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Multiple End Labeling of Oligonucleotides with Terbium Chelate-Substituted Psoralen for Time-Resolved Fluorescence Detection

Andreas Oser, Michael Collasius, and Günter Valet

Arbeitsgruppe Zellbiochemie, Max-Planck-Institut für Biochemie, D-8033 Martinsried, Federal Republic of Germany

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A new procedure for the photochemical functionalization and the subsequent nonradioactive labeling of synthetic oligonucleotides with psoralen derivatives was developed where a double-stranded poly(A–T) tail is attached to the 5′- or 3′-end of the oligonucleotide to be labeled. The double-stranded poly(A–T) tail is covalently crosslinked by psoralen molecules which carry reactive thiol or amino groups for the attachment of labels. A NH_2-specific terbium chelate exhibiting long-lived fluorescence was attached to the functional groups of the intercalated psoralen molecules. Oligonucleotides substituted in this way hybridize readily and can be sensitively detected by time-resolved fluorescence measurements. © 1990 Academic Press, Inc.

Nonradioactively labeled oligonucleotides become more and more important as probes for specific nucleic acid hybridizations. Amplifiable reporter groups like biotin which are attached to nucleotides are frequently used as indirect nucleic acid labels (1,2). Fluorescence- or enzyme-labeled avidin may cause unwanted background signals due to unspecific binding (3) and reduces the number of attached biotins which are detectable due to steric hindrance by their large molecular size (2,4).

Direct labeling of oligonucleotides with fluorescent residues seems, therefore, preferable. The sensitivity of detection is, however, limited because only one label per oligonucleotide is introduced by most chemical labeling procedures (4). The sensitivity of fluorescence detection is further reduced by background autofluorescence, especially in case of *in situ* hybridizations.

The goal of this study was to improve direct fluorescence labeling of oligonucleotides in two ways: first, by the attachment of a higher number of labels per oligonucleotide via psoralen derivatives covalently intercalated into a double-stranded (ds)[1] tail at the 5′- or 3′-end of the oligonucleotide, and second, by the use of a fluorescence marker with long-lived fluorescence which exclude the interference of short-lived background fluorescence.

MATERIALS AND METHODS

Oligonucleotide Synthesis

Oligonucleotides were automatically synthesized on a 381A DNA synthesizer (Applied Biosystems) by the phosphite triester method. The oligonucleotides of interest were prolonged at their 5′-ends by a 16 bases long alternating A–T sequence.

The deprotected oligonucleotides were dissolved in 10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA (pH 8.0) at a concentration of 0.5 mM and heated for 5 min at 96°C. Reannealing of the poly(A–T) tail was accomplished by subsequent overnight incubation at 25°C.

PNH_2 (4′-(9″-amino-2″,6″-diazanonyl)-4,5′,8-trimethylpsoralen, Fig. 1) or PSS (4′-(9″-(3‴-(2⁗-pyridyldithio)propionamido)-2″,6″-diazanonyl)-4,5′,8-trimethylpsoralen, Fig. 1), which is the protected form of the oxidation sensitive thiol-containing psoralen PSH, was synthesized as previously described (5).

Terbium chelate-labeled oligonucleotides were synthesized and stored in buffers prepared with ultrapure water (Millipore Milli-Q system).

[1] Abbreviations used: CPM, 7-diethylamino-3-(4′-maleimidophenyl)-4-methylcoumarin; ds, double-stranded; DTPA, diethylentriaminepentaacetic acid; caDTPA, the cyclic anhydride of DTPA; pAS, 4-aminosalicylic acid; PNH_2, 4′-(9″-amino-2″,6″-diazanonyl)-4,5′,8-trimethylpsoralen; PSH, 4′-(9″-(3‴-mercaptopropionamido)-2″,6″-diazanonyl)-4,5′,8-trimethylpsoralen; PSS, 4′-(9″-(3‴-(2⁗-pyridyldithio)propionamido)-2″,6″-diazanonyl)-4,5′,8-trimethylpsoralen; ss, single-stranded; SSC, standard sodium citrate (150 mM NaCl, 15 mM sodium citrate, pH 7.0); TRF, time-resolved fluorescence.

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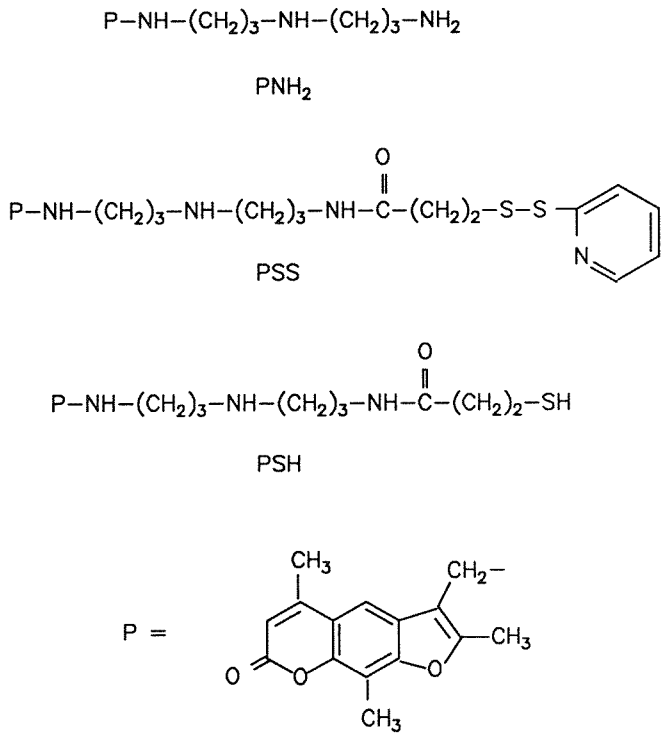

FIG. 1. Structures of functionalized psoralen derivatives containing an amino group (PNH_2) and a protected (PSS) and an unprotected (PSH) thiol group.

Photochemical Functionalization of Partly ds Oligonucleotides by Psoralen Derivatives

The oligonucleotide (1.65 nmol) was incubated in 0.2 ml of 10 mM Tris–HCl, 1 mM EDTA (pH 7.2) with 8.3 nmol PNH_2 or PSS for 15 min at room temperature in the dark to achieve intercalation between the A:T base pairs. The sample was then irradiated for 30 min at 4°C with uv light between 300 and 400 nm (HBO 200-W high-pressure mercury lamp, OSRAM, Augsburg, FRG; UG1 filter, Schott, Mainz, FRG). Addition of fresh psoralen (8.3 nmol), a 15-min incubation in the dark, and a 30-min irradiation were repeated twice to achieve maximum incorporation of the psoralen into the poly(A–T) tail of the oligonucleotide. The last irradiation period was prolonged to 2 h. The reaction progress was followed by measuring the changes in fluorescence emission of psoralen mono- and diadducts (6). In case of PSS, the reactive thiol groups were subsequently deprotected by a 1-h incubation at 4°C in 10 mM dithioerythritol. Low molecular weight components of the sample were removed by Sephadex G-25 chromatography using prepacked NAP-5 columns (Pharmacia-LKB, Freiburg, FRG). The functionalized oligonucleotides were dried in a vacuum centrifuge.

Attachment of Fluorescence Markers

Attachment of 7-diethylamino-3-(4'-maleimidophenyl)-4-methylcoumarin (CPM). Approximately 1.5 nmol of freshly prepared SH-functionalized oligonucleotide were dissolved in 50 μl 50 mM phosphate buffer, 1 mM EDTA (pH 7.8). A 50-nmol sample of CPM (Molecular Probes, Eugene, OR) from a 5 mM stock solution in isopropanol were added and the reaction mixture was shaken for 2 h at room temperature.

The number of bound CPM molecules per oligonucleotide was calculated from fluorescence measurements (Perkin–Elmer LS-5 spectrofluorometer) of the reaction mixture using a fluorescence calibration curve established with CPM-labeled *N*-acetylcysteine (7). Fluorescence excitation and emission wavelengths were set to 390 and 465 nm.

Attachment of terbium chelate Tb^{3+}–DTPA–pAS. The preparation of the chelate and its covalent attachment to the oligonucleotide were performed as previously described for protein labeling (8). A 0.1-ml quantity of a 200 mM solution of the cyclic anhydride of diethylentriaminepentaacetic acid (caDTPA) and 0.1 ml of a 200 mM solution of 4-aminosalicylate (pAS) were mixed and vortexed for 5 min at room temperature. Both solutions were freshly prepared in dimethylsulfoxide which was previously dried by distillation over CaH_2. A 5-μl sample of the reaction mixture (500 nmol DTPA–pAS) was added to the NH_2-functionalized oligonucleotide (1.5 nmol) in 30 μl of 100 mM phosphate buffer (pH 7.0) and the solution was vortexed for 2 min. The addition of 5 μl DTPA–pAS was repeated twice. Excess DTPA–pAS was removed by Sephadex G-25 chromatography. Complexation of oligonucleotide-bound DTPA–pAS with Tb^{3+} was achieved by the addition of 5 μl of a 1 mM solution of $TbCl_3$ (5 nmol) in water to 1.5 nmol DTPA–pAS-labeled oligonucleotide in 0.3 ml of 100 mM phosphate buffer (pH 7.0). The unspecific complexation of Tb^{3+} ions to phosphate groups of the oligonucleotide was diminished by the phosphate buffer.

Purification of Fluorescent Oligonucleotides

Denaturing PAGE. Oligonucleotides were electrophoresed in 15% polyacrylamide gel electrophoresis in 7 M urea at 20 V/cm using 50 mM L-histidine as a high-resolution buffer (9). The fluorescent oligonucleotides were eluted from the gel by a Biotrap BT100 electroelution apparatus (Schleicher & Schuell, Dassel, FRG) as specified by the manufacturer.

HPLC. Since Tb^{3+} ions could have dissociated from the chelate during gel electrophoresis, Tb–DTPA–pAS-labeled oligonucleotides were purified as an alternative by reversed-phase HPLC (Pep-S column, 4 × 250 mm, 5-μm particle size, LKB-Pharmacia). Elution solvents were 0.1 M triethylammonium acetate (pH 7.5; solvent A) and CH_3CN (solvent B). The gradient was 5% B (5 min), 5–10% B (5 min), and 10–25% B (25 min) and the flow rate was 1.0 ml/min.

Absorbance and Fluorescence Measurements

Absorbance measurements of the purified fluorescent oligonucleotides were performed with either a Gilford 240 or a Zeiss DM-4 spectrophotometer. Fluorescence spectra and the lifetime of free Tb^{3+}–DTPA–pAS chelate were determined with a Perkin–Elmer LS-5 spectrofluorometer (Perkin–Elmer, Friedrichshafen, FRG). Tb^{3+} chelate-labeled oligonucleotides were detected by an Arcus 1230 fluorometer (LKB-Wallac, Turku, Finland) which permits time-resolved fluorescence measurements. The original bandpass filter for europium fluorescence emission (615 ± 5 nm) was exchanged for a 435-nm long-pass filter to detect Tb^{3+} fluorescence emission. Delay time and counting time were both set to 400 μs.

Radioactive Oligonucleotide 5′-End Labeling

A 10-pmol sample of the oligonucleotide was incubated for 30 min at 37°C in 10 μl of 50 mM Tris–HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol, 0.1 mM spermidine (pH 8.2) with 10 pmol adenosine 5′-[γ-^{32}P]-triphosphate (50 μCi) and 10 U T4-polynucleotide kinase. Excess free [γ-^{32}P]ATP was removed by Sephadex G-25-chromatography.

Slot-Blot Hybridization with a Psoralen-Modified Oligonucleotide

Target pBH10-R3 plasmid DNA (10) was blotted onto a nitrocellulose filter (Schleicher & Schuell) by a slot-blot apparatus and prehybridized for 4 h at 42°C in 10 ml 5× SSC, 15% formamide, 0.1% bovine serum albumin, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% SDS, 0.2 mg/ml denatured, sonicated salmon sperm DNA. A 5′-^{32}P-labeled PSS-modified oligonucleotide (10 pmol, 10^7 cpm) with the sequence 5′-CCTTCT-TCTTCTATTCCTTCGGGCCTGTCG was added to the prehybridization solution and hybridized for 16 h at 42°C. Then, the filter was washed 2 × 10 min at 42°C in 2× SSC, 0.1% SDS, and 15% formamide.

RESULTS

Oligonucleotides in solution were photochemically labeled via functionalized psoralen derivatives (Fig. 1) following attachment of a poly(A–T) tail to the oligomer (Fig. 2, step I). After denaturation, this poly(A–T) tail folded back under reannealing conditions to form a partly ds oligonucleotide (Fig. 2, step II) showing hypochromicity and an increased ethidium bromide fluorescence (Fig. 3). A head-to-head dimerization of two

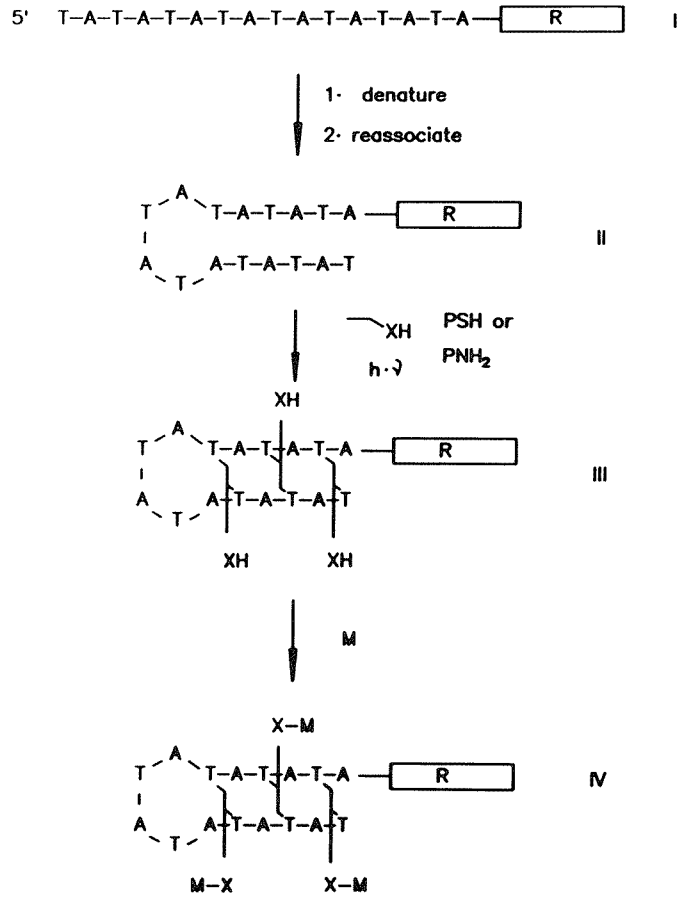

FIG. 2. Scheme for the preparation of end-labeled oligonucleotides via functionalized psoralen derivatives. R is the deprotected oligonucleotide of any sequence of interest. P is the psoralen moiety, X is the reactive group (either S or NH), and M is the label. The structure in step II ensures the most stable loop size of four nucleotides with maximum base pairing (11).

oligonucleotides by their poly(A–T) tails as an alternative to internal double-strand formation did not occur as the electrophoretic mobility of the modified oligonucleotide after covalent psoralen crosslinking (Fig. 2, step III) was only insignificantly reduced (Fig. 5, lanes 1 and 2) in contrast to an expected significant reduction in case of dimerization.

SH- or NH_2-substituted psoralen derivatives PSS or PNH_2 were used for the crosslinking of the poly(A–T) tails to permit the attachment of labels to these reactive groups. A PSS-modified oligonucleotide was labeled at the free 5′-end with ^{32}P and used as probe in a dot-blot hybridization experiment. The hybridization properties of the oligonucleotide were not affected by the psoralen modification and 1 ng (0.13 fmol) of complementary plasmid DNA was detectable after 6 h of autoradiography (Fig. 4).

The incorporation of PSH into oligonucleotides was further demonstrated by the conjugation of the thiol-

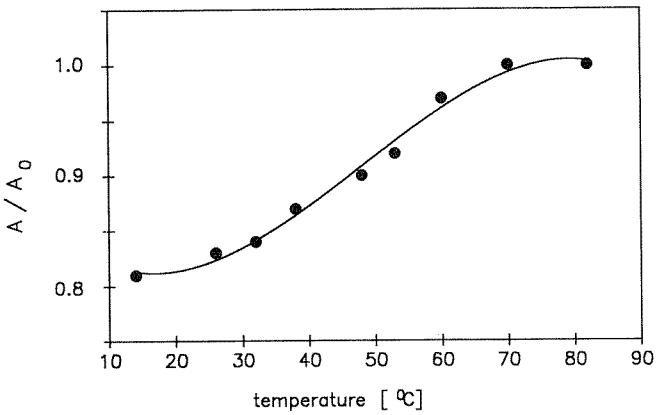

FIG. 3. Transition from denatured, single-stranded to partly double-stranded oligonucleotide was followed by absorption measurements at 260 nm (expressed as the ratio of the absorbance value at each temperature (A) through the absorption value of totally denatured oligonucleotide (A_0)). Oligonucleotide (with R = CGAGTCTAGACTCTGCGGTATTGTGAGGAT) concentration was 0.5 μM in 10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA (pH 8.0). Ethidium bromide fluorescence was 366 arbitrary fluorescence units (afu) for the partly double-stranded form at 20°C as compared to 215 afu for the single-stranded form at 80°C. Fluorescence measurements were performed with 6.6 μM oligonucleotide in the same buffer in the presence of 50 μM ethidium bromide (excitation, 525 nm; emission, 600 nm).

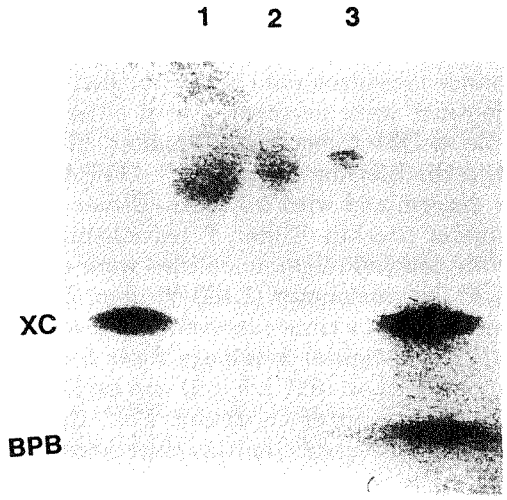

FIG. 5. 15% polyacrylamide/7 M urea gel electrophoresis of oligonucleotide species I (lane 1, 5 nmol), III (lane 2, 3 nmol), and IV (lane 3, approximately 1 nmol) with R as in Fig. 4 and M = CPM. Oligonucleotides were detected by uv shadowing. CPM-labeled oligonucleotide IV in lane 3 showed a blue-green fluorescence upon excitation on a uv transilluminator. The position of the marker dyes, xylene cyanole (XC) and bromphenol blue (BPB), which comigrate with 26-mer and 6-mer oligonucleotides, respectively, are labeled.

specific fluorescent dye, CPM, to an oligonucleotide (steps III and IV in Fig. 2) and a subsequent denaturing polyacrylamide gel electrophoresis. Each modification of the oligonucleotide slightly reduced its electrophoretic mobility (Fig. 5).

CPM was used to quantify the incorporation of psoralen into the oligonucleotide. The number of bound CPM molecules per oligonucleotide varied between 2.5 and 3 molecules as determined by absorbance spectroscopy. This closely corresponds to the theoretical maximum of 3 CPM residues (see Fig. 2). Fluorescence measurements confirmed the dye/oligonucleotide ratio and indicated no fluorescence quenching of CPM by the multiple labeling.

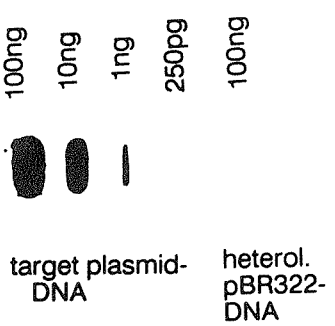

FIG. 4. Slot-blot hybridization of 5′-^{32}P-labeled, PSS-modified oligonucleotide with R = 5′-CCTTCTTCTTCTATTCCTTCGGGCCTGTCG against the complementary sequence present in a 12-kb pBR322-derived plasmid (10). Heterologous pBR322 DNA gave no hybridization signal.

The complex between Tb^{3+} ions and a diethylentriamine pentaacetic acid/4-aminosalicylic acid conjugate (Tb–DTPA–pAS) with a long fluorescence lifetime of 1.58 ms was used as a label for time-resolved fluorescence (TRF) measurements. Tb–DTPA–pAS absorbs light at 260 and 305 nm and emits its long-lived fluorescence with two sharp bands at 490 and 545 nm. The unbound Tb–DTPA–pAS chelate was detectable by TRF down to a concentration of 10^{-11} M in aqueous solutions (Fig. 6B).

The bifunctional cyclic anhydride of DTPA has two carboxy anhydride groups, one of which is used for the preparation of DTPA–pAS and the other for the coupling to the primary amino groups of the PNH_2-functionalized oligonucleotides. After the complexation with Tb^{3+} ions, the labeled oligonucleotide was isolated by reversed-phase HPLC (Fig. 7). The Tb–DTPA–pAS-labeled oligonucleotide eluted between the peaks of free pAS and DTPA–pAS which was earlier than the corresponding PNH_2-functionalized unreacted oligonucleotide. The heterogeneity of the peaks of PNH_2-functionalized and Tb–DTPA–pAS-labeled oligonucleotides can be attributed to different conformations and stoichiometries of psoralen binding. The presence of the fluorescent label in the oligonucleotide was shown by the TRF histogram of the elution profile (Fig. 7) and by uv absorbance measurements of the purified oligonucleotide fraction.

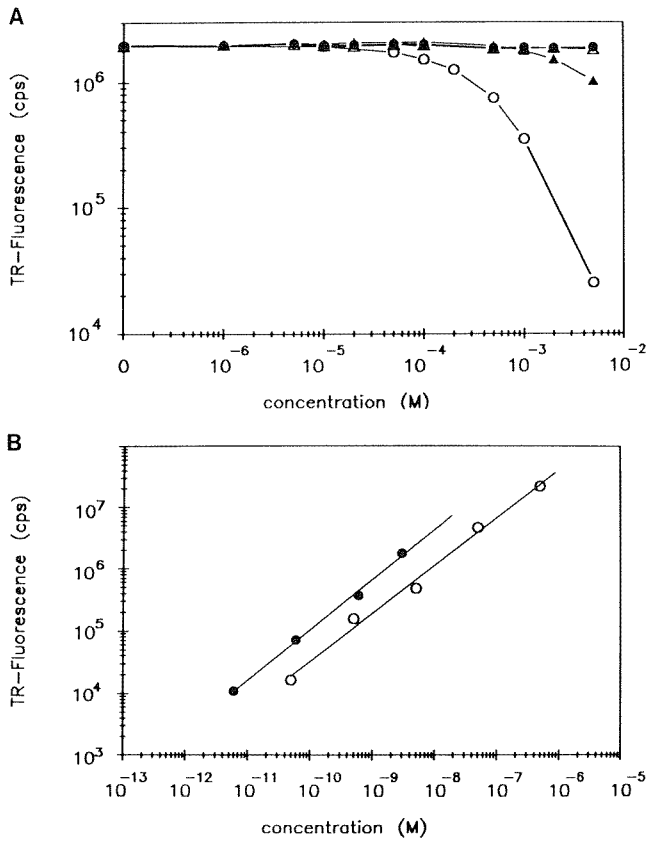

FIG. 6. (A) Stability of Tb–DTPA–pAS-labeled oligonucleotide (with R as in Fig. 3) in the presence of increasing concentrations of Ca^{2+} (●), Mg^{2+} (△), Fe^{3+} (○), and EDTA (▲). The metal ions were added from concentrated chloride stock solutions and EDTA was added from a sodium stock solution to 0.7 nM oligonucleotide in 0.1 M sodium borate buffer (pH 9.2). (B) Time-resolved fluorescence (in counts per second) plotted as a function of concentration of free Tb–DTPA–pAS (○) and Tb–DTPA–pAS-labeled oligomer (with R as in Fig. 3) (●). The fluorescence was measured in 0.1 M sodium borate buffer (pH 9.2) in a sample volume of 1 ml with an Arcus 1230 fluorometer. Fluorescence of a blank buffer solution was between 5×10^3 and 1×10^4 cps.

The ratio of the absorption at 305 and 260 nm was 0.18 in the chelate-modified oligonucleotides, which is between the values for unmodified oligonucleotides (0.05) and free Tb–DTPA–pAS (0.58). From the molar absorption coefficients of ϵ 8.3×10^3 and 6.8×10^4 M^{-1} cm^{-1} at 305 nm for DTPA–pAS and psoralen-modified oligonucleotide, respectively, and of 5×10^5 M^{-1} cm^{-1} at 260 nm for psoralen-modified oligonucleotide, 2.95 Tb–DTPA–pAS moieties were found per oligonucleotide.

Tb^{3+} has a very high affinity to the chelate ligand DTPA with a K_a value of about 10^{23} (12). Complete complexation of all DTPA–pAS ligands with Tb^{3+} can thus be anticipated. To quantify the number of rare-earth ions per oligonucleotide, Tb^{3+} was replaced by Eu^{3+}. The latter allowed the quantitation independantly from the DTPA–pAS ligand by its extraction into a fluorescence enhancement solution (5,16). The fluorescence of this solution was compared to the fluorescence of reference solutions of free Eu–DTPA–pAS. A number of 8 Eu^{3+} ions per oligonucleotide was determined. The number of Eu^{3+} is probably somewhat overestimated because Eu^{3+} ions are more tightly bound to free DTPA–pAS than to oligonucleotide-bound DTPA–pAS but it is difficult to determine exactly the degree of overestimation.

The stability of Tb–DTPA–pAS was demonstrated in dilute aqueous solutions in the presence of other metal ions and other metal chelating agents (Fig. 6A). Ca^{2+}, Mg^{2+}, and EDTA did not interfere with the Tb chelate up to millimolar concentrations. Fe^{3+} was tolerated up to the 10-μM range. The noninterference of EDTA in the millimolar range with the complexation of Tb^{3+} to oligonucleotide-bound DTPA–pAS in the nanomolar range (Fig. 6A) indicates that the K_a of Tb^{3+} for bound DTPA–pAS is about six orders of magnitude higher than the K_a of Tb^{3+} for EDTA, 2×10^{17}.

Tb–DTPA–pAS-modified oligonucleotides are stable at −80°C for at least 6 months. The fluorescence intensity of aqueous solutions of Tb–DTPA–pAS-labeled oligonucleotides decreased, however, to one-half within 8 days when kept at room temperature.

Tb–DTPA–pAS-labeled oligomers were detectable by TRF in a linear dose–response relation down to low picomolar concentrations (Fig. 6B) with fluorescence signals 3.5× higher than equimolar Tb–DTPA–pAS monomer solutions.

The attachment of the Tb chelate to the oligonucleotide had no negative effect on hybridization. This was

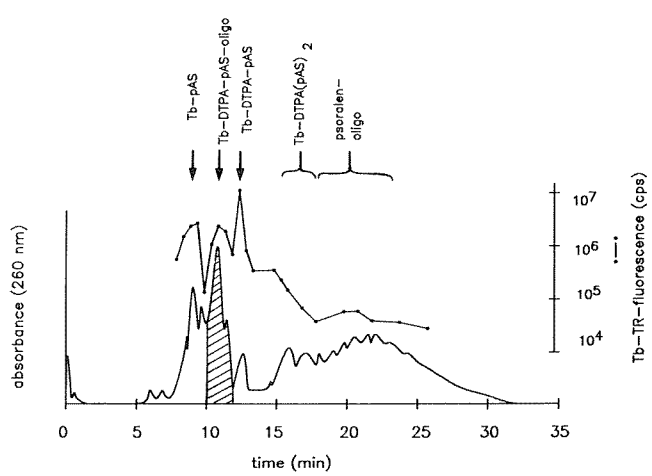

FIG. 7. Reversed-phase HPLC elution profile of the reaction mixture between a PNH_2-functionalized oligonucleotide with R as in Fig. 3 and Tb–DTPA–pAS. The position of all peaks except the Tb–DTPA–pAS–oligonucleotide peak was assigned after individual HPLC runs with the respective single substances. The histogram (● —— ●) shows the presence of Tb^{3+} long-lived fluorescence in the fractions. The hatched area represents the collected fractions of the Tb–DTPA–pAS-labeled product (retention time, 10.0–11.9 min).

shown by the specific hybridization of a 5′-^{32}P and Tb–DTPA–pAS double-labeled oligonucleotide to complementary plasmid DNA. Again, as with the psoralen-substituted oligonucleotide alone (Fig. 4), 1 ng plasmid DNA was readily detectable by autoradiography.

DISCUSSION

Psoralens have been used for the photochemical labeling of recombinant DNA with biotin (13) and metal chelates (5). In this study, psoralens were used for multiple labeling of synthetic oligonucleotides within a ds poly(A–T) tail at the 5′-end (Fig. 2). 3′-End labeling can be achieved in the same way by synthesizing 3′-poly(A–T)-tailed oligonucleotides. This is an improvement over existing chemical oligonucleotide labeling methods (4) yielding usually only one label per oligonucleotide.

The psoralens selectively react with ds DNA by crosslinking two adjacent thymidine residues on opposite strands (Ref. (14); see Fig. 2). This has two important advantages for hybridization reactions. The ss oligonucleotide probe sequence is not altered and the covalent crosslinks prevent the oligonucleotide probe from cross-hybridization to alternating poly(A–T) sequences in the investigated sample. For comparison, cross hybridization is not excluded when biotin–nucleotide-tailed oligonucleotides (1) are used as probes.

The sensitivity of fluorescent oligonucleotide detection can be improved by using TRF instead of fast-decaying fluorescence. TRF permits the rejection of short-lived autofluorescence background or light-scatter signals (15). Lanthanide chelates, predominantly of europium and terbium, emit strong long-lived fluorescence and have been used in numerous fluoroimmunoassays as antibody labels (reviewed in (16)). Europium–DTPA chelates were recently also used as labels for DNA probes with sensitivity in the 0.1-amol range (5,17). The disadvantage of the europium chelate is that hybridizations on gels and *in situ* hybridizations in cells are not possible because the europium ions have to be extracted from the chelate in a nonaqueous environment for fluorescence measurement.

The labeling of oligonucleotide probes with the terbium chelate Tb–DTPA–pAS which is fluorescent in aqueous environment overcomes this problem. This chelate offers a good compromise with respect to long fluorescence lifetime, good sensitivity of detection in aqueous solutions, and ease of synthesis and labeling. The assumed structure of the terbium chelate bound to the psoralen moiety of the oligonucleotide is shown in Fig. 8.

Multiple labeling of oligonucleotides with Tb–DTPA–pAS results in a detection sensitivity of a few femtomoles in a measurement volume of 1 ml (Fig. 6B). This is superior to oligonucleotides labeled with conventional fluorescent dyes having detection limits between

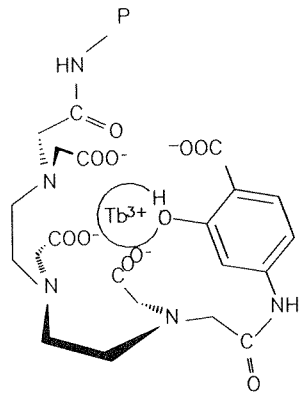

FIG. 8. Presumed structure of Tb–DTPA–pAS attached to the primary amino group of one psoralen moiety in an oligonucleotide (X–M in Fig. 2, IV; P = psoralen moiety). For Tb fluorescence emission the salicylate moiety absorbs light and transfers the energy to the Tb^{3+} ion. The light conversion process is responsible for the delayed fluorescence.

20 and 100 fmol (rhodamine and fluorescein, respectively (18)). Furthermore, these fluorophores are more hydrophobic than the Tb chelate, causing substantial nonspecific binding of the oligonucleotide probes in hybridization assays (18).

Furthermore, if the most established fluorescent dyes like fluorescein or rhodamine are used for multiple labeling, internal concentration quenching diminishes fluorescence yield (19). In case of coumarin derivative CPM and Tb–DTPA–pAS, the fluorescence is not affected by concentration quenching due to their large fluorescence Stoke shifts of 80 and 185 nm, respectively.

Detection limits between 0.02 and 0.2 fmol have been reached for biotinylated oligonucleotides on filters (1,2). The gain in sensitivity by multiple biotin labeling in combination with the second reaction step of biotin detection is, however, limited since not more than one biotinylated nucleotide per 50 or 100 nucleotide residues can be detected due to steric hindrance of avidin binding (2,4). The advantage of the described direct oligonucleotide labeling with Tb–DTPA–pAS is that the number of attached Tb–DTPA–pAS molecules can be easily increased by elongation of the poly(A–T) tail leading to a proportional increase of fluorescence yield.

The use of Tb–DTPA–pAS-labeled oligonucleotide probes is especially promising for flow cytometric measurements of specific DNA or mRNA levels in single cells following *in situ* hybridization in suspension (20). In this application, unspecific binding of the biotin indicator molecule and cellular autofluorescence causes a high fluorescence background. The use of TRF labels for flow cytometry has been proposed earlier (21), but suitable labels were not available at the time.

A further interesting application of the Tb–DTPA–pAS chelate is its use as a label for nucleotide triphos-

phates. These can be subsequently used, e.g., in the polymerase chain reaction which permits the introduction of a high number of labeled nucleotides into specific DNA sequences in analogy to the use of biotin-substituted nucleotides (22).

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