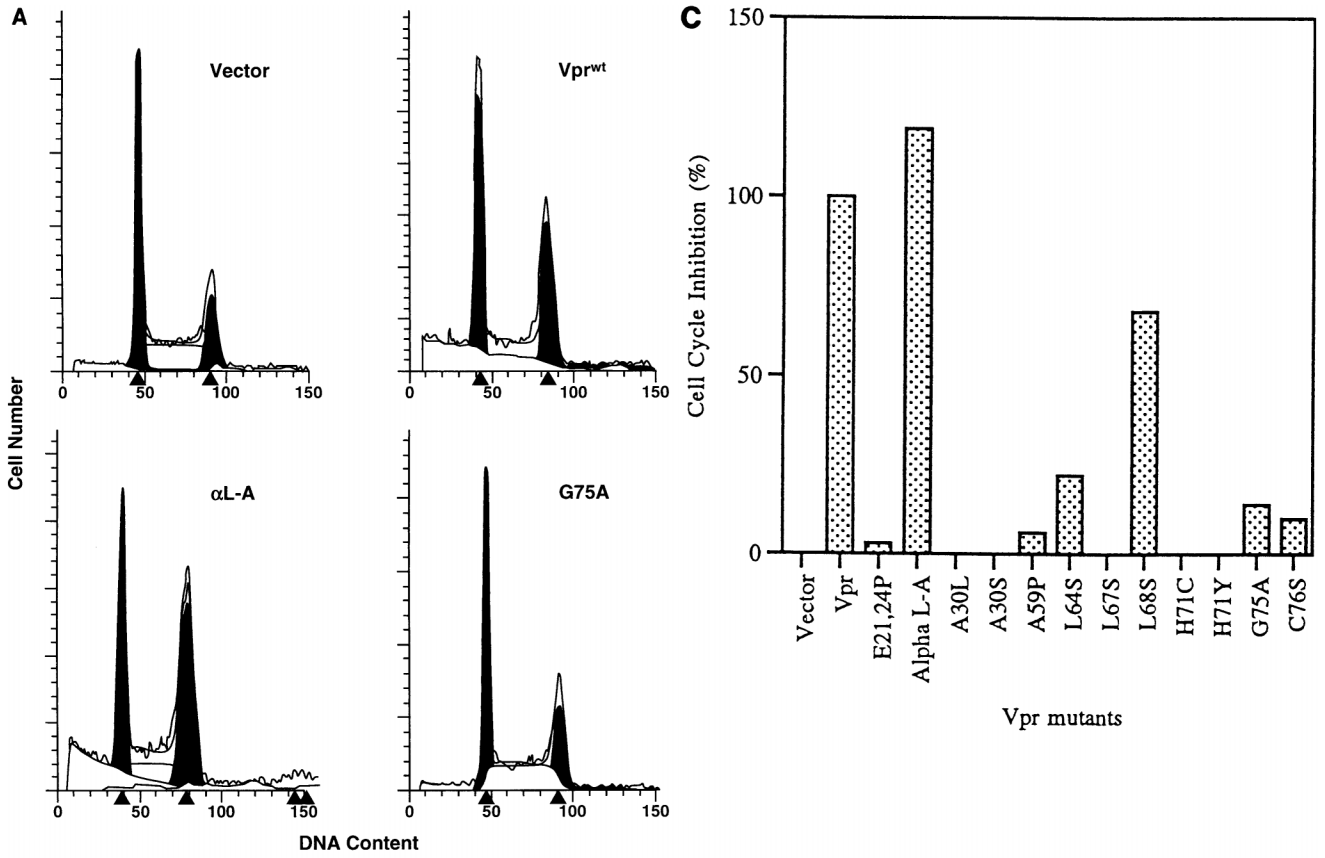

FIG. 4. Expression of HIV-1 *vpr* independent of other viral genes inhibits cell proliferation. RD cells expressing Vpr arrest in the cell cycle with 4N DNA content. (A) Flow cytometric analysis of RD cells stained with propidium iodide. RD cells were transfected with vector alone, wild-type Vpr (Vpr^{wt}), or Vpr mutants. (B) Morphology of RD cells expressing Vpr. RD cells were transfected with vector alone (a) and wild-type Vpr (b). After 2 days, the cells were maintained in DMEM containing puromycin (2 μg/ml). The cells were photographed 5 to 6 days later. (C) Cell cycle arrest activity of Vpr mutants.

shown that Vpr induces cell differentiation (29) and growth arrest and blocks the cell cycle at the G_2/M phase (38, 47). We analyzed a number of Vpr expression plasmids to identify the amino acid residues essential for cell cycle arrest and to define the interrelationship between the virion incorporation, subcellular localization, and cell cycle arrest functions of Vpr. Human rhabdomyosarcoma cells were transiently transfected with Vpr expression plasmids and selected with puromycin. In parallel, cells were transfected with vector alone as controls. Transfected cells were fixed and stained with propidium iodide as whole cells to determine DNA content by flow cytometry. There was a dramatic increase in the proportion of cells in the G_2/M phase of cell division in cells transfected with a wild-type Vpr expression plasmid (Fig. 4A), whereas vector-transfected

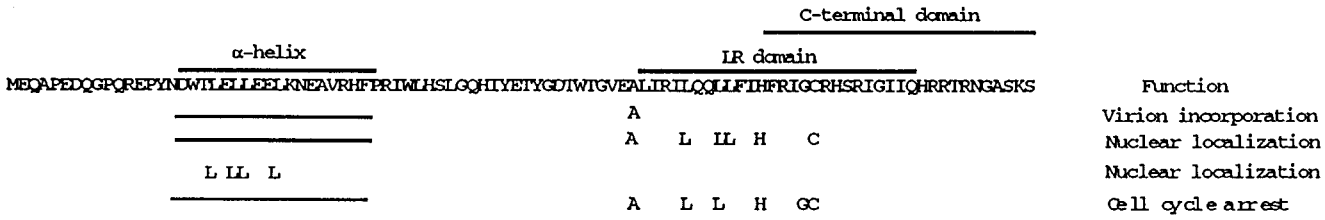

FIG. 5. Domains of Vpr required for virion incorporation, subcellular localization, and cell cycle arrest/differentiation. The amino acid sequence of macrophage-tropic clone 89.6 Vpr is shown, with the α-helical, LR, and C-terminal domains indicated. Critical amino acid residues and domains essential for different functions of Vpr were determined by mutational analysis.

cells showed DNA content similar to that of unsynchronized cycling cells which were grown without puromycin (Fig. 4A). Cells expressing Vpr showed morphological changes such as increased adherence and growth arrest consistent with a terminal differentiated phenotype (Fig. 4B). These results confirm our previous report that Vpr expression in cells is capable of altering cell cycle distribution and morphological status.

We measured the relative cell cycle blocking activity of each mutant Vpr molecule in the transient transfection assay to identify the amino acid(s) important for cell cycle arrest. We noticed some experimental variability in the G_2/M ratio of transfected cells, with both wild-type and mutant Vpr, upon repeated experimentation. Results of the analysis showed that Vpr mutants αL-A and L68S maintained cell cycle blocking activity (Fig. 4C). The cell cycle arrest activity of Vpr mutants E21,24P, A30S, A30L, A59P, L64S, L67S, H71C, H71Y, G75A, and C76S was dramatically reduced (Fig. 4C). We believe that this finding is not likely to be due to instability of the protein, since these mutant molecules are stably expressed (Fig. 1C). Vpr mutants A30L and G75A were inactive in cell cycle arrest while retaining the wild-type nuclear localization (Fig. 3). Of all of the Vpr mutants, αL-A and L68S maintained cell cycle blocking activity, even though these mutant molecules localized in the cytoplasm. In contrast, Vpr mutants E21,24P, L64S, and L67S were inactive in cell cycle arrest and were localized in the cytoplasm (Fig. 3). These results clearly indicate that the helical domain and amino acids in the carboxy-terminal domain control the cell cycle arrest function of Vpr. In support of this inference, Vpr from HXB2 (18-amino-acid deletion in the C terminus) does not induce either cell cycle arrest or morphological differentiation and exhibits the diffused nuclear localization phenotype. These results support the importance of helical domains for the virion incorporation, nuclear localization, and cell cycle arrest functions of Vpr. Importantly, these Vpr mutants clearly segregate the nuclear localization and cell cycle arrest functions as well as morphological differentiation (Table 1).

DISCUSSION

Vpr is unique among the HIV-1 accessory proteins because of its association with virus particles. Recently, we have shown that the protein encoded by the *gag* gene is sufficient for incorporation of Vpr into virus particles (see Fig. 6A) (35, 36). The presence of Vpr in the virion 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 be localized in the nucleus and to arrest cells at the G_2/M phase of cell division (21, 33, 37, 38, 47). To define the interrelationship between the different functions (virion incorporation, nuclear localization, cell cycle arrest, and differentiation) of Vpr, we generated a number of molecules with mutations in different structural domains of Vpr. Our results clearly show that virion incorporation and nuclear localization of Vpr are controlled by putative helical domains and the LR domain, while cell cycle arrest is largely controlled by the C-terminal domain (amino acids 71 to 82) (Fig. 5). Most notable, however, was that incorporation experiments showed that proline substitution mutants were not incorporated into virus-like particles. The helix-destabilizing properties of prolines are well documented (7, 50). Substitution of proline for glutamic acids 21 and 24 (E21,24P) and alanine 59 (A59P) in the helical domains abrogated Vpr incorporation into virus-like particles, suggesting that putative helical domains are required for the virion incorporation of Vpr.

Mutations in alanine 30 (A30S and A30L) resulted in markedly reduced levels of Vpr incorporation into virus-like particles. Furthermore, substitution of alanine for four leucines (αL-A) in the helical domain resulted in a Vpr mutant which was not incorporated into virus-like particles, suggesting the importance of hydrophobic leucines in the helical domains for virion packaging. All of the helical domain mutants except A30L showed impaired virion incorporation and nuclear localization. These impairments may be due to altered structural conformation and/or stability of the mutant Vpr proteins. An analysis of the protein helical motif, correlated with specific biological functions, suggests that amino acid residues in the helices are essential for normal processing and stability by ensuring proper conformation (7, 36, 50). In accordance with these observations, the putative helical domains present in Vpr play an important role in virion packaging into virus particles.

Immunofluorescence analysis clearly indicates that the putative helical and LR domains of Vpr play an important role in the transport of Vpr into the nucleus. The importance of helical domains for nuclear targeting is supported by the impairment of nuclear localization for substitution mutants E21,24P and A59P, which were shown to disrupt the conformation of the helical structure. In contrast, mutation in the hydrophobic face of the helix in Vpr (A30L) resulted in a mutant protein that retained the ability to localize in the nucleus but diminished the virion incorporation function of Vpr. Replacement of leucines with serines in the LR domain abrogated the nuclear localization but retained the virion packaging ability of Vpr. The mechanism by which Vpr is transported to the nucleus and the means by which these residues mediate these functions are unknown. The sequences identified for the nuclear localization of Vpr do not contain a canonical nuclear targeting motif which could be expected to be directly responsible for nuclear targeting. Helical structures are known to support protein-protein interactions (7), and thus it may be through association with cellular protein(s) that Vpr translocates to the nucleus (Fig. 6B). The recent observations from our laboratory and others suggest that Vpr can associate with a cellular factor(s) is consistent with this hypothesis (46, 58). These factors include a

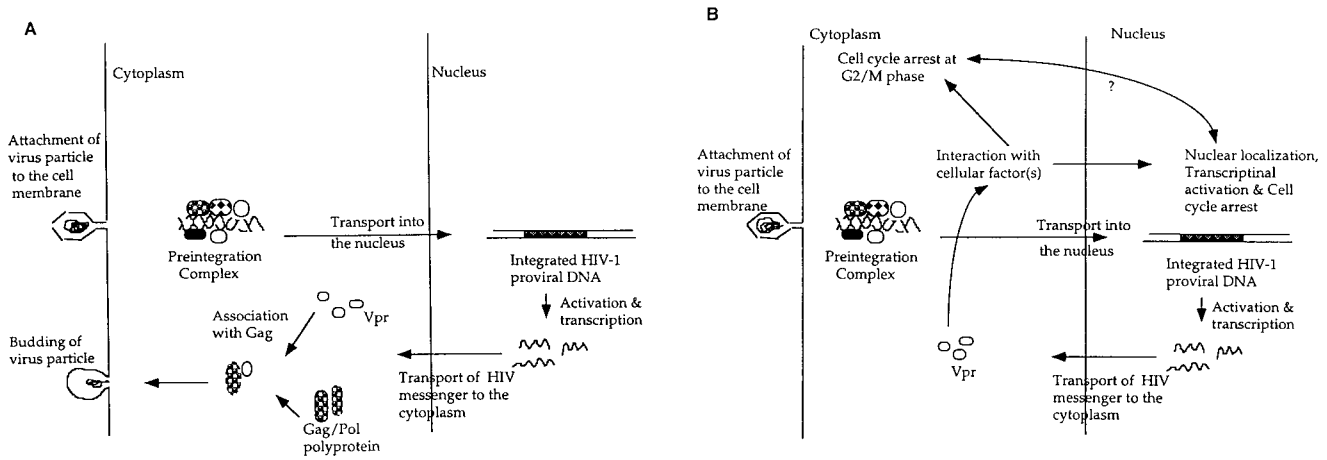

FIG. 6. Model for the different functions of HIV-1 Vpr. (A) Scheme for the incorporation of Vpr into virus particles through interaction with structural polyprotein $p55^{gag}$. (B) Vpr's nuclear localization and cell cycle arrest functions, which were mediated by its interactions with a cellular cofactor(s).

putative glucocorticoid receptor complex which may be translocated to the nucleus with Vpr (Fig. 6B) (46). Zhao et al. (58) recently demonstrated that a mutation in the LR domain, involved in the interaction with a cellular protein (RIP), abolishes the protein-protein interactions. RIP has been shown to be present in the cytoplasm and nucleus. It is possible that the translocation of Vpr into the nucleus is related to its association with the RIP protein or protein complex. In contrast to the helical and LR domains, the conserved Gly75 and Cys76 in the C terminus are dispensable for the nuclear translocation and virion incorporation properties of Vpr. These results suggest that Vpr nuclear translocation does not require a typical NLS. All of the proteins are synthesized in the cytoplasm, and some are transported to the nucleus through recognition of a specific NLS rich in basic amino acid residues. However, others are transported through piggyback binding to another protein which has an NLS (15). It is also possible that nuclear transport of Vpr is not NLS dependent.

Interestingly, of all of the Vpr mutant molecules, A30L and G75A retained the wild-type nuclear localization pattern but were impaired in the cell cycle arrest function. In contrast, Vpr mutants E21,24P, αL-A, L64S, L67S, and L68S retained the ability to arrest the cell at the G_2/M phase but failed to localize in the nucleus. In addition, Vpr mutant A59P was impaired for all functions of Vpr despite detectable expression (Fig. 1C). Presumably, Vpr blocks cell division by modifying a $p34^{cdc2}$-cyclin B protein complex which is involved in cell cycle regulation. Vpr mutants that fail to arrest the cell cycle are not likely to interact with a cellular factor involved in cell cycle regulation or may interact without triggering signals necessary for cell cycle arrest.

The importance of Vpr's ability to arrest the cell cycle, and its direct relevance to HIV-1 replication in monocytes/macrophages or AIDS pathogenesis, is under intensive investigation. Vpr may nonspecifically interfere with the complicated events of cell division. However, results from our laboratory and others suggest that this is not the case, since two Vpr mutants (A30L and G75A) localized in the nucleus but were not involved in the cell cycle arrest function of Vpr. In contrast, Vpr mutant L68S localized in the cytoplasm and maintained the cell cycle arrest activity. Vpr is capable of importing a large preintegration complex into the nuclei of nondividing cells (22). Results obtained from the putative helical and LR domain mutants clearly indicate that nuclear localization is not essential for the cell cycle arrest activity of Vpr. In this context, Fletcher et al. (17) reported that nuclear import of a preintegration complex and cell cycle arrest activities are mediated by two separate genes (*vpx* and *vpr*) in HIV-2/SIV. The identification of the amino acid residues of Vpr required for virion incorporation, nuclear localization, cell cycle arrest, and host cell differentiation may have practical importance. Recently, lentiviruses have been suggested to have utility as gene therapy vectors due to their inherent unique property of nuclear import and genome integration in nondividing cells (42). The segregation of the nuclear import function of Vpr from the cell cycle arrest/differentiation function allows for construction of gene delivery vectors which take advantage of the nuclear import property of Vpr without other, less desirable Vpr activities.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Eastern Technology Council and NIH (SPIRAT) to D.B.W.

REFERENCES

1. **Adachi, A., N. Ono, H. Sakai, K. Ogawa, R., Shibata, T. Kiyomasu, H. Masuike, and S. Ueda.** 1991. Generation and characterization of the human immunodeficiency virus type 1 mutants. Arch. Virol. **117:**45–58.
2. **Akari, H., J. Sakuragi, Y. Takebe, K. Tomonaga, M. Kawamura, M. Fukasawa, T. Miura, T. Shinjo, and M. Hayami.** 1992. Biological characterization of the human immunodeficiency virus type 1 and type 2 mutants in human peripheral blood mononuclear cells. Arch. Virol. **123:**157–167.
3. **Arrigo, S. J., and I. S. Y. Chen.** 1991. Rev is necessary for translation but not cytoplasmic accumulation of HIV-1 vif, vpr, and env/vpu 2 RNAs. Genes Dev. **5:**808–819.
4. **Balliet, J. W., D. L. Kolson, G. Eiger, F. M. Kim, K. A. Mcgann, A. Srinivasan, and R. G. Collman.** 1994. Distinct effects in primary macrophages and lymphocytes of human immunodeficiency virus type 1 accessory genes *vpr*, *vpu* and *nef*: mutational analysis of a primary HIV-1 isolate. Virology **200:** 623–631.
5. **Balotta, C., P. Lusso, R. Crowley, R. C. Gallo, and G. Franchini.** 1993. Antisense phosphorothioate oligodeoxynucleotides targeted to the *vpr* gene inhibit human immunodeficiency virus type 1 replication in primary human macrophages. J. Virol. **67:**4409–4414.
6. **Chae, H. Z., T. B. Uhm, and S. G. Rhee.** 1994. Dimerization of thiol-specific antioxidant and the essential role of cysteine 47. Proc. Natl. Acad. Sci. USA **91:**7022–7026.
7. **Chou, P. Y., and G. D. Fasman.** 1978. Empirical predictions of protein conformation. Annu. Rev. Biochem. **47:**251–276.
8. **Cohen, E. A., E. F. Terwilliger, Y. Jalinoos, J. Proulx, J. G. Sodroski, and W. A. Haseltine.** 1990. Identification of HIV-1 vpr product and function. J. Acquired Immune Defic. Syndr. **3:**11–18.
9. **Cohen, E. A., G. Dehni, J. G. Sodroski, and W. A. Haseltine.** 1990. Human immunodeficiency virus *vpr* product is a virion-associated regulatory protein. J. Virol. **64:**3097–3099.

10. **Connor, R. I., B. K. Chen, S. Choe, and N. R. Landau.** 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology **206:**935–944.
11. **Creighton, T. E.** 1988. Disulfide bonds and protein stability. Bioessays **8:**57–63.
12. **Cullen, B. R.** 1991. Regulation of human immunodeficiency virus replication. Annu. Rev. Microbiol. **45:**219–250.
13. **Dedera, D., W. Hu, N. Vander Heyden, and L. Ratner.** 1989. Viral protein R of human immunodeficiency virus types 1 and 2 is dispensable for replication and cytopathogenicity in lymphoid cells. J. Virol. **63:**3205–3208.
14. **DiMarzio, P., S. Choe, M. Ebright, R. Knoblauch, and N. R. Landau.** 1995. Mutational analysis of cell cycle arrest, nuclear localization, and virion packaging of human immunodeficiency virus type 1 Vpr. J. Virol. **69:**7909–7916.
15. **Dingwall, C., and R. A. Laskey.** 1991. Nuclear targeting sequences—a consensus? Trends Biochem. Sci. **16:**478–481.
16. **Doig, A. J., and D. H. Williams.** 1991. Is the hydrophobic effect stabilizing or destabilizing in proteins? The contribution of disulphide bonds to protein stability. J. Mol. Biol. **217:**389–398.
17. **Fletcher, T. M., B. Brichacek, N. Sharova, M. A. Newman, G. Stivatis, P. M. Sharp, M. Emerman, B. H. Hahn, and M. Stevenson.** 1996. Nuclear import and cell cycle arrest functions of the HIV-1 Vpr protein are encoded by two separate genes in HIV-2/SIVsm. EMBO J. **15:**6155–6165.
18. **Fuerst, T. R., P. L. Earl, and B. Moss.** 1987. Use of a hybrid virus-T7 RNA polymerase system for expression of target genes. Mol. Cell. Biol. **7:**2538–2544.
19. **Garrett, E. D., L. S. Tiley, and B. R. Cullen.** 1991. Rev activates expression of the human immunodeficiency virus type 1 *vif* and *vpr* gene products. J. Virol. **65:**1653–1657.
20. **Gibbs, J. S., A. A. Lackner, S. M. Lang, M. A. Simon, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1995. Progression to AIDS in the absence of a gene for Vpr and Vpx. J. Virol. **69:**2378–2383.
21. **He, J., S. Choe, R. Walker, P. DiMarzio, D. O. Morgan, and N. R. Landau.** 1995. The human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G_2 phase of the cell cycle by inhibiting $p34^{cdc2}$ activity. J. Virol. **69:**6705–6711.
22. **Heinzinger, N. K., M. I. Bukrinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M. A. Lee, H. E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman.** 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in non-dividing host cells. Proc. Natl. Acad. Sci. USA **91:**7311–7315.
23. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
24. **Jowett, J. B. M., V. Planelles, B. Poon, N. P. Shah, M.-L. Chen, and I. S. Y. Chen.** 1995. The human immunodeficiency virus type 1 *vpr* gene arrests infected T cells in the G_2+M phase of the cell cycle. J. Virol. **69:**6304–6313.
25. **Kneller, D. G., F. E. Cohen, and R. Langridge.** 1990. Improvements in protein secondary structure prediction by an enhanced neural network. J. Mol. Biol. **214:**171–182.
26. **Kondo, E., F. Mammano, E. A. Cohen, and H. G. Göttlinger.** 1995. The $p6^{gag}$ domain of human immunodeficiency virus type 1 is sufficient for the incorporation of Vpr into heterologous virus particles. J. Virol. **69:**2759–2764.
27. **Lang, S. M., M. Weeger, C. Stahl-Hennig, C. Coulibaly, G. Hunsmann, J. Müller, H. Müller-Hermelink, D. Fuchs, H. Wachter, M. M. Daniel, R. C. Desrosiers, and B. Fleckenstein.** 1993. Importance of *vpr* for infection of rhesus monkeys with simian immunodeficiency virus. J. Virol. **67:**902–912.
28. **Lavallée, C., X. J. Yao, A. Ladha, H. Göttlinger, W. Haseltine, and E. A. Cohen.** 1994. Requirement of the $Pr55^{gag}$ precursor for incorporation of the Vpr product into human immunodeficiency virus type 1 viral particle. J. Virol. **68:**1926–1934.
29. **Levy, D. N., L. S. Fernandes, W. V. Williams, and D. B. Weiner.** 1993. Induction of cell differentiation by human immunodeficiency virus 1 vpr. Cell **72:**541–550.
30. **Levy, D. N., Y. Refaeli, R. R. MacGregor, and D. B. Weiner.** 1994. Serum Vpr regulates productive infection and latency of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **91:**10873–10877.
31. **Levy, D. N., Y. Refaeli, and D. B. Weiner.** 1995. Extracellular Vpr protein increases cellular permissiveness of human immunodeficiency virus replication and reactivates virus from latency. J. Virol. **69:**1243–1252.
32. **Levy, D. N., Y. Refaeli, and D. B. Weiner.** 1995. The Vpr regulatory gene of human immunodeficiency virus, p. 209–236. *In* I. S. Y. Chen, H. Koprowski, A. Srinivasan, and P. K. Vogt (ed.), Transactivating functions of human retroviruses. Springer-Verlag, Berlin, Germany.
33. **Lu, Y. L., P. Spearman, and L. Ratner.** 1993. Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. J. Virol. **67:**6542–6550.
34. **Macreadie, I. G., L. A. Castelli, D. R. Hewish, A. Kirkpatrick, A. C. Ward, and A. A. Azad.** 1995. A domain of human immunodeficiency virus type 1 Vpr containing repeated H(S/F)RIG amino acid motifs causes cell growth arrest and structural defects. Proc. Natl. Acad. Sci. USA **92:**2770–2774.
35. **Mahalingam, S., S. A. Khan, M. A. Jabbar, C. E. Monken, R. G. Collman, and A. Srinivasan.** 1995. Identification of residues in the N-terminal acidic domain of HIV-1 Vpr essential for virion incorporation. Virology **207:**297–302.
36. **Mahalingam, S., S. A. Khan, R. Murali, M. A. Jabbar, C. E. Monken, R. G. Collman, and A. Srinivasan.** 1995. Mutagenesis of the putative alpha helical domain of HIV-1 Vpr: effect on stability and virion incorporation. Proc. Natl. Acad. Sci. USA **92:**3794–3798.
37. **Mahalingam, S., R. G. Collman, M. P. Patel, C. E. Monken, and A. Srinivasan.** 1995. Role of the conserved dipeptide Gly75 and Cys76 on HIV-1 Vpr function. Virology **210:**495–500.
38. **Mahalingam, S., B. MacDonald, K. E. Ugan, V. Ayyavoo, M. G. Agadjayan, W. V. Williams, and D. B. Weiner.** 1997. In vitro and in vivo tumor growth suppression by HIV-1 Vpr. DNA Cell Biol. **16:**137–143.
39. **Matsuda, Z., X. Yu, Q. C. Yu, T. H. Lee, and M. Essex.** 1993. A virion specific inhibitory molecule with therapeutic potential of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **90:**3544–3548.
40. **Mayers, G., B. Korber, J. A. Berzofsky, R. F. Smith, and G. N. Pavlakis.** 1993. Human retroviruses and AIDS. Los Alamos National Laboratory, Los Alamos, N.Mex.
41. **Mustafa, F., and H. L. Robinson.** 1993. Context-dependent role of human immunodeficiency virus type 1 auxiliary genes in the establishment of chronic virus producers. J. Virol. **67:**6909–6915.
42. **Naldini, L., U. Blomer, P. Gallay, D. Ory, R. Mulligan, F. H. Gage, I. M. Verma, and D. Trono.** 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science **272:**263–267.
43. **Ogawa, K., R. Shibata, T. Kiyomasu, I. Higuchi, Y. Kishida, A. Ishimoto, and A. Adachi.** 1989. Mutational analysis of the human immunodeficiency virus *vpr* open reading frame. J. Virol. **63:**4110–4114.
44. **Paxton, W., R. I. Connor, and N. R. Landau.** 1993. Incorporation of *vpr* into human immunodeficiency virus type 1 virions: requirement for the p6 region of *gag* and mutational analysis. J. Virol. **67:**7229–7237.
45. **Re, F., D. Braaten, E. K. Franke, and J. Luban.** 1995. The human immunodeficiency virus type 1 Vpr arrests the cell cycle in G_2 by inhibiting the activation of $p34^{cdc2}$-cyclin B. J. Virol. **69:**6859–6864.
46. **Refaeli, Y., D. N. Levy, and D. B. Weiner.** 1995. The glucocorticoid receptor type II complex is a target of the HIV-1 Vpr gene product. Proc. Natl. Acad. Sci. USA **92:**3621–3625.
47. **Rogel, M. E., L. I. Wu, and M. Emerman.** 1995. The human immunodeficiency virus type 1 *vpr* gene prevents cell proliferation during chronic infection. J. Virol. **69:**882–888.
48. **Saier, M. H., Jr., and P. McCaldon.** 1988. Statistical and functional analyses of viral and cellular proteins with N-terminal amphipathic α-helices with large hydrophobic moments: importance to macromolecular recognition and organelle targeting. J. Bacteriol. **170:**2296–2300.
49. **Subramanian, R. A., and E. A. Cohen.** 1994. Molecular biology of the human immunodeficiency virus accessory proteins. J. Virol. **68:**6831–6835.
50. **Tacke, E., J. Schmitz, D. Prufer, and W. Rohde.** 1993. Mutational analysis of the nucleic acid-binding 17 KDa phosphoprotein of potato leafroll luteovirus identifies an amphipathic α-helix as the domain for protein/protein interactions. Virology **197:**274–282.
51. **Tristem, M., C. Marshall, A. Karpas, and F. Hill.** 1992. Evolution of the primate lentiviruses: evidence from vpx and vpr. EMBO J. **11:**3405–3412.
52. **Wang, J. J., Y. L. Lu, and L. Ratner.** 1994. Particle assembly and Vpr expression in human immunodeficiency virus type 1 infected cells demonstrated by immunoelectron microscopy. J. Gen. Virol. **75:**2607–2614.
53. **Wu, X., J. A. Conway, J. Kim, and J. C. Kappes.** 1994. Localization of the Vpx packing signal within the C terminus of the human immunodeficiency virus type 2 Gag precursor protein. J. Virol. **68:**6161–6169.
54. **Yao, X. J., R. Subramanian, N. Rougeau, F. Boisvert, D. Bergeron, and E. A. Cohen.** 1995. 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. J. Virol. **69:**7032–7044.
55. **Yu, X., M. Matsuda, M. Essex, and T. H. Lee.** 1990. Open reading frame *vpr* of simian immunodeficiency virus encodes a virion-associated protein. J. Virol. **64:**5688–5693.
56. **Yu, X., Z. Matsuda, Q. C. Yu, T. H. Lee, and M. Essex.** 1993. Vpx of simian immunodeficiency virus is localized primarily outside the virus core in mature virions. J. Virol. **67:**4386–4390.
57. **Yuan, X., Z. Malsuda, M. Matsuda, M. Essex, and T. H. Lee.** 1990. Human immunodeficiency virus vpr gene encodes a virion-associated protein. AIDS Res. Hum. Retroviruses **6:**1265–1271.
58. **Zhao, I.-J., S. Mukherjee, and O. Narayan.** 1994. Biochemical mechanism of HIV-1 Vpr function: specific interaction with a cellular protein. J. Biol. Chem. **269:**15577–15582.
59. **Zhao, L.-J., L. Wang, S. Mukherjee, and O. Narayan.** 1994. Biochemical mechanism of HIV-1 Vpr function: oligomerization mediated by the N-terminal domain. J. Biol. Chem. **269:**32131–32137.