

Solid-phase synthesis of chelate-labelled oligonucleotides: application in triple-color ligase-mediated gene analysis

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ABSTRACT

Oligonucleotides labelled with detectable groups are essential tools in gene detection. We describe here the synthesis of pyrimidine deoxynucleotide-building blocks, modified at their C-5 position with a protected form of a strongly chelating agent. These reagents can be used to introduce multiple metal ions into oligodeoxynucleotides during standard oligonucleotide synthesis. The chelating functions form strongly fluorescent complexes with europium ions, characterized by a wide separation between the excitation and emission spectra. Moreover, the long decay time of the fluorescence permits sensitive time-resolved fluorescence measurements. The chelates also have the stability required to function in triple-color assays involving europium, samarium, and terbium ions. We demonstrate the application of these reagents for ligase-based gene analysis reactions.

INTRODUCTION

Certain lanthanide ions when properly complexed exhibit an intense, extremely long-lived fluorescence, further characterized by a wide distinction between the excitation and emission spectra (large Stokes shift). Because of these properties, chelates of the lanthanide (Ln^{3+}) ions europium (Eu^{3+}), samarium (Sm^{3+}), and terbium (Tb^{3+}) have proven suitable as labels in sensitive immunological (1, 2) and molecular genetic (3, 4, 5) assays.

Lanthanide chelates may be incorporated in probes by conjugation to primary amino groups, introduced in the oligonucleotides during synthesis. This two-stage labelling procedure, however, results in incomplete, often poor coupling of chelates and it requires a time-consuming separation of product from the large excess of label added. This is undesirable in applications where large numbers of different probes are required. In order to simplify probe synthesis, we have constructed phosphoramidites with chelating functions that are suitable for routine, automated introduction of chelates during oligonucleotide synthesis.

A method for coupling alkynes to the C-5 position of 2'-deoxyrimidines (6) was applied to the protected chelating

agent tetramethyl 2,2',2'',2'''-[(4-ethynylpyridine-2,6-diyl)bis-(methylenenitrilo)]tetrakis (acetate) (abbreviated TMEPH) (1) (Scheme 1 A). This protected chelating agent has previously been used successfully in palladium(II)-catalyzed coupling reactions to different iodoarenes, leading to highly fluorescent europium chelates (7, 8). The modified nucleosides were converted to fully protected phosphoramidites that could be coupled efficiently, several in sequence, to an oligonucleotide strand. We illustrate the application of oligonucleotides with this type of chelating groups to the analysis of specific gene sequences in an oligonucleotide ligation assay (OLA).

MATERIALS AND METHODS

General

Anhydrous dimethylformamide (DMF) and dichloromethane were obtained from Aldrich. Pyridine and triethylamine were dried and distilled before use. 5-Iodo-2'-deoxyuridine was purchased from Aldrich. 5-Iodo-2'-deoxycytidine was synthesized according to (9), and converted to N⁴-benzoyl-5-iodo-2'-deoxycytidine by the 'one-pot' procedure (10). TMEPH was synthesized according to (11), and (2-cyanoethoxy) N,N-diisopropylamino chlorophosphine according to (12). All other reagents were of good quality and obtained from commercial sources. Column chromatography was performed on columns of Silica Gel 60 (Merck 230–400 mesh). Thin layer chromatography (TLC) was conducted on Silica-60 F₂₅₄ plates (Merck). Proton nuclear magnetic resonance (NMR) spectra were recorded with a Jeol JNM-FX200 at 200 MHz and ³¹P NMR spectra with Varian Unity 500 operating at 202 MHz. Proton chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard and ³¹P-chemical shifts in ppm (δ) to 85% H₃PO₄ as external standard.

5-TMEP-2'-deoxyuridine (4)

A magnetically stirred mixture of 5-iodo-2'-deoxyuridine (2) (1.35 g, 3.81 mmol), bis(triphenylphosphine)palladium(II) chloride (140 mg, 0.19 mmol), copper(I) iodide (72 mg, 0.38 mmol), dry triethylamine (1.06 ml, 7.62 mmol) in dry DMF was deaerated with argon and TMEPH (1) (2.55 g, 5.72 mmol) was

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added to the reaction mixture. The mixture soon turned dark brown and became warm but cooling was not found necessary at this scale of synthesis. After 15 min, analysis by TLC ($\text{CHCl}_3/\text{MeOH}$ 8:2) revealed complete consumption of 5-iodo-2'-deoxyuridine and formation of a single product of higher R_f . Triethylamine was evaporated and the residue was coevaporated with toluene (20 ml) to remove any traces of this base. DMF was removed by coevaporation with *n*-butanol (3×20 ml). The residue was partitioned between chloroform and saturated sodium bicarbonate, and extracted with $\text{CHCl}_3/\text{EtOH}$ 3:1 (3×100 ml). The organic phase was evaporated in vacuo, dried by coevaporation with toluene (50 ml), dissolved in chloroform, and flash chromatographed using an increasing gradient of ethanol in chloroform as eluent. The product was eluted from the column with 7 % $\text{EtOH}/\text{CHCl}_3$ to give, after evaporation, 2.28 g (89 %) of (4) (R_f 0.27 in $\text{CHCl}_3/\text{MeOH}$ 9:1) in the form of a bright yellow oil that could not be crystallized. ^1H NMR ($\text{CDCl}_3 + \text{CD}_3\text{OD}$): 2.15–2.45 (m, 2 H), 3.39 (m 2H), 3.61 (s, 8 H), 3.71 (s, 12 H), 3.82–3.90 (m, 1H), 4.00 (s, 4 H), 4.52 (m, 1 H), 6.27 (t, 1H), 7.57 (s, 2 H), 8.60 (s, 1 H).

N^4 -benzoyl-5-TMEP-2'-deoxycytidine (5)

This compound was prepared using N^4 -benzoyl-5-iodo-2'-deoxycytidine (3) in a reaction analogous to the preparation of (4), with the exception that a much longer time (120–180 min) was required to consume all starting material. The product (5) was obtained as a yellow oil: (83 %), (R_f 0.32 in $\text{CHCl}_3/\text{MeOH}$ 9:1), ^1H NMR ($\text{CDCl}_3 + \text{CD}_3\text{OD}$): 2.22–2.60 (m, 2 H), 3.35–3.55 (m, 2 H), 3.62 (s, 8 H), 3.70 (s, 12 H), 3.75–3.95 (m, 1 H), 4.02 (s, 4 H), 4.52 (m, 1 H), 6.27 (t, 1 H), 7.20–7.65 (m, 7 H), 8.22 (s, 1 H), 8.89 (s, 1 H).

Synthesis of the 5'-O-dimethoxytrityl-derivatives of the 5'-modified nucleosides

Dimethoxytritylation was performed according to the standard procedure (13). The yield of 5-TMEP-5'-O-dimethoxytrityl-2'-deoxyuridine (6) was 91 %, (R_f 0.52 in $\text{CHCl}_3/\text{MeOH}$ 9:1), ^1H NMR ($\text{CDCl}_3 + \text{CD}_3\text{OD}$): 2.36 (m, 2 H), 3.29 (m 2H), 3.65 (s, 8 H), 3.76 (s, 12 H), 3.79 (s, 6 H), 3.85–3.95 (m, 1H), 4.03 (s, 4 H), 4.56 (m, 1 H), 6.28 (t, 1H), 6.80–6.90 (m, 4 H), 7.20–7.60 (s, 11 H), 8.66 (s, 1 H).

The yield of N^4 -benzoyl-5-TMEP-5'-O-dimethoxytrityl-2'-deoxycytidine (7) was 72 %, (R_f 0.55 in $\text{CHCl}_3/\text{MeOH}$ 9:1), ^1H NMR ($\text{CDCl}_3 + \text{CD}_3\text{OD}$): 2.3–2.65 (m, 2 H), 3.28–3.45 (m, 2H), 3.65 (s, 8 H), 3.71 (s, 12 H), 3.78 (s, 6 H), 3.95 (m, 1 H), 4.09 (s, 4 H), 4.55 (m, 1 H), 6.30 (t, 1 H), 6.80–6.90 (m, 4 H), 7.20–7.65 (m, 16 H), 8.21 (s, 1 H), 8.92 (s, 1 H).

5-TMEP-5'-O-dimethoxytrityl-2'-deoxyuridine-3'-O-(2-cyanoethyl)- N,N' -diisopropylphosphoramidite (8)

Compound (6) (1.69 g, 1.73 mmol) was dried by coevaporation with toluene (10 ml) and dissolved in dry dichloromethane (15 ml). Dry triethylamine (1.20 ml, 8.65 mmol) and (2-cyanoethoxy)- N,N' -diisopropylaminochlorophosphine were added and the mixture was allowed to react for 30 min at room temperature. The mixture was partitioned between dichloromethane (100 ml) and saturated sodium bicarbonate (100 ml), extracted with two additional portions of dichloromethane (50 ml), and the organic phase was evaporated. The residue was dried by coevaporation with toluene (2×20 ml) and purified on a 4×2 cm column of silica gel, previously equilibrated in $\text{CH}_2\text{Cl}_2/\text{Et}_3\text{N}$ 9/1, and run

using the same solvent. Appropriate fractions were pooled, evaporated to dryness, and coevaporated with dry acetonitrile (20 ml) to give 1.71 g (82 %) of oily product (8). This material was stored at -20°C after the addition of Et_3N (50 μl); R_f 0.66 in $\text{CH}_2\text{Cl}_2/\text{EtOAc}/\text{Et}_3\text{N}$ 45:45:10; ^{31}P NMR (CDCl_3 + 2 drops of Et_3N): 148.6 and 149.7.

N^4 -benzoyl-5-TMEP-5'-O-dimethoxytrityl-2'-deoxycytidine-3'-O-(2-cyanoethyl)- N,N' -diisopropylphosphoramidite (9)

This compound was prepared in an analogous reaction. The yield was 85 % oil, (R_f 0.70 in $\text{CH}_2\text{Cl}_2/\text{EtOAc}/\text{Et}_3\text{N}$ 45:45:10), ^{31}P NMR (CDCl_3 + 2 drops of Et_3N): 148.2 and 149.9.

Deprotection of nucleoside derivatives and formation of lanthanide chelates

Modified nucleosides (4) and (5) (20 mg, approximately 0.03 mmol) were dissolved in methanol (2.0 ml). Sodium hydroxide (0.1 M, 0.6 mmol) and the appropriate lanthanide citrate (0.15 mmol) were added to these magnetically stirred solutions and the homogeneous solutions were kept at 20°C for 30 min. The reaction mixture was neutralized by careful addition of 1 M acetic acid. The products of hydrolysis were analyzed and separated chromatographically by FPLC, using a reverse phase PepRPC column (Pharmacia-Sweden) with a gradient of acetonitrile in 0.1 M TEAA. The instrument was fit with on-line coupled UV (254 nm) and fluorescence detectors (Shimadzu RF 535, excitation wavelength 320 nm, emission wavelengths 612 nm for europium chelates and 544 nm for terbium chelates).

In order to remove the N^4 -benzoyl group from the deoxycytidine derivative, the nucleoside solution was evaporated and treated with concentrated ammonia (5.0 ml) overnight. After evaporation of ammonia, the lanthanide complexes were isolated by chromatography. Pure fractions were evaporated to dryness and dissolved in distilled water.

Measurement of direct fluorescence from the nucleoside chelates

The fluorescence properties of the nucleoside chelates were measured in a Perkin-Elmer LS-5 spectrophotometer using the phosphorescence mode. The relative fluorescence yields for europium and terbium chelates are expressed in logarithmic values (log R). The term R is a measure of the total fluorescence of a lanthanide chelate in relation to the fluorescence of the corresponding unchelated lanthanide cation. R is directly proportional to the product $\epsilon \times Q$, where ϵ is the molar absorption coefficient and Q is the emission quantum efficiency. Excitation maxima (λ_{exc}), and emission decay constants, given as reverse values of the decay times (k_{chel}), were also determined for the chelates. All measurements were performed in 0.2 M borate buffer pH 8.5 as described (14).

Solid-phase synthesis of lanthanide-labelled oligonucleotides

Phosphoramidites (8) and (9) were coevaporated with dry acetonitrile (2×5 ml) and diluted to 0.1 M in the same solvent. These solutions were employed for automated DNA synthesis at a 0.2 μmol scale, on a Gene Assembler Synthesizer (Pharmacia). All couplings were performed under the conditions recommended by the manufacturer with a 1 min coupling step. The average coupling yield was greater than 99%, based on colorimetric monitoring of DMT-cation release. The modified nucleotides were incorporated singly or several in sequence either at the 5'- or the 3'-end of the oligonucleotides.

The oligonucleotides were deprotected by first treating the solid support with NaOH (0.1 M, 1.0 ml) for 2 hr at room temperature. Ammonium chloride (1.0 M, 0.1 ml) was then added and the solution was evaporated to dryness. The residue was treated with concentrated ammonia (1.0 ml) for 16 hr at 60°C. The appropriate lanthanide citrate (approximately 10 eq.) was added to the ammonia solution at 20°C and the mixture was incubated for 90 min, followed by desalting over a NAP-10 Sephadex column (Pharmacia), and preparative reverse phase FPLC separation.

Measurement of indirect fluorescence

Europium, samarium, or terbium ions, carried as chelates, were detected after release and uptake by another chelating agent in a fluorescence enhancement solution. The chelate-modified oligonucleotides were transferred to a flat-bottom polystyrene microtiter plate (Nunc, Denmark), containing 180 μ l of 0.1 M acetate-phthalate pH 3.2, 15 μ M 2-naphthoyl trifluoroacetone, 50 μ M tri-*n*-octylphosphine oxide, and 0.1% Triton X-100 (Wallac, Finland). At this pH, lanthanide ions are released from the oligonucleotides and the fluorescence from the europium and samarium chelates that form in the enhancement solution was quantitated in a DELFIA Plate Reader Research Fluorometer (Wallac). Next, 20 μ l of a terbium fluorescence enhancement solution (100 μ M 4-(2,4,6-trimethoxyphenyl)-pyridine-2,6-dicarboxylic acid and 1% cetyltrimethylammonium bromide in 1.1 M NaHCO₃ (modified from 15) was added to the wells and after a further 10 minute incubation the terbium-specific fluorescence was recorded.

Investigation of chelate stability

Two oligonucleotides were synthesized, each comprising 10 T residues and one chelate-modified base at the 5' end. One of these oligonucleotides also incorporated 5 residues derived from the 6-(dimethoxytrityloxy)hexyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (**10**) at the 3' end (Fig. 2). The longer and the shorter oligonucleotides were deprotected in the presence of europium citrate or terbium citrate, respectively.

The two oligonucleotides labelled with europium or terbium ions were mixed at 5 μ M each in water or in 0.1 M NaOH, and incubated at different temperatures. After appropriate times, 100 μ l aliquots were removed and analyzed by chromatography using a PepRPC 5/5 FPLC column with both UV and fluorescence detection. Samples diluted in NaOH were injected without neutralization in order to avoid the risk of exchange between the metal ions due to local acidification. Metal ion exchange in both directions was examined by monitoring the specific fluorescence of terbium ions under the peak containing material originally labelled with europium ions. Conversely, in a separate run europium fluorescence was measured under the peak representing material originally labelled with terbium.

The area of the peak of terbium fluorescence from the material originally labelled with europium was compared to the total terbium fluorescence and used as a measure of the exchange of ions. The transfer of europium ions was calculated in a similar manner, after correction for europium readings in the terbium channel. The measurements of exchange of terbium ions and of europium ions did not differ significantly.

PCR

Three human DNA sequences were amplified by PCR. The target sequences were derived from the genes for α_1 antitrypsin

(α_1 AT), the cystic fibrosis transmembrane conductance regulator gene (CFTR), and from an anonymous DNA segment located in the q28 region of the X chromosome (316L). This fragment was derived from the left-hand end of the yeast artificial chromosome XY316 (16).

The following primers were used to amplify these three segments. α_1 AT: 5' TCAGCCTTACAACGTGTCTCTGCTT 3' and 5' GTATGGCCCTCTAAAAACATGGCCCC 3'. CFTR: 5' GTTTTCCTGGATTATGCCTGGCAC 3' and 5' GTTGG-CATGCTTTGATGACGCTTC 3'. 316L: 5' GTACCTTGTC-AGTAAGAGACTGCC 3' and 5' GTCTTAGAAGTCCTTA-TGACCCCC 3'.

The fragments were amplified in a volume of 100 μ l of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 5 mg/ml bovine serum albumin, 2 mM dNTP, PCR primers at 1 μ M each, and including 200 ng human genomic DNA and 2U Taq polymerase. The temperature profile was 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min repeated in 30 cycles. After the last cycle, a final extension was performed at 72°C for 7 min.

Ligase-mediated gene detection

Oligonucleotides used for ligase-mediated analysis included variable numbers of chelates of europium, samarium, or terbium ions, with a biotin (B) residue, or with a 5' phosphate (P). The pair of oligonucleotides used to detect the P₁Z mutation (17) of the α_1 AT gene were 5' Sm₁₅ GGCTGTGCTGACCATCGACA 3' and 5' P AGAAAGGGACTGAAGCTGT B 3' (5). The CFTR sequence was detected using 5' Tb₁₀ ATTAAGAAA-ATATCATCTT 3' and 5' P TGGTGTTCCTATGATGAAT B 3' (5). Finally, segment 316L was detected using the oligonucleotide pair 5' Eu₁₀ GTACCTTGTCAGTAAGAGACTGC-C-3' and 5' P TTTTAAAGTTCTTTTGATAT B 3'.

In a separate experiment, variants of the latter oligonucleotide pair were also used including either 10 or a single europium chelate or where the position of the biotin and the ten chelate-modified nucleotides in the oligonucleotide pair had been reversed.

Before ligase-mediated analyses, the amplification reactions were diluted in water to a final volume of 10 μ l and added to wells of a flexible microtiter plate, overlaid with mineral oil, and denatured for 5 min at 93°C. After quick cooling to a temperature below 60°C, a 10 μ l ligation mixture was added to each of the samples and these were incubated for 45 min at room temperature. The composition of the ligation mixture was: 0.4

Table I. Fluorescence properties of some nucleoside-coupled chelates of europium or terbium ions.

Nucleoside residue	λ_{exc} (nm)	$\log R_{Eu^{3+}}$	$k_{Eu^{3+}}$ (ms ⁻¹)	$\log R_{Tb^{3+}}$	$k_{Tb^{3+}}$ (ms ⁻¹)
Deoxyuridine-C-5-yl-	334	5.51	2.60	2.37	0.87
Deoxycytidine-C-5-yl-	330	5.47	2.59	n.d.	n.d.
N-4-Bz-deoxycytidine-C-5-yl-	330	5.40	2.62	n.d.	n.d.

The chelating function shown in Scheme 1A was attached to the deoxyuridine, deoxycytidine, or to a base-protected form of deoxycytidine. The optimal wavelengths for exciting the chelates are given as λ_{exc} and the logarithm of the fluorescence of the chelated ions, compared to that of the free lanthanide ions, are given as $\log R_{Ln^{3+}}$. Finally the emission decay constants of the fluorescence signal are given as $k_{Ln^{3+}}$. Emission maxima for both free and chelated ions were, respectively, 615 nm (europium) and 545 nm (terbium). The slit used for excitation was 15 nm and for emission 10 nm.

M NaCl, 0.018 M Tris-acetate pH 7.5, 0.018 M magnesium acetate, 0.09 M potassium acetate, the two ligation probes at 0.6 μ M each, and 0.32 U T4 DNA-ligase (Pharmacia).

After the ligation reaction, 20 μ l of buffer A (0.1 M Tris-HCl pH 7.5, 1 M NaCl, 0.1 % (v/v) Triton X-100) was added to each well. Next, a manifold-solid support with 96 prongs, coated with avidin-coupled Sepharose particles (18), was incubated in the microtiter wells on a shaking platform for 30 min at room temperature. The set of solid supports with the captured biotinylated molecules was washed twice with buffer A, once with 0.1 M NaOH to denature DNA, and again twice with buffer A. Each washing step took 10 min and the set of supports was then transferred to another microtiter plate for time-resolved fluorescence measurements.

RESULTS

Synthesis of nucleoside-phosphoramidites incorporating a chelating function

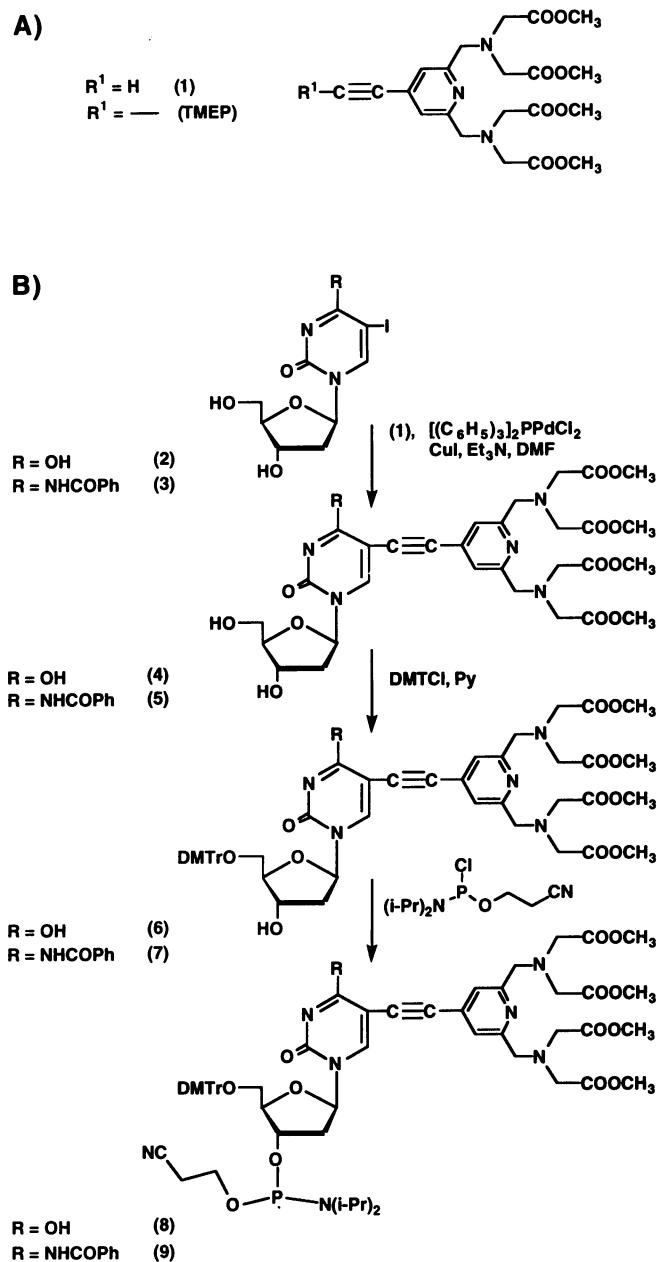
The strategy for synthesizing nucleotide-phosphoramidites with a function capable of complexing lanthanide ions, is outlined in Scheme 1. This strategy relies on commercially available 5-iodo-2'-deoxyuridine (2) or on N⁴-benzoyl-C-5 iodo-2'-deoxycytidine (3) that can be obtained in two standard reactions, starting from 2'-deoxycytidine. The chelating function, protected as a tetramethyl ester and substituted at the pyridine ring with an ethynyl-group (1), was synthesized in a 5-step reaction, starting from chelidamic acid, in 42% overall yield.

5-Iodo-2'-deoxycytidine (19) and other halogenated nucleosides (20) can be coupled to a variety of terminal alkynes by treatment with bis(triphenylphosphine)palladium(II) chloride and copper(I) iodide in triethylamine. DMF is a suitable solvent in this reaction, since it increases the solubility of all reagents and improves the reaction kinetics. Furthermore, it also suppresses the formation of cyclic side products (21). Such cyclic compounds, formed as a result of nucleophilic attack by the pyrimidinic C-4 amine (cytidine) or C-4 hydroxyl (uridine) on the C-5 ethynyl group, have been frequently observed among reaction products obtained in the absence of DMF (22).

Treatment of each of the above C-5 iodo nucleosides with (1) in the presence of bis(triphenylphosphine)palladium(II) chloride, copper(I) iodide and triethylamine in dry DMF resulted in complete conversion of both starting nucleosides to single products as determined by TLC. After work-up and silica gel column separation the labelled nucleosides were isolated in yields of 89% (4) and 83% (5).

A remarkable difference in reactivity was observed between derivatives (2) and (3). Under the above conditions 2'-deoxyuridine couples with release of heat in less than 10 min, while the corresponding 2'-deoxycytidine-derivative reacts more than 10-fold slower. This is at variance with previous observations of base-unprotected starting materials, indicating that all of the investigated iodonucleosides react at approximately the same rate (20). The dependence of the reaction rate on base-protection probably reflects steric hindrance by the N-4-benzoyl protecting group of the interaction between the bulky palladium complex and the C-5-iodonucleoside, required for successful coupling.

Finally, the 5'-hydroxyls of (4) and (5) were protected as their dimethoxytrityl derivatives (6) and (7) and the 3'-hydroxyls were activated as their (2-cyanoethoxy)-N,N-diisopropyl phosphoramidites, affording the key reagents (8) and (9).



Scheme 1. Synthesis of modified nucleotide phosphoramidites, capable of chelating lanthanide ions. (A) The protected chelating function. (B) Construction of the phosphoramidites. For convenience, uridine is presented in its unusual tautomeric form.

Synthesis of lanthanide-labelled oligonucleotides

Chelate-modified residues were incorporated at the 5'- or the 3'-end of oligonucleotides. In the latter case, synthesis began with one of the standard DNA supports. There is no obvious limit to the number of nucleotides with chelating functions that can be successfully introduced in an oligonucleotide. We have synthesized in good yield probes including up to 30 chelating groups.

Sodium hydroxide (0.1 M) was used at the first deprotection step in order to prevent undesired ammonolysis of carboxylic

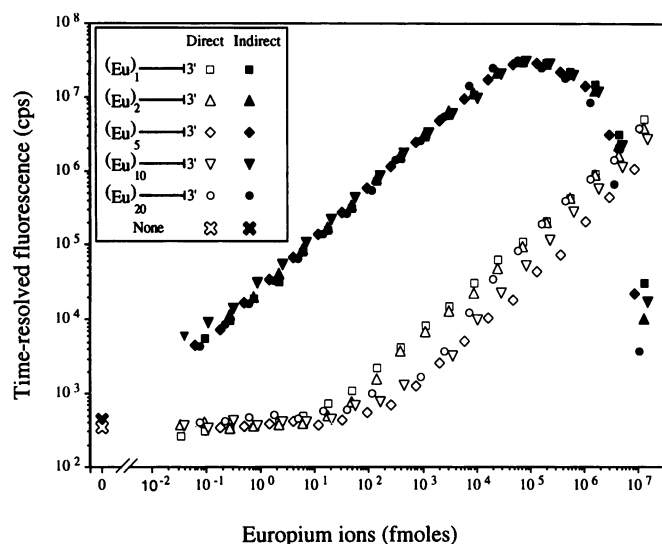


Figure 1. Additivity of the direct fluorescence from chelate-modified nucleotides incorporated in oligonucleotides. Oligonucleotides composed of the same 20 nucleotides additionally included 1, 2, 5, 10, or 20 europium chelate-modified nucleotides at the 5' end. These oligonucleotides were serially diluted, distributed in microtiter wells, and assayed for direct fluorescence (open symbols), and subsequently, after the addition of an enhancement solution, for indirect fluorescence (filled symbols). The time-resolved fluorescence in counts per second (cps) was plotted as a function of the amount of europium ions in the microtiter wells.

esters on the chelating functions, as would be expected if concentrated aqueous NH_3 was applied directly. This was followed by standard ammonia deprotection. At the end of this treatment, an excess of the appropriate lanthanide citrate was added to form the desired labelled product and to prevent complexation of extraneous metal ions that may be present at micromolar concentrations in the separation media. It is essential to add the lanthanide ions as a weak citrate chelate and not as free salt in order to prevent precipitation of oligonucleotides carrying chelating functions in the presence of a large excess of free metal ions.

The number of lanthanide ions incorporated in each oligonucleotide was assayed after chromatographic purification of the oligonucleotides. Lanthanide ion concentrations in the oligonucleotide preparations were determined by measurements of indirect fluorescence, compared to standard curves from known concentrations of the salts. Oligonucleotide concentrations were obtained through measurements of absorbance at 260 nm, after correction for the presence of the known number of deoxy U-derivatives, characterized by the specific absorbance at 350 nm.

On the basis of the above measurements we conclude that the number of lanthanide ions per oligonucleotide were within 10 % of the expected.

Fluorescence properties of the nucleoside-chelates

Fluorescence data for the new nucleoside-derivatives are presented in Table 1. The 5-deoxyuridyl chelate and the 5-deoxycytidyl chelate and its N^4 -benzoyl derivative all exhibited strong fluorescence as europium complexes and rather low fluorescence as the corresponding terbium complexes. Earlier

studies of samarium complexes of similar chelating agents demonstrated very low fluorescence (M.K. unpublished). Therefore, samarium measurements were omitted in this study.

Minimal differences were observed between the fluorescence intensity or the wavelength of the excitation maxima of the three investigated europium chelates. The fluorescence yield of these europium chelates compares favourably with that of other chelates that have been used to modify macromolecules (23).

As is true for other lanthanide chelates (14), the optimal excitation wavelength of the compounds (here at around 330 nm) is far removed from the fluorescence emitted from europium and terbium ions at 615 nm and 545 nm, respectively. These emission maxima are identical for the free and the chelated forms of each of the two metal ions. Accordingly, the light used for excitation can be efficiently filtered out during detection. The long decay time of the fluorescence from the lanthanide chelates permits further reduction of the background in detection by time-resolved measurements, avoiding contributions by the exciting light source and by nonspecific short-lived fluorescence from the sample.

Oligonucleotides labelled with these compounds may thus be useful in genetic assays where the fluorescence emitted from the complexes incorporated in probes is directly detected. Chelate-modified oligonucleotides can also be used to transport the lanthanide ions through an assay for subsequent transfer to a fluorescence enhancement solution. In the low pH of this solution the lanthanide ions are released and complexed by another chelating agent. By taking advantage of the fluorescence emitted from these complexes, increased fluorescence yield may be obtained. Moreover, enhancement solutions are available that permit highly sensitive detection and distinction of fluorescence from europium, samarium, and terbium ions (see below). This type of indirect fluorescence measurement does not, however, permit localized detection of chelate-modified probes.

Additive fluorescence from multiple chelates incorporated in oligonucleotides

Conventional fluorophores, exemplified by fluorescein, exhibit the phenomenon of fluorescence quenching when located at short distances from each other. The reason for this is that, due to the overlapping excitation and emission spectra, the energy emitted from one fluorophore may be absorbed by near-by fluorophores. Because of this effect, only a limited number of such organic fluorophores, maybe as few as a single one, can be added to an oligonucleotide before the fluorescence of the molecules decreases or is lost altogether (24, 25).

By contrast, for lanthanide chelates having large Stokes shifts quenching was expected to be less of a problem. In order to investigate if the inclusion of more chelates in oligonucleotides results in proportionally increased direct fluorescence, we compared the fluorescence from oligonucleotides, 20 nucleotides long and additionally including either 1, 2, 5, 10, or 20 europium chelate-modified nucleotides, derived from compound (8). Serial dilutions of these oligonucleotides were analyzed for direct fluorescence and, after the addition of a fluorescence enhancement solution, for indirect fluorescence emitted by the europium chelates forming in this solution. The concentrations of the oligonucleotides, incorporating known numbers of europium ions, were calculated by comparing the indirect fluorescence from the europium ions with that from standard dilutions of EuCl_3 .

In Figure 1 the fluorescence has been plotted as a function of the number of europium ions in the wells. As expected, the direct fluorescence from the oligonucleotides is weaker than the indirect

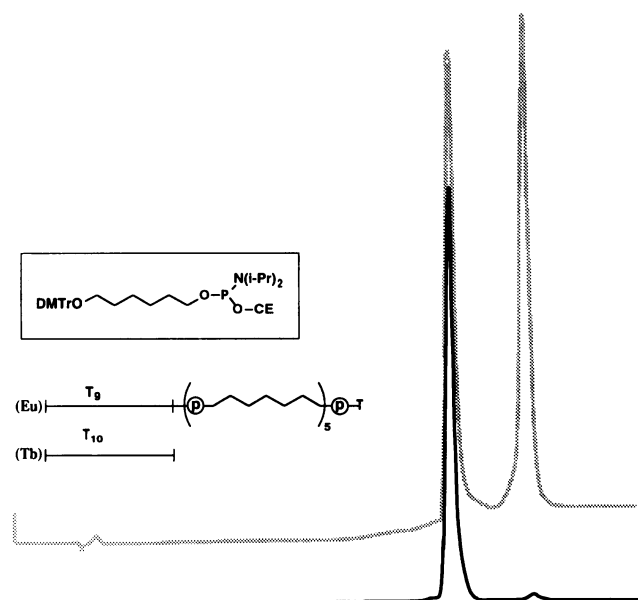


Figure 2. Stability of lanthanide chelates. One of two oligonucleotides, both containing a single 5' lanthanide chelate and 10 T-residues, additionally included 5 hydrophobic residues of the reagent (10) (boxed) incorporated at the 3' end. The chelate of the longer oligonucleotide included an europium chelate and the shorter a terbium chelate. The Figure illustrates the FPLC tracings of the two oligonucleotides, having been incubated together for 36 hrs at pH 7.0 and 20°C. The UV₂₅₄ signal is shown as a grey line while the direct terbium fluorescence recorded from the chelates is indicated by a black line. The left-most peaks are derived from the shorter, less hydrophobic oligonucleotide, originally labelled with terbium ions, while the right peaks represent the longer, europium-labelled material. A minimal terbium-specific signal is observed in the peak representing material originally labelled with an europium chelate, indicating a low level of exchange under these incubation conditions.

fluorescence. However, the difference between the direct and indirect fluorescence is similar for all of the oligonucleotides examined. At high concentrations of europium ions the indirect fluorescence can be seen to level off or to be reduced due to saturation of the photomultiplier detector and because the chelating capacity of the enhancement solution is exceeded, resulting in the formation of predominantly nonfluorescent complexes.

From this analysis it is clear that the direct fluorescence, per europium ion, is independent of the number of chelates present in each oligonucleotide. We therefore conclude that for this type of fluorophores it is possible to increase the direct, as well as the indirect, fluorescence signal from an oligonucleotide in proportion to the number of chelates added.

Chelate stability

In assays where more than one type of lanthanide label is used it is important that negligible exchange of ions occurs between probes labelled with different lanthanide-chelates. On the other hand, in indirect fluorescent assays, where the chelated ions are released and taken up in solution by another complexing agent before the fluorescence measurement, the lanthanide ions must be readily dissociated under the appropriate conditions.

In order to investigate the kinetic stability of the chelates, we used two oligonucleotides, containing equal numbers of UV-absorbing bases but with distinct hydrophobic properties. The two oligonucleotides were labelled either with a europium chelate

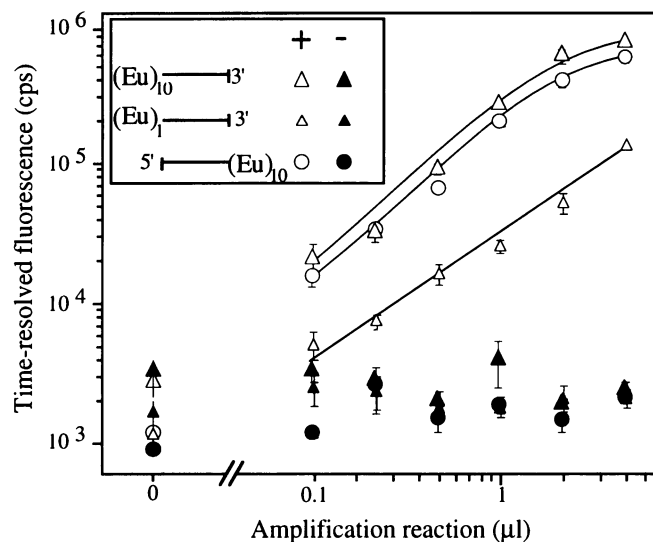


Figure 3. Oligonucleotides labelled with europium chelates were used in ligase-mediated gene detection of variable amounts of a positive (open symbols) or negative (filled symbols) amplification reaction of a DNA segment derived from the human X chromosome. The chelating functions were either added at the 5'-end of one of the members of an oligonucleotide-pair used for ligation-based detection (triangles), or at the 3'-end of the other member of the pair (circles). Either ten chelates (larger symbols) or a single chelate (smaller triangles) were added per chelate-modified oligonucleotide. The results are given as mean and standard deviation (SD) of quadruplicate determinations

or with a terbium chelate, introduced during synthesis by means of reagent (8). After incubation under various conditions, the pair of oligonucleotides were separated by FPLC and the exchange of metal ions was investigated by fluorescence measurement.

The exchange of lanthanide ions after a 36 h incubation at pH 7.0 and 20°C was found to be 0.45 % (Figure 2). Clearly, this is sufficiently low to allow the simultaneous use of probes labelled with different lanthanide ions in most genetic analyses. This rate of exchange may, however, prohibit long-term storage of premixed probes. Incubation at 20°C in 0.1 M NaOH resulted in 1.4 % exchange after a 30 min incubation. Finally, incubation at 95°C and neutral pH for 30 min caused 16.5 % of metal exchange, precluding the use of probes labelled with different chelates during extensive thermal cycling.

The chelating agents efficiently released lanthanide ions in a 30 min incubation at pH 3.2, permitting the uptake of the ions by another chelating agent, present in the low pH of the enhancement solution.

Chelate-modified oligonucleotides used in ligation-based gene detection

Oligonucleotides labelled with lanthanide chelates were used in an oligonucleotide ligation assay (26) to identify specific human DNA sequences amplified by PCR. In this assay, DNA sequences are detected through their ability to assist in the ligation of pairs of oligonucleotides, hybridizing in immediately adjacent positions on a target sequence. The two remote ends of the oligonucleotide probes, not engaged in the ligation reaction, may be modified to permit capture via biotin and detection through time-resolved fluorescence analysis, as previously described (5).

We applied a series of oligonucleotides, modified with europium chelates incorporated using reagent (8), in a ligase-based assay for the detection of a human DNA marker, amplified

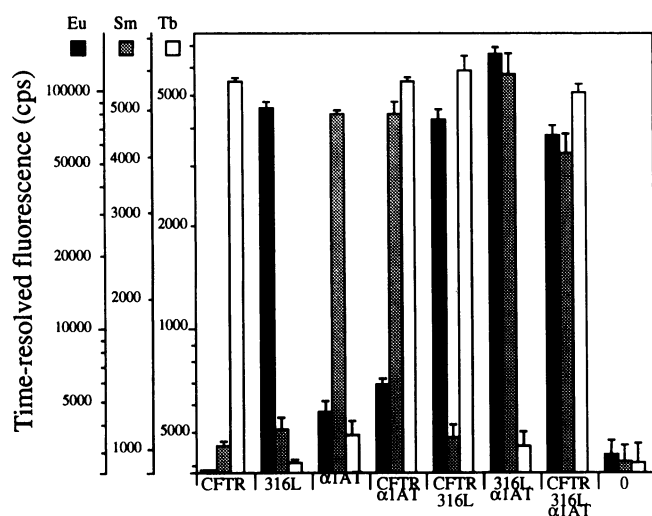


Figure 4. Triple-color detection of three different amplified DNA sequences. Aliquots of 1.3 μ l of three different amplification reactions were added in various combinations and analyzed by ligation-mediated analysis, using a ligation mixture containing probes specific for each of the three amplified DNA sequences. An europium-labelled probe was used for the detection of a DNA sequence, derived from the Xq28-region of the human X chromosome (316L)(the europium signal is shown as filled bars). A samarium-labelled probe was designed to participate in the detection of a sequence, derived from a mutant variant of the α_1 AT-gene (the samarium signal is shown as grey bars). The terbium-specific signal (open bars) was taken as a measure of the presence of an amplified sequence from the gene involved in the causation of cystic fibrosis (CFTR) since one of the probes specific for this product was labelled with terbium ions chelates. The mean and SD of quadruplicate determinations are shown.

from the q28 region of the X chromosome. One of the members in each pair of ligation probes included one or more lanthanide chelates and the other oligonucleotide carried a biotin residue. After the ligation reaction, ligation products were isolated on an avidin-modified manifold-support (18) and, after washes, oligonucleotides bound to the supports were subjected to indirect time-resolved fluorescence measurements.

In Figure 3, the results of an analysis is presented where variable amounts of PCR products were analyzed in the ligation assay. Using an oligonucleotide modified with 10 europium chelates in combination with another, biotinylated oligonucleotide, 0.1 μ l of an amplification reaction was sufficient for a clearly detectable signal. The signal rises approximately proportionally up to 4 μ l of the PCR sample, providing quantitative information on the success of the amplification reaction. The positions where the europium chelates and the biotin residues were added could be reversed without any significant consequences for the results of the assay. Using an oligonucleotide labelled with a single europium chelate, we obtained correspondingly weaker signals and a reduced sensitivity of detection.

Triple-color ligase-mediated detection of PCR products

The chelating agents described herein can be used to complex any of several lanthanide ions to oligonucleotide probes used in gene detection. Europium, samarium, and terbium ions can all be distinguished through indirect fluorescence measurement with good sensitivity of detection.

We constructed reagents for the ligase-mediated detection of three different human DNA sequences, amplified by PCR. One

member of each of the three pairs of ligation probes was labelled with either europium, samarium, or terbium chelates. Aliquots of the three different amplification reactions were added in various combinations to reactions including all ligation probes. After the ligation reactions, products were collected by binding to an avidin-modified support, washed, and transferred by binding to a fluorescence enhancement solution in order to determine the presence of europium or samarium ions. Next, terbium ions were detected after the addition of another enhancement solution. Figure 4 demonstrates that the assay efficiently detects the presence of 1.3 μ l of each of the three amplification reactions added in any combination. Oligonucleotides labelled with lanthanide chelates thus lend themselves to convenient analyses of three different sequences in a DNA sample.

DISCUSSION

Highly sensitive detection is of crucial importance in advanced gene analytic assays. The possibility to simultaneously analyse more than one property of the sample by using several probes labelled with distinct detectable groups can speed up analyses and permits internally controlled quantitative measurements of DNA sequences. We demonstrate herein the construction of building-blocks for oligonucleotide synthesis that permit the incorporation of multiple chelating functions during standard oligonucleotide synthesis. As little as 10^{-17} mol of an oligonucleotide, labelled with these europium chelates, can be detected in microtiter wells by indirect fluorescence analysis. Moreover, the chelating functions can be used to complex — in order to separately detect — at least three more lanthanide ions, samarium, terbium, and dysprosium, albeit with a ten- to hundred-fold lower sensitivity of detection (15). We demonstrate herein that the stability of the chelates is adequate to permit the use of probes labelled with three lanthanide ions together in one assay. Even more distinguishable colors can be obtained by also labelling individual probes with combinations of lanthanide ions.

The chelating functions described here can be used to transport lanthanide ions through various assays for subsequent release and uptake by other chelating reagents in an enhancement solution. This detection offers sensitive detection but the spatial resolution of detected molecules is lost. Since the europium chelates attached to oligonucleotides exhibit direct fluorescence, an alternate strategy would be to study this localized signal. The long decay time of this type of fluorophores limits the number of excitations of a single fluorophore that are possible in a given time interval. However, the extreme properties of the present fluorophores; the long Stokes shift and duration of the fluorescence and the possibility to add many chelates over a short distance with a proportionate increase of the fluorescence, may open possibilities for the highly sensitive in situ detection of localized probe molecules, modified with chelates, in e.g. tissue sections or in metaphase chromosomes.

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