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Sequence-specific Methyltransferase-Induced Labelling (SMILing) of plasmid DNA for studying cell transfection

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Abstract—Plasmid DNA (pUC19 and pBR322) was sequence-specifically, covalently labelled with Cy3 fluorophores using a newly synthesised *N*-adenosylaziridine cofactor and the DNA methyltransferase M.TaqI. The fluorescently labelled plasmids were used for transfection of mammalian cells and their intracellular distribution was visualised by epifluorescence and confocal fluorescence microscopy. Although these prokaryotic plasmids do not contain nuclear import sequences, translocation into the nuclei was observed.
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1. Introduction

Despite huge progress in bioconjugation chemistry sequence-specific labelling of native DNA remains a challenging task. Methods for producing, for example, sequence-specific fluorescently labelled plasmid DNA are of great importance to study DNA transport and localisation processes inside cells using fluorescence microscopic techniques.[1] Depending on the context of the study, DNA can be delivered by transfection (non-viral vectors), transduction (viral vectors) or microinjection into different compartments of the cell (cytoplasm or nucleus). Especially, the process of cell transfection using non-viral vectors is still poorly understood and DNA import into the nucleus of non-dividing cells is a matter of curiosity and intensive debate.[2–7]

So far, mainly two DNA labelling approaches have been applied to follow the intracellular pathway of transfected or microinjected plasmid DNA by fluorescence microscopy. The first approach is based on fluorescently labelled triple helix-forming peptide nucleic acids (PNA clamps).[8–11] PNA clamps form non-covalent triplex invasion complexes (two PNA strands bind to one strand of double-stranded DNA) with high specificity and stability.[12] However, complex formation is not efficient and the use of a 50-fold excess was necessary to achieve quantitative labelling.[10] In addition, it is often required to introduce a suitable target sequence into the plasmids.

In the second approach DNA is labelled covalently which avoids any doubts about label dissociation from the DNA under physiological conditions. This approach is based on reactive chemicals able to modify DNA components and to deliver fluorophores or other reporter groups of interest to the DNA.[13–19] However, labelling with such reactive chemicals is random allowing no control of the position and number of the labels on individual plasmids. Indeed, it was demonstrated that the average number of labels per plasmid plays an important role in transfection and that increasing label density reduces the transfection efficiency.[14,18] In particular, in vitro transcription/translation experiments using randomly labelled DNA indicated that a high labelling density inhibits transcription which might explain the lower transfection efficiency observed.[18]

To circumvent potential problems associated with non-covalent or random labelling we employed an enzymatic

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method for sequence-specific and covalent labelling of DNA with fluorophores. This method makes use of the ability of DNA methyltransferases (MTases) to couple sequence-specifically the synthetic cofactor *N*-adenosylaziridine with their DNA recognition sequences.[20] By attaching reporter[21–23] or functional groups[24,25] to *N*-adenosylaziridine at the adenine 8-position cofactors are obtained which can be used in combination with DNA MTases having different recognition sequences for Sequence-specific Methyltransferase-Induced Labelling of DNA (SMILing DNA).

Herein, we report the synthesis of the novel *N*-adenosylaziridine cofactor derivative 6Cy3Az (**1**) containing a Cy3 fluorophore attached via a linker at the 6-position of the adenine ring and its successful quantitative coupling with plasmid DNA by the DNA MTase M.TaqI. In addition, we demonstrate that the fluorescently labelled DNA is suitable for intracellular tracking after transfection of CHO-K1 cells.

2. Results

2.1. Synthesis

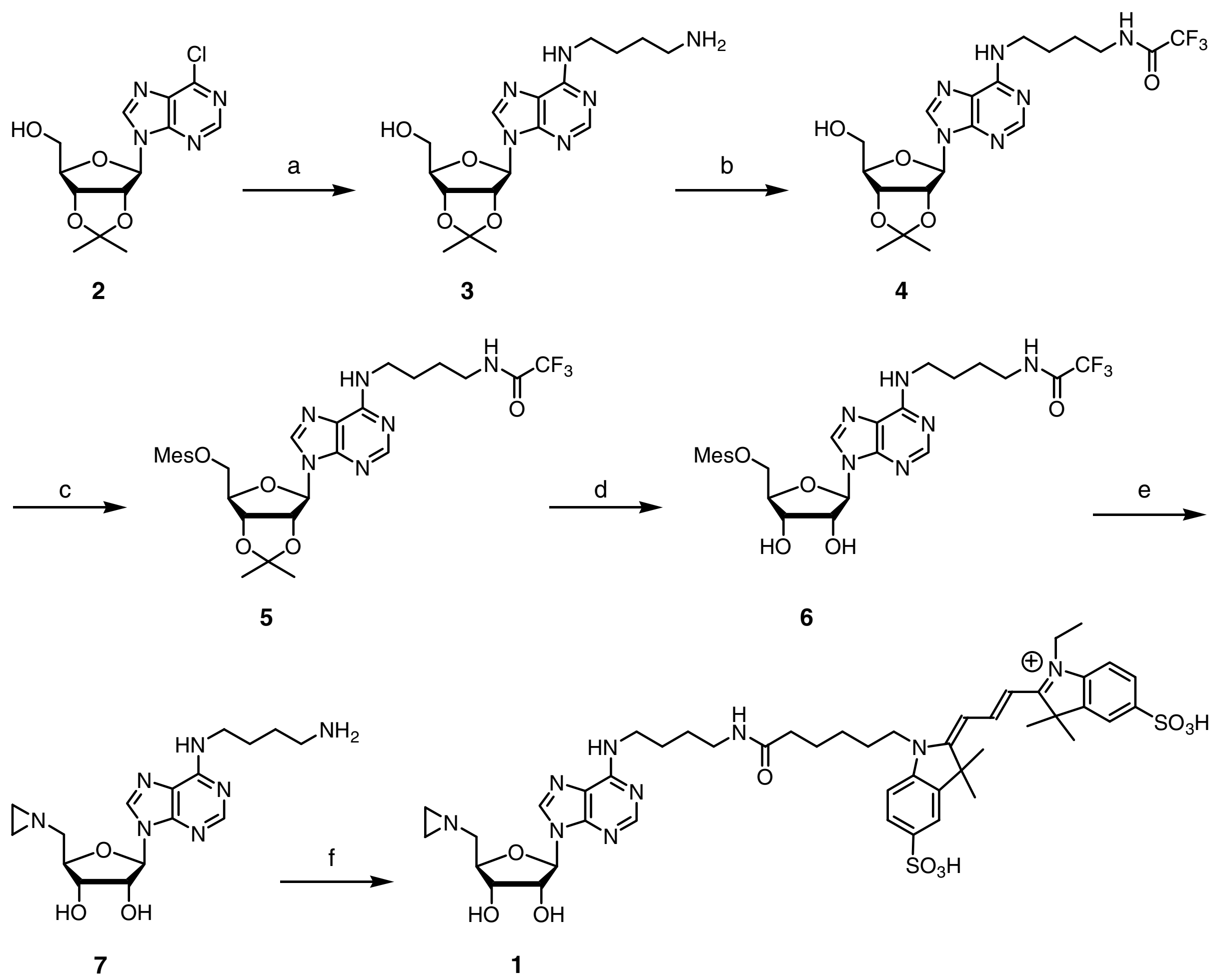
The rationale for attaching the reporter group at the 6-position instead of the 8-position of *N*-adenosylaziridine was that bulky C8-substituents in adenosine analogues have a strong tendency to alter the rotation around the glycosidic bond from the naturally preferred *anti* into the *syn* conformation.[26–30] Such a *syn* conformation should have two disadvantages: first, DNA MTases bind their natural cofactor *S*-adenosyl-L-methionine (AdoMet) in the *anti* conformation as observed in several crystal structures,[31–37] and cofactor binding affinity would be lowered by the energetic cost for rotating the aziridine cofactor from the *syn* into the *anti* conformation. Second, nucleophilic substitution of 5′ activated adenosines is often accompanied by *cyclo* nucleoside formation resulting from intramolecular attack of the N_3 nitrogen onto the activated 5′-position[38] and this undesired side reaction should be more pronounced with adenosine analogues preorientated in the *syn* conformation. In addition, inspection of available three-dimensional structures of DNA MTases in complex with the natural cofactor AdoMet or cofactor analogues[22] revealed that attaching reporter groups via a flexible linker to the 6-position should not block cofactor binding by M.TaqI and most other DNA MTases. As fluorescent reporter group we choose Cy3 because of its brightness, photostability, excitation maximum (~548 nm) and emission maximum (~562 nm).[39] At these wavelengths an overlap with the fluorescence of the utilised nuclei staining agents is avoided and the autofluorescence signal from the cells is low.

The synthesis of the novel aziridine cofactor 6Cy3Az (**1**) with the Cy3 fluorophore attached to the 6-position is summarized in Scheme 1. 6-Chloro-2′,3′-*O*-isopropylideneadenosine (**2**), which was obtained from commercially available 6-chloroadenosine according to a literature procedure by Kappler and Hampton,[40] was reacted with 1,4-diaminobutane for introducing the linker at the 6-position. The primary linker amino group of **3** was protected as trifluoroacetamide and the 5′ hydroxy group of **4** activated as mesylate. In contrast to 5′ activation of a corresponding protected adenosine with a linker at the 8-position[23] *cyclo* nucleoside formation was not a problem in this case as indicated by the high yield of the reaction. The isopropylidene group of **5** was removed under acidic conditions before aziridine was introduced into the mesylate **6** by nucleophilic substitution. During basic workup the primary amino group was deprotected and the resulting *N*-adenosylaziridine derivative **7** purified by reverse-phase HPLC. For coupling of the primary amine with Cy3 the collected fractions of eluted intermediate **7** were directly supplemented with Cy3 *N*-hydroxysuccinimidyl ester (Cy3 NHS ester) to furnish the desired *N*-adenosylaziridine derivative 6Cy3Az (**1**). An important feature of this synthetic route is that Cy3 is introduced in the last step which minimises material loss of this expensive fluorophore. In addition, solutions of the eluted intermediate **7** can be stored at −80 °C for several months allowing various amine-reactive reporter groups to be attached when desired.

2.2. Fluorescent labelling of plasmid DNA by SMILing DNA

Labelling of the commercially available plasmids pUC19 and pBR322 was performed with the synthetic cofactor 6Cy3Az (**1**) and the DNA MTase M.TaqI. Naturally, M.TaqI catalyses the nucleophilic attack of the exocyclic amino group of adenine within the 5′-TCG**A**-3′ double-stranded DNA sequence onto the activated methyl group of AdoMet. With the aziridine cofactor 6Cy3Az (**1**) the course of the reaction is changed and nucleophilic attack onto the aziridine ring leads to sequence-specific coupling of the whole fluorescent cofactor with DNA (Scheme 2A). This enzymatic reaction was used for directed labelling of pUC19 DNA with four Cy3 fluorophores (pUC19/4Cy3) and pBR322 with seven Cy3 fluorophores (pBR322/7Cy3) at their M.TaqI recognition sequences (Scheme 2B).

The plasmids were incubated with an excess of M.TaqI in the presence of 6Cy3Az (**1**) at 60 °C for 3 h. Since M.TaqI remains tightly bound to the reaction product, the enzyme was removed by incubation with Proteinase K and guanidinium hydrochloride as denaturing agent. After plasmid purification the degree of labelling was analysed in a DNA protection assay using the cognate restriction endonuclease R.TaqI. This enzyme readily cleaves unmodified 5′-TCGA-3′ sequences but is unable to fragment the modified DNA and occurrence of fragmentation was analysed by agarose gel electrophoresis (Fig. 1). Plasmids incubated with M.TaqI and 6Cy3Az (**1**) were nearly fully protected against fragmentation (i.e., cutting of both strands) by R.TaqI (Fig. 1A and B, lanes 1) indicating almost quantitative labelling of the 5′-TCGA-3′ sequences. The observed predominant transformation of supercoiled plasmids into relaxed plasmids upon incubation with R.TaqI can be explained by our previous observation that M.TaqI can only deli-



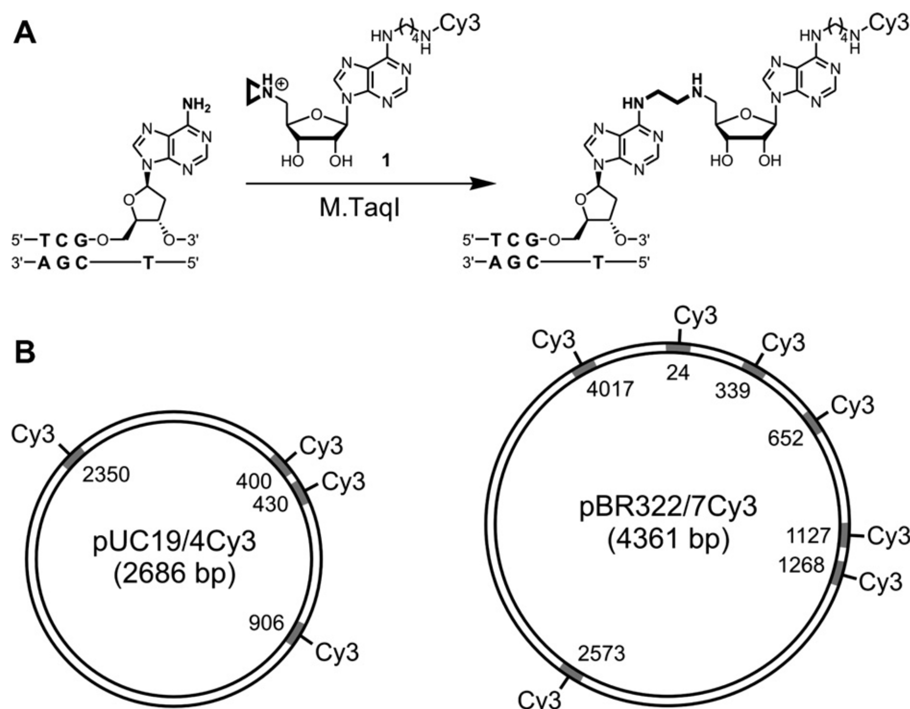
Scheme 1. Synthesis of 6Cy3Az (**1**). Reagents and conditions: (a) 1,4-diaminobutane, NEt_3, EtOH, 60 °C, 18 h (95%); (b) trifluoroacetic acid ethylester, NEt_3, MeOH, room temperature, overnight (66%); (c) MesCl, DMAP, NEt_3, CH_2Cl_2, 0 °C, 2 h (86%); (d) 50% formic acid (aq), room temperature, 3 d (96%); (e) 1—aziridine, EDIA, room temperature, 3 d, 2—$HNEt_3HCO_3$ (aq, pH 8.6), room temperature (10%); (f) Cy3 NHS ester, $HNEt_3HCO_3$ (aq, pH 8.6), DMSO, room temperature, 1 h (50%).

ver one molecule of aziridine cofactor to its palindromic recognition sequence containing two target adenines (unpublished result). This leads to hemi-alkylated recognition sequences which might be susceptible to nicking (i.e., cutting of one strand) by R.TaqI and relaxation of the plasmids. In contrast to the labelling reactions, controls with plasmids incubated in the presence of 6Cy3Az (**1**) only (Fig. 1A and B, lanes 3), in the presence of M.TaqI only (Fig. 1A and B, lanes 5) or in the absence of both M.TaqI and 6Cy3Az (**1**) (Fig. 1A and B, lanes 7) were completely fragmented by R.TaqI. It is also interesting to note that most of the plasmids stayed in their native supercoiled form (about 70%) after labelling and purification (Fig. 1A and B, lanes 2).

2.3. Cell transfection with fluorescently labelled plasmid DNA

For transfection experiments with the fluorescently labelled plasmids pUC19/4Cy3 and pBR322/7Cy3 CHO-K1 cells were grown on polyornithine-coated coverslides overnight and treated with a mixture of Lipofectamine 2000 and labelled plasmids. The transfection mixture was replaced by serum containing medium after 5 h and the cells were grown for another 19 h. The cells were fixed using paraformaldehyde and their nuclei stained with DAPI (4′,6-diamidino-2-phenylindol) for epifluorescence microscopy (Figs. 2 and 3) or with the cyanine dye TO-PRO-3 (4-[3-(3-methyl-2(3H)-benzothiazolylidene)-1-propenyl]-1-[3-(trimethylammonio)propyl]-quinolinium diiodide) for confocal laser scanning microscopy (Fig. 4). Since both plasmids do not contain reporter genes for expression in mammalian cells, our objective was to use them as simple models for studying the intracellular distribution of DNA in transfected cells.

As seen in Figure 2, the Cy3 fluorescence was observed both in large bright clumps located in the extracellular medium and in small spots localised inside the cells. The fluorescence pattern was identical for the two labelled plasmids pUC19/4Cy3 and pBR322/7Cy3 (Fig. 2B and E). The large fluorescent aggregates are characteristic for DNA condensation by cationic lipids.[17] These large-sized particles contain many DNA molecules and are known to sediment on the cell



Scheme 2. Sequence-specific coupling of 6Cy3Az (**1**) with the 5′-TCG**A**-3′ target sequence by the adenine-specific DNA MTase M.TaqI (A). Schematic representation of labelled plasmids pUC19/4Cy3 (left) and pBR322/7Cy3 (right) showing the attachment positions (M.TaqI recognition sequences) of the Cy3 fluorophores (B).

membrane thereby facilitating the cellular uptake of DNA.[41] In contrast, the small spots observed in the cytoplasmic compartment are consistent with the disruption of the transfection particles into small vesicles during or following endocytosis. It is currently admitted that the plasmid DNA-lipid particles follow a complex multistep pathway (endosome entrapment, lysosomic sequestration, transport by microtubules) that ultimately routes them to the nuclear membrane.[3,4,6] In the presented experiments, the formation of a rim of fluorescent particles around the nuclei of transfected cells (Fig. 2C and F) illustrates this fate.

In addition, about 25% of the cells which had internalised pUC19/4Cy3 or pBR322/7Cy3 exhibited Cy3 fluorescence in the nucleus. This is demonstrated by a perfect overlap of the Cy3 fluorescence with the blue fluorescence of the DNA staining reagent DAPI (Fig. 3A and B). The localisation of the Cy3 fluorescence in the nuclei was also confirmed by laser scanning confocal microscopy using TO-PRO-3 as DNA staining reagent (Fig. 4). Both fluorescence microscopic techniques showed that the Cy3 fluorescence is distributed quite homogeneously suggesting that plasmid DNA is organized differently once inside the nucleus. A similar distribution of pBR322 was already reported after microinjection into the nucleus.[42]

3. Discussion

SMILing DNA is an attractive method for site-specific covalent labelling of DNA. By virtue of the sequence specificity of the applied DNA MTase, the method enables full control over the position and number of labels on the DNA substrate. In addition, a variety of fluorophores or other reporter groups can be delivered to the DNA due to the versatility of the cofactor preparation that permits introduction of the label in the last step of the synthesis. Here, we have shown that this method can be applied for labelling supercoiled plasmid DNA and most of the plasmids retained their native supercoiled form. The quantification of the labelling reaction can be performed with good accuracy by analysis with a cognate restriction endonuclease. This easy and sensitive quantification by DNA protection is a clear advantage of the SMILing DNA procedure compared to other labelling methods that make use of classical spectroscopic techniques. Finally, the fluorescent plasmids can be purified using standard methods and can be produced at microgram-scale suitable for biological use.

Two labelled plasmids bearing four and seven Cy3 fluorophores, respectively, were introduced in cells by lipofection and intracellular tracking was carried out by fluorescence microscopy. Our observations allow to conclude that the internalisation process of the fluorescent plasmids is consistent with the current knowledge in the field.[3,4,6] Clearly, large lipofectamine-DNA microparticles are seen on the cell surfaces, whereas small vesicles are detected in the cytoplasm which are mostly clustered in the perinuclear space. The number of cells that internalised the fluorescent plasmids was in the expected range for lipofection of CHO-K1 cells (15–20%).

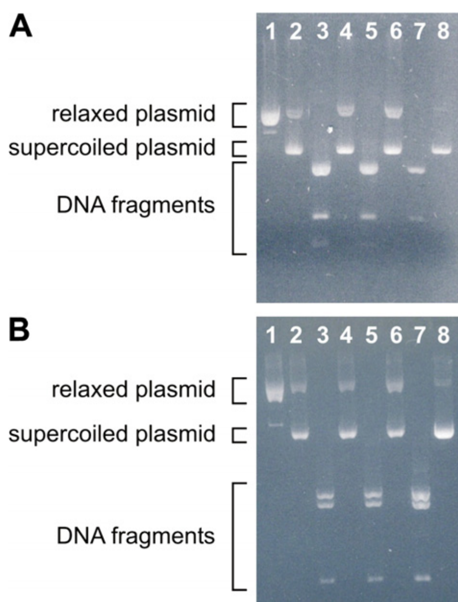


Figure 1. DNA protection analysis of pUC19 (A) and pBR322 (B) labelling with 6Cy3Az (**1**) and M.TaqI. (A) Lane 1: pUC19 incubated with M.TaqI and 6Cy3Az (**1**) followed by incubation with R.TaqI; lane 2: pUC19 incubated with M.TaqI and 6Cy3Az (**1**); lane 3: pUC19 incubated with 6Cy3Az (**1**) and cleaved by R.TaqI; lane 4: pUC19 incubated with 6Cy3Az (**1**); lane 5: pUC19 incubated with M.TaqI and cleaved by R.TaqI; lane 6: pUC19 incubated with M.TaqI; lane 7: pUC19 cleaved by R.TaqI; lane 8: pUC19. (B) Lane 1: pBR322 incubated with M.TaqI and 6Cy3Az (**1**) followed by incubation with R.TaqI; lane 2: pBR322 incubated with M.TaqI and 6Cy3Az (**1**); lane 3: pBR322 incubated with 6Cy3Az (**1**) and cleaved by R.TaqI; lane 4: pBR322 incubated with 6Cy3Az (**1**); lane 5: pBR322 incubated with M.TaqI and cleaved by R.TaqI; lane 6: pBR322 incubated with M.TaqI; lane 7: pBR322 cleaved by R.TaqI; lane 8: pBR322.

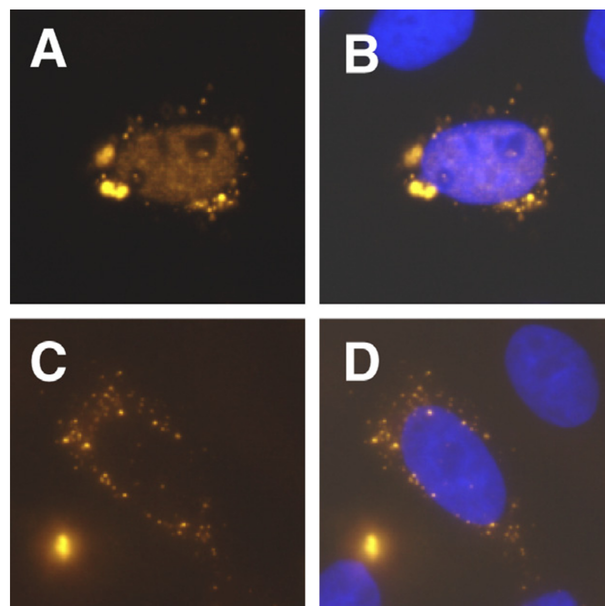


Figure 3. Two examples of cells from transfection with pUC19/4Cy3 containing fluorescence inside. Analysis was performed by epifluorescence microscopy and Cy3 fluorescence was found in the cytoplasm and in the nucleus (A and B) or only in the cytoplasm (C and D). In (B) and (D) the Cy3 fluorescence is superimposed with the blue DAPI fluorescence.

Interestingly, a subpopulation of about 25% of the cells which had internalised the plasmids showed a high Cy3 fluorescence intensity within the nucleus perfectly overlapping with the signal of the counterstaining agent and co-localisation has been confirmed by confocal laser scanning microscopy. It is well admitted that nuclear entry of DNA is the major obstacle in the gene transfer process. This is why transfection is considerably more efficient in actively dividing cells where breakage of the nuclear membrane occurs. In this respect, passive inclusion of DNA due to cell division could explain to some extent our observation. However, it is well known that transfection leads to stalling of cell growth and cells

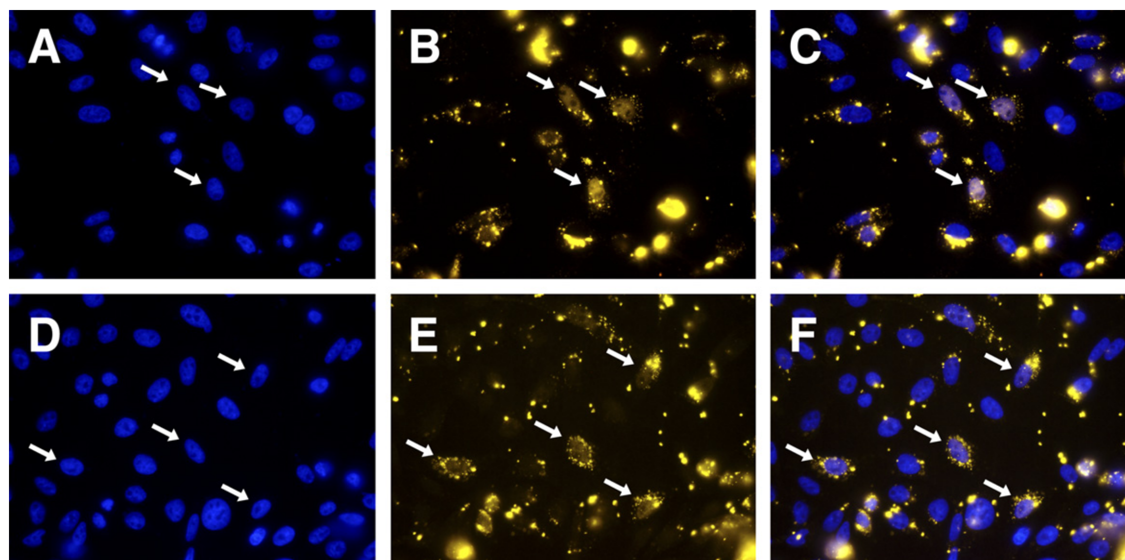


Figure 2. Transfection of CHO-K1 cells with pUC19/4Cy3 (A–C) or pBR322/7Cy3 (D–F) analysed by epifluorescence microscopy. The cell nuclei were stained using DAPI (A and D). The Cy3 fluorescence of the labelled DNA is clearly visible (B and E) and cells exhibiting Cy3 fluorescence inside their nuclei are indicated by arrows. Pictures (C) and (F) show superimpositions of DAPI and Cy3 fluorescence.

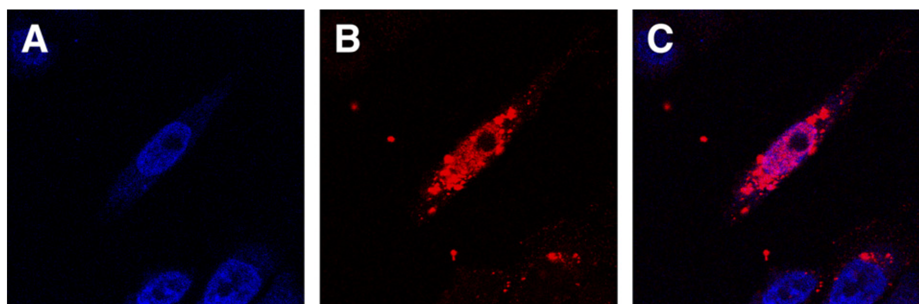


Figure 4. Confocal laser scanning fluorescence microscopic analysis of a cell containing Cy3 fluorescence in the nucleus after transfection with pUC19/4Cy3. TO-PRO-3 fluorescence of stained nuclei is shown in (A) and Cy3 fluorescence is shown in (B). The superimposition in (C) demonstrates co-localisation of the Cy3 and TO-PRO-3 fluorescence. Fluorescence signals are shown in false colours (TO-PRO-3 is blue and Cy3 is red).

recover very slowly. Thus, it seems reasonable to consider that the nuclear localisation of the plasmids might only partially be explained by cell division. In this line, our results are not consistent with studies reporting that unmodified pUC19 or pBR322 remained in the cytoplasm after microinjection, whereas plasmids encoding the SV 40 nuclear import sequence were found in the nucleus.[43,44] Although the sequence-dependent mechanism involving passage through the nuclear pore complexes (NPC) is viewed as the consensus model,[5] a number of questions related to the physical and molecular basis of nuclear import remain unanswered.[45,46] In particular, in some circumstances (electroporation, transfection with linear PEI) the use of biological nuclear import elements has been shown to be less important.[47] Inter alia, this points out to the importance of the delivery method which determines the intracellular status of the DNA (naked or associated with lipid). In the above-mentioned studies[43,44] pUC19 and pBR322 plasmids were microinjected into the cytoplasm, whereas we used lipofection. This could be an important difference because it has been suggested that DNA complexed by cationic lipids might be released into the nucleus by fusion of the lipid with the nuclear membrane.[17] However, more investigations are clearly needed to unambiguously establish a sequence-independent nuclear uptake in our experimental system. Nevertheless, the inherent complexity of the DNA trafficking process already points out a strong need for developing new tools for studying the multitude of parameters influencing the fate of DNA delivered into the cell.

In summary, our study demonstrates that SMILing DNA is a valuable technique for studying the various aspects of plasmid trafficking in the cell. Compared to other commonly used methods for DNA labelling SMILing DNA offers important advantages. First, the labelled plasmids are mainly obtained in their native supercoiled form which is believed to be important for cell transfection experiments. Second, the plasmid DNA is covalently modified which circumvents uncertainties related to label dissociation in the cell and labelling is site-specific. The latter aspect not only allows full control of the number of labels but also to direct the labels to non-functional sequences which will be of importance when plasmids with biological relevant regions like reporter genes, gene enhancing or localisation sequences are studied.

4. Experimental

4.1. General

6-Chloro-2′,3′-*O*-isopropylideneadenosine (**2**)[40] and aziridine[48,49] were prepared according to literature procedures. *N*-Ethyldiisopropylamine (EDIA) was purchased from Fluka. 4-(Dimethylamino)-pyridine (DMAP) and methanesulfonyl chloride (MesCl) were purchased from Merck. All reagents were of p.a. grade. Dry solvents were either purchased or obtained by drying using common laboratory techniques.[50] Proteinase K was obtained from Qiagen. The plasmids and the restriction endonuclease R.TaqI were purchased from MBI Fermentas. The DNA MTase M.TaqI was overexpressed, purified and separated from the natural cofactor as described before.[34,51] The CHO-K1 cells were a generous gift of Hervé Chneiweiss of the Neuropharmacology Department at the Collège de France.

All air or water sensitive chemical reactions were carried out in dried glassware under argon atmosphere. TLC glass plates coated with silica gel 60 F_{254} (Merck) were used for TLC. Flash chromatography was carried out using Merck silica gel 60 (40–63 µm). HPLC was performed using a Waters Breeze System equipped with the binary programmable pump system 1525, the dual wavelength absorbance detector 2487 and a Waters inline degasser. NMR spectra were recorded using a Mercury 300 (300 MHz and 75 MHz for ^{1}H and ^{13}C, respectively) or an Inova 400 (400 MHz, 100 MHz and 376 MHz for ^{1}H, ^{13}C and ^{19}F, respectively) (all Varian) in the NMR spectroscopy facility of the Institute of Organic Chemistry at the RWTH Aachen. $CDCl_3$ ($\delta_H = 7.24$ ppm and $\delta_C = 77.0$ ppm) or [D_6]DMSO ($\delta_H = 2.49$ ppm and $\delta_C = 39.5$ ppm) were used as solvents and for calibration. Assignment of ^{13}C signals is based on ^{1}H, ^{13}C-correlated 2D NMR and on ^{13}C DEPT spectra. Electrospray ionization mass spectra (ESI-MS) were obtained using a Finnigan LCQ DECA XP Plus in the mass spectrometry facility of the Institute of Organic Chemistry at the RWTH Aachen. UV

absorption measurements were performed in methanol or water using a Varian Cary 3E spectrometer.

4.2. Synthesis

4.2.1. *N*6-[1″-(4″-Aminobutyl)]-2′,3′-*O*-isopropylideneadenosine (3). To a solution of 1,4-diaminobutane (1.05 g, 11.9 mmol) and triethylamine (1.70 mL, 11.9 mmol) in ethanol (5 mL) was added slowly a solution of 6-chloro-2′,3′-*O*-isopropylideneadenosine (**2**)[40] (0.97 g, 2.97 mmol) in ethanol (50 mL) and the reaction mixture was stirred at 60 °C for 18 h. The solvent and excess of reagents were removed under reduced pressure to give the crude nucleoside **3** (1.07 g, 95%) as a white solid. ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 1.33$ (s, 3H, isopropylidene-CH$_3$a), 1.56 (s, 3H, isopropylidene-CH$_3$b), 1.58–1.70 (m, 4H, H2″, H3″), 2.55–2.62 (m, 2H, H4″), 3.46–3.54 (m, 2H, H1″), 3.54–3.60 (m, 2H, H5′), 4.22–4.26 (m, 1H, H4′), 4.98 (dd, $^3J = 2.48$ Hz, $^3J = 6.19$ Hz, 1H, H3′), 5.34 (dd, $^3J = 2.97$ Hz, $^3J = 6.18$ Hz, 1H, H2′), 6.13 (d, $^3J = 2.97$ Hz, 1H, H1′), 8.00 (s, br, 1H, 6-NH), 8.23 (s, 1H, H8), 8.37 (s, 1H, H2).

4.2.2. *N*6-[1″-(4″-Trifluoroacetamido)butyl]-2′,3′-*O*-isopropylideneadenosine (4). To a solution of nucleoside **3** (1.05 g, 2.77 mmol) and triethylamine (0.96 mL, 6.92 mmol) in methanol (50 mL) was added trifluoroacetic acid ethylester (1.8 mL, 16.6 mmol). The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (silica gel, ethyl acetate) to give nucleoside **4** (0.87 g, 66%) as a white solid (R_f 0.20, ethyl acetate). ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 1.33$ (s, 3H, isopropylidene-CH$_3$a), 1.55 (s, 3H, isopropylidene-CH$_3$b), 1.57–1.61 (m, 4H, H2″, H3″), 3.18–3.24 (m, 2H, H4″), 3.43–3.62 (m, 4H, H5′, H1″), 4.20–4.24 (m, 1H, H4′), 4.97 (dd, $^3J = 2.47$ Hz, $^3J = 6.18$ Hz, 1H, H3′), 5.24 (t, $^3J = 5,57$ Hz, 1H, 5′-OH), 5.34 (dd, $^3J = 2.97$ Hz, $^3J = 6.19$ Hz, 1H, H2′), 6.13 (d, $^3J = 2.97$ Hz, 1H, H1′), 7.92, (s, br, 1H, 6-NH), 8.23 (s, 1H, H8), 8.34 (s, 1H, H2), 9.41 (t, $^3J = 5.44$ Hz, 1H, 4″-NH); ^{19}F NMR (282 MHz, [D$_6$]DMSO): $\delta = -74.353$; ESI-MS (positive ion mode): *m*/*z* (relative intensity): 475.7 (100) [M+H]$^+$, 303.7 (27) [*N*6-[1′-(4′-trifluoroacetamido)butyl]-adenine+H]$^+$.

4.2.3. *N*6-[1″-(4″-Trifluoroacetamido)butyl]-2′,3′-*O*-isopropylidene-5′-*O*-mesyladenosine (5). To a solution of nucleoside **4** (0.85 g, 1.79 mmol) in dry methylene chloride (60 mL) under argon atmosphere were added 4-(dimethylamino)-pyridine (DMAP) (0.22 g, 1.79 mmol) and triethylamine (6.24 mL, 45.0 mmol) and the solution was cooled to 0 °C in an ice bath. Methanesulfonyl chloride (1.39 mL, 17.9 mmol) was slowly added and the reaction mixture was stirred at 0 °C for 2 h. The reaction was quenched by adding an ice-cold, saturated sodium hydrogen carbonate solution (15 mL) and the organic layer was removed. The aqueous layer was extracted with ice-cold chloroform (3× 25 mL) and the combined organic layers were dried over magnesium sulfate. After filtration the solvent was removed under reduced pressure and the crude product was purified by column chromatography (silica gel, methanol/methylene chloride 5:95) to give nucleoside **5** (0.85 mg, 86%) as a light yellow solid (R_f 0.21, methanol/methylene chloride 5:95). ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 1.34$ (s, 3H, isopropylidene-CH$_3$a), 1.56 (s, 3H, isopropylidene-CH$_3$b), 1.56–1.64 (m, 4H, H2″, H3″), 3.11 (s, 3H, mesyl-CH$_3$), 3.16–3.23 (m, 2H, H4″), 3.25–3.36 (m, 2H, H1″), 4.36–4.50 (m, 3H, H5′, H4′), 5.07–5.11 (m, 1H, H3′), 5.45 (dd, $^3J = 2.23$ Hz, $^3J = 6.43$ Hz, 1H, H2′), 6.25 (d, $^3J = 2.23$ Hz, 1H, H1′), 7.95 (s, br, 6-NH), 8.24 (s, 1H, H8), 8.31 (s, 1H, H2), 9.37–9.46 (m, 1H, 4″-NH); ^{19}F NMR (282 MHz, [D$_6$]DMSO): $\delta = -74.35$; ESI-MS (positive ion mode): *m*/*z* (relative intensity): 591.1 (10) [M+K]$^+$, 575.1 (15) [M+Na]$^+$, 553.3 (17) [M+H]$^+$, 457.5 (100) [*cyclo* nucleoside].

4.2.4. *N*6-[1″-(4″-Trifluoroacetamido)butyl]-5′-*O*-mesyladenosine (6). Nucleoside **5** (500 mg, 0.905 mmol) was dissolved in aqueous formic acid (50%, 35 mL) and the reaction mixture was stirred at room temperature for 3 d. The solvents were removed under reduced pressure and remaining solvent co-evaporated with water/methanol (1:1, 3× 10 mL). After drying in high vacuum nucleoside **6** (447 mg, 96%) was obtained as a white solid (R_f 0.17, methanol/methylene chloride 5:95). ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 1.66$–1.86 (m, 4H, H2″, H3″), 3.16 (s, 3H, mesyl-CH$_3$), 3.18–3.24 (m, 2H, H4″), 3.25–3.39 (m, 2H, H1″), 4.13–4.18 (m, 1H, H4′), 4.23–4.28 (m, 1H, H3′), 4.40–4.55 (m, 2H, H5′), 4.64–4.69 (m, 1H, H2′), 5.48 (s, br, 1H, OH), 5.63 (s, br, 1H, OH), 5.95 (d, $^3J = 5.50$ Hz, 1H, H1′), 7.95 (s, br, 1H, 6-NH), 8.22 (s, 1H, H8), 8.32 (s, 1H, H2), 9.36–9.46 (m, 1H, 4″-NH); ^{19}F NMR (376 MHz, [D$_6$]DMSO): $\delta = -78.01$; ESI-MS (positive ion mode): *m*/*z* (relative intensity): 535.2 (5) [M+Na]$^+$, 513.3 (100) [M+H]$^+$, 417.7 (9) [*cyclo* nucleoside].

4.2.5. 5′-*N*-Aziridinyl-*N*6-[1″-(4″-aminobutyl)]-5′-deoxyadenosine (7). Nucleoside **6** (150 mg, 0.292 mmol) was dissolved in a mixture of dry aziridine[48,49] (0.9 mL, 17.9 mmol) (CAUTION: aziridine is hazardous and should be handled with care in a fume hood) and *N*-ethyldiisopropylamine (EDIA) (3 mL, 18 mmol) under argon atmosphere and stirred at room temperature for 3 d. The reaction progress was monitored by analytical reverse-phase HPLC (Prontosil-ODS, 5 μm, 120 Å, 250 × 4.6 mm, Bischoff, Leonberg, Germany). Compounds were eluted with acetonitrile (14% for 5 min, followed by linear gradients to 31.5% in 10 min, to 35% in 10 min and to 70% in 5 min) in triethylammonium acetate buffer (0.1 M, pH 7.0) at a flow of 1 mL/min. The product **7** eluted with a retention time of 4.4 min (UV detection at 280 nm and 300 nm). Volatile compounds were removed under reduced pressure and the crude product was dissolved in triethylammonium hydrogen carbonate buffer (0.01 M, pH 8.6, 2 mL) before purification by preparative reverse-phase HPLC (Prontosil-ODS, 5 μm, 120 Å, 250 × 8 mm, Bischoff, Leonberg, Germany). Compounds were eluted with acetonitrile (7% for 5 min, followed by linear gradients to 21% in 15 min and to 70% in 5 min) in triethylammonium hydrogen carbonate buffer (0.01 M, pH 8.6) at a flow

of 3 mL/min. Fractions containing product **7** (retention time 17.4 min, UV detection at 280 nm and 300 nm) were stored at −80 °C. The amount of product **7** (10.1 mg, 10%) in the combined fractions (30 mL) was determined by UV spectroscopy using the published extinction coefficient $\varepsilon^{267} = 16{,}000\ \mathrm{L\ mol^{-1}\ cm^{-1}}$ of *N*6-[1″-(4″-aminobutyl]-2′,3′-*O*-(methoxyethylidene)adenosine.[52] ESI-MS (positive ion mode): *m*/*z* (relative intensity): 364.3 (11) $[M+H]^+$.

4.2.6. 5′-*N*-Aziridinyl-*N*6-[1″-(*N*″-Cy3)- 4″-aminobutyl]-5′-deoxyadenosine (1). To a solution (1 mL) of nucleoside **7** (190 µg, 0.52 µmol) in triethylammonium hydrogen carbonate buffer (0.01 M, pH 8.6) containing acetonitrile was added Cy3 *N*-hydroxysuccinimidyl ester (Cy3 NHS ester) (161 µg, 0.22 µmol) in dimethylsulfoxide (600 µL). The reaction mixture was stirred at room temperature for 1 h. The crude product was purified by preparative reverse-phase HPLC (Prontosil-AQ, 5 µm, 120 Å, 250 × 8 mm column equipped with a Prontosil-AQ, 5 µm, 120 Å, 33 × 8 mm pre-column, Bischoff, Leonberg, Germany) and compounds were eluted with acetonitrile (20% for 5 min, followed by linear gradients to 45% in 5 min, to 50% in 7 min and to 100% in 2 min) in triethylammonium hydrogen carbonate buffer (0.01 M, pH 8.6) at a flow of 3 mL/min. Fractions containing the product **1** (retention time 12.5 min, UV detection at 280 nm and 550 nm) were combined and dried by lyophilization. The aziridine cofactor **1** (107 µg, 50% with respect to Cy3 NHS ester) was obtained as a red solid. For enzymatic transformations **1** was used from a stock solution in dimethylsulfoxide stored at −80 °C. ESI-MS: *m*/*z* (relative intensity): 976.6 (6) $[M]^+$ (positive ion mode), 974.7 (4) $[M-2H]^-$ (negative ion mode).

4.3. Labeling of pUC19 and pBR322 plasmid DNA

The plasmid (5 µg) and aziridine cofactor **1** (20 µM) were incubated in buffer (100 µL; 20 mM Tris–HCl, pH 6.0, 50 mM KOAc, 50 mM $Mg(OAc)_2$, 0.01% Triton X-100, 1 mM DTT) in the presence of M.TaqI (2- to 5-fold excess over recognition sequences) at 60 °C for 3 h. After labelling guanidinium hydrochloride (12 µL, 5 M) and Proteinase K (8 µL, 600 mAU/mL) were added and the mixture was incubated at 60 °C for 1 h. The labelled plasmid was purified using the QIAquick PCR purification Kit (Qiagen) according to the instructions supplied by the manufacturer. The plasmid was eluted in 50 µL of the supplied elution buffer and the concentration determined using a GeneQuant pro DNA/RNA calculator (BioChrom).

4.4. Restriction analysis of labelled plasmids pUC19/4Cy3 and pBR322/7Cy3

The labelled plasmid (200 ng) was incubated with R.TaqI (1 U) in buffer (10 µL) supplied by the manufacturer at 60 °C for 1 h. Loading buffer (2 µL; 0.25% bromophenol blue, 30% glycerol) was added and the samples were analysed by agarose gel electrophoresis (0.75% agarose gel prestained with SYBR Safe™, Molecular Probes). Electrophoresis was carried out at 80 V for 2 h in 1×TBE buffer. Bands were visualised using UV light and gels photographed using a polaroid camera. Pictures were digitalised and bands corresponding to supercoiled and relaxed form of plasmids quantified using the image analysing software ImgeJ (http://rsb.info.nih.gov/ij/).

4.5. Cell transfection

CHO-K1 cells (approx 10^5 cells) were seeded on glass coverslides (16 mm diameter) coated with polyornithine in a 24-well plate and grown in F12 medium (Gibco) containing foetal bovine serum (6.5%) overnight. Transfection was carried out using serum-free Opti-MEM medium (Gibco) and Lipofectamine 2000 (Invitrogen). Cells in each well were transfected with 250 ng of labelled plasmid according to the instructions by the supplier. After 5 h-exposure to the transfection mix, the transfection medium was replaced by F12 medium (Gibco) containing foetal bovine serum (6.5%) and the cells were cultured for another 19 h before they were fixed with paraformaldehyde (4%). The cell nuclei were stained with DAPI (Molecular Probes) for fluorescence microscopy (Epifluorescence microscope Nikon Eclipse E 800 with 60×, 1.4 objective, oil immersion, Nikon Digital Camera DXM 1200; Nikon) or with TO-PRO-3 (Molecular Probes) for confocal laser scanning microscopy (Confocal laser scanning microscope DM6000 with SP2 unit, 63×, 1.4 objective, oil immersion; Leica). For quantification of transfection with pUC19/4Cy3 2338 CHO-K1 cells from three independent experiments were visually analysed. Four hundred and fifty-three cells contained fluorescence inside and 114 of these cells additionally contained fluorescence in the nucleus. Analysis of transfection with pBR322/7Cy3 included 1255 cells from one experiment. Two hundred and three cells with fluorescence inside were found and 55 of these cells contained additional fluorescence in the nucleus.

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