The Cationic Amphipathic α-Helix of HIV-1 Viral Protein R (Vpr) Binds to Nucleic Acids, Permeabilizes Membranes, and Efficiently Transfects Cells*

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Viral protein R (Vpr) is a small protein of 96 amino acids that is conserved among the lentiviruses human immunodeficiency virus type 1 (HIV-1), HIV-2, and simian immunodeficiency virus. We recently sought to determine whether the karyophilic properties of Vpr, as well as its ability to bind nucleic acids, could be used to deliver DNA into cells. We have found that the C-terminal domain of Vpr-(52–96) is able to efficiently transfect various cell lines. Here, we show that the shortest active sequence for gene transfer corresponds to the domain that adopts a α-helix conformation. DNA binding studies and permeabilization assays performed on cells demonstrated that the peptides that are efficient in transfection condense plasmid DNA and are membranolytic. Electron microscopy studies and transfection experiments performed in the presence of inhibitors of the endocytic processes indicated that the major entry pathway of Vpr-DNA complexes is through endocytosis. Taken together, the results show that the cationic C-terminal α-helix of Vpr has DNA-condensing as well as membrane destabilizing capabilities, both properties that are indispensable for efficient DNA transfection.

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The human immunodeficiency virus type 1 (HIV-1) is a member of the lentivirus subfamily of retroviruses. In addition to the gag, pol, and env genes found in all retroviruses, the HIV-1 genome contains six accessory genes: tat, rev, vif, vpr, vpu, and nef. Viral protein R (Vpr), a 96-amino acid protein, is produced late in the virus life cycle and is packed in the viral particle (1, 2). Although Vpr is dispensable for viral replication in cell culture, several critical activities have been attributed to this small protein. In particular, Vpr is known to play an important role in facilitating infection of macrophages (3, 4) as well as in inducing cell cycle arrest in the G2 phase of infected cells (5, 6). Other biological functions ascribed to Vpr include:

(i) transcriptional activation of the HIV-1 long terminal repeat and of various heterologous promoters (7), (ii) co-activation of the human glucocorticoid receptor (8), (iii) induction of apoptosis (9, 10), and (iv) formation of ion channels in lipid bilayers (11).

Unlike other retroviruses, HIV-1 is able to replicate in non-dividing cells. How the preintegration complex is imported in the nucleus remains unclear. Four viral components of the preintegration complex reportedly have karyophilic properties, namely the integrase, the matrix protein, Vpr, and the central DNA flap (12–16). Active transport of proteins into the nucleus requires specific peptide signals referred to as nuclear localization signals. Although Vpr does not contain a canonical nuclear localization signal, it localizes to the nucleus, it can interact with host proteins related to nuclear transport such as importin-α and nucleoporins, and it promotes nuclear entry of viral nucleic acids in nondividing macrophages (17–19). Finally, it was reported that Vpr can disrupt the nuclear envelope, thereby providing a possible entry route for the preintegration complex (20).

The different activities of Vpr may require distinct functional domains which remain ill characterized. Future structure-function studies, however, will be facilitated because recent investigations allowed the determination of the structure of Vpr (21–24). It is characterized by a flexible N-terminal region, a middle domain that spans the N-terminal α-helix-(40–48)–γ turn-(49–54)–α helix-(55–83), and it ends with a flexible C-terminus (23). Of note, the N-terminus is negatively charged, whereas numerous basic amino acids are found in the C-terminal domain of Vpr (Fig. 1). Moreover, the amphipathic α helix3-(55–83) overlaps with a leucine-rich domain that contains a short leucine zipper-like motif (Fig. 1).

The goal of nonviral gene transfer is to mimic the successful viral mechanisms for overcoming cellular barriers while minimizing the problems associated with the use of biological vectors. Most nonviral vectors are able to complex DNA and facilitate its entry into the cell as well as its escape from the endosome. Yet, nuclear transport remains the major bottleneck in successful gene transfer with synthetic DNA carriers (25, 26). Considering the karyophilic properties of Vpr as well as its ability to bind nucleic acids (27, 28), we have recently explored the possibility of using Vpr as a DNA transfection agent. We have found that the C-terminal fragment-(52–96) of Vpr, but not the whole protein, is able to deliver DNA efficiently into different cell lines (29). It was the first example of a peptide derived from a natural protein displaying such a high transfection activity in the absence of auxiliary agents. In the present work, we have determined the shortest Vpr sequence with gene transfer activity and studied the different steps of Vpr-
mediated transfection from DNA compaction to endosomal escape of complexes.

EXPERIMENTAL PROCEDURES

Materials—Dimethyldialcitrile, cytochalasin B, methyl-β-cyclodextrin, cholesterol-charged methyl-β-cyclodextrin, and poly-l-lysine-HBr (PLL; degree of polymerization (dp) = 180) were from Sigma. DOTAP was from Avanti Polar Lipids Inc., and polyethylenimine (PEI), 25 kDa, was from Aldrich. PS-MD2-Luc3TR (7.6 kb) and PAAV-NLS-LacZ (8.3 kb) are expression plasmids encoding, respectively, the firefly luciferase and the β-galactosidase under the control of the human cytomegalovirus immediate-early promoter (CMV) promoter. Human recombinant protein of HIV-1 strain 89.6, which has the following sequence: MEQA–, was synthesized by Syntem. Melittin was purchased from Sigma.

These results were then expressed as light units/10 s/mg of protein. The results were normalized by a Bradford protein quantification assay.

Electrotransfection—DNA binding was studied by means of agarose gel retardation assays. One μg of DNA and increasing amounts of peptide were each diluted in 25 μl of 150 mM NaCl and mixed. After 20 min, samples were electrophoresed through a 1% agarose gel using Tris borate-EDTA buffer, and DNA was visualized after ethidium bromide staining.

Cell Culture—The culture medium Dulbecco’s modified Eagle’s medium Glutamax (DMEM, Invitrogen) was supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (Hyclone). We used the following two cell lines in our experiments: human hepatocarcinoma cells (HepG2, ATCC) and transformed human embryonic kidney cells (HEK-293, ATCC).

The complexes—Cells were plated 1 or 2 days before transfection to obtain a confluence of 60–80% at the time of the experiment. For experiments performed in 24- or 48-well plates, 4 or 2 μg, respectively, of plasmid DNA and the desired amount of peptide, PLL, DOTAP, or PEI were diluted into 100 or 50 μl, respectively, of 150 mM NaCl and gently mixed. After 20 min of incubation, the mixture was diluted with serum-free medium to a final volume of 1 or 0.4 ml, respectively; 0.5 or 0.2 ml, respectively, of the transfection mixture was then put on each well of the duplicate for 3 h. The transfection medium was then replaced with DMEM, 10% fetal calf serum, and transgene expression was evaluated 24–48 h after the beginning of the transfection. Each experiment was carried out several times; within a series, experiments were done in duplicates.

Transfection in the presence of dimethyldialcitrile (final concentration 62.5–250 μM) and cytochalasin B (final concentration 5–40 μM) were performed as described above except that the drug was added to the cells in serum-free medium prior to the addition of complexes (10 and 30 min before transfection for dimethyldialcitrile and cytochalasin B, respectively). For transfections with methyl-β-cyclodextrin (MβCD) or cholesterol-charged methyl-β-cyclodextrin (MβCD-Chol; at a final concentration of 5–10 μM), cells were incubated for 1 h with the drug in serum-free medium before transfection. The transfection experiments involving chloroquine (Sigma) at a final concentration of 100 μM were done as described above except that the drug was added after dilution of the complexes with DMEM, just prior to the addition of the transfection medium to the cells.

Transgene Expression—The luciferase assay was performed as described previously (29). Luciferase background was subtracted from each value, and the transfection efficiency, expressed as light units/10 s/well (with 1 light unit = 10 counts), is the mean of duplicates. When drugs (dimethyldialcitrile, methyl-β-cyclodextrin, and cholesterol-charged methyl-β-cyclodextrin) were present during transfection, the results were normalized by a Bradford protein quantification assay. These results were then expressed as light units/10 s/mg of protein. The LacZ activity was measured by chemiluminescence as recommended by the manufacturer (Tropix).

Exclusion Assay—One μg of DNA was complexed with increasing amounts of peptide in a final volume of 50 μl. Fifty μl of a 150 mM NaCl solution containing ethidium bromide (8 μg/ml) was then added to the complexes. The fluorescence resulting from ethidium bromide intercalation in DNA was measured with a 96-well fluorimeter (Spectramax, Gemini; excitation 485 nm, emission 590 nm). Results were expressed as the percentage of the maximum fluorescence signal when ethidium bromide was bound to DNA in the absence of competition.

Erythrocyte Lysis Assay—After centrifugation of 10 ml of fresh human blood for 10 min at 1000 × g, the plasma and the white layer of leukocytes were removed. The erythrocytes were washed five times with 11 ml sodium citrate in Hepes-buffered saline, pH 7.3. The solution was then divided into two aliquots, which were washed three times and resuspended in assay buffer with the appropriate pH (200 mM sodium citrate, pH 5, or 11 mM sodium citrate in Hepes-buffered saline, pH 7.3) at a concentration of 107 cells/ml. A 75-μl aliquot of erythrocytes was added to each well of a 96-well one-type microtiter plate containing 75 μl of a serial dilution of the compound to be tested in assay buffer. The plate was then gently shaken for 1 h at 37°C. Controls (100% lysis) were obtained by incubating erythrocytes with 4 μl of Triton X-100 or assay buffer. After removal of the unlysed erythrocytes by centrifugation for 5 min at 1000 × g, 75 μl of the supernatant was transferred to a new microtiter plate (flat-bottom), and hemoglobin absorption was determined at 450 nm (background correction at 750 nm). The lysis percentage was given by the following formula:

\[ \frac{1}{1 + \text{OD}_{450}} \times \text{OD}_{450} - \text{OD}_{750} \cdot \text{tRNA} = \text{OD}_{450} - \text{OD}_{750} \cdot \text{hemoglobin} \cdot \text{OD}_{450} - \text{OD}_{750} \cdot \text{Triton X-100} - \text{OD}_{750} \cdot \text{hemoglobin} \]

Cell Permeabilization—HepG2 cells, plated in 24-well plates, were incubated for 1 h at 37°C to room temperature to block endocytosis. The test compound, diluted in 250 μl of PBS containing 5 μg of ethidium bromide, was then added to the cells. After a 30-min incubation at room temperature in the dark under gentle shaking, cells were washed once with PBS, harvested with 1 mM EDTA/PBS, and analyzed by flow cytometry (FACScalibur, BD Biosciences).

Electron Microscopy—Peptides were mixed with 0.02 μg/ml DNA in a final volume of 50 μl of NaCl, 150 mM. Five μl of the mixture was deposited onto an electron microscope grid covered with a thin carbon film previously activated by a glow discharge in the presence of pentylene. The grids were then stained with 2% aqueous uranyl acetate, drained, and blotted. The observations were done with the annular dark-field mode in a Zeiss 902 EM, filtering out inelastically scattered electrons for enhanced contrast and resolution. For intracellular trafficking studies, 20 μg of plasmid DNA was mixed with peptide in a 150 mM NaCl solution in a final volume of 1 ml. After 20 min, serum-free medium was added, and the solution was pipetted onto the cells plated 1 day earlier in a 15-cm dish. After 3 h, the transfection medium was replaced with DMEM containing 10% serum. At different times, cells were fixed with medium containing 10% fetal calf serum and 2% glutaraldehyde, harvested, and centrifuged. The pellet was resuspended in Sorenson’s buffer (67 mM phosphate buffer, pH 7.4) and then postfixed in 2% osmium tetroxide, dehydrated with ethanol and propylene oxide, and embedded in Epon. Ultrathin sections were prepared with an LKB Ultrotome. Sections of cells were colored with uranyl acetate and lead citrate and observed with a LEO 920 microscope.

RESULTS AND DISCUSSION

Definition of the Shortest Vpr-derived Peptide with Transfection Activity—To find the shortest sequence allowing efficient gene transfer, different subfragments of Vpr-(52–96) were synthesized, and their transfection activity was evaluated on two cell types. Increasing amounts of peptides were complexed to a luciferase expression plasmid (CMV-Luc) and incubated with the cells for 3 h. Luciferase activity was measured 30 h later. The cationic polymer 25-kDa PEI (30), one of the most efficient transfection reagents, was included as a positive control. On human HEK-293 cells, Vpr-(55–91), Vpr-(55–86), and Vpr-(55–82) allowed for gene transfer levels comparable with those obtained with PEI and about 10 times higher than those of Vpr-(52–96) (not shown). On human HepG2 cells, Vpr-(55–91) was the most active fragment, resulting in luciferase levels 1 log above those obtained with PEI. Vpr-(52–96), Vpr-(55–86), and Vpr-(55–82) were at least as active as PEI (Fig. 1). When five amino acid residues were removed from the N terminus of Vpr-(55–91) (Vpr-(60–91)), the transfection activity was significantly reduced, indicating that this stretch is indispensable. On the other hand, the efficiency of Vpr-(52–75), which is seven residues shorter on the C-terminal end than Vpr-(55–82), was very low. Thus, among the different subfragments, the shortest active sequence for gene delivery is Vpr-(55–82) (Fig. 1). Inter-
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estingly, this sequence corresponds to the C-terminal domain, which adopts a α-helix conformation in Vpr-(1–96) (23). The Leu60 and Leu67 side chains are located on the hydrophobic side of the helix, and it was shown that they are involved in Vpr dimerization through a leucine zipper-type mechanism. Although the replacement of these two leucines by alanine residues eliminates Vpr dimerization (21), it preserved the helical structure of the peptide and had no effect on the transfection activity of Vpr-(52–96) subfragments. We therefore wondered whether different structures of the DNA complexes could be related to differences in transfection efficiency between subfragments. We therefore evaluated the relative affinity of three peptides (Vpr-(55–91), (60–91), and (55–86)) for plasmid DNA by performing an ethidium bromide exclusion experiment. This was achieved by preparing complexes of DNA at different charge ratios and adding an excess of ethidium bromide prior to spectroscopic analysis. DNA accessibility can be evaluated with this assay because a large increase in fluorescence is observed when the phenanthridinium moiety of ethidium bromide intercalates DNA. Fig. 2A shows that maximal DNA condensation was reached for all the compounds tested, including poly-L-lysine, at a +/− charge ratio between 1 and 2. Moreover, no differences were observed between transfecting (Vpr-(55–91) and Vpr-(55–86)) and poorly transfecting (Vpr-(60–91)) peptides.

The structure of the DNA complexes was further characterized by electron microscopy. Fig. 2B, panel a, shows that DNA complexes obtained with the optimal amount of Vpr-(55–91) for gene transfer are large aggregates, likely to contain high copy numbers of plasmid DNA. Aggregates formed with subfragment-(60–91) and (55–86) were similar (Fig. 2B, panels b and c), although sometimes unbound DNA fibers that extended outward from the condensed region were observed (panels d and e). In contrast, a large part of the DNA remained uncondensed in complexes generated with Vpr-(52–70), a fragment that contains only one positively charged amino acid (Fig. 2B, panel f).

The formation of large aggregates with cationic amphipathic peptides may be explained as follows (31): positive charged residues in the peptides interact electrostatically with the negative charge of phosphate in DNA, whereas the opposite hydrophobic side creates interactions between peptide-DNA complexes, resulting in aggregation.

Taken together, the results obtained by electron microscopy and the ethidium bromide exclusion assay are in good agreement. They show that DNA compaction, even when complexes have similar structures (as judged by electron microscopy for Vpr-(55–91) and (60–91)), is not sufficient for efficient transfection.

**Entry Pathway into the Cell**—We have previously shown that Vpr-(52–96) delivers high numbers of DNA molecules into the cells (29). However, the mechanism by which the delivery is done is unknown. To evaluate this process, we used electron microscopy. At early times (i.e., after 2 h; Fig. 3) the Vpr-(52–96)-DNA complexes appeared as electron-dense particles at the cell surface. Then, as the duration of incubation increased, the complexes were taken up into the cell by an endocytic process. Once in the cytoplasm, the DNA particles were found exclusively within large vesicles and were no longer visible after 24 h (not shown).
HEK-293 cells were transfected in 15-cm plates with 150 μg of Vpr-(55–96)/20 μg of DNA complexes (charge ratio (+/-) = 3.3). The transfection was stopped after different periods of time (2, 6, 10, and 14 h). The cells were then used for electron microscopy observations. Control cells are devoid of any electron-dense material (not shown). By contrast, in transfected cells (2, 6, 10, and 14 h) electron-dense particles can be visualized (arrows). The scale bar represents 1 μm. Arrowheads and open arrows indicate endocytic vesicles and nuclei, respectively.

To investigate the uptake mechanism of DNA complexes, cells were treated before or during transfection with chemical agents that interfere with the endocytic processes. HepG2 cells were transfected with Vpr-(55–91) in presence of cytochalasin B, which inhibits phagocytosis and pinocytosis but not receptor-mediated endocytosis (32). The results show that the luciferase levels were slightly increased in the presence of the drug (data not shown). Similar observations have been reported by others using DNA formulations containing either a cationic lipid or a lipid/peptide mixture (33, 34).

Because we observed very large vesicles containing DNA complexes (Fig. 3), we further examined the uptake mechanism by using dimethylamiloride, a compound that inhibits macropinocytosis (35). In these experiments, performed with HEK-293 cells, we used a human recombinant adenovirus (Ad-LacZ) as negative control because macropinocytosis is not essential for viral uptake (36). We observed a 4-fold decrease in reporter gene expression in the presence of 250 μM dimethylamiloride for Vpr-(55–91) and PEI-mediated transfection, whereas the adenoviral transduction efficiency was slightly increased (data not shown). Thus, macropinocytosis is not an essential entry pathway for Vpr-(55–91)-DNA complexes. Finally, transfection was performed following cholesterol depletion of the plasma membrane with MβCD (37). Cholesterol depletion results in the inhibition of clathrin-mediated endocytosis, although it also affects the structure and function of invaginated caveolae, including caveolae-dependent endocytosis (38). When HepG2 cells, which lack caveolae (39), were depleted of cholesterol prior to transfection by a 1-h treatment with MβCD, the luciferase levels were decreased about 100-fold for Vpr-(55–91) and PEI-mediated transfection (Fig. 4). The efficiency of the cationic lipid DOTAP was scarcely altered (Fig. 4), whereas that of the cationic lipid/DOPE formulation, LipofectAMINE, was strongly reduced (not shown). In fact, a decrease in DOTAP activity was detected only with higher concentrations of MβCD. A similar significant reduction of the transfection efficiency after MβCD treatment was recently reported with the lipidic formulation SAINT-2/DOPE (40).

The results show that the presence of cholesterol in the membranes is essential for efficient Vpr (but also PEI and lipid)-mediated transfection, suggesting that the major entry pathway is through clathrin-mediated endocytosis.

**Homolytic Activity of Vpr Fragments**—The results described above do not exclude the possibility that DNA may also enter the cytoplasm directly. Indeed, peptides from the C-terminal region of Vpr including the conserved HFRIGCRHSRIG motif (Fig. 1) can cause permeabilization of yeast cells (41). Recent results showing that Vpr can gain access to intracellular compartments independently from the infection process support the idea that Vpr is membrane-active (24).

This opens the possibility that “active” DNA complexes could enter the cell by membrane permeabilization. Alternatively, the permeabilization activity of Vpr could be required for endosomal escape. To determine whether the membrandlytic activity is required for efficient gene transfer, we evaluated the capacity of different subfragments of Vpr to lyse freshly prepared human erythrocytes. Increasing amounts of either Vpr-(52–96), -(55–91), -(60–91), or -(1–51) were incubated with erythrocytes at neutral pH. After 1 h, the amount of hemoglobin released was measured by spectrophotometry. Fig. 5A
activity was evaluated for the subfragment Vpr-(55–91) and Vpr-(60–91), whereas C-terminal subfragments were all hemolytic at various degrees ((55–91) > (52–96) > (60–91)). This activity was maintained in the presence of DNA. Interestingly, it was strongly inhibited at acidic pH (Fig. 5B).

These results indicate that there is a correlation between hemolysis and the activity of Vpr derivatives as transfection agents. Such a correlation has been described for other peptides such as those derived from influenza HA2, which potentiate pLys-DNA complexes and destabilize membranes at acidic pH (42). In contrast to these peptides, Vpr subfragments act alone and are inactivated at acidic pH, suggesting a different mechanism for membrane permeabilization.

Cell Membrane Permeabilization Activity of Vpr Derivatives—The permeabilization activity of Vpr fragments was also evaluated on HepG2 cells. Cells were first incubated for 1 h at room temperature to reduce endocytosis, and the peptide was then added together with ethidium bromide, a poorly membrane-permeant molecule that becomes strongly fluorescent upon binding to DNA. Positive control was obtained by incubating the cells with melittin, a highly permeabilizing peptide (43), whereas incubation with ethidium bromide alone was used as negative control. The results show that several peptides, including Vpr-(55–91), -(60–91), -(52–70) (Fig. 6), and -(55–86) and -(55–82) (not shown), induced an increase of the cell fluorescence. Among the three peptides shown in Fig. 6, Vpr-(55–91) in the absence of DNA had the highest activity followed by Vpr-(60–91) and Vpr-(52–70). In the presence of DNA, the permeabilization efficiency of the peptides was not altered, except for Vpr-(55–91) (Fig. 6). These results demonstrate that Vpr subfragments are able to permeabilize plasma membranes of mammalian cells in the presence of DNA.

Influence of Cholesterol Content on Permeabilization Activity—As described above, reduction of the membrane cholesterol content reduces the transfection efficiency of Vpr-(55–91). Besides inhibiting clathrin-mediated endocytosis, cholesterol depletion can modulate the membrane disruption activity of peptides (44). To evaluate the influence of cholesterol content on the membrane disruption activity of Vpr, we pretreated HepG2 cells with either MβCD, to deplete plasma membrane of cholesterol, or with MβCD-Chol complex, to enrich the membranes with the sterol. The MβCD ± Chol treatments did not significantly modify the permeabilization activity of Vpr-(55–91) (not shown). We then checked whether MβCD-Chol treatment results in an enhanced transfection efficiency. HepG2 membranes were enriched with cholesterol before transfection. The results show that Vpr-(55–91)-mediated transfection was slightly increased under these conditions compared with control, whereas the efficiency of DOTAP and PEI was slightly reduced (not shown).

These results indicate that the membrane cholesterol content only moderately modifies the permeabilization activity of Vpr subfragments. Thus, the 2-log decrease, observed on transfection efficiency after MβCD treatment (Fig. 4), is not due to the inhibition of the permeabilization activity.

Vpr as Helper for Polylysine-mediated Transfection—pLys-DNA complexes escape rather inefficiently from internal vesicles, but endosomolytic agents such as chloroquine or fusogenic peptides can be used as helper during pLys-mediated transfection (42, 45). We reasoned that if Vpr subfragments are able to disrupt membranes, then they should be able to enhance pLys-mediated transfection. To evaluate this, pLys-CMV-Luc complexes were pre-formed, and Vpr or Vpr complexed with salmon sperm carrier DNA was added. Fig. 7 shows that in the presence of Vpr-(55–91) or Vpr-(60–91), the luciferase activity obtained with pLys was increased 23- and 37-fold, respectively, whereas the addition of an excess of pLys (±DNA) did not enhance transfection. The helper effect of the two Vpr deriva-
The smallest active fragment corresponds to the C-terminal domain, which adopts a helix conformation in Vpr(1–96).

This may suggest that this particular conformation is important for efficient gene transfer. In fact, it is interesting to note that other cationic amphipathic peptides with high transfection activities, such as KALA (46) or pPTG20 (47), are also characterized by their capacity to bind DNA, destabilize membranes, and aggregate the complexes or a more efficient nuclear transport of DNA. As the major entry pathway is through endocytosis, our results suggest that the permeabilizing activity of Vpr peptides allows endosomal escape of DNA. Release of the DNA probably takes place before acidification of the endosome occurs, because the membranolytic activity is strongly reduced at acidic pH. The escape of DNA from endosomes remains, however, a rate-limiting step with complexes being trapped in endocytic vesicles even at 10–14 h post-transfection (Fig. 3). Once released into the cytosol, DNA must be protected against enzymatic degradation and transported into the nucleus. We showed previously that when Vpr-(52–96)-DNA complexes are incubated with DNase I for 1 h at 37°C, the integrity of the plasmid is not preserved (29). Thus, DNA degradation may be another limiting step. As indicated by electron microscopy, the Vpr-DNA complexes tend to form multimolecular aggregates, which are too large to cross an intact nuclear membrane. Therefore, the ability of Vpr to transfect nondividing cells has to be investigated. However, it is possible that smaller particles are also generated. Given that reporter expression is due to a minority of plasmids entering the nucleus (29), DNA transfection may be mediated by small particles, whereas aggregates, representing the major population of the complexes, are not productive. Alternatively, the results obtained by de Noronha et al. (20) open the possibility that aggregates enter the nucleus by disrupting the nuclear envelope.
and adopt an α-helical conformation that positions lysines or arginines on one side of the helix. These features thus seem to be essential for efficient gene transfer.

Because the sequence of a peptide can be modified easily, we can envision further improvement of the transfection efficiency of Vpr either by sequence alteration or by introducing a motif that provides cell specificity.

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REFERENCES

1. Paxton, W., Connor, R. I., and Landau, N. R. (1993) J. Virol. 67, 7229–7237
2. Jenkins, Y., Pornillos, O., Rich, R. L., Myzak, D. G., Sundquist, W. I., and Malim, M. H. (2001) J. Virol. 75, 10537–10542
3. Conner, R. I., Chen, B. K., Choe, S., and Landau, N. R. (1995) Virology 206, 935–944
4. Eckstein, D. A., Sherman, M. P., Penn, M. L., Chin, P. S., De Noronha, C. M., Moore, M. S., Blobel, G., and Bukrinsky, M. (1998) EMBO J. 17, 3008–3017
5. Henklein, P., Bruns, K., Sherman, M. P., Tessmer, U., Licha, K., Kopp, J., de Noronha, C. M., Greene, W. C., Wray, V., and Schubert, U. (2000) J. Biol. Chem. 275, 32016–32026
6. Zhu, B., Fischinger, A. J., Moninger, T., Poellinger, K. A., and Walsh, M. J. (1995) J. Biol. Chem. 270, 18997–19007
7. Brunner, S., Sauer, T., Carotta, S., Cotten, M., Saltik, M., and Wagner, E. (2000) Gene Ther. 7, 401–407
8. Zhang, S., Pointer, D., Singer, G., Feng, Y., Park, K., and Zhao, L. J. (1998) Gene 212, 55–61
9. Stewart, S. A., Poon, B., Jowett, J. B., and Chen, I. S. (1997) J. Virol. 71, 5579–5592
10. Paxton, W., Connor, R. I., and Landau, N. R. (1993) J. Virol. 67, 7229–7237
11. Piller, S. C., Ewart, G. D., Jans, D. A., Gage, P. W., and Cox, G. B. (1999) J. Exp. Med. 194, 1407–1419
12. Bartz, S. R., Rolger, M. E., and Emerman, M. (1996) J. Virol. 70, 2324–2331
13. Goh, W. C., Rolger, M. E., Kinsey, C. M., Michael, S. F., Fultz, P. N., Nowak, M. A., Hahn, B. H., and Emerman, M. (1999) Nat. Med. 5, 65–71
14. Cohen, E. A., Terwilliger, M. E., Kalinos, Y., Proulx, J., Sodroski, J. G., and Haseltine, W. A. (1990) J. Acquired Immune Defic. Syndr. 3, 11–18
15. Kres, T., Gragovaj, A., Kopp, J., Ruzsics, B. R., Pavlakis, G. N., and Chrousos, G. P. (1999) J. Exp. Med. 189, 51–62
16. Stewart, S. A., Poon, B., Jowett, J. B., and Chen, I. S. (1997) J. Virol. 71, 5579–5592
17. Pacold, E., Wiedmeyer, K., Deininger, P., and Wiede, B. (1999) J. Virol. 73, 4238–4238
18. Chomel, J. C., Leborgne, C., Druillennec, S., Lenoir, C., Coulaud, D., Spitz, L., Lewis, P., Goldfarb, D., Emerman, M., and Stevenson, M. (1993) Nature 365, 666–669
19. Nie, Z., Bergeron, D., Subramanian, R. A., Yao, X. J., Checrone, F., Rougeau, N., and Cohen, E. A. (1998) J. Virol. 72, 4104–4115
20. Jenkins, Y., Pornillos, O., Rich, R. L., Myzak, D. G., Sundquist, W. I., and Malim, M. H. (2001) J. Virol. 75, 10537–10542
21. Conner, R. I., Chen, B. K., Choe, S., and Landau, N. R. (1995) Virology 206, 935–944
22. Eckstein, D. A., Sherman, M. P., Penn, M. L., Chin, P. S., De Noronha, C. M., Moore, M. S., Blobel, G., and Bukrinsky, M. (1998) EMBO J. 17, 909–917
23. Vodicca, M. A., Koepp, D. M., Silver, P. A., and Emerman, M. (1998) Genes Dev. 12, 175–185
24. de Noronha, C. M., Sherman, M. P., Lin, H. W., Cavrois, M. V., Moir, R. D., Goldman, R. D., and Greene, W. C. (2001) Science 294, 1105–1108
25. Schuler, W., Wecker, R., de Roquigny, H., Baudat, Y., Sire, J., and Roques, B. P. (1999) J. Biol. Chem. 274, 2105–2117
26. Wecker, K., and Roques, B. P. (1999) Eur. J. Biochem. 266, 359–369
27. Wecker, K., Morellet, N., Bouaziz, S., and Roques, B. P. (2002) Eur. J. Biochem. 269, 3779–3788
28. Henklein, P., Bruns, K., Sherman, M. P., Tessmer, U., Licha, K., Kopp, J., de Noronha, C. M., Greene, W. C., Wray, V., and Schubert, U. (2000) J. Biol. Chem. 275, 32016–32026
29. Zahn, F., Faubender, A. J., Moninger, T., Poellinger, K. A., and Walsh, M. J. (1995) J. Biol. Chem. 270, 18997–19007
30. Brunner, S., Sauer, T., Carotta, S., Cotten, M., Saltik, M., and Wagner, E. (2000) Gene Ther. 7, 401–407
31. Zhang, S., Pointer, D., Singer, G., Feng, Y., Park, K., and Zhao, L. J. (1998) Gene 212, 55–61
32. de Roquigny, H., Caneparo, A., Delaunay, T., Bischler, J., Mousadet, J. F., and Roques, B. P. (2000) Eur. J. Biochem. 267, 3654–3669
33. Kichler, A., Pages, J. C., Leborgne, C., Druillennec, S., Lenoir, C., Coudal, D., Delain, E., Le Cam, E., Roques, B. P., and Danos, O. (2000) J. Virol. 74, 5424–5431
34. Bousif, O., Lezoualch, F., Zanta, M. A., Meredith, N., Scherman, D., Demeneix, B., and Behr, J. P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7297–7301