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# Esterase-Triggered Fluorescence of Fluorogenic Oligonucleotides

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In the prooligonucleotide approach, a step of activation by cellular esterases is necessary for the removal of internucleoside phosphate masking groups and subsequent intracellular delivery of active antisense oligonucleotides. The efficacy of this approach implies that prooligonucleotides, once they are taken up by cells, are demasked by esterases during their course to their nucleic acid targets. In this regard, a method for labeling oligomers with esterase-activable fluorogenic tag was designed. The two phenolic functions of carboxyfluorescein were protected by pivaloyl groups, yielding a nonfluorescent lactone which was further activated as a *N*-hydroxysuccinimide ester. Two nuclease-resistant phosphorothioate 18-mer and methylphosphonate 19-mer oligodeoxynucleosides were attached to this biprotected fluorescein derivative via an amino linker at the 5'-end of the oligomers. The two conjugates were assayed for their carboxyesterase substrate ability in different biological media. In the presence of purified esterases or when incubated in serum or cell extracts, both oligonucleotide conjugates became fluorescent. In addition, the phosphorothioate oligoconjugate was microinjected into the cytoplasm of human fibroblasts, and a fast cytoplasmic release of fluorescence was observed with a rapid translocation of the fluorescent oligomer into the nucleus.

## INTRODUCTION

The prooligonucleotide concept has been developed by us and others during the past few years (1–5). Prooligonucleotides were designed as a new class of antisense analogs with internucleosidic phosphates masked by enzymolabile protecting groups. Suppression of negative charges and increase of lipophilicity are expected to improve the cellular uptake of these oligonucleotide prodrugs (6–8) and to induce a completely different bioavailability pattern in relation with the route of administration. After intracellular delivery, these uncharged and nuclease-resistant analogs may be converted by cellular esterases to phosphodiester or phosphorothioate oligonucleosides able to elicit RNase H (9). The efficacy of this approach implies that prooligonucleotides, once they are taken up by cells, are demasked by esterases during their course to their nucleic acid targets.

In this regard, the aim of our work was to design a method for labeling oligomers with an esterase-activable fluorogenic tag and to visualize esterase activity within the cell. We report here the synthesis of the 5(6)-carboxyfluorescein derivative protected on the two phenolic functions by pivaloyl groups, yielding the nonfluorescent lactone form. Cleavage of the ester groups by the cellular carboxyesterases would open the lactone to the fluorescent xanthen-3-one derivative. The *N*-hydroxysuccinimide (NHS) ester of the biprotected carboxyfluorescein was then prepared and reacted with the amino linker at the 5'-end of two nuclease-resistant phosphorothioate (ps) 18-mer and methylphosphonate (mp) 19-mer oligonucleosides, yielding the fluorogenic oligonucleotide conjugates **1** and **2**, respectively.

To assess the structure of the fluorogenic conjugates, **1** and **2** were treated with ammonia or pig liver esterase (PLE) and the reaction products were analyzed by HPLC. Furthermore, the fluorescence released upon PLE hydrolysis was measured by fluorescence spectrophotometry. We examined the carboxyesterase substrate ability of the two oligoconjugates **1** and **2** in different biological media including cell culture medium and cell extracts. Finally, we studied the fate of the fluorogenic phosphorothioate oligoconjugate **1** in human fibroblasts after microinjection into the cytoplasm and nucleus.

## EXPERIMENTAL PROCEDURES

**Materials and Methods.** Except as noted, reagents and solvents were commercially obtained and used without further purification. Amine-free *N,N*-dimethylformamide (DMF) (puriss) was purchased from Fluka. Anhydrous methylene chloride (CH<sub>2</sub>Cl<sub>2</sub>) was distilled from phosphorus pentoxide. Silica gel TLC was carried out on Kieselgel 60 F<sub>254</sub> plates (Merck), and compounds were visualized by UV shadowing. Silica gel 60 (Merck 230–400 mesh) was used for flash column chromatography. FAB mass spectra (MS) were recorded on a JEOL DX300 spectrometer operating with a JMA-DA 5000 mass data system in positive ion mode; the ESI mass spectrum was obtained with a Finnigan MAT SSQ 7000 mass spectrometer, and the MALDI-TOF spectrum was recorded on a PerSeptive Biosystems Voyager mass spectrometer. The <sup>1</sup>H-NMR spectra were recorded with a Bruker DRX 400 MHz spectrometer, and chemical shifts were measured relative to CHCl<sub>3</sub> fixed at 7.24 ppm. Reversed-phase HPLC was performed on a Waters 600 E system equipped with a Model 990 photodiode array detector and using a Macherey-Nagel EC Nucleosil 5μ C<sub>18</sub> column (150 × 4.6 mm) for analytical purposes and a Waters Delta Pak 15μ C<sub>18</sub> 100 Å column (300 × 7.8 mm) for preparative work. The fluorescence spectra were recorded on a Spex-Fluorolog spectrofluorometer Model 1681 (Jobin-Yvon).

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**3',6'-Bis(*tert*-butyloxy)-3-oxospiro[isobenzofuran-1(3*H*),9'(9*H*)-xanthene]-5(6)-carboxylic Acid (3).** To a cooled (ice bath) and stirred solution of 5(6)-carboxy-fluorescein (1 g, 2.6 mmol) in dry pyridine (10 mL) was added dropwise pivaloyl chloride (1.6 g, 1.6 mL, 13.3 mmol). The mixture was stirred at room temperature overnight. The excess of pivaloyl chloride was hydrolyzed with cold water (10 mL), and the volume of the reaction mixture was reduced to 5 mL under reduced pressure. Methylene chloride (10 mL) was then added, and the resulting solution was washed four times with water (50 mL), dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. The residue (1.54 g) was fractionated by flash chromatography on a silica gel (48 g) column (3.5 cm i.d.) using 10% diethyl ether in CH<sub>2</sub>Cl<sub>2</sub> (700 mL) and then a stepwise gradient of 1–20% methanol in CH<sub>2</sub>Cl<sub>2</sub> as eluants. Fractions containing pure **3** as a mixture of regioisomers (*R<sub>f</sub>* 0.35 for regioisomer **5** and *R<sub>f</sub>* 0.42 for regioisomer **6**, eluant system CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH 4:1 v/v) were combined and evaporated to give a colorless foam (766 mg, 1.4 mmol, 53% yield): <sup>1</sup>H NMR (CDCl<sub>3</sub>) (regioisomer **6**) δ 1.36 (1s, 18 H, *t*Bu), 6.79 and 7.08 (2m, 6H), 7.85 (s, 1 H, *H<sub>I</sub>*), 8.11 (d, 1 H, *H<sub>4</sub>*, *J<sub>4-5</sub>* = 8 Hz), 8.32 (dd, 1 H, *H<sub>5</sub>*, *J<sub>5-4</sub>* = 8 Hz, *J<sub>5-1</sub>* = 1 Hz), 10.57 (br s, 1 H, -CO<sub>2</sub>H); (regioisomer **5**) δ 1.40 (1s, 18 H, *t*Bu), 6.84 and 7.11 (2m, 6H), 7.32 (d, 1H, *H<sub>I</sub>*, *J<sub>1-6</sub>* = 8 Hz), 8.43 (d, 1H, *H<sub>6</sub>*, *J<sub>6-1</sub>* = 8 Hz), 8.81 (s, 1H, *H<sub>4</sub>*); MS (+ FAB, glycerol/thioglycerol 50:50, v/v) [*M* + *H*]<sup>+</sup> = 545.

**1-[3',6'-Bis(*tert*-butyloxy)-3-oxospiro[isobenzofuran-1(3*H*),9'(9*H*)-xanthene]-5(6)-yl]carbonyloxy]-2,5-pyrrolidinedione (4).** *N*-[3-(Dimethylamino)propyl]-*N*-ethylcarbodiimide hydrochloride (EDC) (377 mg, 1.96 mmol) and NHS (178 mg, 1.55 mmol) were successively added to a solution of compound **3** (766 mg, 1.4 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (10 mL). The reaction mixture was stirred at room temperature for 4 h. EDC (54 mg, 0.28 mmol) was then added and stirring was carried on for 3 h. The reaction mixture was evaporated to give a yellowish foam. The residue was dissolved in ethyl acetate (50 mL), and the solution was washed four times with water (100 mL). The organic layer was then dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue (982 mg) was fractionated on a silica gel (30 g) column (3.5 cm i.d.) using first a gradient of CH<sub>2</sub>Cl<sub>2</sub> (10–100%) in cyclohexane and then a gradient of methanol (1–2%) in CH<sub>2</sub>Cl<sub>2</sub> as eluants. Pooled fractions containing pure product **4** were evaporated to dryness. The residue was dissolved in dioxane, and the solution was lyophilized to afford a white powder (595 mg, 0.92 mmol, 47% yield): *R<sub>f</sub>* 0.5 (TLC solvent: CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH/CH<sub>3</sub>COOH 97:2.5:0.5 v/v/v); <sup>1</sup>H NMR (as a mixture of regioisomers **5** and **6** in 2:1 ratio) (CDCl<sub>3</sub>) δ 1.36 (br s, 18 H, *t*Bu), 2.95 [m, 4H, -(CH<sub>2</sub>)<sub>2</sub>], 6.78 and 7.11 (2m, 6H), 7.36 (d, <sup>2</sup>/<sub>3</sub> H, *H<sub>I</sub>* of isomer **5**, *J<sub>1-6</sub>* = 8 Hz), 7.92 (s, <sup>1</sup>/<sub>3</sub> H, *H<sub>I</sub>* of isomer **6**), 8.19 (d, <sup>1</sup>/<sub>3</sub> H, *H<sub>4</sub>* of isomer **6**, *J<sub>4-5</sub>* = 8 Hz), 8.40 (dd, <sup>1</sup>/<sub>3</sub> H, *H<sub>5</sub>* of isomer **6**, *J<sub>5-4</sub>* = 9 Hz, *J<sub>5-1</sub>* = 1.3 Hz), 8.42 (dd, <sup>2</sup>/<sub>3</sub> H, *H<sub>6</sub>* of isomer **5**, *J<sub>6-1</sub>* = 8 Hz, *J<sub>6-4</sub>* = 1.5 Hz), 8.83 (s, <sup>2</sup>/<sub>3</sub> H, *H<sub>4</sub>* of isomer **5**); MS (+ FAB, glycerol/thioglycerol 50:50, v/v) [*M* + *H*]<sup>+</sup> = 642.

**Synthesis of 5'-Amino-Linked Oligonucleotides 7 and 8.** The phosphorothioate oligonucleoside **5** (sequence 5' AACGTTGAGGGGCATCGT 3') and the methylphosphonate oligo-**6** (sequence 5' TpAACGTTGAGGGGCATCGT 3') were prepared on 1 μmol scale with an Applied Biosystems Model 381A DNA synthesizer. The standard solid-phase β-cyanoethyl phosphoramidite chemistry in combination with Beaucage's reagent (10) was applied to the phosphorothioate oligo-**5** synthesis. The methylphosphonate oligomer **6** was prepared with the ap-

propriately protected nucleoside methylphosphonamidite synthons purchased from Glen Research and according to published procedures (11). Functionalization at the 5'-end of both phosphorothioate (ps) 18-mer and methylphosphonate (mp) 19-mer oligonucleosides **5** and **6** by 1,4-diaminobutane was manually performed on the solid support as previously described (12). After deprotection of **7** by standard ammonia treatment and deprotection of methylphosphonate oligo-**8** according to literature conditions (13), the 5'-amino oligomers **7** and **8** were lyophilized three times and then were reacted with **4** without further purification.

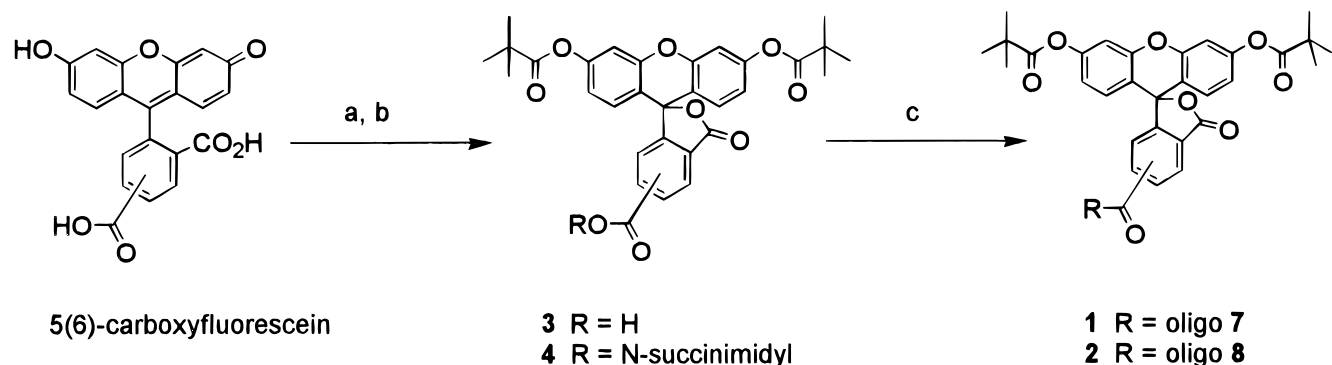
**Synthesis and Purification of Fluorogenic Oligonucleotide Conjugates 1 and 2.** To a mixture of DMF (105 μL) and 0.1 M NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> buffer (pH 9) (495 μL) were added successively a solution of 5'-amino-linked oligo-**7** (1.6 mM in water, 100 μL, 28.5 A<sub>260</sub> units) and a solution of the activated ester **4** (12.5 mM in DMF, 500 μL, 40-fold molar excess). The turbid mixture was stirred vigorously at room temperature for 1.5 h in the dark. The reaction was monitored by HPLC analysis (solvent A = 0.05 M triethylammonium acetate, pH 7; solvent B = 80% acetonitrile in buffer A; linear gradient, from 7% to 63% of B over 20 min and then to 100% of B over 20 min; flow rate = 1 mL min<sup>-1</sup>). The reaction mixture was loaded onto a Sephadex G-25 column (2 × 33 cm) equilibrated with 20% ethanol in water, and the crude oligoconjugate **1** was eluted with the same eluant. The oligoconjugate **1** was further purified by preparative reverse-phased HPLC (same eluting conditions as above except 2 mL min<sup>-1</sup> as flow rate). The pooled fractions were directly desalted through a Sep-Pak C<sub>18</sub> (Waters) cartridge following the manufacturer's instructions. The oligoconjugate **1** was eluted with a mixture of acetonitrile and water (50:50, v/v) and then the solvent was evaporated. Yield (measured by UV absorbance at 260 nm) was 25% after purification and desalting with respect to the crude 5'-amino-oligo-**7**: UV spectrum λ<sub>max</sub> = 256.4 nm; MS (negative ion mode ESI, methanol/water/triethylamine 49:50:1, v/v/v) *m/z* calcd 6493.4, found 6495.5.

The same procedure as described above was applied for the synthesis and purification of the oligoconjugate **2** except for the following modifications: to a mixture of DMF (170 μL) and 0.1 M NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> buffer (pH 9) (495 μL) were added successively a solution of 5'-amino-linked oligo-**8** (1.4 mM in water/acetonitrile 50:50, v/v, 100 μL, 25 A<sub>260</sub> units) and a solution of the activated ester **4** (12.5 mM in DMF, 438 μL, 40-fold molar excess). The turbid mixture was stirred vigorously at room temperature for 0.5 h in the dark. Yield was 23% with respect to the crude 5'-amino-oligo-**8**: UV spectrum λ<sub>max</sub> = 255 nm; MS (positive ion mode MALDI-TOF, saturated solution of hydroxypropylamine in 0.3M diammonium acetate in acetonitrile) *m/z* calcd 6487.6, found 6485.3.

**Esterase Substrate Ability of Fluorogenic Oligoconjugates 1 and 2.** *a. HPLC Analysis.* Oligonucleotide conjugate **1** or **2** (18 μM) was incubated at 37 °C with PLE (80 units mL<sup>-1</sup>) (Aldrich Chemical Co.) in 25 mM Tris-HCl buffer (pH 7.5). DMSO (6‰ final concentration in the incubation medium) was added for solubilization of the methylphosphonate derivative **2**. The reaction was monitored by HPLC analysis with the same eluting conditions described above.

*b. Fluorescence Spectrophotometry.* Oligonucleotide conjugate **1** or **2** (1.1 μM for PLE experiments, otherwise 4.8 μM) was incubated at 37 °C either in the presence of PLE (8 units mL<sup>-1</sup>) in 25 mM Tris-HCl buffer (pH 7.5), in RPMI 1640 containing 10% (v/v) heat-deactivated fetal calf serum (FCS; Gibco BRL), or in total CEM-SS cell extracts (14). DMSO (6‰ final concentration in the

### Scheme 1. Synthesis of Fluorogenic Conjugates 1 and 2<sup>a</sup>



<sup>a</sup> Key: (a) pivaloyl chloride, pyridine; (b) NHS, EDC, anhydrous  $\text{CH}_2\text{Cl}_2$ ; (c) oligo-7 or oligo-8,  $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$  buffer, pH 9, 0.1 M, DMF. Oligo-7,  $\text{H}_2\text{N}(\text{CH}_2)_4\text{NHC}(\text{O})(\text{A}_2\text{CGT}_2\text{GAG}_4\text{CATCGT})_3'$ . Oligo-8,  $\text{H}_2\text{N}(\text{CH}_2)_4\text{NHC}(\text{O})(\text{TpA}_2\text{CGT}_2\text{GAG}_4\text{CATCGT})_3'$ . No mark indicates a phosphorothioate linkage, the underscored nucleic bases a methylphosphonate linkage, and the (p) a phosphodiester linkage.

incubation medium) was added for solubilization of the methylphosphonate derivative **2**. The intensity of fluorescence emission (527 nm) was measured as a function of time by fluorescence spectrophotometry upon excitation at 494 nm.

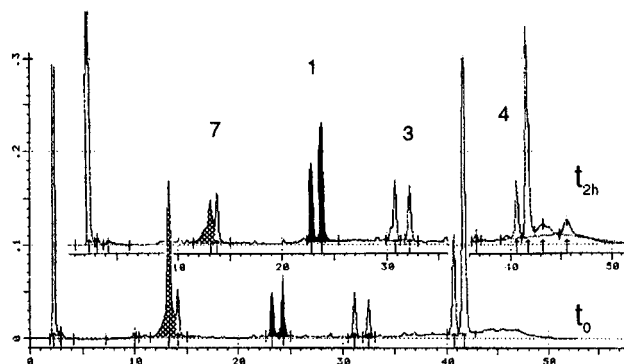
**Microinjection and Confocal Fluorescence Microscopy.** Human foreskin fibroblasts Hs68 were cultured in  $\text{CO}_2$  incubators at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 6–10% FCS on 25 mm diameter acid-washed glass coverslips. Cells were microinjected into the cytoplasm and nucleus with a few femtoliters of a solution of 77  $\mu\text{M}$  oligoconjugate **1** in 100 mM HEPES (pH 7.5). Cells were fixed in 3.7% formalin in phosphate-buffered saline (PBS) and were observed with a Leica confocal laser scanning microscope, equipped with a krypton–argon dual-wavelength laser (excitation at 488 nm) (15).

### RESULTS AND DISCUSSION

In the present work, we selected the carboxyfluorescein which can exist as a fluorescent xanthen-3-one form or as a nonfluorescent lactone form when the two phenolic functions are esterified. In our approach, we wanted the lactone form to persist all along the conjugate synthesis; for this reason the strategy of coupling the fluorogenic label to the oligomer required the carboxyfluorescein protecting groups to be maintained intact.

Various methods for labeling oligonucleotides with nonradioactive tags have been extensively reported in the literature (16). One of the commonly used approaches is to attach the label at the 5' terminus of the oligomer either by incorporating the suitably protected label phosphoramidite during solid-phase oligonucleotide synthesis or by derivatizing the deprotected oligonucleotide in aqueous medium. In our case, the second procedure of postsynthesis functionalization was more convenient to avoid exposure of the biprotected carboxyfluorescein residue to conditions used during oligonucleotide deprotection (ammonium hydroxide, 55 °C for 5 h). This strategy required the synthesis of a 5'-end-modified oligonucleotide with a primary amino group available for subsequent coupling with the activated ester of the protected carboxyfluorescein **4**.

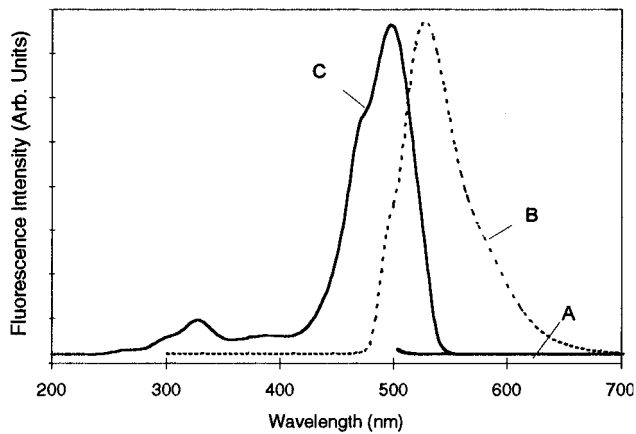
The NHS ester of the 3',6'-diacetyl-5(6)-carboxyfluorescein is commercially available, but the acetyl groups were not sufficiently stable during the coupling reaction between the oligo derivative and the active ester. We prepared the NHS ester **4** of the more stable 3',6'-dipivaloyl-5(6)-carboxyfluorescein **3**, which has been previously mentioned in the literature (17), but no



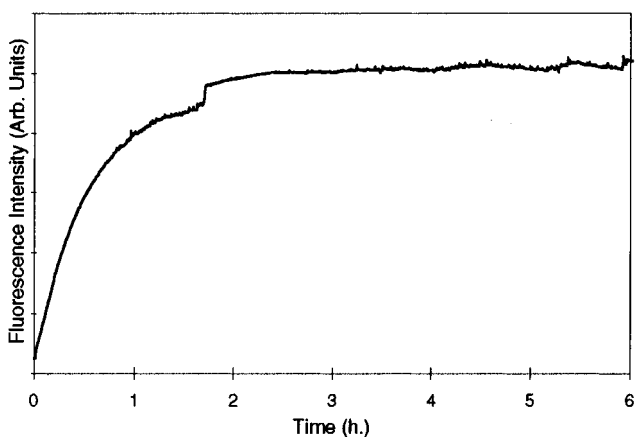
**Figure 1.** Reversed-phase HPLC chromatogram ( $\lambda_{260\text{nm}}$ ) of the reaction mixture between 5'-aminophosphorothioate oligonucleotide (18-mer) **7** and NHS ester of bis(pivaloyl)carboxyfluorescein **4** at  $t = 0$  and 2 h. **1**, fluorogenic phosphorothioate oligoconjugate. **3**, bis(pivaloyl)carboxyfluorescein arising from hydrolysis of **4**.

experimental procedures and data have been reported so far. First, the commercial 5(6)-carboxyfluorescein was reacted with pivaloyl chloride to give the nonfluorescent lactone **3**, which was subsequently reacted with EDC and NHS to yield the NHS ester **4** as described under Experimental Procedures (Scheme 1). In our hands, attempts to synthesize the fluorogenic conjugate with the 5'-amino-linked oligomer prepared with commercially available 5'-amino-modifier C6 (Glen Research) were not satisfactory. Therefore, the functionalization at the 5' end of two nuclease-resistant phosphorothioate **5** and methylphosphonate **6** oligomers was performed manually on solid support after activation of the 5'-hydroxyl group by *N,N*-carbonyldiimidazole (CDI) and then reaction with 1,4-diaminobutane (12). The 5'-amino oligo-7 and -8 obtained were used without purification in the subsequent coupling step with the fluorogenic tag **4**.

The fluorogenic conjugate synthesis was carried out in the dark for 0.5–1.5 h using a 40-fold molar excess of **4** in a 1:1 mixture of DMF and  $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$  buffer, pH 9 (18). This coupling reaction was run at pH 9, which was a good compromise to achieve synthesis without loss of pivaloyl groups. The conversion of starting 5'-amino-linked oligonucleotide **7** or **8** into oligoconjugates **1** and **2**, respectively, was monitored by HPLC analysis (Figure 1). Two peaks at  $R_T = 23.4$  and 24.2 min corresponding to more lipophilic compounds than the 5'- $\text{NH}_2$ -oligonucleotide **7** ( $R_T = 13.3$  min) were tentatively assigned to the isomeric mixture of oligoconjugate **1**. We noticed a similar increase in retention time between the oligoconjugate **2** ( $R_T = 25.5$  and 26.3 min) and the starting



**Figure 2.** Fluorescence emission spectra upon excitation at 494 nm of the phosphorothioate oligoconjugate **1** before incubation (A) and after 15 h of incubation (B) in cellular extract at 37 °C. (C) represents the fluorescence excitation spectrum of the oligoconjugate **1** after 15 h of incubation in cellular extract.



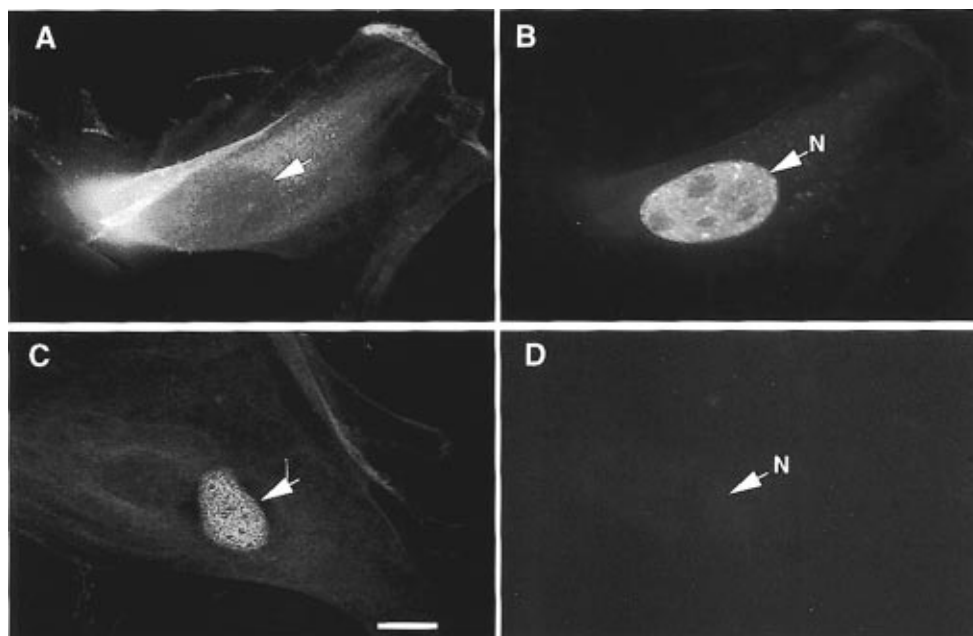
**Figure 3.** Time course fluorescence at 527 nm (upon excitation at 494 nm) during incubation of **1** at 37 °C with PLE (8 units mL<sup>-1</sup>). The initial concentration of oligoconjugate **1** was 1 μM in Tris-HCl buffer (25 mM), pH 7.5.

oligonucleotide **8** ( $R_T = 15.8$  min). Two extra peaks corresponding to the hydrolysis of succinimidyl ester **4** to carboxylic acid **3** ( $R_T = 31$  and 32.5 min) were observed (19). The formation of the oligoconjugates **1** and **2** was monitored at three different wavelengths (245, 260, and 494 nm), and no absorption at 494 nm [characteristic of the unprotected fluorescein derivative (20)] was detected, which indicated that the fluorogenic label was still intact.

Unbound fluorescein derivatives were removed by size exclusion gel chromatography, and oligoconjugates **1** and **2** were further purified by reversed-phase HPLC. These oligonucleotide conjugates were characterized by ESI mass spectrometry for the oligoconjugate phosphorothioate **1** and MALDI-TOF/MS for the methylphosphonate derivative **2**; the molecular weight determined for each compound was found to be in agreement with that calculated.

For further characterization, two samples of oligoconjugate **1** were incubated with either ammonia or PLE. For both chemical and enzymatic hydrolysis, HPLC chromatograms indicated conversion of the starting material into a more polar product. In both cases, products had the same retention time and exhibited an absorption at 494 nm similar to that present in fluorescent conjugates (20). Maximum excitation wavelength was 494 nm, and maximum emission was determined at 514 nm. These experiments were performed with oligoconjugate **2**, and the same data were obtained. These results indicate that fluorogenic oligoconjugates **1** and **2** were deprotected to yield the corresponding fluorescent oligonucleotides.

To determine the carboxyesterase substrate ability of the two oligoconjugates **1** and **2** in biological media, the oligoconjugates **1** and **2** were incubated with PLE or in RPMI 1640 supplemented with 10% heat-deactivated FCS or in total CEM-SS cell extracts, and the fluorescence emission spectra (Figure 2) and the fluorescence curves as a function of time were recorded (Figure 3). All of the curves reach a plateau that corresponds to the deprotected conjugate, and their emission spectra were similar in the three experiments. The fluorescence



**Figure 4.** Microinjection of fluorogenic phosphorothioate oligoconjugate **1**. Oligoconjugate **1** was co-injected with an inert rabbit antibody into either the cytoplasm (A and B) or the nucleus (C and D) of human fibroblasts Hs68. Live cells were analyzed 30 min after injection using fluorescence confocal microscopy. Marker antibodies were visualized after staining with Texas-Red conjugated goat anti-rabbit antisera (A and C). Carboxyfluorescein-labeled material is displayed in panels B and D. Scale bar is 5 μm.

intensity increased with a higher rate in cell extracts ( $t_{1/2} = 2$  h for **1**,  $t_{1/2} = 1$  h for **2**) than in 10% deactivated FCS RPMI 1640 ( $t_{1/2} = 6$  h for **1**,  $t_{1/2} = 7$  h for **2**), probably because of the higher carboxyesterase content of the former (14). These in vitro experiments confirmed that the oligonucleotide conjugates **1** and **2** are substrates of the esterases.

To investigate the fate of these fluorogenic oligonucleotides within the cell and to visualize the intracellular esterase activity, phosphorothioate oligoconjugate **1** was microinjected into either the cytoplasm or the nucleus of living human fibroblasts. After microinjection into the cytoplasm, confocal fluorescence microscopy revealed primarily a fast emission of fluorescence localized in the cytoplasm (Figure 4). Ten minutes after microinjection, the fluorescence was diffusely distributed in the cytoplasm with some more fluorescent spots attributed to confinement of the fluorescent oligomer to vesicles. This can be explained by an important esterase activity in the vesicles and/or an accumulation of the labeled oligomer in these cytoplasmic organelles. Thirty minutes after microinjection, fluorescence could no longer be observed in the cytoplasm and was essentially located in the cell nucleus. The persistence of fluorescence for > 16 h within the nucleus demonstrated the stability of the linkage between the label and the oligomer in the cells (21, 22). In contrast, when oligonucleotide conjugate **1** was microinjected into the cell nucleus, no detectable fluorescence was observed even after 30 min. This result indicates that esterase activity was predominantly localized in the cytoplasm and that a translocation of fluorescent oligomer occurred from the cytoplasm to the nucleus as already observed with fluorescent oligonucleotides (23). This last result shows that the fluorogenic tag has no effect on the behavior of fluorogenic oligonucleotides as compared to other fluorescent oligomers.

In conclusion, we described the synthesis of fluorogenic oligonucleotides that were obtained by coupling the NHS ester of the bis(pivaloyl)carboxyfluorescein to 5'-amino-phosphorothioate or methylphosphonate oligonucleoside. We provided chromatographic and spectroscopic evidence of the synthesized conjugate identity. We demonstrated that the designed fluorogenic oligonucleotides are substrates of esterases present in serum and in cell extracts. Additionally, we showed that the fluorogenic phosphorothioate conjugate become fluorescent upon esterase-mediated hydrolysis within the cell and that esterase activity was mainly localized in the cytoplasm.

The labeling of prooligonucleotides with this fluorogenic tag is under current investigation as is their cellular uptake in comparison to that of anionic phosphorothioate conjugate **1** and uncharged methylphosphonate conjugate **2**.

#### ACKNOWLEDGMENT

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