The ultimate destination for most gene therapy vectors is the nucleus and nuclear import of potentially therapeutic DNA is one of the major barriers for non-viral vectors. We have developed a novel approach of attaching a nuclear localization sequence (NLS) peptide to DNA in a non-essential position, by generating a fusion between the tetracycline repressor protein TetR and the SV40-derived NLS peptide. The high affinity and specificity of TetR for the short DNA sequence tetO was used in these studies to bind the NLS to DNA as demonstrated by the reduced electrophoretic mobility of the TetR-tetO-DNA complexes. The protein TetR-NLS, but not control protein TetR, specifically enhances gene expression from lipofected tetO-containing DNA between 4- and 16-fold. The specific enhancement is observed in a variety of cell types, including primary and growth-arrested cells. Intracellular trafficking studies demonstrate an increased accumulation of fluorescence labeled DNA in the nucleus after TetR-NLS binding. In comparison, binding studies using the similar fusion of peptide nucleic acid (PNA) with NLS peptide, demonstrate specific binding of PNA to plasmid DNA. However, although we observed a 2–8.5-fold increase in plasmid-mediated luciferase activity with bis-PNA-NLS, control bis-PNA without an NLS sequence gave a similar increase, suggesting that the effect may not be because of a specific bis-PNA-NLS-mediated enhancement of nuclear transfer of the plasmid. Overall, we found TetR-NLS-enhanced plasmid-mediated transgene expression at a similar level to that by bis-PNA-NLS or bis-PNA alone but specific to nuclear uptake and significantly more reliable and reproducible.

Nuclear translocation of a DNA-vector complex is a crucial limiting step in non-viral transfection (1, 2). Several studies with different transfection agents have shown that plasmid is efficiently internalized into cells but less than 1% of the DNA present in the cytoplasm reached the nucleus (3, 4). In addition, several groups provided experimental evidence that cells undergoing mitosis are far more readily transfected than cell cycle arrested or quiescent cells, suggesting that the dissociation of the nuclear membrane during mitosis greatly facilitates nuclear entry (5, 6).

The nuclear membrane is a tight barrier and transport of large macromolecules from the cytoplasm to the nucleus occurs through a specialized structure of the nuclear envelope, the nuclear pore complex (for review see Refs. 7 and 8). Transport generally occurs by an energy-dependent process involving the interaction of specific highly basic nuclear localization sequences (NLS) with the nuclear pore complex (9, 10). The nuclear membrane is also a tight barrier for exogenous DNA or RNA and many proteins of the karyophilic viruses contain NLS sequences, which are involved in active transport of the viral genome through the nuclear pore (11).

Several studies have shown that the addition of an NLS peptide to non-viral gene transfer complexes can increase their transfection efficiency (12, 13). However, when the highly basic NLS peptide binds to DNA by electrostatic interaction, the transfection efficiency is modest (14, 15) and it seems that the direct interaction of the NLS peptide with DNA can impair its nuclear import capacity (16, 17). Chemical modification and random covalent coupling of peptides to a gene expression cassette can also have detrimental effects (18, 19). Little increase in nuclear targeting was obtained by use of an NLS peptide linked to a DNA by triple helix formation with a single DNA target (20), whereas covalently linking an NLS peptide to one end of capped linear DNA produced promising results (13). However, this technique is time consuming, expensive, and difficult to scale up.

To be efficient the NLS peptide should be able to bind with high affinity to a non-essential DNA sequence and remain accessible for recognition by the nuclear pore complex. Here, we compare two approaches for site-specific binding of an NLS peptide to DNA. The first system exploits the tetracycline repressor protein TetR, a homodimer, with high affinity and specificity for a palindromic short DNA sequence known as the tetracycline operator sequence tetO. In bacteria, in the absence of tetracycline, the repressor binds to this sequence (association constant $K_a = 10^{11} M^{-1}$) inhibiting the expression of the two genes it controls (21, 22). This system has also been widely applied to control gene activities in eukaryotic cells (23). In this study, we have used its affinity for the operator sequence to attach an NLS peptide to plasmid vector DNA (Fig. 1A) by producing a fusion protein between the TetR protein and the well known NLS peptide from the SV40 large tumor antigen (13, 15) and have investigated the ability of the fusion protein TetR-NLS to increase DNA nuclear import.

For comparison we have also exploited the ability of a dimeric homopyrimidine bis-PNA (peptide nucleic acid) (24, 25) to strand invade double stranded plasmid DNA and bind to a

The abbreviations used are: NLS, nuclear localization sequence; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; MSC, mesenchymal stem cells; PNA, peptide nucleic acid.
target site in a triplex interaction. Previously, binding of duplex forming PNA-NLS to 11 target sites of plasmid DNA has been observed using either bis-PNA-NLS or bis-PNA alone at low and high PNA:DNA binding ratios suggesting that this is not because of NLS-mediated nuclear localization.

**MATERIALS AND METHODS**

**Construction of the TetR-NLS Fusion Protein and the Reporter Gene Plasmid**—Plasmids expressing the TetR-NLS protein or the TetR protein were constructed using the pMal-c2 vector (New England Biolabs). To construct the pMal-TetRLNS plasmid, the tetO sequence was PCR amplified from pTetO-1 using a primer encoding the NLS from the SV40 large tumor antigen (pkkkrkvedp) with an XbaI and EcoRI site at the 5' and 3' end of the NLS. The amplification product was digested with EcoRI and inserted into the XmnI/EcoRI-digested pMal-c2 vector using blunt end and EcoRI ligation, resulting in the pMal-TetRLNS plasmid. The control plasmid pMal-TetRI was obtained by digestion of the plasmid pMal-TetRNLS by XbaI and self-ligation to remove the untranslated region of the expression cassette. Plasmid pGL3-2X7 contains the luciferase reporter gene under control of the SV40 promoter and seven TetR-binding sites (tetO) in the 3' end of the NLS. The amplification product was digested with BglII and SalI. A primer that inserts a BamHI site at the 5' end of the NLS was used to create the XhoI/SacI-digested pGL3 vector. The desired amount of DNA (plasmid or linear fragment), which contains the tetO fragment was incubated for 2 h at room temperature in 20 mM Tris-HCl (pH 8.0) containing 5 mM MgCl2, with the fusion protein TetR-NLS or with the control protein TetR at different molar ratios of dimer protein to plasmid. The same amount of protein was used with the linear DNA fragment.

The protein-DNA complexes were loaded on a 1% agarose gel made up in 1× TBE and containing 0.5 μg/ml ethidium bromide. For the inhibition binding experiment, the complex buffer was supplemented with a tetracycline analogue, the doxycycline hydrochloride (Sigma).

**PNA-NLS Molecules and Plasmid Constructs**—J-bis-PNA H-(k),TTT- TTTTTC-3, TTTTCCCTCTT-NH2 was the kind gift of Dr. Peter Nielsen (Center for Biomolecular Genkendelse, Copenhagen, Denmark). Control bis-PNA H-(k),TTTCTCTTCTT(L),TTTTCTCTTCTK-NH2 and bis-PNA-NLS H-pkkkrkvedpyk(G),TTTCTCTTCTT(L),TTTTCTCTTCTK-NH2 were synthesized by Oswel (Southampton, UK). Peptide nucleic acids are listed above with PNA Watson-Crick bases capitalized and amino acids in lowercase. The lysines (k) added to the NLS are designed to improve PNA stability and hoist gene binding to DNA because of their positive charge, and to give these PNAs a slight net positive charge. L refers to a flexible AEEA linker (amino 2,6-diazoacidoic acid), whereas, eg refers to a similar flexible hydrophilic linker (8-amino 3,6 diazoacidoic acid). J refers to the synthetical nucleoside pseudouridine, which exhibits a positive charge. The SV40-modified NLS alone, H-pkkkrkvedpyk-NH2, was synthesized by Affiniti Research Products Ltd. (Manheed, Exeter, UK).

Primers PNAxFwd 5′-TGGACTTTTCTTCTTGTG-3′ and PNAxRev 5′- TGGCAAGAGAAAAAGG-3′ were used to insert a PNA-binding site, specific for all of the PNAs, flanked by dual SalI sites in pDlox3 (28), making primers that corresponded to the TetR-NLS cassette (not shown). The TetR-NLS cassette was excised by BamHI and BglII digestion and inserted into the single BamHI site of pNOX3 to create two constructs. The final constructs PnIX2 and PnIX10 contain the PNA-binding site at the end of the luciferase gene, or in front of the cytomegalovirus promoter, respectively.

**PNA Binding and Assay for Restriction Digest Blocking**—1 μl of PNA in 0.1% trifluoroacetic acid, with a final concentration of 0-20 μM, was added to 1 μl of template DNA (usually 40 or 200 ng). The reaction was incubated at room temperature for 1 h, to achieve binding of PNA to DNA by strand invasion. The salt concentration was then adjusted to 10 mM NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 100 mM NaCl (Inivitrogen) and 1 μl of either SalI (flanking the PNA-binding site) or EcoRI (80 bp downstream of the PNA binding site), and incubated at 37 °C for 1 h. This reaction was then run on a 1% agarose gel to visualize digestion of DNA.

**Cell Transfection and Transgene Detection**—NIH 3T3, Hela, A549, COS7, and N18 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum. Primary mesenchymal stem cells (MSC) were derived from the femur of a neonatal lamb. Briefly, whole marrow was flushed in phosphate-buffered saline and homogenized by fine chopping of the calcified material. A red blood cell lysis and filtration was performed and cells were washed in buffered saline containing 1% fetal calf serum and resuspension in human mesencult medium (StemCell Technologies) according to the manufacturer’s instructions for human MSC derivation. Sheep MSCs were derived by their ability to adhere to tissue culture dishes and their fibroblastic properties and cultured for a maximum of two passages in human mesencult medium (StemCell Technologies) prior to transfection.

Cells were seeded, 24 h before transfection, in 48- or 24-well tissue culture plates to reach 60 to 70% confluence during transfection. The complexes TetR-NLS-DNA or PNA-DNA were prepared as described above with 0.25 or 0.4 μg of plasmid (depending on the tissue culture plate) or the desired amount of linear fragment to give an identical gene copy number were diluted in Opti-MEM (Invitrogen) and LipofectAMINE (Invitrogen) was added to the pre-formed DNA complexes at a DNA:liposome ratio of 1:12 (w/w) and incubated for 30 min at room temperature to complete the liposome formation before adding to cells. After 3 h, transfection mixtures were replaced with 100 μM NaCl, 1 mM EDTA and the protein of interest was eluted in the same buffer with 10 μM maltose. The protein-containing fractions were pooled, concentrated in an Amicon centric unit and dialyzed overnight against 20 mM Tris-HCl (pH 8.0). The purified samples were then analyzed by SDS-polyacrylamide gel electrophoresis and quantified by BCA protein assay (Pierce), before storage at −20 °C. An average of 3 mg of purified protein can be obtained per liter of bacteria culture.
complete medium. The transgene activity was measured after 24 h using a luciferase reporter assay kit (Roche Diagnostics) or a chemiluminescent/β-galactosidase reporter assay kit (Roche Diagnostics). The protein concentration of the cell lysates was determined using the BCA protein assay (Pierce) and the enzyme activities were expressed as relative light units per milligram of cellular protein. Each experiment was performed at least three times.

For the in situ/β-galactosidase visualization, cells were fixed with paraformaldehyde/glutaraldehyde solution and X-gal staining was performed. Cells were then counted, at least three separate times for each well to determine the transfection efficiency and each transfection experiment was repeated at least three times.

For transfection on cell cycle-arrested cells, cells were grown to 60–70% confluence and the medium was replaced by Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and containing aphidicolin at 5 μg/ml (Sigma). After an incubation of 24 h, transfections were carried out as described above with medium containing aphidicolin. For the experiment in the presence of doxycycline, the complex buffer and the cell medium were supplemented with doxycycline hydrochloride (Sigma) at a final concentration of 1 μg/ml.

Cell Trafficking Study—To label the fragment Frag2X7tetO, without damaging the TetR-NLS binding recognition motifs tetO, the DNA was digested with MluI and SalI producing a 5' protruding end. The recessed 3’ end of the fragment was then filled using exonuclease-free Klonev-2DNA polymerase (Amersham Biosciences) in the presence of 20 μM unlabeled dATP, dCTP, dGTP, and Cy3-UTP for 1 h at 37°C. Unincorporated nucleotides were removed using a QiaQuick purification column (Qiagen).

Cells were plated on glass coverslips for 1 day and transfections were performed as described using 0.5 μg of labeled DNA. After 1 or 2 h at 37°C, cells were fixed with a solution of paraformaldehyde (3%) for 20 min at room temperature. After several washes with phosphate-buffered saline, the slides were mounted in fluoro mounting medium (ICN Biomedicals) and observed on a fluorescence microscope.

RESULTS

Construction, Expression, and Purification of the Recombinant TetR-NLS Protein—A plasmid designed for bacterial expression and purification of the protein TetR-NLS, which contains the TetR repressor in fusion with the SV40-derived NLS-peptide or the control protein TetR alone were constructed in the pMal-c2 vector (New England Biolabs) (Fig. 1A). In this vector, the cloned gene was inserted downstream from the malE gene of Escherichia coli, which encodes the maltose-binding protein, resulting in expression of TetR-NLS as an maltose-binding fusion protein. After induction by isopropyl-1-thio-β-D-galactopyranoside the protein was overexpressed in bacteria and the fusion protein can be purified easily from the bacterial lysate on an amylose resin column using the affinity of the maltose-binding protein for maltose. As shown in Fig. 1B the resulting product can be found as a major band at 67 kDa on an SDS-polyacrylamide gel. Crucially, after dialysis in an appropriate buffer, the recombinant TetR proteins are able to form functional homodimers equivalent to the wild type TetR repressor protein (29) (Fig. 1C).
TetR-NLS Protein Binds Specifically to tetO Containing DNA—To investigate whether the TetR-NLS fusion protein could specifically bind the tetO operator sequence as effectively as the TetR protein, we performed a gel retardation assay using plasmid pGL3–7tetO carrying seven operator sequences (7tetO) at the 3′ end of the luciferase gene (Fig. 2A). In accordance with the established uses of TetR/tetO interaction in gene expression studies (30) we constructed this vector with 7 TetR operator sequences (7tetO). Both TetR and TetR-NLS proteins, respectively, appear as one major band of reduced electrophoretic mobility corresponding to protein-DNA complexes. A small fraction of DNA was still able to migrate into the gel as non-complexed DNA. As already described for the wild type Tet repressor (21), binding of the fusion protein is more efficient in the presence of a low concentration of MgCl₂ (TetR-NLS w/o MgCl₂) or in the presence of 5 mM MgCl₂ (TetR-NLS). The experiment was performed in parallel with DNA lacking the 7tetO sequence. Binding of the fusion protein TetR-NLS in the presence of doxycycline (Dox) at a molar ratio for Dox:TetR-NLS of 1 or 5. As a further demonstration of specificity, we investigated the effect of the antibiotic tetracycline on binding of the TetR-NLS to the tetO sequence fragment. The Tet repressor affinity for the operator sequences is abolished by complexing it with the antibiotic (23, 31). In the TetR-NLS system, we found that tetracycline inhibits the binding only partially (data not shown) and therefore applied the more potent tetracycline analogue, doxycycline (23, 32). At a molar ratio Dox:TetR-NLS of 1:1, the binding of the Tet repressor to the DNA was partially abolished. When the concentration of doxycycline was increased to a ratio 5:1, the DNA was completely released from the TetR-NLS protein and migrated freely into the gel (Fig. 2B). This indicates that the formation of the TetR-NLS-DNA complex is indeed because of the binding between the TetR-NLS and the tetO sequence and not the result of nonspecific interaction.

TetR-NLS Enhances Lipofection—To investigate the ability of the TetR-NLS fusion protein to enhance gene transfer, protein-DNA complexes were formed at several different molar

![Fig. 2. Gel retardation assay of fusion protein-DNA complexes. A, 1 μg of plasmid (plasmid pGL3–7tetO) or 3-kb fragment DNA carrying seven operator sequences (7tetO) were incubated with the fusion protein TetR-NLS, at a molar dimer protein:DNA ratio of 5, without MgCl₂ (TetR-NLS w/o MgCl₂) or in the presence of 5 mM MgCl₂ (TetR-NLS). The experiment was performed in parallel with DNA lacking the 7tetO sequence. B, fragment DNA carrying 7tetO was incubated with the fusion protein TetR-NLS in the presence of doxycycline (Dox) at a molar ratio for Dox:TetR-NLS of 1 or 5. Non-complexed DNA (DNA) or DNA complexed with the Tet repressor without NLS sequence (TetR) were used as a control. The complexes were then electrophoresed on a 1% agarose gel.](image-url)
ratios (1–10 corresponding to 10:250 ng to 100:250 ng) of protein dimer to plasmid using the luciferase expression plasmid pGL3–7tetO and then adding the cationic lipid LipofectAMINE to the complexes. Because the addition of the protein can modify the size of the lipid-DNA complexes and could thereby influence transfection, the size profile of the different complexes was studied by photon correlation spectroscopy. After 30 min, the size of the lipid-DNA complexes were 188 ± 30 nm. A slight increase in size was observed when the protein TetR-NLS or TetR was added to the complexes resulting in an average size of the complexes of 204 ± 30 nm at a ratio of 5 and 216 ± 30 nm at a ratio of 10.

The LipofectAMINE-protein-DNA complexes formed at different ratios were used to transfect NIH 3T3 cells (Fig. 3). At a molar ratio of protein dimer/plasmid of 5:1, the fusion protein TetR-NLS increases the transfection efficiency of the LipofectAMINE more than 4-fold, whereas no enhancement of transfection efficiency was observed with the control plasmid pGL3. These data indicate that the TetR-NLS protein enhances gene transfer in a specific manner involving binding to the tetO operator sequence and that this enhancement is because of the presence of the NLS peptide as it was not observed with the Tet repressor alone. Higher ratios of protein-DNA did not improve the gene transfer.
Because the fusion protein binds the linear fragment more efficiently and because we assume that efficient nuclear targeting of non-viral constructs also depends on a large extent on the size of the transferred constructs, the same transfection experiments were done with a 2.7-kb DNA fragment containing only the luciferase expression cassette with the 7 tetO sites at its 3’ end (Frag7tetO). The amount of DNA used (125 ng) was calculated to provide an identical number of transgenes as the intact plasmid (Fig. 3). The lipofection efficiency of the linear fragment was about 1 order of magnitude lower than plasmid lipofection. This is most likely because of degradation of the unprotected DNA ends by exonucleases. However, the enhancement of transfection by the TetR-NLS protein was significantly higher than with the DNA plasmid, about 8-fold of LipofectAMINE alone. The highest increase was achieved with the Frag2X7tetO, which contains recognition motifs at both ends of the fragment. With this fragment, the TetR-NLS protein at a ratio of TetR-NLS:DNA of 20 enhanced the transfection efficiency of the lipoplexes up to about 16-fold over LipofectAMINE only. This increase is not just because of the protection of the DNA ends by the proteins because only a two times increase is observed with the LipofectAMINE/TetR control. With the corresponding plasmid, pGL3-2X7tetO, containing the tetO fragment both upstream and downstream from the expression cassette, the increase is around 5-fold that of LipofectAMINE, at a protein dimer:plasmid ratio of 20:1. When the antibiotic doxycycline is added during transfection, the enhancement provided by the NLS fusion protein was ablated (Fig. 4), suggesting that specific binding of the TetNLS to the tetO recognition motifs is required for the enhancement of lipofection.

**TetR-NLS Increases Nuclear Localization of Transfected DNA**—To study the intracellular localization of the complexes, we labeled the DNA Frag2X7tetO with the Cy-3 fluorophore. The label was applied at the end of the fragment in order not to destroy the binding site of the fusion protein. Two hours after lipofection of NIH 3T3 cells with the DNA-TetR-NLS complexes, some localized fluorescence was observed by fluorescence microscopy in the nuclei of many cells, whereas with LipofectAMINE alone the DNA was generally only observed in the cytoplasm at this time point (Fig. 5). The proportion of cells containing DNA in the nuclei was evaluated by counting 100 cells in two separate experiments. After 1 h lipofection, with the TetR-NLS protein 35% of cells contained fluorescent DNA in the nucleus in contrast to 11% cells with LipofectAMINE only. After 2 h, these numbers reached to 51% with the TetR-NLS and 15% with the LipofectAMINE.

**TetR-NLS Increases Transfection of Cell Cycle Arrested and Primary Cells**—To exclude cell division in the process of nuclear entry, transfection experiments were also performed on cells pretreated with aphidicolin, which blocks cells in G1/S phase (33) (Fig. 6). A dramatic decrease of the level of lipofection with DNA only on these cell cycle-arrested cells is observed. However, addition of the fusion protein at the optimum dimer protein:plasmid ratio of 20 increases the transfection efficiency 12-fold. An increase of 8-fold is also still obtained with the Frag2X7tetO. The enhancement of lipofection by TetR-NLS on arrested cells is a further indication of an NLS-mediated energy-dependent nuclear transfer of the TetR-NLS-DNA involving the nuclear pore complex.

To quantify the effect of TetR-NLS on transgene expression in terms of the percentage of transgene expression as well as by overall protein determination, we transfected several cell lines, including primary sheep MSC with a β-galactosidase plasmid. TetR-NLS enhanced β-galactosidase expression in all cell lines; about 3-fold on the easily transfected 3T3 cells and about 5-fold on the other cell lines. This activity increase is always associ-

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**Fig. 4. Inhibition of transfection by doxycycline.** LipofectAMINE/TetR-NLS-DNA complexes were prepared at the optimum ratio using the Frag2X7tetO as described in the legend to Fig. 3 in the presence of doxycycline (DOX). Transfections were performed in NIH 3T3 cells pretreated with doxycycline at a final concentration of 1 μg/ml. Luciferase activity was analyzed 24 h after transfection and expressed in relative light units (RLU/mg of protein).

**Fig. 5. Fluorescence microscopy with Cy3-labeled DNA.** The linear fragment Frag2X7tetO labeled with Cy3 was used to transfect NIH 3T3 cells in combination with TetR-NLS-LipofectAMINE (top) or with LipofectAMINE alone (bottom). Two hours after transfection, cells were fixed and observed by fluorescent microscope (magnification ×100).
ated with an increase of the percentage of transfected cells. Interestingly, the best result was obtained in the primary sheep cell line, where the addition of the TetR-NLS enhanced the percentage of transfected cells 3.5-fold (Fig. 7).

**bis-PNAs Specifically Bind to a PNA Target Site in Plasmid DNA at Physiological pH**—To compare our TetR-NLS system with another nuclear targeting system we tested the ability of a PNA-NLS to increase DNA gene transfer. For this study, we used a bis-PNA displaying the SV40-derived NLS peptide (bis-PNA-NLS). The capacity of bis-PNA to strand invade double stranded plasmid DNA and bind to a target site by triplex formation was exploited for binding of the NLS sequence to the DNA. J-bis-PNA (24) is known for its pH-independent triplex formation and clamping properties because of the presence of pseudoisocytosine bases (J) on the second clamping strand. The presence of pseudoisocytosine bases (J) is an important component of hoogsteen strand (triplex) binding kinetics (34), particularly at a physiological pH. Therefore, to further protonate the hoogsteen strand of our bis-PNAs that lack Js we added 3 lysine residues in the case of the control bis-PNA and with another nuclear targeting system we tested the ability of bis-PNA to strand invade double stranded plasmid DNA and bind to a target site by triplex formation was exploited for binding of the NLS sequence to the DNA.

Enhancement of Lipofection by bis-PNA-NLS—We then investigated the effect of bis-PNA-NLS on the transfection efficiency of LipofectAMINE at several molar ratios of bis-PNA-NLS-DNA from 10:1 to 10,000:1 using a plasmid containing a PNA-binding site after the luciferase expression cassette (Fig. 9). Addition of bis-PNA-NLS to DNA preparations at a low ratio (10:1) consistently produced a significant (p < 0.05) and reproducible 2–8.5-fold increase in luciferase activity over LipofectAMINE alone on N18 and HeLa cells, respectively, whereas this increase was not always reproducible on other cell lines. When higher ratios of bis-PNA-NLS to DNA were used (10,000:1), a highly significant (p < 0.01) 1.5–3 log increase in luciferase activity was observed in all cells tested. However, the addition of the control bis-PNA (without an NLS sequence) or of the control bis-PNA alone mixed with NLS peptide produced a
similar increase at ratios of 10:1 or 10,000:1, respectively. These data suggest that enhancement effects may not be because of increased nuclear localization mediated by specific binding of the NLS. As the larger effect occurred at binding ratios (10,000:1) causing retardation of DNA in agarose gels, this suggests that luciferase activity enhancement was most likely a result of nonspecific DNA condensation by bis-PNA-NLS or bis-PNA alone. Moreover, an almost identical enhancement of transgene expression was observed using these conditions with a control plasmid, which does not contain a PNA-binding site (data not shown). This emphasizes further that the enhancement is not because of a specific bis-PNA-NLS binding interaction but most likely a result of nonspecific DNA condensation by PNA.

**DISCUSSION**

The aim of this study was to improve DNA nuclear translocation with non-viral vectors. It is often difficult in NLS studies to establish the difference between an increase in gene expression because of a genuine increase in active nuclear import of DNA rather than as a result of DNA condensation by the cationic NLS sequence (17). The results of this study indicate
that the novel TetR-NLS fusion protein enhances lipofection in an NLS-specific manner by facilitating nuclear import. First, binding of the TetR-NLS fusion protein to DNA requires both the presence of the TetR domain in the fusion protein and of the tetO recognition sequences in the DNA. The electrophoretic mobility shift assay demonstrated binding of the TetR-NLS to the tetO recognition motifs without interference of the cationic NLS sequence. This binding is not because of electrostatic interaction with the DNA via the NLS, because no gel retardation of DNA without the tetO sequence is observed. Second, TetR-NLS binding can be completely abolished by doxycycline as observed in the original TetR/IRES system (32). And finally, the 8–12-fold increase in gene expression also observed on cell cycle-arrested cells suggests that this increase is not dependent on cell mitosis but is an energy-dependent process involving the nuclear pore complex and the NLS sequence. The TetR protein alone enhances the lipofection efficiency about two times, which may be because of the protection of DNA against exonuclease activity notably for the linear DNA, and this increase is abolished by aphidicolin. In contrast, the TetR-NLS protein increases the transfection efficiency of all DNA constructs containing the tetO sequence.

Enhancement of transgene expression was shown here on several cell lines including primary mesenchymal stem cells. As these cells have stem cell properties (36) this may be important for ex vivo gene therapy strategies. A comparison of the enhancement of gene transfer efficiency by determination of total transgenic protein and the percentage of transfected cells showed a less pronounced increase in percentage transduction. This is not surprising, as TetR-NLS would not increase the rate of cell entry of the construct but enhance nuclear transport once entry has occurred.

The enhancement of transfection with the TetR-NLS protein is dependent on the size or structure of the DNA construct. For the same copy number of transgenes, the increase is about 4 times with the plasmid and about 16 times with the Frag2X7tetO on cell cycle active cells. The size of the DNA construct seems to be a crucial parameter in nuclear transfer even with an active process. These data are in accordance with results of other teams (37). The most successful study using the SV40 NLS peptide achieved an around 100-fold increase, using short DNA fragments (13), whereas only a 2–8-fold increase was observed with plasmid (26, 38). It may be worth exploring different pathways using our TetR-based system for the nuclear import of larger DNA fragments. Increases of transfection efficiency of plasmid up to 60-fold have been obtained using a non-classical NLS sequence from the heterogenous nuclear ribonucleoprotein, the peptide M9, which utilizes a different nuclear protein import pathway involving transportin (39, 40).

Specific plasmid DNA binding could be achieved in our bis-PNA-NLS studies, refuting our initial concern regarding the binding capacity of these bis-PNAs lacking pseudouracil nucleosides J. Specific digestion inhibition by bis-PNA-NLS constructs at a ratio of 200:1, PNA:DNA, although somewhat reduced compared with J containing PNA constructs, was clearly effective, suggesting continued PNA binding to DNA at physiological pH. This is in agreement with other demonstrations of successful binding of non-protonated bis-PNA at low pH (41), or J-bis-PNA at high pH values (42), and suggests that we may be most likely to obtain a dual PNA binding conformation at the specific binding site on our plasmid (41), thus providing two PNA-NLS peptides per plasmid molecule.

However, the specificity of bis-PNA-NLS enhancement on gene expression from a luciferase expressing plasmid was less convincing. A 2–8-fold enhancement of luciferase expression was observed by complexing of bis-PNA-NLS with DNA at a low ratio (10:1) that is considerably lower than that required for observable PNA binding by restriction analysis. Although this effect was variable between and within cell types, it was often statistically significant. However, control bis-PNA + NLS alone, added at this ratio to DNA, also produced similar levels of luciferase enhancement (data not shown). The enhancing effect of PNA or NLS at this ratio cannot be because of condensation of DNA at such low PNA concentrations as seen from our restriction inhibition and retardation studies, suggesting an alternative mechanism of this enhancement of luciferase activity.
The high concentration effect of PNA:DNA enhancement of luciferase activity appeared to be solely because of charge/charge interactions leading to condensation of DNA. The enhancing effect of oligolysine on cationic liposome-mediated gene expression, by charge condensation of DNA (14, 43), supports the hypothesis that the enhancement by high concentrations of PNA is because of the same effect. Interestingly NLS alone bound to DNA does not have an enhancing effect on luciferase activity at a 10,000:1 ratio. This is in agreement with data on oligolysine enhancement of gene transfer, which shows that oligolysines containing shorter lysine chains (<13) do not condense DNA as well as, for example, Lys-18 (44, 45). Our data are in line with these observations because the NLS possesses 3 overall positive charges, and bis-PNA-NLS has 7. However, the control bis-PNA, containing solely 4 positive charges was also able to enhance luciferase activity at a 10,000:1 ratio, suggesting a PNA specific enhancement effect, not related to specific PNA binding, nor solely to charge ratio. Indeed high molar ratio (10,000:1) control bis-PNA-DNA complexes transfected without LipofectAMINE into cells achieved by optimizing the DNA construct using capped improvement of the transfection efficiency of this system may also be achieved by mixing the DNA and fusion protein.

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