Specific 3′-Terminal Modification of DNA with a Novel Nucleoside Analogue that Allows a Covalent Linkage of a Nuclear Localization Signal and Enhancement of DNA Stability

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We report a straightforward method for the site-specific modification of long double-stranded DNA by using a maleimide adduct of deoxycytidine. This novel nucleoside analogue was efficiently incorporated at the 3′-termini of DNA by terminal deoxynucleotidyl transferase (TdT). Thiol-containing compounds can be covalently linked to the maleimide moieties. We added a nuclear localization signal peptide to the 3′-terminal of a 350 bp-long DNA that encoded short-hairpin RNA, and these modifications resulted in the enhancement of silencing activity by RNA interference. This enhancement is mainly attributed to increased stability of the template DNA.

Introduction

The vectors used as gene-delivery systems play a very important role in gene therapy. Two types are available: viral and nonviral vectors. Viral vectors provide the more efficient gene-delivery systems, but their use is associated with high cost, immunogenicity, and—in particular—high potential toxicity,[1] so the development of nonviral vectors as safe gene-delivery systems is of great interest. However, there are also problems with nonviral gene-delivery systems, such as low cell-specificity of gene delivery, inefficient uptake by cells, inefficient delivery to nuclei, limited stability, and inefficient expression.[2] Further improvements in certain properties such as cell-specific delivery, cellular uptake efficiency, controlled intracellular distribution, stability against degradation, and target specificity are required. Many methods for attaining these desired improvements are being investigated.

The chemical modification of DNA might help to solve some of the problems associated with nonviral vectors. For example, peptides and/or proteins have been conjugated to DNA as enhancers of biological functions. Because covalent linkage gives a consistent product in which the desired properties can be carefully controlled, it is preferable to link these molecules to DNA covalently rather than through ionic interactions. A major method for the covalent modification of DNA at a specific position involves solid-phase synthesis. In efforts to enhance antisense technology,[3] methods have been developed for linking single-stranded oligonucleotides covalently to peptide,[4] lipid,[5] cholesterol,[6] and carbohydrate[7] moieties. Such modifications enhanced certain properties of the DNA, such as uptake by cells and nuclear localization of oligonucleotides. However, methods for modification in a DNA synthesizer are subject to limitations with respect to the length of the DNA oligomer that is synthesized. Moreover, since the reaction conditions—oxidation with iodide and/or deprotection with ammonia, for example—are so harsh, the introduction of certain functional groups that are reactive under these conditions can be very difficult.

In contrast to the modification of an oligonucleotide, post-synthesis modification of long double-stranded DNA (linear or plasmid DNA) is both difficult and complicated. Some methods, such as diazocoupling[8–10] and photocoupling,[11–16] that allow the modification of DNA through covalent bonding have been developed, but the limited or nonexistent specificity of such reactions—varying numbers of a particular modification are introduced at a variety of sites in the target DNA—has been a serious problem.[17–19] The excessive extent of the chemical modifications introduced by these methods results in the inhibition of transcription. In contrast to these unspecific chemical methods, the specific modification of the end of a DNA molecule appears not to inhibit transcription and has even been reported to enhance the nuclear import[20] and the expression of the DNA.[21] The cited reports indicate that modification of DNA at a specific position can allow the effective delivery of genes to the nucleus. However, the method for conjugating the peptide to the DNA adapted by Zanta et al. requires tedious synthesis of a DNA-NLS peptide conjugate (NLS = nuclear localization signal); this excludes the possibility

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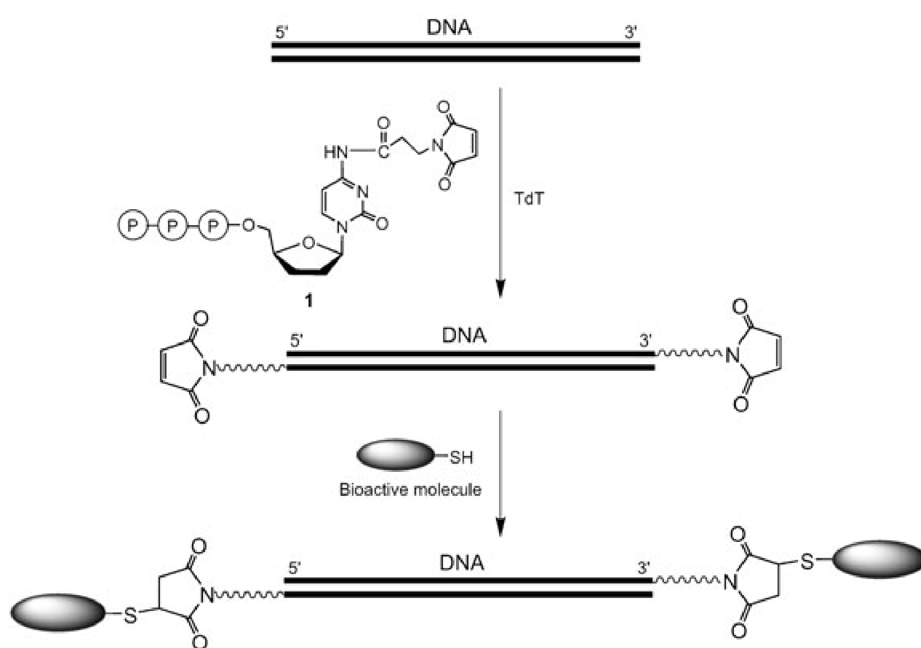
of scaling up to the microgram range.[21] Therefore, it is necessary to develop methods for efficient and site-specific modification of long double-stranded DNA (dsDNA). The challenge is to develop a novel, convenient method that can be easily scaled up if necessary.

Considering the site-specific modification, we decided to modify the 3′-termini of linear DNA because, in plasma, hydrolysis of DNA is the result of exonucleolytic activity and occurs exclusively in the 3′ to 5′ direction.[22] Therefore, protection of DNA from such enzymatic degradation by modifying its 3′-termini should be advantageous in a clinical setting. To the best of our knowledge, however, there are no reports of general methods that allow the efficient modification of DNA at 3′-termini with covalently linked peptides, proteins, and other molecules, and so we have attempted to develop a method for the enzymatic modification of DNA at its 3′-termini. An outline of our strategy is shown in Scheme 1. A maleimide adduct of deoxycytidine is incorporated at each 3′-end of a long double-stranded DNA through the action of terminal deoxynucleotidyl transferase (TdT). In contrast to the other forms of DNA polymerase, such as the Klenow fragment, TdT is a template-independent polymerase that catalyzes the addition of deoxynucleotides at the 3′-hydroxy termini of DNA[23] and allows modification of DNA at its 3′-termini. The maleimide moiety is very useful for linking biomacromolecules because it reacts with thiol groups, such as the cysteine residues in proteins. Such reactions proceed even under very mild conditions and yields are usually very high. Here we report the synthesis of a novel nucleic acid analogue (**1**) and its efficient incorporation at DNA 3′-termini. We have applied this novel technique for enhancement of the silencing activity by RNA interference (RNAi).



Scheme 1. *Selective modification of the 3′-termini of long double-stranded DNA. The novel nucleic acid analogue containing a maleimide moiety is introduced at the 3′-termini of DNA by the action of terminal deoxynucleotidyl transferase (TdT).*

Results and Discussion

We synthesized a novel nucleoside analogue (**1**) that contained a maleimide moiety, as shown in Scheme 2. The amino and 5′-hydroxy groups of the deoxycytidine starting material were protected with acetic anhydride and with 4,4′-dimethoxytrityl chloride (DMTrCl), respectively. The oxygen of the 3′-hydroxy group was then removed in order to avoid the introduction of multiple maleimide moieties at the 3′-termini of the DNA.[24] After deprotection of the 5′-amino group, a single maleimide moiety was introduced. Finally, deprotection of the hydroxy group yielded the novel analogue **1**. The 5′-triphosphate of this dideoxynucleoside (**7**) was synthesized by a previously described method.[25]

We examined the enzymatic incorporation of **7** into DNA (9-mer ODN) using radiolabeled oligodeoxynucleotides (ODNs, Figure 1) and found that **7** was added very efficiently to the 3′-termini of DNA (Figure 1, lane 3). It is noteworthy that as much as 200 pmol of DNA were labeled by only four units of TdT. TdT catalyzes both the phosphorylation and the phosphonylation of 3′-hydroxy moieties and it allows the introduction of various kinds of compound into DNA.[26] It is likely that a strategy similar to ours should allow the incorporation into DNA of a variety of functional groups and not only maleimide.

In order to check the reactivity of the maleimide moiety in DNA with a thiol group, we added various concentrations of cysteine to a reaction mixture containing the modified DNA (Figure 2, lanes 4, 5 and 6). An increase in the concentration of cysteine increased the yield of the conjugate (Figure 2). At 50 mM cysteine, formation of the conjugate was quantitative. The obvious efficiency of the conjugation reaction is a major practical advantage because it facilitates purification and scaling up of the reaction.

To the best of our knowledge, only one method for the introduction of maleimide into DNA has been reported: namely, the coupling of a free primary alkylamine to an activated ester/maleimide bifunctional crosslinker, such as an ester of 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid and *N*-hydroxysuccinimide (SMCC).[21,27–29] However, the yield of this reaction is usually low ($\approx$30%). Alternatively, DNA can be modi-

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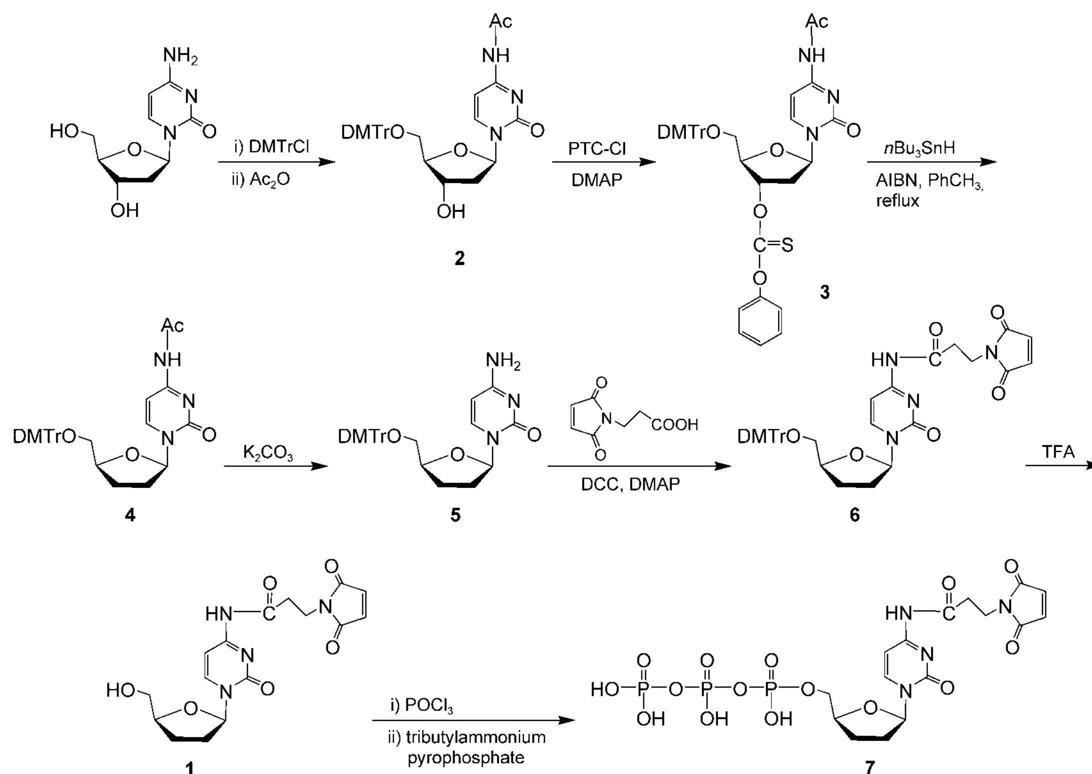

Scheme 2. *Incorporation of a maleimide moiety into a nucleoside analogue (**1**).*

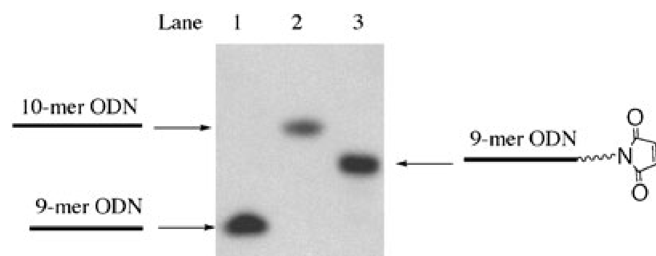

Figure 1. *Incorporation of **7** into an oligodeoxynucleotide by the action of TdT. Products were analyzed on a denaturing 20 % polyacrylamide gel. Lane 1: 9-mer oligodeoxynucleotide (ODN) + TdT (control). Lane 2) 10-mer ODN. Lane 3: same as in lane 1 after treatment with **7** (2 mM).*

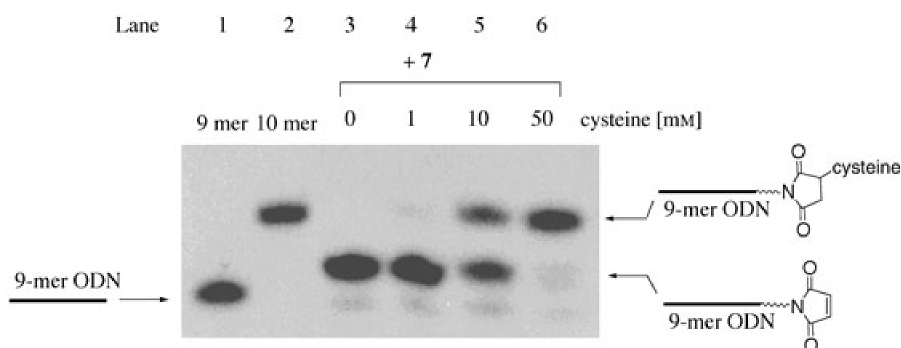

Figure 2. *Covalent attachment of cysteine to the 3′-termini of maleimide-modified DNA. Products were analyzed on a denaturing 20 % polyacrylamide gel. Lane 1) 9-mer ODN + TdT (control). Lane 2) 10-mer ODN. Lane 3) same as in lane 1 after treatment with **7** (2 mM). Lanes 4, 5, and 6) lane same as in 3 plus 1 mM, 10 mM, and 50 mM of cysteine, respectively.*

fied with a thiol and then conjugated with peptides functionalized with maleimide.[30–32] Our method has many advantages over these: i) the yield is almost quantitative and 200 pmol of DNAs were modified with only 4 U of TdT, so even milligram quantities of modified DNA can easily be prepared in the laboratory, ii) our method does not involve any extra modification of the DNA, such as the preparation of alkylamino-containing DNA, and iii) our method can be applied to any unmodified double-stranded DNA (dsDNA), prepared by PCR or by other methods, and there is no limit to the length of the DNA that can be modified.

This reaction can be used for the conjugation of DNA with other thiol-containing compounds, and so we tried to use it to link long dsDNA covalently with peptides, introducing a maleimide moiety into dsDNA (350-mer, PCR product) with the aid of TdT, as described above. After removal of excess **7**, we treated the modified DNA with a cationic peptide including an NLS[21] and a cysteine residue at its carboxyl terminus (PKKKRKVE-DPYC). In order to avoid precipitation of the DNA, the amount of peptide in the reaction mixture was kept below the level that would approach the

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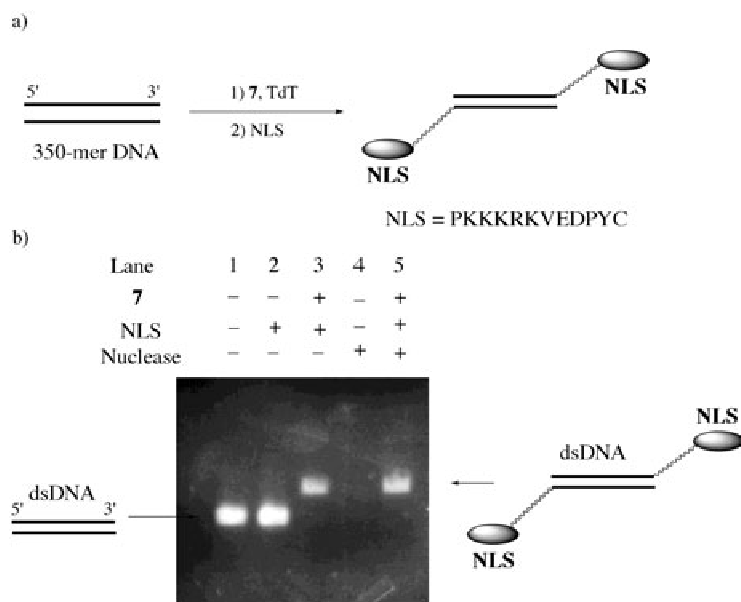

Figure 3. *a) Covalent attachment of a nuclear localization signal (NLS) to long double-stranded DNA (dsDNA). b) Products were analyzed on a 3% agarose gel. Lane 1) untreated 350 bp dsDNA. Lane 2) 350 bp dsDNA after incubation with the NLS peptide. After incubation, excess peptide was removed by washing with NaCl (2 M). Lane 3) 350 bp dsDNA, modified with **7** by TdT, after incubation with the NLS peptide. Excess peptide was removed after incubation by washing with NaCl (2 M). Lanes 4 and 5) DNA with free termini (lane 4) or with covalently attached NLS (lane 5) was incubated with exonuclease for 1 hour. Unlike the unmodified DNA (lane 4), DNA modified with NLS peptide was stable under these conditions.*

charge-neutralizing ratio. The reaction was allowed to proceed in phosphate buffer (20 mM, pH 7.0) for 6 h. Excess peptide was removed by two washes with NaCl (2 M), which eliminated unspecific electrostatic interactions. The peptide-conjugated DNA was analyzed for altered mobility on a 3.0% agarose gel (Figure 3). The mobility of the dsDNA that had reacted with the peptide after modification with maleimide was reduced (lane 3), while the migration of unmodified dsDNA that had been incubated with the peptide was similar to that of untreated dsDNA (compare lanes 1 and 2). This result indicated the successful covalent linkage of maleimide-modified dsDNA and the NLS, and the reaction was almost complete.

Next, we checked the stability of dsDNA covalently conjugated with NLS at its 3′-termini. The linear PCR product and the NLS-dsDNA conjugates were independently treated with exonuclease III and the resulting mixtures were analyzed on agarose gel (Figure 3). DNA with free termini was totally degraded after incubation with the exonuclease for 1 hour (lane 4), whereas the dsDNA covalently attached to the NLS clearly resisted nucleolytic degradation under the same conditions (lane 5).

Finally, we investigated the effect of the covalently linked NLS on the suppressive activity of RNAi. RNAi is a natural phenomenon by which double-stranded RNA (dsRNA) or short-hairpin RNA (shRNA) induce the sequence-dependent degradation of a cognate mRNA in cells.[33] We attached the NLS to the 3′-termini of a dsDNA 350-mer encoding a shRNA. The target of the shRNA was the firefly gene for luciferase.[34] We transfected HeLa S3 cells with an expression vector encoding *Renilla* luciferase, an expression vector encoding firefly luciferase, and dsDNA with or without the conjugated peptide, or with control vector DNA [pU6[34]]. As shown in Figure 4, dsDNA that expressed shRNA directed against firefly luciferase disrupted the expression of the firefly gene for luciferase without altering the activity of *Renilla* luciferase serving as an internal control (compare lanes 1 and 2). This gene-specific silencing activity by RNA interference was enhanced when the NLS was linked to the 3′-termini of the DNA (compare lanes 1 and 4). This enhancement can be attributed to two factors: i) protection of the DNA from attacks by nucleases by the modification of the DNA at 3′-terminus, and/or ii) enhancement of the import of the DNA into the nucleus by the attached NLS. With regard to the former factor, a similar conclusion had been reached by detection of enhancement of silencing activity by increasing the stability of DNA templates against nucleases by formation of dumbbell-shaped DNA.[21,35] To check the latter factor, we conjugated mutated NLS (mNLS; PKTKRKVEDPYC[21]) to the 3′-terminus of the DNA. As shown in Figure 4, only a small difference (5%) between the silencing activities of DNA-NLS and DNA-mNLS conjugates could be detected (compare lanes 3 and 4). This enhancement was smaller than we had

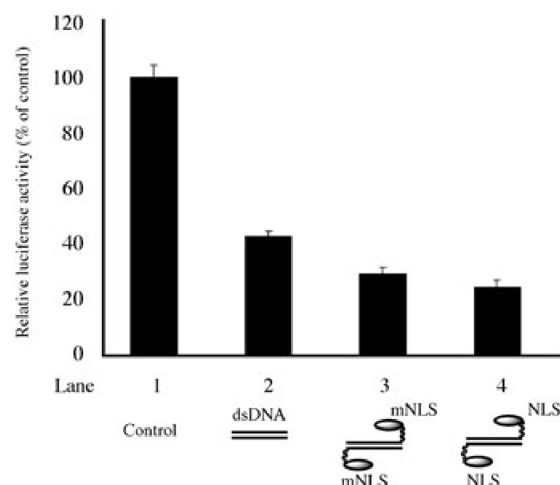

Figure 4. *Covalently linked NLS enhanced the suppressive activity of RNA interference. HeLa S3 cells were transfected with the empty vector pU6 (control) or with dsDNA or with the NLS-DNA or mNLS conjugate. See text for details.*

expected, a likely explanation for such weak enhancement being the apparent loss in the nuclear import ability of the positively charged NLS to interact with transport receptors after conjugation to negatively charged DNA. The loss of function of NLS is most probably due to electrostatic interactions between the negatively charged phosphate groups of the DNA and the cationic NLS peptides. The positive charges of the lysine and arginine residues of the NLS peptides are critical

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for recognition by the transport receptors, and their neutralization would probably diminish the import abilities of the NLS. Possible ways to overcome this problem include increasing the spacer length separating the NLS peptide from the DNA, and the use of other types of NLSs, such as the M9 nuclear localization signal, which does not interact with DNA so strongly, or the longer NLS of SV40 which has phosphorylation sites within the NLS signal, because phosphorylation reduces the positive charge of the NLS.[20]

Conclusion

We have described a novel method for conjugating dsDNA with thiol-containing compounds. In our system, DNA can be efficiently labeled with maleimide, allowing large-scale synthesis of the product. Our method allows the selective modification of the 3′-termini of DNA, and so inactivation of transcription is apparently avoided. Moreover, NLS covalently attached to the 3′-termini of DNA enhanced the suppressive activity of NLS-DNA-driven siRNA. Modification of the 3′-termini of DNA increased the stability of the DNA against enzymatic degradation, which is one of the problems associated with construction of an efficient delivery system.[2]

The goal of our work is the development of artificial viruses expressing short RNAs, such as shRNA and micro RNA (miRNA).[36,37] Modification of DNA with small molecules and/or large biomacromolecules holds promise because such modifications can enhance the biological deliverability of the DNA into cells. Conjugation of DNA with a bioactive peptide or protein appears to be a very attractive strategy, and in this study we have shown that a covalently linked NLS enhanced RNAi activity, although to a limited extent. We are now attempting to conjugate DNA with other proteins or longer peptides prepared in *E. coli* or yeast cells. Since our novel system enables one to attach any molecule specifically to the end of DNA, this system should contribute to the development of artificial viruses.

Experimental Section

Materials: All solvents and reagents were purchased commercially and were used without further purification. ^{1}H NMR spectra (400 MHz) were recorded on a Varian AS400 spectrometer. Column chromatography was performed on silica gel (Wako gel-C200), while Wako 70F silica gel was used for thin layer chromatography. Terminal deoxynucleotidyl transferase (TdT) was purchased from New England Biolabs (Beverly, MA, USA). Peptides were purchased from Genenet (Fukuoka, Japan).

Synthesis of a novel nucleoside analogue, 2′,3′-dideoxy-*N*^{4}-(3-maleimidopropylamino)cytidine (1 in Scheme 2)

Synthesis of N^4*-acetyl-2′-deoxy-*O$^{5'}$*-di-*p*-methoxytrityl cytidine (**2**)*: A solution of the 2′-deoxycytidine monohydrate starting material (5.0 g, 19.0 mmol) in dimethylformamide (190 mL) was treated with acetic anhydride (1.79 mL, 19.0 mmol) and triethylamine (5.3 mL, 38.0 mmol). After the mixture had been stirred at room temperature for 6 h, the solvent was removed under vacuum. The resulting residue was redissolved in pyridine and the solvent was removed by evaporation. After this step had been repeated once, the crude product was dissolved in pyridine (180 mL), and 4,4′-dimethoxytrityl chloride (DMTr-Cl; 6.4 g, 18.9 mmol) was added. After this mixture had been stirred at room temperature for 3 h, the solvent was evaporated and the residue was partitioned between $CHCl_3$ and 5% aqueous $NaHCO_3$. The organic phase was dried over Na_2SO_4, concentrated by evaporation, and applied to a column of silica gel. Evaporation of appropriate fractions yielded **2** (7.8 g, 69% over two steps). ^{1}H NMR (400 MHz; $CDCl_3$): $\delta = 2.20$ (s, 3H; $CH_3C{=}O$), 2.24 (m, 1H; H-2′), 2.75 (m, 1H; H-2′), 3.40 (dd, $J = 3.7$, 10.8 Hz, 1H; H-5′), 3.47 (dd, $J = 3.0$, 10.8 Hz, 1H; H-5′), 3.78 (s, 6H; OCH_3), 4.15 (m, 1H; H-4′), 4.52 (m, 1H; H-3′), 6.25 (t, $J = 5.8$ Hz, 1H; H-1′), 6.86–7.40 (m, 13H), 7.19 (d, $J = 7.4$ Hz, 1H; H-5), 8.26 ppm (d, $J = 7.4$ Hz, 1H; H-6), 9.59 (brs, 1H; NH).

Synthesis of N^4*-acetyl-2′-deoxy-*O$^{5'}$*-di-*p*-methoxytrityl-*O$^{3'}$*-phenoxythiocarbonyl cytidine (**3**)*: A solution of **2** (7.8 g, 13.2 mmol) in CH_3CN (130 mL) was treated with 4-(dimethylamino)pyridine (DMAP; 3.3 g, 27.0 mmol) and phenyl chlorothionoformate (2.2 mL, 14.5 mmol). The solution was stirred at room temperature for 16 h, the solvent was evaporated, and the residue was partitioned between $CHCl_3$ and 5% aqueous $NaHCO_3$. The organic phase was dried over Na_2SO_4, concentrated by evaporation, and applied to a column of silica gel. Evaporation of appropriate fractions yielded **3** (7.0 g, 73%). ^{1}H NMR (400 MHz; $CDCl_3$): $\delta = 2.24$ (s, 3H; $CH_3C{=}O$), 2.44 (m, 1H; H-2′), 3.10 (m, 1H; H-2′), 3.54 (dd, $J = 2.7$, 10.7 Hz, 1H; H-5′), 3.58 (dd, $J = 3.6$, 10.7 Hz, 1H; H-5′), 3.79 (s, 6H; OCH_3), 4.54 (m, 1H; H-4′), 5.88 (m, 1H; H-3′), 6.37 (dd, $J = 5.7$, 7.8 Hz, 1H; H-1′), 6.83–7.45 (m, 13H), 7.10 (d, $J = 7.4$ Hz, 1H; H-5), 7.30–7.49 (m, 5H; Ph), 8.09 (d, $J = 7.4$ Hz, 1H; H-6), 8.64 ppm (brs, 1H; NH).

Synthesis of N^4*-acetyl-2′,3′-dideoxy-*O$^{5'}$*-di-*p*-methoxytrityl-cytidine (**4**)*: A solution of **3** (7.0 g, 9.62 mmol) in anhydrous toluene (192 mL) was heated at reflux and treated dropwise over the course of 5 min with a mixture of tributyltin hydride (nBu$_3$SnH; 7.5 mL) and 2,2′-azobisisobutylnitrile (AIBN; 607 mg). After the system had been heated at reflux for a further 30 min, the solvent was removed under vacuum. The resulting residue was partitioned between $CHCl_3$ and 5% aqueous $NaHCO_3$, and the organic phase was dried over Na_2SO_4, concentrated by evaporation, and applied to a column of silica gel. Evaporation of appropriate fractions yielded **4** (4.12 g, 78%). ^{1}H NMR (400 MHz; $CDCl_3$): $\delta = 1.95$ (m, 2H; H-3′), 2.21 (m, 1H; H-2′), 2.22 (s, 3H; $CH_3C{=}O$), 2.50 (m, 1H; H-2′), 3.35 (dd, $J = 4.1$, 11.0 Hz, 1H; H-5′), 3.55 (dd, $J = 2.7$, 11.0 Hz, 1H; H-5′), 3.81 (s, 6H; OCH_3), 4.27 (m, 1H; H-4′), 6.09 (m, 1H; H-1′), 6.85–7.43 (m, 13H), 7.10 (d, 1H; $J = 7.0$ Hz, 1H; H-5), 8.42 (d, $J = 7.0$ Hz, 1H; H-6), 8.58 ppm (brs, 1H; NH).

*Synthesis of 2′,3′-dideoxy-*O$^{5'}$*-di-*p*-methoxytrityl-cytidine (**5**)*: A solution of **4** (4.12 g, 7.5 mmol) in K_2CO_3 in methanol (50 mM, 75 mL) was stirred at room temperature for 6 h and the solvent was then removed under a vacuum. The resulting residue was partitioned between $CHCl_3$ and 5% aqueous $NaHCO_3$, and the organic phase was dried over Na_2SO_4, concentrated by evaporation, and applied to a column of silica gel. Evaporation of appropriate fractions yielded **5** (2.54 g, 66%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.87$ (m, 1H; H-3′), 1.95 (m, 1H; H-3′), 2.15 (m, 1H; H-2′), 2.42 (m, 1H; H-2′), 3.31 (dd, $J = 3.8$, 10.7 Hz, 1H; H-5′), 3.54 (dd, $J = 2.4$, 10.7 Hz, 1H; H-5′), 3.80 (s, 6H; OCH_3), 4.22 (m, 1H; H-4′), 5.32 (d, $J = 7.3$ Hz, 1H; H-5), 6.10 (m, 1H; H-1′), 6.83–7.44 (m, 13H), 8.11 (d, $J = 7.0$ Hz, 1H; H-6), 8.58 ppm (brs, 1H; NH).

*Synthesis of 2′,3′-dideoxy-*O$^{5'}$*-di-*p*-methoxytrityl-*N^4*-3-maleimidopropionylamino cytidine (**6**)*: A solution of 3-maleimidopropionic acid (796 mg, 4.71 mmol) in CH_2Cl_2 (20 mL) was treated with anhydrous 1-hydroxybenzotriazole (HOBT; 636 mg, 4.69 mmol), DMAP (26 mg,

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0.21 mmol), and 1,3-dicyclohexylcarbodiimide (DCC; 927 mg, 4.49 mmol). After the solution had been stirred at room temperature for 30 min, undissolved material was removed by filtration. The filtrate and triethylamine (596 μL, 4.28 mmol) were added to **5** (1.1 g, 2.14 mmol) with stirring at room temperature over 30 min. The reaction mixture was partitioned between CH_2Cl_2 and brine, and the organic phase was dried over Na_2SO_4, concentrated by evaporation, and applied to a column of silica gel. Evaporation of appropriate fractions yielded **6** (1.0 g, 70%). ^{1}H NMR (400 MHz; $CDCl_3$): $\delta=1.91$ (m, 2H; H-3′), 2.18 (m, 1H; H-2′), 2.50 (m, 1H; H-2′), 2.86 (t, $J=6.9$ Hz, 2H; $CH_2C{=}O$), 3.35 (dd, $J=4.0$, 10.9 Hz, 1H; H-5′), 3.54 (dd, $J=2.5$, 10.9 Hz, 1H; H-5′), 3.82 (s, 6H; OCH_3), 3.88 (t, $J=6.9$ Hz, 2H; CH_2N), 4.28 (m, 1H; H-4′), 6.06 (m, 1H; H-1′), 6.68 (s, 2H; CH=CH), 6.85–7.44 (m, 13H), 7.09 (d, $J=7.0$ Hz, 1H; H-5), 8.41 (d, $J=7.0$ Hz, 1H; H-6), 9.72 ppm (br s, 1H; NH).

*Synthesis of 2′,3′-dideoxy-*N^4*-3-maleimidopropionylamino cytidine (**1**)*: A solution of **6** (500 mg, 0.75 mmol) in CH_2Cl_2 (75 mL) was treated at 0 °C with trifluoroacetic acid (TFA; 750 μL). After it had been stirred at 0 °C for 3 min, the reaction mixture was neutralized with 5% aqueous $NaHCO_3$. The neutralized solution was partitioned between CH_2Cl_2 and brine, and the organic phase was dried over Na_2SO_4, concentrated by evaporation, and applied to a column of silica gel. Evaporation of appropriate fractions yielded **1** (165 mg, 61%). ^{1}H NMR (400 MHz; $CDCl_3$): $\delta=1.93$ (m, 2H; H-3′), 2.19 (m, 1H; H-2′), 2.49 (m, 1H; H-2′), 2.89 (t, $J=6.8$ Hz, 2H; $CH_2C{=}O$), 3.78 (dd, $J=3.0$, 1.2 Hz, 1H; H-5′), 3.88 (t, $J=6.9$ Hz, 2H; CH_2N), 4.05 (m, 1H; H-5′), 4.25 (m, 1H; H-4′), 6.05 (m, 1H; H-1′), 6.70 (s, 2H; CH=CH), 7.33 (d, $J=7.4$ Hz, 1H; H-5), 8.43 (d, $J=7.0$ Hz, 1H; H-6), 10.05 ppm (br s, 1H; NH); elemental analysis calcd (%) for $C_{16}H_{18}N_4O_6$: C 53.02, H 5.01, N 15.47; found: C 52.94, H 4.97, N 15.03.

*Synthesis of 2′,3′-dideoxy-*N^4*-3-maleimidopropionylamino cytidine 5′-triphosphate (**7**)*: Compound **1** (100 mg, 0.28 mmol) was dissolved in trimethyl phosphate (1.4 mL), and the solution was cooled to 0 °C. This solution was treated with 1,8-bis(dimethylamino)naphthalene (90 mg) and phosphorus oxychloride (30 μL), and was then stirred for 2 h at 0 °C.[26] Tributylamine (410 μL) and tributylammonium pyrophosphate (240 mg in 3.2 mL DMF) were then added, and the solution was stirred for 1 min before the addition of triethylammonium bicarbonate (1 M, 28 mL, pH 7.5) to quench the reaction. After further stirring for 20 min at room temperature, the reaction mixture was lyophilized. Purification by reversed-phase (C18) HPLC (CH_3CN in 0.1 M triethylammonium bicarbonate, pH 7.5) yielded the desired triphosphate (**7**). Phosphorus NMR was taken in D_2O with Tris (50 mM, pH 7.5) and EDTA (2 mM); an external phosphoric acid standard was used. ^{31}P NMR (140 MHz): $\delta=-7.34$ (α-phosphate), −21.48 (β-phosphate), −4.44 ppm (γ-phosphate).

Covalent attachment of cysteine to oligodeoxynucleotides: The 9-mer oligodeoxynucleotides (ODNs) were radiolabeled at their 5′-termini by use of [γ-^{32}P]ATP and T4 polynucleotide kinase, and labeled ODNs were purified on PAGE. We used 100 pmol of ODNs, including a small amount of DNA-[5′-^{32}P primer, for each experiment. Each assay mixture (10 μL) contained buffer as indicated, TdT (4 U), and the triphosphate (2 mM). Cysteine was added to the reaction mixture at various concentrations, the reaction mixtures were incubated for 1 h at 37 °C, and the resulting conjugates were analyzed for altered mobility on a 20% polyacrylamide denaturing gel.

Covalent attachment of a peptide to DNA: A 350-mer DNA (2 μg) that encoded a short hairpin RNA (shRNA) directed against the firefly gene for luciferase was prepared by PCR as follows. Two synthetic DNA primers (1 pmol each) were mixed with the plasmid vector, which included a U6 promoter and encoded shRNA targeted to the firefly gene for luciferase.[34] The target sequence is 5′-GTG CGC TGC TGG TGC CAA C-3′.[34] Amplification by PCR was then performed with *ExTaq* DNA polymerase (Takara, Kyoto, Japan). The sequences of the sense and antisense primers were 5′-CAA GCT-TGC ATA AAA AGT GCG-3′ and 5′-CCA GTG AAT CAA GGT CGG G-3′. The PCR product was isolated by electrophoresis on a 1% agarose gel, and the purified product was labeled with the adduct by TdT as described above. Excess adduct was removed by ultrafiltration (Ultrafree-MC 30 000, Millipore, MA, USA). The recovered DNA that had been modified with the maleimide adduct at its 3′-termini was dissolved in distilled water to a final concentration at 0.2 g L^{-1}. In order to avoid precipitation of the DNA, the appropriate peptide was added to a level that did not approach the charge-neutralizing ratio, wherein the ratio of positive to negative charge is 0.9:1.0. The reaction mixture, in phosphate buffer (20 mM, pH 7.0), was incubated for 6 h. Excess peptide was removed by two washes with NaCl (2 M) in the ultrafiltration unit, which were followed by three washes with distilled water. The peptide-DNA conjugate was analyzed for altered mobility on a 3.0% agarose gel, which was stained with ethidium bromide after electrophoresis.

The treatment of DNA-peptide conjugate with exonuclease: The linear PCR product and DNA-peptide conjugate were independently treated with exonuclease III (TaKaRa). Aliquots (100 pmol) of substrates were incubated with exonuclease (100 U) for 1 hour. The products were analyzed on agarose.

Cell culture, transfection, and luciferase assays: HeLa S3 cells (30 000 cells per well) were seeded 24 h before transfection in 48-well tissue culture plates. Transfections were performed with Lipofectamine 2000 reagent (Invitrogen, Germany) according to the manufacturer's instructions. HeLa S3 cells were transfected with an expression vector encoding *Renilla* luciferase (pRL-RSV; 25 ng), an expression vector encoding firefly luciferase (25 ng), and dsDNAs with or without the conjugated peptide (25 ng), or control vector DNAs (pU6[34]). Luciferase activities were analyzed after 24 h by use of the dual luciferase system (Promega, Madison, WI, USA). Cells were lysed and the activities of firefly and *Renilla* luciferases were measured sequentially with a Berthold luminometer (Lumat LB9501, Berthold, Wildbad, Germany). The firefly luciferase activities were normalized by reference to the activity of *Renilla* luciferase.

Keywords: bioconjugates · gene technology · nuclear localization signal · nucleic acids · peptides

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