Efficient DNA Transfection Mediated by the C-Terminal Domain of Human Immunodeficiency Virus Type 1 Viral Protein R

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Viral protein R (Vpr) of human immunodeficiency virus type 1 is produced late in the virus life cycle and is assembled into the virion through binding to the Gag protein. It is known to play a significant role early in the viral life cycle by facilitating the nuclear import of the preintegration complex in nondividing cells. Vpr is also able to interact with nucleic acids, and we show here that it induces condensation of plasmid DNA. We have explored the possibility of using these properties in DNA transfection experiments. We report that the C-terminal half of the protein (Vpr52−96) mediates DNA transfection in a variety of human and nonhuman cell lines with efficiencies comparable to those of the best-known transfection agents. Compared with polylysine, a standard polycationic transfection reagent, Vpr52−96 was 10- to 1,000-fold more active. Vpr52−96-DNA complexes were able to reach the cell nucleus through a pH-independent mechanism. These observations possibly identify an alternate pathway for DNA transfection.

The accessory genes of lentiviruses are not essential for viral replication in tissue culture but can be critical for the establishment of a productive infection in their natural hosts. The vpr gene is found in human immunodeficiency virus type 1 (HIV-1), HIV-2, and simian immunodeficiency virus and encodes the 15-kDa viral protein R (Vpr), which is produced late in the virus life cycle and is assembled into the virion through binding to Gag (11, 38, 44). A variety of activities have been associated with Vpr. It enhances the replication of HIV-1 in lymphocytes and monocyte-derived cell lines (9, 30), it is a cofactor of the human glucocorticoid receptor (27). Vpr has also been implicated in transport of the viral preintegration complex (PIC) into the nucleus, a property that may help HIV-1 infect nondividing cells (18, 21, 39, 48). In the absence of other HIV-1 proteins, Vpr is localized predominantly in the nucleus, although it does not contain a typical nuclear localization signal (12, 31).

Genetic and structural studies have assigned the various functions of Vpr to overlapping domains within the molecule. Nuclear accumulation of Vpr was reported to depend on the presence of α-helices in both the N- and C-terminal halves of the protein (Fig. 1) (34, 46). The N-terminal helices are believed to be involved in incorporation of Vpr into virions. The C-terminal domain, which is rich in basic amino acids, contains elements essential for G2 arrest (33, 34) and nucleic acid binding activities (10, 55).

The ability of Vpr to interact with nucleic acids and to enter the nucleus, either in the context of PICs or independent of other viral proteins, prompted us to test its ability to deliver plasmid DNA into mammalian cells. An efficient carrier of DNA for transfections must package the DNA into compact particles which can be readily taken up by cells. It must also protect DNA from cellular nucleases, allow its release from endocytic vesicles, and favor its nuclear import. While existing carriers can fulfill some of these criteria, none of them provides all of the necessary functions. The nuclear import of DNA remains the major hurdle for efficient transfection (6, 13, 53).

We report here that the C-terminal domain of Vpr (Vpr52−96), but not the N-terminal part, is able to interact with plasmid DNA and that it mediates DNA transfection with efficiencies comparable to those of the best transfection reagents.

MATERIALS AND METHODS

Plasmids. SMD2-LucΔITR (7.6 kb) and PCIneo-Luc (7.3 kb) are expression plasmids encoding the firefly luciferase gene under the control of the human cytomegalovirus (CMV) immediate-early promoter. eGFP-C1 (4.7 kb; Clontech) was used. Plasmids were linearized with BamHI and purified (Qiagen). The eGFP and CMV-LacZ constructs are plasmids expressing LacZ under the control of the phosphoglycerate kinase (PGK) promoter and the CMV immediate-early promoter, respectively.

Peptides. Peptide Vpr1−96 from HIV-1 strain LAI, with the sequence MEQAPDDOPQPRPYDWTTEELKNEAVRHFPRIWLSLGHONYEYG DTWTVGREALIQQLLOHFHRRSRRIGICSRIQOQRTRNGASKS, was used. The peptides were synthesized as described previously (11). Electrospray mass spectrometry was used to confirm the identities of peptides Vpr1−51 (theoretical molecular mass [MMth] = 6,167; calculated molecular mass [MMcalc] = 6,167), Vpr52−96 (MMth = 5,247.3; MMcalc = 5,249), Vpr97−111 (MMth = 11,394.9; MMcalc = 11,394.9), Vpr112−150 (MMth = 13,921.26; MMcalc = 13,921.26), Vpr151−185 (MMth = 3,148.66; MMcalc = 3,148), Vpr186−220 (MMth = 3,148; MMcalc = 3,148), Vpr221−257 (MMth = 2,578.97; MMcalc = 2,578.97), and Arg49Vpr52−96Ala54 (MMth = 5,162.02; MMcalc = 5,162). Peptides Vpr52−75, Vpr80−96, and Vpr52−96 were synthesized by SYNTHEME. The peptides were stored at −80°C as 1-mg/ml solutions in MilliQ water.

Cell culture. The culture medium Dulbecco’s modified Eagle medium (DMEM; GIBCO-BRL) was supplemented with 2 mM l-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 10% heat-inactivated fetal calf serum (FCS; HyClone). We used human hepatocarcinoma cells ( HepG2; Amer...
American Type Culture Collection [ATCC]), human epithelial carcinoma cells (HeLa 229; ATCC), human embryonic retinoblasts (cell line 911; kindly given by Introgene), transformed human embryonic kidney cells (cell line 293; ATCC), and Swiss mouse embryo cells (NIH 3T3; ATCC).

DNA retardation assay and sensitivity of the polyplexes to DNase I. DNA binding was studied by means of agarose gel retardation assays. Plasmid DNA (1 µg) and increasing amounts of peptide were each diluted in 25 µl of 150 mM NaCl and then mixed together. After a period of 25 min, samples (25 µl) were electrophoresed through a 1% agarose gel, using Tris-borate-EDTA buffer, and DNA was visualized after ethidium bromide staining.

electron microscopy. Vpr peptides at a final concentration of 0.08 µg/µl for Vpr1–96 and 25 µM for Vpr70–96 were mixed with 0.02 µg of plasmid DNA (3.4 ng/ml) in a final volume of 50 µl of 150 mM NaCl. Five microliters 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 pentane. The grids were then stained with 2% aqueous uranyl acetate, drained, and blotted. The observations were done in the annular dark-field mode with a Zsiss model 902 electron microscope, filtering out inelastically scattered electrons for enhanced contrast and resolution (28).

RESULTS

Interactions between Vpr derivatives and plasmid DNA. Full-length Vpr (Vpr1–96) and several Vpr subfragments (Table 1 and Fig. 1) were synthesized and tested for their capacity to interact with plasmid DNA at a physiological ionic strength (150 mM NaCl). Peptide-DNA interactions can be measured by determining the amount of peptide required to retard the migration of plasmid DNA toward the cathode during agarose gel electrophoresis of the plasmid DNA. The indicated amounts of peptides are the minimal amounts required to retard completely 1 µg of plasmid DNA.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amt of peptide needed for complete DNA retardation (µg)</th>
<th>Plusminus charge ratio of the peptide-DNA complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vpr1–96</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>Vpr1–51</td>
<td>&gt;30*</td>
<td>NA</td>
</tr>
<tr>
<td>Vpr2–96</td>
<td>2</td>
<td>0.8</td>
</tr>
<tr>
<td>Vpr2–93</td>
<td>2</td>
<td>0.77</td>
</tr>
<tr>
<td>Vpr2–75</td>
<td>20</td>
<td>2.1</td>
</tr>
<tr>
<td>Vpr60–80</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Vpr70–96</td>
<td>2</td>
<td>1.4</td>
</tr>
<tr>
<td>Vpr77–96</td>
<td>&gt;5–</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* The indicated amounts of peptides are the minimal amounts required to retard completely 1 µg of plasmid DNA.

\*

The largest amount of peptide tested was 30 µg.

\*

With 5 µg of peptide, most of the DNA was retarded, but complete retardation was not attained even with a significantly larger amount of peptide (i.e., 30 µg).

\*

NA, not applicable.
DNA molecule. Figure 2 shows that in a single Vpr77–96-DNA which was less active in the retardation assay, induced an comparable to that observed with other transfection reagents. Vpr1–96 mediated significantly less gene transfer than did pLys. PEI is a potent transfection reagent even in the absence of endosomolytic agents (2, 3), whereas pLys cannot efficiently mediated transfection resulted in up to 3 orders of magnitude higher luciferase activities on 911 and HepG2 cells than did pLys under optimal conditions, thus reaching efficiencies comparable to those obtained with PEI or DOTAP (Fig. 3C). For transfections into NIH 3T3 cells and HeLa cells, Vpr52–96 was only 3 to 10 times more efficient than pLys. The leucine-rich region and the C terminus of Vpr can stimulate reporter gene expression directed from the HIV-1 long terminal repeat as well as from heterologous viral promoters (17, 19, 50). Importantly, this stimulation is usually under 10-fold and is linked to G2 arrest activity (16, 17, 19). To rule out the possibility that Vpr52–96 transactivated reporter gene expression in our experiments, we performed a series of control studies. First, we determined whether the externally added peptide Vpr52–96, alone or mixed with plasmid DNA, was able to induce cell cycle arrest. Although less than 30% of the cells expressed the reporter gene following transfection, most of the cells took up polyplexes, as shown by flow cytometry after incubation of the cells with fluorescent Vpr52–96-DNA complexes (Fig. 4B). The cell cycle was analyzed after propidium iodide staining of DNA 24 and 30 h after transfection (see Materials and Methods). Control cells and cells incubated with Vpr52–93 (data not shown) or with Vpr52–93–CMV-Luc complexes showed normal cell cycle profiles, with about 50% of the cells being in $G_0/G_1$. The experiment was controlled by treatment of 293 cells with thymidine and nocodazole, which resulted in accumulation of cells in the G2 phase (to >70%) (Fig. 4A). We concluded that Vpr52–96, alone or complexed with DNA did not mediate G2 arrest in our system. In a second control experiment, we used the mutant Arg80Ala, which is unable to arrest cells in G2 (16, 17). We compared the mutated peptide Arg80Vpr52–96Ala93 to wild-type Vpr52–96 with regard to the ability to form complexes with plasmid DNA and to transfect 293 cells. The mutated peptide behaved like Vpr52–96 in the DNA retardation assay and was as active as in DNA transfections (data not shown).

Finally, we looked at a possible transactivation effect of Vpr52–96 by applying complexes to 293 cells previously transfected with a CMV-driven reporter gene. Cells first received PEI-CMV-Luc complexes, and after 43 h they were transfected again with either Vpr52–93–CMV-LacZ, DOTAP-CMV-LacZ, or PEI–CMV-LacZ. Thirty hours after the second transfection, the luciferase and β-galactosidase activities were measured. Figure 5 shows that none of the secondary transfections led to enhanced luciferase expression. Thus, we concluded that the high levels of reporter gene expression obtained with Vpr52–96 cannot be explained by transactivation of the CMV promoter.

Intracellular fate of the transfection complexes. To determine whether the higher transfection levels obtained with Vpr52–96, compared to pLys were due to more-efficient DNA delivery, we harvested transfected 293 cells 7, 24, 48, and 96 h after transfection and isolated low-molecular-weight DNA. Southern blot analysis indicated that significant amounts of plasmid DNA were present at 7 h posttransfection in both Vpr52–96– and pLys-transfected cells (Fig. 6). One day after Vpr52–96-mediated transfection, the amount of plasmid present in the 293 cells was dramatically decreased whereas cells transfected with pLys still contained large amounts of DNA. After
FIG. 2. Electron microscopy of Vpr<sub>52–96</sub>-DNA and Vpr<sub>77–96</sub>-DNA complexes. The visualization of Vpr-DNA complexes was performed by positive staining and annular dark-field electron microscopy. The Vpr<sub>52–96</sub>-DNA and Vpr<sub>77–96</sub>-DNA complexes were prepared in 150 mM NaCl as described in Materials and Methods. (a and b) Vpr<sub>52–96</sub> induces the formation of aggregates of plasmid DNA. The aggregates in panel b, which represent a large majority of the events that can be observed on the grid, result from the incorporation of several DNA molecules. (c and d) Vpr<sub>77–96</sub> induces a compaction of the DNA molecules, without collapse and effective aggregations, but an ordered condensation with lamellar structures. Such condensation can lead to the formation of rods or toroids. (d) Illustration of toroidal compaction of plasmid DNA mediated by Vpr<sub>77–96</sub>. Scale bars, 100 nm.
4 days, no DNA was detectable in Vpr<sub>52–96</sub>-transfected cells whereas the signal remained in the case of pLys. Although much less DNA accumulated following Vpr<sub>52–96</sub> transfection, reporter gene activity was superior to that of pLys at all time points (Fig. 6, lower panel). Of note, the high level of luciferase activity observed at day 4 cannot be explained by the accumulation of enzyme during the first hours because the half-life of this enzyme in mammalian cells is only 3 h (47).

This experiment indicates that most of the delivered DNA does not contribute to transgene expression. This DNA is trapped either in vesicles or in the cytosol and is eventually degraded at a rate that depends on the capacity of its carrier to provide protection against nucleases (29). To evaluate the capacity of Vpr<sub>52–96</sub> to protect DNA against degradation, we incubated Vpr<sub>52–96</sub>-DNA complexes with DNase I for 37°C, and after elimination of Vpr by SDS treatment, DNA was analyzed by agarose gel electrophoresis. Vpr<sub>52–96</sub> was not able to preserve the integrity of the plasmid (data not shown), in contrast to pLys (7). Thus, the rapid disappearance of the transfected DNA (Fig. 6) may reflect the sensitivity of Vpr<sub>52–96</sub>-DNA complexes to nucleases.

Ligand-pLys-mediated gene transfer can be significantly enhanced in the presence of chloroquine (15, 54), a weak lysosomotropic base which accumulates in acidic endocytic vesicles, neutralizes their pH, and destabilizes their membranes. Another beneficial effect of chloroquine in pLys-mediated transfection experiments may be the dissociation of polyplexes (15). Figure 7 shows that Vpr<sub>52–96</sub>-mediated transfection of HepG2 cells was 2- to 10-fold more efficient in the presence of 100 μM chloroquine. This enhancement is comparable to that observed with pLys (Fig. 7). We concluded that at least some of the Vpr<sub>52–96</sub>-DNA complexes are localized in chloroquine-sensitive endocytic vesicles.

Several peptides known to enhance pLys-mediated DNA transfection are believed to act by disrupting the endosome membrane at low pH values (35, 49). Their fusogenic activity is triggered when protonation of their side-chain carboxy groups induces a change from a random conformation to an α-helix. This process is inhibited by bafilomycin A1, a specific inhibitor of the vacuolar proton pump (4, 52). To test the possibility that Vpr<sub>52–96</sub> was acting along a similar pathway, we transfected HepG2 cells in the presence of increasing amounts of bafilomycin A1. Figure 7 shows that gene transfer was not significantly altered under these conditions. This indicates that if Vpr<sub>52–96</sub> complexes are released from endosomes, it happens via a pH-independent mechanism.

In summary, our study of the intracellular fate of Vpr<sub>52–96</sub>-DNA complexes indicates that only a minority of them efficiently find their way to the nucleus whereas most are trapped in vesicles or in the cytosol and are quickly degraded. The active fraction of complexes ending up in the nucleus either avoids the endocytic route or can be released from endosomes by a pH-independent mechanism.

**DISCUSSION**

Vpr is incorporated into HIV virions through interactions with at least two gag-encoded proteins, p6 and Ncp7. It is known to bind RNA and DNA (10, 55) and therefore may be an integral component of the PIC following reverse transcription. The presence of Vpr has been shown to facilitate nuclear import of the complex, possibly through interactions with...
Karyopherins (40). Considering these properties, we tested the possibility of using Vpr directly as a DNA transfection agent.

We first analyzed the DNA binding activities of a variety of Vpr-derived peptides in gel retardation assays. Our study confirmed that peptide fragments from the C-terminal domain (residues 52 to 96), but not those from the N terminus (residues 1 to 51), are able to form complexes with DNA. We showed that Vpr52–96 induces the compaction of plasmid DNA, leading to the formation of aggregates with irregular sizes and shapes. Rather unexpectedly, the positively charged peptide Vpr80–96 was found to be unable to bind DNA efficiently. Instead, residues between positions 70 and 80 appear to play a key role in the formation of a DNA binding domain.

Transfection experiments identified Vpr52–96 and Vpr52–93 as agents with higher transfection efficiencies than pLys. Surprisingly, full-length Vpr, which also binds DNA, was always significantly less efficient than pLys in these experiments. Recent observations indicate that Vpr1–51 can partially hinder nucleic acid recognition by Vpr52–96 (10). Our results suggest that the transfection activity of the Vpr52–96 region is not available in the context of the whole Vpr protein. It is not known whether an active configuration related to the transfection activity described here is unveiled at any step of HIV infection. Yet, it is tempting to speculate that the ability of Vpr52–96 to act as a DNA carrier is related to the role of Vpr as an enhancer of the nuclear import of viral DNA.

Multiple activities have been associated with Vpr, including cell cycle arrest in G2 and transactivation of viral promoters. We believe that the expression of the luciferase reporter gene which we used in our experiments to measure the transfection efficiency was not influenced by these activities, for the following reasons: (i) no cell cycle arrest was observed following transfection with Vpr52–96-DNA complexes, (ii) a Vpr52–96 peptide containing a mutation that eliminates G2 arrest was as active as the wild-type peptide, and (iii) Vpr52–93-mediated transfection did not transactivate a CMV promoter already present in the transfected cells (Fig. 5).

The mechanism of cellular uptake of Vpr-DNA complexes still needs to be elucidated. Positively charged DNA complexes

![Diagram](http://jvi.asm.org/)

**FIG. 4.** Study of possible Vpr52–96-induced G2/M phase arrest. (A) The cell cycles of untreated (a), thymidine- and nocodazole-treated (b), and Vpr52–96-transfected (c) 293 cells were analyzed by flow cytometry as described in Materials and Methods. (B) Evaluation of the association and/or endocytosis of fluorescent DNA-Vpr52–96 complexes on 293 cells after 4 h of incubation at 37°C. The autofluorescence of the cells is shown by the broken line. The x axis represents the relative fluorescence intensity.

![Diagram](http://jvi.asm.org/)

**FIG. 5.** Does Vpr52–96 transactivate the CMV promoter of the reporter plasmid? 293 cells transfected with PEI 25 kDa-CMV-Luc were transfected again (except the control) 43 h later with (+) or without (−) the following polyplexes: Vpr52–93-CMV-LacZ, DOTAP-CMV-LacZ, and PEI 25 kDa-CMV-LacZ. Thirty hours after the second transfection, the luciferase (solid gray bars) and the β-galactosidase (stippled bars) activities were determined by measuring luminescence.
can enter cells after binding to membrane-associated sulfated proteoglycans (36). Under our experimental conditions, with a plus/minus charge ratio of around 2.5, the complexes could follow this pathway. We have observed that although higher levels of gene expression are reached when Vpr52–96 is used, smaller amounts of DNA are found within the cells than when pLys is employed. This can be related to the higher susceptibility to DNase displayed by the Vpr-DNA complexes. It also indicates that a large number of DNA molecules do not participate in the expression of the reporter gene. Most likely, these molecules are trapped in acidic vesicles, and we showed that a proportion are released from these vesicles upon chloroquine treatment. It is not clear, however, whether endocytosis is an obligatory pathway used by Vpr-DNA complexes for access to the cytoplasm. Alternatively, the active complexes may enter the cell directly after membrane permeabilization. Indeed, Macreadie et al. have reported that the C-terminal part of Vpr causes permeabilization in different yeast strains (32) and might allow the design of a new class of peptides for gene transfer.

FIG. 6. Efficiency of delivery of plasmid DNA by Vpr52–96–DNA complexes. Six-well plates were transfected, or not (mock), with either Vpr52–96 or pLys complexes. Polyplexes were prepared by mixing equal volumes of CMV-Luc and DNA (4 μg/ml) with Vpr52–96 or pLys. After 24 h of incubation, cells were harvested and lysed and the low-molecular-weight DNA was recovered. At the same time, the luciferase activity was measured. For each set of conditions, two different volumes of the Hirt extracts (i.e., one-fourth and one-half of the volume of one well) were used. The graph below the blot presents the levels of luciferase activity obtained at different time points after transfection with Vpr52–96 or pLys.

FIG. 7. Transfection of HepG2 cells with Vpr52–96–CMV-Luc in the presence of chloroquine or bafilomycin A1. HepG2 cells plated in 24-well plates were transfected with 24 μg of Vpr52–96 and 4 μg of CMV-Luc (stippled bars) or 4 μg of pLys and 4 μg of CMV-Luc (solid gray bars) in the presence (+) or absence (−) of either 100 μM chloroquine or 175 nM bafilomycin A1. After 2.5 h of incubation, the transfection medium was removed and replaced with DMEM containing 10% FCS. The luciferase assay was performed 2 days after transfection.

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Replication of Herpes Simplex Virus Type 1 within Trigeminal Ganglia Is Required for High Frequency but Not High Viral Genome Copy Number Latency

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Volume 74, no. 2, p. 964–974, 2000. Page 971, column 2, 2nd full paragraph, lines 8–11: The sentence beginning “In mouse TG,” should read “In mouse TG, Coen et al. (4) observed reduced numbers of LAT \( ^* \) neurons in mice latently infected with a TK\(^{–} \) mutant and Jacobson et al. (21a) showed that this reduction was due to a TK mutation that does not affect the UL24 gene or its expression. In addition, quantitative PCR analysis of the viral genome has revealed a significant decrease in the amount of latent DNA present in the TG of mice latently infected with TK-null mutants (22, 25, 26).” Page 973, column 1, last paragraph of the text. Line 8: “(1, 44)” should read “(1, 14a, 44).” The following should be added to the References:


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Volume 73, no. 12, p. 9969–9975, 1999: In Table 1, the Results, and the Discussion, strain 0667-CHN-85 was isolated in The People’s Republic of China in 1987 and not The Republic of China in 1985. The correct designation in Fig. 1 should, therefore be 0667-CHN-87. The Acknowledgments should note that this strain was kindly provided by Zhi-Ming Zheng.
AUTHORS’ CORRECTIONS

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