Oligomers of the Arginine-rich Motif of the HIV-1 TAT Protein Are Capable of Transferring Plasmid DNA into Cells* [S]

We constructed multimers of the TAT-(47–57) peptide. This polycationic peptide is known to be a protein and particle transduction domain and at the same time to comprise a nuclear localization function. Here we show that oligomers of the TAT-(47–57) peptide compact plasmid DNA to nanometric particles and stabilize DNA toward nuclease degradation. At optimized vector compositions, these peptides mediated gene delivery to cells in culture 6–8-fold more efficiently than poly-L-arginine or the mutant TAT2-M1. When DNA was precompacted with TAT peptides and polyethyleneimine (PEI), Superfect, or LipofectAMINE was added, transfection efficiency was enhanced up to 390-fold compared with the standard vectors. As early as after 4 h of transfection, reporter gene expression mediated by TAT-containing complexes was higher than the 24-h transfection level achieved with a standard PEI transfection. When cells were cell cycle-arrested by serum starvation or aphidicolin, TAT-mediated transfection was 3-fold more efficient than a standard PEI transfection in proliferating cells. In primary nasal epithelial cells and upon intratracheal instillation in vivo, TAT-containing complexes were superior to standard PEI vectors. These data together with confocal imaging of TAT-DNA complexes in cells support the hypothesis that the TAT nuclear localization sequence function is involved in enhancing gene transfer.

Poor escape of nonviral gene vectors from the endosomal compartment after cellular uptake and inefficient translocation into the nucleus substantially limit their efficiency (1, 2). In this study, the arginine-rich motif of the HIV-1 TAT protein should be used to overcome these obstacles. The 101-amino acid HIV-1 TAT protein regulates transcriptional activation of the human immunodeficiency virus type 1 long terminal repeat promoter element by binding to a short nascent stem-loop leader RNA, trans-activation response (TAR), recruiting a positive transcription elongation complex (P-TEFb) (3). Binding of HIV-1 TAT protein to the TAR RNA is substantially mediated through the arginine-rich motif (amino acids 47–57), which represents a basic stretch of amino acids located to domain 4 of the HIV-1 TAT protein (3). Besides its importance for binding to the TAR RNA, the arginine-rich motif of the HIV-1 TAT protein (TAT peptide) has been shown to function as a protein transduction domain, penetrating cell membranes in a manner different from classic endocytosis (4–6). This uptake mechanism was also observed for heterologous proteins when they were chemically coupled (7) or genetically fused (8) to the TAT peptide. In addition, the conjugation of the TAT peptide to various structures of nanometric size, such as superparamagnetic nanoparticles (9), liposomes (10), and A phage (11), led to their uptake into cells in a manner apparently different from endocytosis. Besides this unique feature of the TAT-peptide, the TAT peptide represents a nuclear localization sequence (12). Upon binding of the TAT peptide itself or its conjugates to the nucleocytoplasmic shuttle protein importin β, nuclear import through the nuclear pore has been observed (4, 6–10).

The objective of the present study was to examine whether the unique features of the TAT peptide (protein transduction domain and nuclear localization sequence) and its polycationic nature are suitable to enhance nonviral gene delivery. Chemical properties of cationic polymers, such as the degree of polymerization, the type of cationic groups present in the polymer, or the amino acid composition of cationic oligopeptides influence the biophysical properties of their polyelectrolyte complexes with plasmid DNA. DNA binding affinity, surface charge, and particle size have pronounced effects on their efficacy in gene delivery and on biodistribution in vivo (13–15). Since compaction of DNA into stable microparticulate structures by oligopeptides suitable for gene transfer requires a minimum chain length of 6–10 cationic amino acids (13), we synthesized the TAT peptide as oligomers. The dimer, trimer, and tetramer of identical repeats with intervening glycine residues comprise 16, 24, and 32 positive charges, respectively.

We attempted to find a correlation between the degree of oligomerization of the TAT peptide, the resulting biophysical characteristics of peptide-DNA complexes, and their gene delivery efficiency in context with the unique functions of the TAT peptide.

Experimental Procedures

Peptide Synthesis

The following peptides were synthesized. Peptide C[YGRK-KRRQRRRG]2–4 (TAT2–4) contained the arginine-rich motif of the human immunodeficiency virus type 1 TAT protein. Peptides were synthesized on an Applied Biosystems 431A automatic synthesizer according to a standard Fmoc ([N-(9-fluorenyl)methoxycarbonyl] proto-
Oligomers of the TAT Peptide for Gene Transfer

col. Peptide C(YGRKERRQERRG)₂ (TAT₂-M1) was synthesized by the Department of Medicine (Charité) (Institute of Biochemistry, Humboldt-University, Berlin). The free sulfhydryl groups were modified by dithiopyridine reaction (13).

### Plasmid

pCMV-Luc containing firefly luciferase cDNA driven by the cytomegalovirus promoter was generously provided by Dr. E. Wagner (Department of Pharmacy, Ludwig-Maximilians-University Munich) and amplified as described in Ref. 16.

### Size and ζ Potential Measurement

The particle sizes were determined by dynamic light scattering, and ζ potentials were measured electrophoretically (Zetasizer 3000HS, Malvern Instruments, Herrenberg, Germany). Gene vector solutions in distilled water or HBS were generated at a DNA concentration of 10 μg/ml (17).

### Fluorescence Quenching Assay

Plasmid DNA (pCMVLuc) was labeled with TOTO-1 (dielectric base pair ratio of 1:20). The TAT oligomers were serially diluted in HBS in a 96-well plate corresponding to the indicated charge ratios. 100 μl of a solution of TOTO-1-labeled DNA (0.25 μg of DNA in HBS) was pipetted to either 100 μl of TAT oligomer solution or 100 μl of HBS (100%) and thoroughly mixed. The excitation filter was set at 485 nm, and the emission filter was set at 535 nm (SPECTRAFluor Plus, Tecan, Germany). Measurements were performed in quadruplicates.

### Cell Culture

Human bronchoepithelial cells (16HBE140) were provided by Dieter C. Gruener (University of Vermont, Burlington, VT). Cells were grown in FCS (10%)-supplemented minimum essential medium (Invitrogen GmbH, Karlsruhe, Germany) at 37 °C in a 5% CO₂ humidified atmosphere. COS-7 cells were cultivated in FCS (10%)-supplemented Dulbecco’s modified Eagle’s medium (Invitrogen). Fresh nasal respiratory epithelium was obtained from patients through surgery and immediately processed as follows. Briefly, epithelial cells were detached from connective tissue after 1-h incubation at 37 °C in 0.05% trypsin/0.5% (v/v) EDTA (Gibco, Carlsbad, CA). After treatment, the suspension was centrifuged at 150 g for 5 min, mixed vigorously by pipetting and centrifuged at 150 g for 5 min, ambient temperature). The pellet was resuspended in 8 ml of RPMI plus 10% FCS containing antibiotics, and cells were seeded into 35-mm dishes. The next day, the medium was replaced by bronchial epithelial cell growth medium (catalog no. C21160; Promocell).

### Preparation of Gene Vector Complexes

Gene vector complexes for one well were formulated as follows. 1 μg of DNA and the corresponding amount of vector were diluted in HBS (150 mM NaCl, 10 mM HEPES, pH 7.4) to 75 μl, respectively. The DNA solution was pipetted to the vector solution and mixed vigorously by pipetting up and down. The complexes were incubated for 20 min at ambient temperature before use. Ternary gene vector complexes for one well were generated in the same manner, but DNA, TAT oligomer, and standard cationic transfection agent (PEI, average molecular mass of 25 kDa; Aldrich, Deisenhofen, Germany; dialyzed against water, 12 kDa molecular mass cut-off and adjusted to pH 7; fractured dendrimers; SuperFect (Qiagen, Hilden, Germany) or LipofectAMINE (Invitrogen, Carlsbad, CA)). Gene vector solutions in water and HBS were added, mixed vigorously, and incubated at ambient temperature for 10 min. Alternatively, DNA was first pipetted to the standard vector solution, and the TAT oligomer solution was then added.

### Transfection Procedure and Luciferase Activity Measurement

150 μl of gene vector solution, corresponding to 1 μg of DNA, were pipetted onto cells (COS-7, 30,000 cells/well; 16HBE, 100,000 cells/well), which had been seeded into 24-well plates 1 day before, and then covered with 850 μl of medium in the absence of FCS. After 4 h of incubation at 5% CO₂ and 37 °C, the medium was replaced with 10% FCS-containing medium supplemented with 0.1% (v/v) penicillin/streptomycin and 0.5% (v/v) gentamycin (Invitrogen). When transfections were performed at 4 °C, cells were incubated for 1 h at 4 °C before transfection and incubation with the gene vectors was performed at 4 °C. When transfections were performed in the presence of a mixture of endocytosis inhibitors, the transfection medium was supplemented with antimycin A (1 μg/ml; Sigma), sodium fluoride (10 mM; Sigma), and sodium azide (0.1% (m/v); Sigma), respectively. After 4 h, the medium was replaced with 10% FCS-containing medium supplemented with antibiotics (see above).

### Fluorescence in Situ Hybridization and Confocal Laser-scanning Microscopy

A three-dimensional fluorescence in situ hybridization was done following the protocol of Solovei and Cremer (18) with several modifications for our specific needs.

**Probe Generation**—A DNA probe was generated from the pEGFP plasmid (Clontech) by nick translation. A digoxigenin hapten was inserted with Dig Nick Translation Mix (Roche Diagnostics) generating a probe with a 300-bp median length. The reaction mixtures were cleaned of small fragments and nucleotides with the Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany). 1 μg of probe was precipitated with 50 μg of salmon testes DNA (Sigma). The dried pellet was resuspended in 20 μl of formamide at 37 °C for 2 h and stored until use at −20 °C.

**Hybridization**—The probe was brought to 37 °C and mixed 1:1 with “Fish Mix” (4× SSC, 40% dextran sulfate), resulting in a hybridization solution of 25 ng/μl probe in 2× SSC, 20% dextran sulfate, 50% formamide. The hybridization solution was denatured at 75 °C for 5 min and
briefly kept at 37°C prior to slide application. The slides were removed one at a time from the storage solution, 50 μl of hybridization solution was placed on each slide, a coverslip was applied, and finally rubber cement was used as a sealant. The sealed slides were heated to 75°C for 3 min to denature the cellular DNA, also further denaturing the probe DNA. The slides were placed in a moist chamber within a 37°C oven and were hybridized for 2-3 days.

Image Acquisition—Light optical sections were generated for each nucleus with a three-channel confocal laser-scanning microscope (TCS 4D; Leica Inc., Deerfield, IL) equipped with a Plan Apo 63×/1.32 oil immersion lens. Using the 488 and 567 lines of an argon/krypton laser for visualization of the Sytox and rhodamine, signals respectively, stacks of 256×256 equidistant 8-bit grayscale images were generated at an axial distance of 250 nm, pixel size of 1 nm. Each series consisted of ~20–25 images. Lines were averaged eight times. The different fluorochromes were imaged sequentially in identical nuclear planes. Laser power and voltage for the rhodamine channel were maintained at the same level for all conditions, whereas the voltage of the Sytox channel was adjusted for variations in nuclear counterstain. Cells were randomly selected when a signal appeared to be in the cell, and the midnuclear sections were used to detect the distribution of plasmid DNA.

Animals and Delivery of Gene Vectors to the Lung

Ternary gene vector complexes used for in vivo experiments were generated as described above, but the TAT oligomer and DNA solution were diluted in double-distilled water (Fresenius AG, Bad Homburg, Germany), respectively. 60 μl of gene vector solution containing 20 μg of DNA were applied per mouse. The gene vector application was performed as described in Ref. 16. In brief, mice were anesthetized intraperitoneally with pentobarbital and directly intubated with a single 60-μl bolus of the indicated gene vector using a 22-gauge intravascular cannula sleeve, needle removed (25 mm, 0.9-mm outer diameter, 0.6-mm inner diameter; Baxter, Germany). At 24 h post-transfection, mice were anesthetized intraperitoneal with pentobarbital, and mice peritoneal were opened by midline incision. In order to wash blood from the lungs and to avoid interference with the subsequent luciferase assay, a posterior vena cava exit was cut, and 1 ml of an isotonic sodium chloride solution was slowly perfused into the mice right cardiac ventricle. The lungs were dissected from animals, frozen in liquid nitrogen, and stored at -70°C. At assay time, the tissue was thawed on ice, and 500 μl of ice-cold lysis buffer (25 mM glycylglycine, 15 mM magnesium sulfate, 4 mM EDTA, 0.1% Triton X-100 (m/V), 1 mM phenylmethylsulfonyl fluoride, 0.15 units/ml buffer aprotinin) was added to each sample and homogenized for 20 s using a Polytron Pt 2100 homogenizer (level 5 corresponding to 26,000 rpm; Kinematica, Litau/Luzern, Switzerland). Samples were centrifuged at 10,000 × g at 4°C for 10 min, and 40 μl of the supernatant were measured for luciferase activity in a Lumat LB 9507 instrument (Berthold) injecting 100 μl of luciferase reagent (Promega) to each sample, and the light emitted over 10 s was measured. The background was subtracted from the reported values. 1 × 10⁶ relative luciferase units/10 s correspond to 1.25 ng of luciferase.

**FIG. 2.** Transfection efficiency of TAT oligomers or pLa gene vectors at different charge ratios under various conditions (COS-7 cells). *A*, in the absence of chloroquine; *B*, in the presence of chloroquine (300 μM); *D*, at different temperatures; *E*, in the absence or presence of endocytosis inhibitors; *C*, in comparison with the mutant TAT2-M1 (16HBE140r cells); *F*, in comparison with various nonviral gene vectors (data show a representative experiment, n = 4, ± S.D.; transfection rates of TAT oligomers significantly different from the pLa or TAT2-M1 complexes are indicated by an asterisk (p < 0.05), TAT2-M1 (p < 0.01)).
All animal procedures were approved and controlled by the local ethics committee and carried out according to the guidelines of the German law of protection of animal life.

**Statistical Analysis**

Results are reported as means ± S.D. The statistical analysis between different groups has been determined with a nonpaired t test. \( p \leq 0.05 \) was considered significant. All statistical analyses were performed using the program StatView 5.0. (SAS Institute Inc., Cary, NC).

**RESULTS**

**Biophysical Properties of the TAT Oligomer Gene Vectors—**The condensation of DNA into particulate structures is a prerequisite for gene delivery. For this reason, we first examined the capability of the TAT oligomers to condense DNA and to form particulate complexes. Laser light scattering showed that all of the TAT oligomer gene vectors had a diameter of \( \sim 70 \) nm in water, which increased to 700 nm in HBS at charge ratios of \( \pm 1 \) and 5, respectively (Table 1). pLa (control) gene vectors showed a similar diameter. TAT oligomer and pLa gene vectors had a positive \( \zeta \) potential of approximately \( +40 \) mV in water and negative \( \zeta \) potential of approximately \( -20 \) mV at \( \pm 1 \) in HBS, which changed into a positive \( \zeta \) potential of 20 mV at 5 and slightly increased with the degree of oligomerization (Table 1). The extent of complexation was independent of the degree of polymerization as examined by electrophoretic mobility shift assay and DNase I protection assay (see Supplementary Material). The only difference among TAT oligomer gene vectors depending on the oligomer size could be observed when complexes were analyzed by a fluorescence quenching assay (Fig. 1). Fluorescence quenching strongly increased at low charge ratios, reaching a plateau at approximately \( \pm 2 \). Higher charge ratios only slightly increased further DNA condensation. Fluorescence quenching induced by pLa was shifted to a higher charge ratio compared with the TAT peptides. Fluorescence quenching at a given charge ratio increased with the degree of oligomerization, indicating that the DNA was more tightly packed in the complex when the size of the TAT oligomer increased.

**Transfection Efficiency of TAT Oligomer Gene Vectors—**Plasmid DNA was complexed with each of the TAT oligomers at different charge ratios and transfection efficiency was examined in vitro. In addition, pLa gene vectors were prepared under the same conditions as control to assess the possibility of a sequence-dependent process (Fig. 2A). For each charge ratio tested, a significant improvement of transfection by TAT\(_2\) and TAT\(_3\) gene vectors compared with pLa gene vectors was observed. No statistical difference between TAT\(_1\) and pLa gene vectors was observed. The transfection rates mediated by each of the oligomers did not correlate with the degree of oligomerization of the TAT peptide. Intermediate length of the TAT\(_3\)-oligomer mediated the highest level of transgene expression. To further characterize sequence dependence, gene transfer efficiency mediated by the nuclear transport-deficient TAT\(_2\)-M1 peptide (12) was examined. Gene transfer efficiency mediated by the TAT\(_2\)-M1 peptide (± charge = 10) was 6-fold lower as compared with the TAT\(_2\) peptide (± charge = 10, Fig. 2C, \( p < 0.01 \)).

**The Influence of Endocytosis on Transfection Efficiency of TAT Oligomer Gene Vectors and Comparison with Standard Cationic Transfection Agents—**To examine endocytosis as an uptake mechanism, transfections were performed in the presence of chloroquin (19), at 4 °C (6, 10, 11) or in the presence of metabolic inhibitors (antimycin A, sodium azide, and sodium fluoride) (20–22). For each of the TAT oligomer gene vectors, transfection efficiencies in the presence of chloroquine strongly increased (Fig. 2B; compare with Fig. 2A). Gene transfer mediated by TAT\(_2\) and TAT\(_3\) gene vectors was significantly higher as compared with pLa gene vectors. This was not the case for TAT\(_4\) vectors. Overall transfection efficiency increased in the following order: TAT\(_2\) > TAT\(_3\) > TAT\(_4\). In the presence of chloroquin, the transfection efficiency of TAT\(_2\) gene vectors was enhanced 40-fold, whereas the transfection efficiency of pLa gene vectors was only enhanced 3-fold. Transfections performed at 4 °C led to a 20–90-fold decrease of transgene expression (Fig. 2D). Transfection rates in the presence of the endocytosis inhibitors decreased even more (Fig. 2E). Transgene expression mediated by TAT\(_3\)-DNA complexes in the presence of chloroquin was 3- and 4-fold higher as compared with PEI and SuperFect polyplexes and at the same level as LipofectAMINE polyplexes (Fig. 2F). These data suggest that the cellular uptake mechanism of the TAT oligomer gene vector complexes was predominated by the endosomal pathway and could not be precisely differentiated from endocytosis. However, due to the much stronger increase of gene transfer of the TAT oligomer complexes in the presence of chloroquin, as compared with pLa complexes, one could suggest that gene transfer of the TAT oligomers, in particular TAT\(_2\), was peptide sequence-dependent. This is supported by the higher gene transfer efficiency of the TAT\(_3\) gene vectors as compared with the TAT\(_2\)-M1 gene vectors (Fig. 2C).

**Intracellular Localization of Plasmid DNA after Transfection with TAT Oligomer Gene Vectors—**To further investigate the localization of plasmid DNA electrostatically bound to each of the TAT oligomers within the cell, COS-7 cells were transfected with TAT oligomer or pLa gene vectors, and transfections were stopped after 4 h. Images were generated with a 63× objective by confocal laser-scanning microscopy. Green emission signal represents the cell nuclei stained with Sytox 16; red emission signal shows the distribution of pEGFP in the same microscope field using a digoxigenin-labeled DNA probe. The probe was detected with anti-digoxigenin rhodamine antibody.

![Fig. 3. Intracellular localization of plasmid DNA (pEGFP).](image-url)
served for pLa gene vectors.

Ternary Gene Vector Complexes: Combination of TAT Oligomers with Various Standard Cationic Transfection Agents—The previous experiments suggest that the TAT oligomers could facilitate the transport of DNA into the nucleus. Next we examined whether the TAT oligomers were able to enhance gene transfer efficiency of poly- or lipoplexes that lack efficient nuclear plasmid transport (2, 23, 24). Ternary gene vector complexes consisting of TAT oligomer, standard cationic transfection agent, and DNA were formulated in two different manners. Either TAT oligomer was added to preformed gene vectors consisting of DNA and standard cationic transfection agent, or complexes were generated vice versa through the addition of standard cationic transfection agent to preformed TAT oligomer gene vector complexes. When one charge equiv-

![Figure 4](image1)

**FIG. 4.** Transfection of COS-7 cells with various ternary TAT oligomer gene vector complexes. Either DNA was first complexed with the TAT oligomer peptides at a charge ratio of 1 and PEI (N/P = 10), fractured dendrimers (N/P = 4.5), or LipofectAMINE (w/w = 10:1) were added afterward (A), or DNA was first complexed with PEI (N/P = 10), fractured dendrimers (N/P = 4.5), or LipofectAMINE (w/w = 10:1) and the TAT oligomer peptides (+1) were added (B) (data show a representative experiment, n = 4, ± S.D.; transfection rates significantly different from the standard vector are indicated by an asterisk (p < 0.05)).

![Figure 5](image2)

**FIG. 5.** Kinetics of transgene expression. 16HBE cells were transfected with the ternary gene vector complexes or PEI polyplexes, and luciferase activity was measured at the indicated time points (n = 3, ± S.D.; transfection rates mediated by the ternary complexes were significantly different from PEI at each time point (p < 0.01)).

![Figure 6](image3)

**FIG. 6.** Transfection efficiency of ternary gene vector complexes on growth-arrested cells. 16HBE cells were either blocked by aphidicolin incubation or by serum starvation and transfected with ternary gene vector complexes or PEI polyplexes (n = 3, ± S.D.; transfection rates significantly different from PEI are indicated by an asterisk (p < 0.05)).
alent of each of the TAT oligomers or pLa was added to pre- 
formed standard cationic gene vector complexes, the level of 
transgene expression remained approximately at the same 
level as of the standard cationic gene vectors (Fig. 4). In con- 
trast, when the ternary gene vector complexes were generated 
vice versa (i.e. the DNA was first complexed with one charge 
equivalent of each of the TAT oligomers, and then the standard 
transfection agents were added), a strong increase of transgene 
expression depending on the degree of oligomerization and the 
type of standard transfection agent was observed (Fig. 4). The 
presence of TAT<sub>3</sub>, TAT<sub>4</sub>, and TAT<sub>5</sub> enhanced transgene expression 
of PEI 130-, 80-, and 40-fold, respectively. pLa did not 
enhance PEI-mediated gene transfer. An analog behavior could 
be observed when the TAT oligomers were combined with ei- 
ther fractured dendrimers or LipofectAMINE.

Kinetics of Transgene Expression Mediated by Ternary TAT<sub>n</sub>- 
PEI-DNA Complexes—To characterize in more detail the trans- 
fection mechanism of the ternary TAT<sub>n</sub>-PEI-DNA complexes, 
the kinetics of transgene expression was examined on 16HBE 
cells (Fig. 5). At the 4-h time point, the transgene expression of 
transfected 16HBE cells was 220-, 170-, and 100-fold higher for 
the TAT<sub>2</sub>-, TAT<sub>3</sub>-, and TAT<sub>4</sub>-derived ternary gene vectors as 
compared with PEI polyplexes, respectively. At the 8-h time 
point, the enhancement in transfection efficiency increased to 
390-, 240-, and 140-fold, respectively. After 24 h, the level of 
transfection efficiency mediated by the ternary TAT<sub>2</sub>-4 gene 
vecors was 104-, 56-, and 45-fold higher as compared with PEI 
polyplexes, respectively. The transfection efficiency of the 
TAT<sub>2</sub>-PEI-DNA complex at as early as the 4-h time point was 
4-fold higher as compared with the transfection efficiency me- 
diated by PEI polyplexes after 24 h (p < 0.01). These data 
suggest rapid accumulation of the DNA in the cell nucleus 
when TAT oligomers were incorporated into the PEI 
polyplexes.

Transfection Efficiency of Ternary TAT<sub>n</sub>-PEI-DNA on Growth-arrested Cells—To further assess whether the 
enhancement of transfection efficiency mediated by the TAT ol- 
gomers could be due to facilitated nuclear transport of the 
transgene, transfection experiments were performed on growth-arrested cells. Transfection experiments were per- 
formed on 16HBE cells either arrested by aphidicolin incuba- 
tion (25) or through serum starvation (26). Under both condi- 
tions, the transfection efficiency of the ternary gene vector 
complexes was significantly higher as compared with PEI poly- 
plexes (Fig. 6). The incorporation of TAT<sub>2</sub>, TAT<sub>3</sub>, and TAT<sub>4</sub> led to 
a 35-, 20-, and 12-fold higher transfection rate on aphidico- 
lin-treated cells and a 12-, 10-, and 8-fold higher transfection 
rate on cells treated under conditions of serum starvation, 
respectively. In both cases, transgene expression mediated by 
the ternary complexes (e.g. TAT<sub>2</sub>-PEI-DNA) was 2–3-fold 
higher as compared with transgene expression mediated by 
PEI polyplexes under standard conditions (i.e. no growth-ar- 
rested cells). pLa improved PEI-mediated transfection 13- and 
3.5-fold.

Transfection Efficiency of Ternary TAT<sub>n</sub>-PEI-DNA on Pri- 
mary Cells and in Vivo—To better mimic the in vivo situation, 
transfection with the ternary TAT<sub>2</sub> or pLa gene vector com- 
plexes has been performed on primary nasal respiratory epi- 
thelium (Fig. 7A). Transgene expression mediated by the ter- 
nary TAT<sub>2</sub>-PEI-DNA complexes was 10-fold higher as 
compared with transgene expression mediated by PEI. The 
incorporation of pLa improved PEI-mediated transfection as 
well, but only 1.6-fold. Gene transfer efficiency of ternary TAT<sub>2</sub> 
gene vectors has been examined in vivo and compared with 
ternary pLa and standard PEI gene vectors. Mice were intra- 
tracheally instilled with gene vector solutions, and luciferase 
gene expression was measured after 24 h (Fig. 7B). Whereas 
luciferase expression mediated by ternary TAT<sub>2</sub> gene vectors 
was 4-fold higher as compared with PEI polyplexes, pLa in- 
duced only a 2-fold increase in luciferase gene expression. 
These data indicate that TAT<sub>2</sub> can improve gene delivery effi- 
ciency of PEI polyplexes in vivo.

DISCUSSION

In this study, we examined whether the unique features of the arginine-rich motif of the HIV-1 TAT protein (TAT peptide) 
(i.e. protein transduction domain and nuclear localization se- 
quence) would enhance nonviral gene transfer. We examined 
these unique features in the context of biophysical parameters 
of the gene vectors. The biophysical parameters of synthetic 
vectors have been shown, on the one hand, to influence their 
gene transfer efficiency (13, 27, 28); on the other hand, they are 
influenced by the molecular weight of the cationic polymer (13, 
14). Thus, to control the biophysical parameters (i.e. also to 
control in parts the gene transfer efficiency), the TAT peptide 
was synthesized as oligomers of different molecular weights. 
Interestingly, the biophysical complex parameters of the 
TAT<sub>2</sub>-4 gene vectors were similar. The degree of oligomeriza- 
tion only correlated with the degree of DNA condensation as 
examined by fluorescence quenching assay and increased with 
the higher degree of oligomerization. Gene vectors formulated 
with pLa exhibited similar biophysical parameters. These data 
show that the size and surface characteristics of both the TAT 
oligomer and pLa-derived gene vectors were similar, whereas
DNA compaction of the gene vectors slightly varied depending on the degree of oligomerization. From this, we conclude that differences in transfection efficiency among the TAT oligomers and when compared with the pLa can be primarily attributed to the TAT sequence itself rather than to different biophysical gene vector parameters. The results obtained from transfection experiments were further confirmed by confocal laser-scanning microscopy, which showed higher accumulation of DNA in the cell nucleus mediated by Tat$_2$- and Tat$_3$- but not by Tat$_1$- and pLa. In addition, these data could suggest that the affinity of the Tat oligomers to the nuclear import machinery decreases with the higher degree of oligomerization and is minimal for pLa.

The presence of chloroquine during transfection has been shown to increase the level of transgene expression of several nonviral gene vectors due to reduced endolysosomal entrapment and degradation of the gene vector complexes (19). Since transgene expression strongly increased in the presence of chloroquine, this indicated that endosomes were involved in cellular uptake of the gene vectors. However, it is conceivable that two separate uptake mechanisms (i.e., endosomal uptake and direct membrane penetration) take place independently in parallel at the same time. To examine this possibility, the transfection process has been analyzed when endocytosis was blocked (i.e. at 4°C (6, 10, 11)) or in the presence of metabolic inhibitors (20–22). Both transfections performed at 4°C and in the presence of endocytosis inhibitors led to a strong reduction of transgene expression. Therefore, the main uptake mechanism of Tat oligomer gene vectors was apparently through endocytosis and not mediated by a proposed protein transduction domain. A possible explanation could be due to the size of the gene vectors (~70 nm). Constructs that have been delivered into cells via the conjugation of the Tat peptide so far have been much smaller in size (45–200 nm) (9, 10). Thus, simple electrostatic binding of DNA by the Tat peptide might lead to particles inappropriate for membrane penetration.

The higher gene transfer efficiency mediated by the Tat$_2$ peptide as compared with the nuclear transport-deficient Tat$_3$-M1 peptide (12) and the strong increase of gene transfer of the Tat oligomer complexes in the presence of chloroquine as compared with pLa complexes suggests that gene transfer of the Tat oligomers was peptide sequence-dependent, which could be due to facilitated nuclear translocation mediated by the nuclear localization sequence function. This concept is supported by the behavior of ternary gene vector constructs. Transfection efficiency of pLa in the presence of the Tat oligomers was strongly enhanced on growth-arrested and primary cells as well as in vivo and at early time points after transfection. In all of these cases, only a minor fraction of cells underwent mitosis (i.e., breakdown of the nuclear membrane could be excluded as the major mechanism for nuclear DNA localization). Consequently, these experiments suggest that the Tat oligomers could promote nuclear translocation of the DNA. An increase was observed when pLa was combined with Lipo-fectAMINE and when experiments were performed on growth-arrested cells and in vivo. These observations could be due to partial activity of pLa as a nuclear localization sequence, which has been reported recently (29).

The formulation order of vector complexes was found to have an enormous effect on the gene delivery efficiency. A 390-fold increase of transgene expression was only observed when the DNA was first complexed with the Tat oligomer and PEI was added afterward. When the complexes were generated vice versa, transgene expression remained on the same level as of PEI polyplexes. These differences could be explained by analysis of the structure of the resulting gene vectors. When DNA was first complexed with the Tat oligomers at a charge ratio of ±1, the resulting intermediate complexes had a z potential of ~20 mV (Table 1), whereas DNA complexed with PEI at a nitrogen of PEI per phosphate of the DNA ratio of 10 resulted in a z potential of ±32 mV. Thus, preformed negatively charged Tat gene vectors allow the binding of positively charged PEI to their surface through electrostatic interaction, which was indicated by a change of the z potential from negative to positive (~40 mV) and by size reductions of 600 nm. In contrast, such changes were not observed when DNA vectors were formulated vice versa. Thus, the formulation method could result in a shell-like ternary complex with the Tat oligomers bound to the DNA in the core of the complex and a layer of PEI at the periphery of the complex (see Supplementary Material Fig. 3A). In contrast, binding of the Tat oligomers on the surface of positively charged PEI polyplexes should not be possible due to electrostatic repulsion. In this case, Tat oligomers could rather be homogeneously distributed in the solution (Supplementary Material Fig. 3B). From these data, we infer the following model, which could explain the exceptional efficiency of the ternary gene vector complexes. The tertiary complexes, which apparently look like a plain PEI complex, could behave like a plain PEI polyplex concerning the first steps that are involved in gene transfer (i.e., the complexes are apparently taken up into the cell via receptor-mediated endocytosis (hepane-sulfate proteoglycan receptor) (30) and are located to the endosomal compartment. In the endosomes, the “proton-sponge” effect of PEI (31) could induce their disruption and release the complexes into the cytosol. Within the next steps, the PEI shell could be released, probably due to nonspecific interaction with cytosolic components, and the core complex could slowly diffuse toward the cell nucleus. Binding of the Tat oligomer to the nucleocytoplasmic shuttle protein importin b could then facilitate DNA translocation into the nucleus, resulting in high transgene expression. In this model, PEI would function as an endosomal disrupting agent such as chloroquin but incorporated into the complex itself.

In conclusion, we showed that oligomers of the Tat peptide mediate efficient gene delivery. In particular, their combination with powerful standard cationic transfection reagents (e.g. PEI) resulted in very efficient gene transfer. This effect correlated inversely with the degree of oligomerization, and we suggest that facilitated nuclear localization could be involved. Further studies will focus on improved formulation methods of Tat-DNA complexes to overcome size restrictions that could limit the functionality of the protein transduction domain and nuclear localization sequence so far.

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