Coupling of a targeting peptide to plasmid DNA by covalent triple helix formation

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Abstract The nuclear localization signal (NLS) of the SV40 large T antigen efficiently induces nuclear entry of proteins. We have developed a strategy for covalent coupling of one or a controlled number of NLS peptides to plasmid DNA at a specific site by triple helix formation. A psoralen-oligonucleotide-NLS peptide conjugate was synthesized and characterized by proteolysis with trypsin. This conjugate was used to covalently associate one NLS peptide to plasmid DNA by triple helix formation and photoactivation. The oligonucleotide-NLS peptide conjugate interacted with the NLS-receptor importin α. The reporter gene was expressed after transfection of the modified plasmid in NIH 3T3 cells, indicating no loss of the gene expression functionality of the plasmid. On the other hand, no increase in expression was observed as a result of the NLS peptide. This site-specific coupling technology can be used to couple to a plasmid other ligands targeting to a specific receptor.

Key words: Nuclear localization signal; DNA; Triple helix; Gene transfer; Importin; Gene therapy

1. Introduction

Plasmids used for non-viral gene therapy are generally delivered as a complex with a variety of chemical or biochemical vectors such as cationic lipids, in order to obtain maximal transfection efficiency [1]. Non-viral gene transfer with cationic lipids can be divided into several steps including internalization of the DNA via fusion at the cellular membrane or via endocytosis, followed by plasmid diffusion to the nuclear envelope and entry into the nucleus, probably through the pore complexes [2,3]. One of the steps limiting the efficiency of non-viral gene transfer into non-dividing cells is the entry of plasmid DNA from the cytoplasm into the nucleus [2,4].

The role of signal sequences for protein import into the nucleus is well documented [5,6]. Proteins larger than 60 kDa are excluded from the nucleus unless they harbor a nuclear localization signal (NLS). The NLS peptides usually comprise stretches of highly basic amino acids [5]. Among the different NLSs, the NLS of the simian virus 40 (SV40) large T antigen has been extensively studied. This short peptide possesses five basic amino acids and it efficiently induces nuclear targeting when conjugated to a non-karyophilic protein of molecular weight up to 465 kDa [7]. Karyopherins are involved in nuclear protein import through association with nuclear localization signals in the cytoplasm, then binding to the nuclear pore complex [8]. The 58 kDa mouse karyopherin α (called importin α or m-importin) binds to the NLS sequence and then interacts with karyopherin β (also called importin β). The resulting complex binds to the nuclear pore [9], and is translocated through the pore in a mechanism involving the small GTPase Ran and other proteins [10].

In order to determine if the nuclear import of plasmid DNA can be increased by association with the protein import machinery, it is straightforward to covalently link a NLS peptide to DNA. Non-specific covalent linkage of multiple NLS peptides on linearized or circular DNA has been described [11,12]. However, in these strategies, NLS peptides were associated with non-specific sites on DNA, leading to the loss of gene expression. In the present work, we have developed a new methodology to covalently associate a cationic NLS peptide with plasmid DNA at a specific site on the plasmid, by triple helix formation. We report here the synthesis and characterization of these conjugates. This site-specific coupling technology can be used for other purposes, for instance to couple to a plasmid other ligands targeting to a specific receptor.

2. Materials and methods

2.1. Analytical and semipreparative HPLC

Analytical and semipreparative HPLC were performed on a Merck-Hitachi gradient pump equipped with an AS-2000A autosampler, a L-6200A intelligent pump and a UV-Vis detector L-4000. Method 1: The HPLC system used a C18 Vydac-218 TP 1022 (10 mm diameter) column, with a gradient of acetonitrile/water 0.1% TFA (from 5 to 50% acetonitrile in 40 min) and a flow rate of 7 ml/min. Detection was by UV absorbance at 254 nm. Method 2: The HPLC system used a 250×4.6 mm C4 Vydac column and a gradient of 5–50% acetonitrile over 40 min in 0.1 M triethylammonium acetate, pH 7.5 (flow rate = 1 ml/min). Detection was by UV absorbance at 260 nm.

2.2. Peptide synthesis

The following peptide was synthesized: FmocK(N^{ε}-maleoyl)-GAGPKKKRKV-NH_2 which contains the wild type SV40 large T NLS (PKKKRKV). The peptide was synthesized on an Applied Biosystems 431A automatic synthesizer using a Rink amide resin as solid support and Fmoc strategy for amino acid assembly. Fmoc-Lys(N^{ε}-maleoyl)OH was coupled manually in a separate flask at the N-terminal position of the peptide with a solution of dichloromethane containing 4 equivalents of Fmoc-Lys(N^{ε}-maleoyl)OH, 4 equivalents of BOP reagent and DIEA (pH 10). The peptide was cleaved from the Rink resin in the usual manner and purified by semipreparative HPLC, as described above (method 1). The final purified products were analyzed by mass spectroscopy. FmocK(N^{ε}-maleoyl)-GAGPKKKRKVNH_2; HPLC: semipreparative, R_t = 22–27 min; MH^+: 1499.

2.3. Oligonucleotide synthesis

Purified oligonucleotides with the sequence 5′-AAGGAGAG-GAGGGAGGGAA-3′ were purchased (Eurogentec, Seraing, Bel-

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Abbreviations: NLS, nuclear localization signal; SV40, simian virus 40; GST, glutathione *S*-transferase

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gium). They were modified with either a thiol group at the 5′ or 3′ terminus and a C6 spacer arm, or a psoralen group at the 5′ terminus and a C6 spacer arm. The abbreviations for these oligonucleotides are given in Table 1.

2.4. Preparation of oligonucleotide-peptide conjugate

Lyophilized GA_{19}-SH or Pso-GA_{19}-SH oligonucleotides were dissolved in 0.1 M triethylammonium acetate, pH 7.5, at a concentration of 0.5 mg/ml. Lyophilized FmocK(N^{ε}-maleoyl)GAGPKKKRKV-NH_2 peptide was dissolved in 100 mM triethylammonium acetate, pH 7.5, at a concentration of 0.4 mg/ml. Equimolar quantities of oligonucleotide and peptide were mixed at room temperature. The reaction was monitored by HPLC, and, after 2 h, the conjugate was purified by HPLC as described above (method 2).

2.5. Degradation of oligonucleotide-peptide conjugates by trypsin

The two oligonucleotide-peptide conjugates, GA_{19}-NLS and Pso-GA_{19}-NLS, were analyzed after proteolytic treatment with trypsin. Solutions containing 1 μg of the conjugate in 7 μl of water were combined with 1 μl of trypsin (5 mg/ml; Boehringer Mannheim), 1 μl of 0.1 M Tris-HCl, pH 9 and 1 μl of 0.5 M EDTA. After 1 h of incubation at 37°C, samples were analyzed on 15% denaturing polyacrylamide gel and oligonucleotides were stained according to the Bio-Rad Silver Stain kit (Bio-Rad).

2.6. Covalent association of oligonucleotide-peptide conjugate to dsDNA

Plasmid pCMVLacZ (7257 bp) carries a cassette containing the enhancer-promoter from the immediate-early gene of cytomegalovirus (CMV) and the β-galactosidase gene. Plasmid DNA was purified using Wizard Megaprep kit (Promega, Madison, WI, USA). The sequence 3′-AAGGAGAGGAGGGAGGGAA-5′ was cloned between positions 7238 and 7256, out of the expression cassette. Plasmid (3 pmol, 15 μg) was incubated overnight at 37°C with oligonucleotide-peptide conjugate (150 pmol) in 0.1 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$. The mixture was cooled in an ice-water bath and illuminated for 15 min in the dark using a sun lamp (Rad Free UV lamp, 365 nm; Schleicher and Schuell, Ecquevilly, France). Excess oligonucleotide-peptide conjugates were removed by ion-exchange chromatography (PCR purification kit, Qiagen, Hilden, Germany).

2.7. Analysis of the photoproducts

Plasmids (3 pmol, 15 μg) were incubated one night at 37°C with various concentrations of oligonucleotide-peptide conjugates in 0.1 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$. The mixture was cooled in an ice-water bath and illuminated for 15 min in the dark using a sun lamp (Rad Free UV lamp, 365 nm; Schleicher and Schuell, Ecquevilly, France). The plasmid was then digested at 37°C with *Mfe*I and *Spe*I endonucleases. Samples were electrophoresed on a denaturing 15% polyacrylamide gel and DNA was stained according to the Bio-Rad Silver Stain kit (Bio-Rad).

2.8. Transgene expression studies

NIH 3T3 cells were obtained from the American Type Culture Collection (ATCC CRL-1658). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 4.5 g/l glucose, supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum. Cells were grown at 37°C in a 5% CO_2/air incubator. One day before transfection, 24 well culture plates were seeded with 90 000 cells per well. Cationic lipid RPR 120535 [13] was used for the transfection assays. Plasmids (20 ng/μl) were diluted in 150 mM NaCl and mixed with a cationic lipid solution (0.12 mM). The DNA/cationic lipid complex was diluted in culture medium (DMEM) in the absence of serum, and then added to the cells (0.5 μg of DNA and 3 nmol lipid/well). After 2 h at 37°C in a 5% CO_2/air incubator, 10% fetal bovine serum was added to the cells. The cells were incubated for 48 h at 37°C in a 5% CO_2/air incubator. The cells were washed twice with PBS and lysed with 250 μl of cell culture lysis reagent (Promega, Madison, WI, USA). The β-galactosidase expression was measured according to the Lumigal β-galactosidase genetic reporter system (Clontech, Palo Alto, CA, USA). Light emission was measured by integration over 10 s using a Lumat LB9501 luminometer (EG and G Berthold, Evry, France). Light emission was normalized to the protein concentration determined using the Pierce BCA assay (Pierce, Rockford, IL, USA).

2.9. Preparation of recombinant importin α-GST protein

Mouse importin α was cloned into pGEX-2T to produce a GST (glutathione *S*-transferase) fusion protein: importin α-GST [8]. The plasmid encoding the fusion protein was kindly provided by Dr. Y. Yoneda (Osaka University Medical School, Osaka, Japan). Importin α-GST was overexpressed and purified as previously described [8] and kindly provided by Dr. F. Blanche and Dr. V. Thuillier (Rhône-Poulenc Rorer Gencell, Vitry-sur-Seine, France).

2.10. Importin α binding assay

All assays were performed using importin α-GST recombinant protein. Binding to importin α was performed according to [12], with the following modifications: the bead slurry (80 μl) was incubated with 2 μg of oligonucleotide-peptide conjugate, in 0.5 ml of binding buffer, for 30 min at room temperature. The beads were collected by centrifugation at 2000×*g* for 30 s. The supernatant was removed, and 30 μl were used to analyze the presence of the unbound conjugate (this fraction is called the unbound fraction). The beads were washed five times with 0.5 ml of binding buffer and centrifuged at 2000×*g* for 30 s. Finally, beads, corresponding to the bound fraction, were resuspended with 15 μl of electrophoresis loading buffer (0.05% bromophenol blue, 40% sucrose, 0.1 M EDTA pH 8, 0.5% sodium lauryl sulfate). The bound and unbound fractions were analyzed by electrophoresis on a denaturing 15% polyacrylamide gel and DNA was stained according to the Bio-Rad Silver Stain kit (Bio-Rad).

3. Results

3.1. Preparation of oligonucleotide-peptide conjugates

A peptide containing wild type SV40 large T NLS sequence

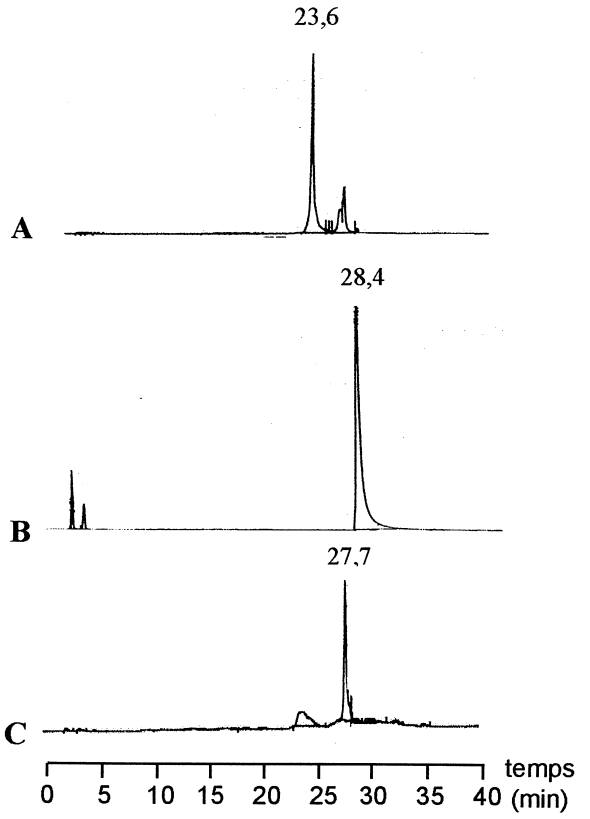


Fig. 1. HPLC chromatograms of oligonucleotide-NLS peptide conjugates. HPLC profiles of starting material and conjugate obtained after 2 h of reaction are shown. The molecules were analyzed by reversed phase HPLC using method 2 described in Section 2.1 (absorption wavelength: 260 nm). A: Pso-GA_{19}-SH oligonucleotide. B: NLS peptide. C: Pso-GA_{19}-NLS conjugate after 2 h of incubation.

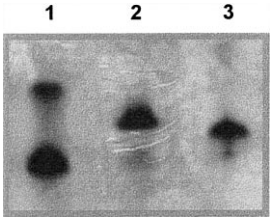


Fig. 2. Characterization of oligonucleotide-NLS peptide conjugates. Pso-GA_{19} oligonucleotide (lane 1), Pso-GA_{19}-NLS conjugate (lane 2), Pso-GA_{19}-NLS treated with trypsin (lane 3) were analyzed by 15% denaturing polyacrylamide gel electrophoresis and silver staining.

(NLS) was used. Scheme 1 illustrates the chemistry used to prepare oligonucleotide-NLS peptide conjugates. The coupling step involves reaction of the NLS peptide bearing a maleimide group with the oligonucleotide bearing a nucleophilic thiol residue (oligonucleotide Pso-GA_{19}-SH or GA_{19}-SH) [14]. Equimolar quantities of the two reagents were used. The NLS peptide was chemically modified to bear a Fmoc group. Since this group absorbs at 260 nm, the conjugation chemistry was easily monitored by reversed-phase HPLC. After 2 h, the reaction was finished (Fig. 1). The yield of this reaction was 40% with oligonucleotide Pso-GA_{19}-SH and 75% with oligonucleotide GA_{19}-SH, as shown by the almost complete disappearance of starting materials.

3.2. Characterization of oligonucleotide-peptide conjugates

The purity of the oligonucleotide-peptide conjugate was confirmed by polyacrylamide gel electrophoresis. It appeared as one major band (Fig. 2, lane 2). The presence of the peptide residue within Pso-GA_{19}-NLS conjugate was further confirmed by electrophoresis in denaturing polyacrylamide gel and by proteolytic treatment using trypsin. Trypsin catalyzes hydrolysis at the carboxyl side of lysine or arginine residues in peptides and proteins. The oligonucleotide-peptide conjugate migration distance was delayed as compared to non-modified oligonucleotide migration distance (Fig. 2, lane 2). The oligonucleotide-peptide conjugate migration distance was modified after proteolytic treatment using trypsin (Fig. 2, lane 3). Polyacrylamide gel electrophoresis indicated incomplete proteolysis of the oligonucleotide-peptide conjugates. Similar results were obtained with the GA_{19}-NLS conjugate (data not shown).

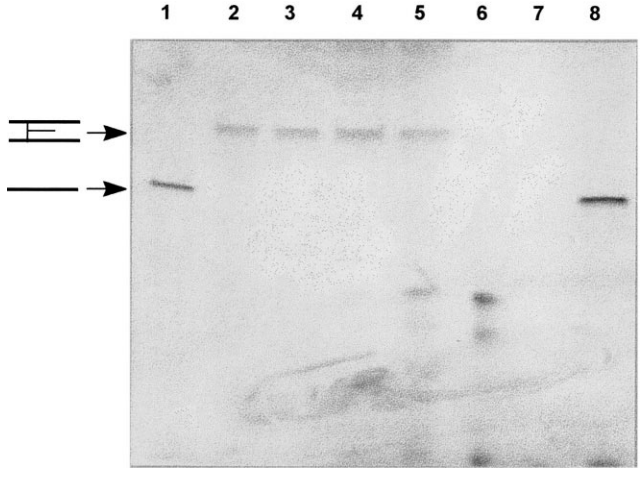


Fig. 3. Triple helix formation. Pso-GA_{19}-NLS conjugate and plasmid were incubated overnight at 37°C in 100 mM Tris-HCl pH 7.5, 50 mM $MgCl_2$. Molar excess (conjugate/plasmid) was 15 (lane 2), 50 (lanes 3 and 8), 100 (lane 4), 200 (lane 5). Plasmid alone (lane 1) and oligonucleotide alone (lanes 6 and 7) were used as controls. After triple helix formation, the samples were irradiated except for lanes 7 and 8, then digested with two endonucleases, *Mfe*I and *Spe*I, and analyzed by 15% denaturing polyacrylamide gel electrophoresis and silver staining.

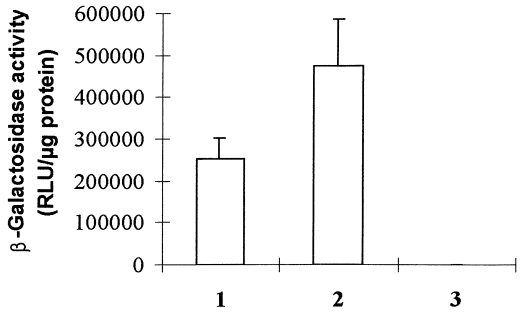


Fig. 4. Transgene expression after transfection of plasmid-NLS conjugate. Non-modified plasmid (1) and plasmid covalently modified with Pso-GA_{19}-NLS conjugate (2) were used for transfecting NIH 3T3 cells with cationic lipid RPR 120535. No plasmid was used in 3. The β-galactosidase expression was evaluated after 24 h of incubation. Values are means ± S.D. ($n = 3$).

3.3. Covalent triple helix formation between oligonucleotide-peptide conjugates and plasmid

The efficiency of triple helix formation and photoactivation was studied on plasmid using the psoralen-oligonucleotide-peptide conjugate. Pso-GA_{19}-NLS conjugate and plasmid were mixed with a molar conjugate/plasmid excess varying between 15 and 200. After triple helix formation and irradiation, the plasmid was digested with the two endonucleases *Mfe*I and *Spe*I. The digestion released a 70 bp fragment containing the triple helix site. On a denaturing polyacrylamide gel, this 70 bp plasmid fragment, when covalently associated with an oligonucleotide-peptide conjugate, has a shorter distance of migration than the non-associated fragment. With an excess of Pso-GA_{19}-NLS conjugate/plasmid of 15, all plasmid could be covalently associated with a Pso-GA_{19}-NLS conjugate (Fig. 3, lane 2). Without photoactivation, no covalent association could be detected in the same conditions after electrophoresis in denaturing conditions (Fig. 3, lane 8). The specificity of the psoralen reaction at the duplex-triplex junc-

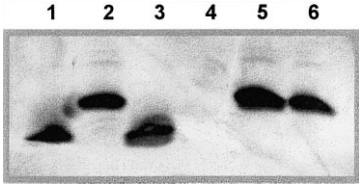
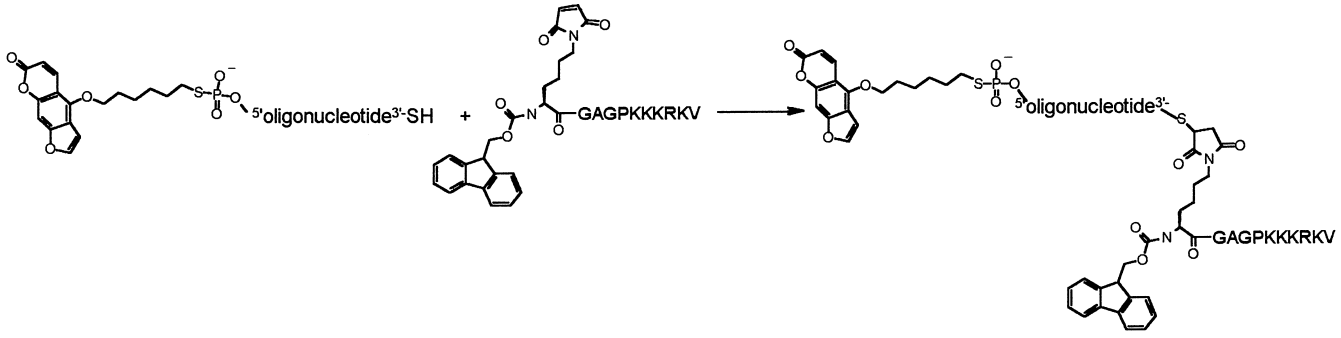


Fig. 5. Interaction between importin α-GST and oligonucleotide-NLS peptide conjugates. Immobilized importin α-GST (2 μg) was incubated for 30 min at room temperature with Pso-GA_{19} (2 μg) (lanes 3 and 4), Pso-GA_{19}-NLS (lanes 5 and 6). Free Pso-GA_{19} (lane 1), free Pso-GA_{19}-NLS (lane 2) and fractions bound to importin α-GST (lanes 4 and 6) and unbound (lanes 3 and 5) were analyzed by 15% denaturing polyacrylamide gel electrophoresis and silver staining.



Scheme 1. Synthesis of oligonucleotide-NLS peptide conjugates. The scheme illustrates synthesis using Pso-GA_{19}-SH oligonucleotide and NLS peptide.

tion directed by oligonucleotide binding to its target sequence was confirmed: plasmid covalently modified by triple helix formation and non-modified plasmid were digested with various endonucleases and the digestion products compared on a denaturing polyacrylamide gel (data not shown).

3.4. Transfection with peptide-plasmid conjugate

The functionality of the reporter plasmid was studied after transfection of NIH 3T3 cells using a lipopolyamine, RPR 120535 [13]. Plasmid covalently associated with Pso-GA_{19}-NLS conjugate by triple helix formation and irradiation led to β-galactosidase expression (Fig. 4). The increase in expression over non-modified plasmid was not significant.

3.5. Importin α binding assay

Binding of the oligonucleotide-peptide conjugate to importin α was studied using the importin α-GST fusion protein binding assay with glutathione-Sepharose beads, followed by polyacrylamide gel electrophoresis. Pso-GA_{19}-NLS conjugate was detected in the bound fraction (interacting with the importin α-GST beads) whereas Pso-GA_{19} oligonucleotide was recovered in the supernatant (Fig. 5). Similar results were obtained with GA_{19}-NLS conjugate and GA_{19} oligonucleotide (data not shown). However, using the same assay, we could not detect binding to importin α of the plasmid covalently associated with Pso-GA_{19}-NLS conjugate by triple helix formation and irradiation (data not shown).

4. Discussion

Several methods for covalent attachment of molecules to nucleic acids have been developed and reported so far [11,12,15,16]. Among them, the use of photoactive molecules leads to efficient but non-specific covalent modification of DNA [12,17]. A suitable method for site-specific labeling of plasmid DNA involves the formation of a triple helix consisting of an oligonucleotide bound within the major groove of a double-stranded target sequence [18]. The triple helix used in our study was based on oligopurine probes. The oligonucleotide binds in an antiparallel orientation with respect to the duplex DNA [19].

Table 1
Oligonucleotides used

Oligonucleotide	Modification(s)
GA_{19}	none
GA_{19}-SH	thiol group at the 5′ terminus
Pso-GA_{19}	psoralen group at the 5′ terminus
Pso-GA_{19}-SH	psoralen group at the 5′ terminus, thiol group at the 3′ terminus

Psoralen-based reactions can be used to form covalent links at the end of double helices [20], to attach triple helix forming oligonucleotides to specific sites in DNA [21], to inhibit gene expression [22], or to perform targeted DNA mutagenesis [23]. The interstrand crosslinks are between the furan or the pyrone sides of the psoralen and the 5-6 double bond of one or two thymines in the DNA [23]. The psoralen preferred reaction sites are 5′-TpA or 5′-ApT. We constructed a plasmid containing a triple helix target site adjacent to the psoralen reaction site, upstream of the transgene expression cassette, and used a GA-rich oligonucleotide to form a triple helix at the target site. Using a psoralen-oligonucleotide conjugate thus makes it possible to form a covalent triple helix at a specific site.

Covalent attachment of molecules to oligonucleotides is developed in order to promote the uptake of oligonucleotides by cells, and/or to improve their resistance to nucleases. The preparation of oligonucleotide-peptide conjugates presents a challenge because the two molecules, oligonucleotide and peptide, have different chemical stability conditions. Different methods have been described [14]. We decided to couple a purified oligonucleotide modified with a thiol residue to a NLS peptide bearing a maleimide group. Oligonucleotides with or without a psoralen group were easily coupled to the NLS peptide.

Using a binding assay, we showed that Pso-GA_{19}-NLS and GA_{19}-NLS conjugates interacted with the NLS binding protein, importin α. Our results show that NLS peptides are recognized, in spite of the oligonucleotide electrostatic properties.

Pso-GA_{19}-NLS conjugate could form a stable and covalent triple helix with the plasmid target site, and a low molar excess of conjugate was needed. The modified plasmid expressed the transgene at the same level as non-modified plasmid. This was consistent with the working hypothesis of inserting the triple helix target site upstream of the transgene expression cassette.

In a previous study, we attached several NLS peptides to plasmid DNA by photoactivation using a *p*-azido-tetrafluorobenzyl-NLS peptide conjugate, and showed that the modified plasmid could interact with importin α using the same binding assay [12]. At least 10 NLS peptides must be attached per plasmid DNA in order to detect association of the conjugates with importin α [12]. Since the conjugates used in the present

study bear only one NLS peptide per plasmid DNA, it is consistent that their interaction with importin α could not be observed, being under the detection limit.

We did not observe any significant increase in expression as a result of the NLS peptide. In a recent study [24], transfection enhancement was observed using a capped 3.3 kb linear plasmid tagged with a single nuclear localization signal peptide. We covalently coupled one NLS peptide to plasmid DNA by a different method and chose to keep plasmid integrity and size. These differences can explain why we did not observe any significant effect on transfection efficiency.

In conclusion, we have designed a novel simple method for covalently coupling one NLS peptide to plasmid DNA using a triple helix. This technology makes it possible to control the number of peptides linked to DNA, which is strictly dependent on the number of inserted triple helix-forming sequences, and to control also the site of association, which is determinant for maintaining plasmid integrity and expression. The modified plasmid can be produced on a large scale. Moreover, we show in the present work that oligonucleotide-NLS peptide conjugates are recognized by the importin α NLS receptor. The present strategy could be used for coupling other ligands to plasmid DNA, and to study plasmid-ligand conjugate interaction with receptors, while maintaining transgene expression.

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