Nonradioactive Assay of DNA Hybridization by DNA-Template-Mediated Formation of a Ternary Tb^{III} Complex in Pure Liquid Phase

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The identification of specific nucleic acid sequences by the hybridization technique[1] is usually carried out in a heterogeneous two-phase system. The nucleic acid material being investigated is immobilized on a solid matrix and then incubated in individual steps (prehybridization, hybridization, washings, detection) with various solutions.[2] Homogeneous hybridizations in pure liquid phase exhibit more favorable hybridization kinetics and, moreover, make the prehybridization and washing steps superfluous. This appreciable simplification is of great interest for the development of automated procedures. However, it requires special labeling of the nucleic acid probes, so that a measurable signal—by fluorescence resonance energy transfer (FRET),[3] for example—is only obtained after hybrid formation with the complementary sequence. Here we describe such a system involving suitable pairs of oligonucleotide probes, chemically modified to form a ternary Tb^{III} complex after hybridization with the template nucleic acids. Upon photoexcitation, the salicylate group of one oligonucleotide, serving as a ligand, transfers the excitation energy to the lanthanide ion of the second oligonucleotide[4] and a $Tb^{3\oplus}$-specific, long-lived fluorescence is then emitted.

The chemical modification involved attachment of the energy donor salicylate (pAS) to the 3′ end of one oligonucleotide (3′ON-pas) and a diethylenetriaminepentaacetate (DTPA) ligand bearing the energy acceptor and fluorescence emitter $Tb^{3\oplus}$ to the 5′ end of the other oligonucleotide (5′ON-DTPA-Tb) (Fig. 1). The oligonucleotide sequences were chosen in such a way that, after hybridization to the complementary DNA strand, the energy donor and acceptor were positioned in direct proximity.[5] Compared with ternary complex formation in free solution, the formation of a complex between DTPA-Tb on the one hand and salicylate on the other is strongly favored on the DNA template (Fig. 1).

The 3′-end labeling of 3′ON with 4-aminosalicylic acid (pAS) was accomplished by established methods of 3′-end

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  0570-0833/90/1010-1167 $ 3.50+.25/0

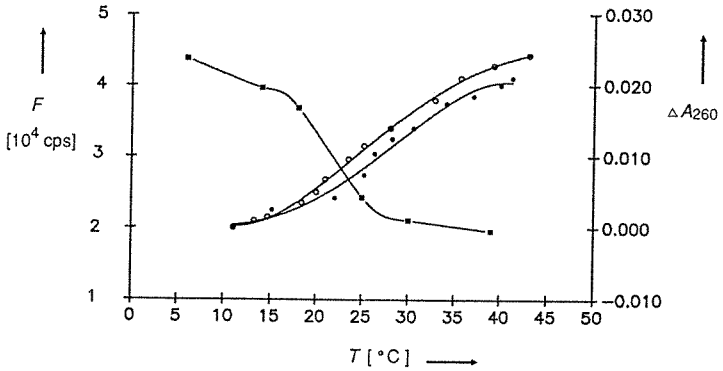

Fig. 3. Time-delayed fluorescence F (■—■) and change of the absorption ΔA_{260} (●–●) for the system template/3'ON-pAS/5'ON-DTPA-Tb (each 100 nM) as a function of temperature T in 10 mM Tris-HCl/0.5 M NaCl at pH 8.0. A T_m value of 27 °C was determined from the variation in hypochromicity; by contrast, the system without 5'-end labeling (template/3'ON-pAS/5'ON, o—o) has a T_m value of 26 °C. The T_m values of 3'ON and 5'ON alone with the template DNA are 19.5 and 20 °C, respectively.

The dependence of the energy-transfer efficiency on the distance of the oligonucleotide pairs was investigated by shortening 5'ON by one nucleotide. The DTPA-Tb label was then located on the adenine residue of 5'ON (see Fig. 1). For the same concentration of template, the hybridization signal thereby decreased by 54%. Lengthening of the spacer between the 5'-phosphate group and the DTPA-Tb chelate from an ethyl to a hexyl arm[15] resulted in restoration of 88% of the original energy-transfer efficiency.

Complex formation between energy donor and acceptor guarantees a more efficient energy transfer in comparison to a system in which energy transfer only occurs owing to spatial proximity of donor and acceptor—as was the case for the earlier described hybridization assays using the FRET principle.[3]

A further advantage of the pAS/Tb donor/acceptor system results from the *long-lived* $Tb^{3\oplus}$ fluorescence with a lifetime of 1.58 ms,[4b] which allows the measurement of delayed fluorescence.[16] The direct donor and acceptor fluorescence emission, which is short-lived and independent of the energy-transfer process, leads to an appreciable, unspecific background fluorescence in the usual FRET systems.[17] Measurement of the delayed fluorescence, however, allows reliable suppression of this background fluorescence. The sensitivity of the DNA-hybridization assay described here might be further increased by a better choice of excitation and emission wavelengths of fluorescence and by the use of other ligand/lanthanide FRET systems.

Besides simplifying the hybridization process compared with two-phase systems, the method described here is of special significance for the rapid identification of mutations, insertions, deletions, chromosome translocations, and splicing sites, since the two oligonucleotide probes must be in direct proximity.

Experimental Procedure

Synthesis of 3'ON-pAS: 16 nmol of 3'ON containing a 3'-ribouridyl residue [3b, 9] was treated for 2 h on ice with $NaIO_4$ (2 μmol) in 0.2 mL of 50 mM sodium acetate buffer at pH 4.75. After addition of ethylene glycol (10 μL, 90 μmol) and Sephadex G-25 chromatography, the oxidized 3'ON was incubated with pAS (4 μmol) and $NaBH_3CN$ (4 μmol) in 0.15 mL of 0.2 M sodium phosphate buffer at pH 8.0 for about 15 h. After Sephadex G-25 purification, 3'ON-pAS was isolated by denaturing polyacrylamide gel electrophoresis (20%, 8 M urea) [18a].

Synthesis of 5'ON-DTPA-pAS: 160 nmol of the 5'-aminolink-functionalized oligonucleotide [10] was treated twice in 50 μL of 0.2 M potassium hydrogen carbonate buffer at pH 8.2 with DTPA dianhydride (4 μmol; 0.2 M solution in anhydrous dimethyl sulfoxide). After Sephadex G-25 purification, the 5'ON-DTPA was isolated by reversed phase HPLC [18b] and then treated with an equimolar amount of $TbCl_3$.

Received: May 25, 1990;
supplemented: August 20, 1990 [Z 3981 IE]
German version: *Angew. Chem. 102* (1990) 1197

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[6] The molecular modeling system consisted of an Evans & Sutherland PS390 Workstation and the graphics programs "Helix" (Dr. U. Heinemann, Institut für Kristallographie, Freie Universität Berlin) and "Insight" (Biosym, München). We gratefully acknowledge the friendly support of Dr. *J. Köhler* (Genzentrum, München).
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Volume 29 · Number 10
October 1990
Pages 1167–1169

International Edition in English

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