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Hybridization Assays Using an Expressible DNA Fragment Encoding Firefly Luciferase as a Label

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We report the use of a new label, an expressible enzyme-coding DNA fragment, for nucleic acid hybridization assays. The DNA label contains a firefly luciferase coding sequence downstream from a T7 RNA polymerase promoter. The target DNA (200 bp) is denatured and hybridized simultaneously with two oligonucleotide probes. One of the probes is immobilized in microtiter wells, via the digoxigenin/anti-digoxigenin interaction, and the other probe is biotinylated. After completion of the hybridization, the hybrids are reacted with a streptavidin−luciferase DNA complex. Subsequently, the solid-phase bound DNA is expressed by coupled transcription/translation. The synthesized luciferase catalyzes the luminescent reaction of luciferin with O_2 and ATP. The luminescence is linearly related to the amount of target DNA in the range of 5−5000 amol. The CVs obtained for 20 and 100 amol of target are 6.5% and 10.8%, respectively ($n = 4$).

The specific and strong interaction between two complementary nucleic acid strands forms the basis for the development of hybridization assays. Hybridization methodology is emerging as the most promising area in laboratory medicine and has transformed the way clinical testing is realized. Previous tests have been based on the monitoring of gene products, i.e., phenotypic markers, such as oncoproteins, viral antigens, etc. In contrast, current laboratory tests that are based on hybridization allow the analysis of disease at the nucleic acid level. Thus, pre- or postnatal diagnosis of genetic disease can be accomplished by hybridization of the patient's DNA with allele-specific oligonucleotide probes that recognize mutations, deletions, or insertions causing the disease. Also, the various infectious agents can be measured in biological fluids by hybridization with specific probes. In forensic science, hybridization of DNA with minisatellite probes allows the unique identification of individuals (DNA fingerprinting).[1,2]

Radioactive probes (usually labeled with ^{32}P), in combination with autoradiographic detection, dominated in the field of hybridization assays for more than 2 decades and provide the highest sensitivities. However, the short half-life of ^{32}P, the health hazards and problems associated with its use and disposal, and the long exposure times (many hours to days) required for detection have placed limitations on the routine use of hybridization assays in the clinical laboratory. The current trend in this area is toward novel nonradioactive alternatives.[2,3]

The labels can be incorporated into the probes either enzymatically (e.g., using DNA polymerase or deoxynucleotidyl transferase and modified deoxynucleoside triphosphates) or by chemical conjugation (e.g., introduction of NH_2 groups into the probe via cytidine transamination and then conjugation to the reporter molecule).[4] Nonisotopic hybridization assays based on fluorescent, chemiluminescent, or enzyme labels have been developed. Generally, there are two strategies for the analysis of hybrids. Either the reporter molecule is directly conjugated to the probe,[5,6] or a ligand is attached to the probe and the hybrids are measured in a subsequent step by adding a specific, labeled binding protein. The ligand may be biotin or a hapten (e.g., digoxigenin). Labeled (strept)avidin or antihapten antibodies may then be employed for detection.[7,8] Enzymes (such as alkaline phosphatase and horseradish peroxidase) are the most widely used nonradioisotopic labels because they provide amplification through the high turnover of their substrates to detectable products.[2]

Recently, we reported[9] that a DNA fragment (DNA template) coding for an enzyme can be used as a novel label for the development of highly sensitive immunoassays (expression immunoassays). In these assays, after completion of the immunoreaction, the DNA template (a luciferase-coding DNA) is expressed by in vitro transcription/translation and the activity of the synthesized enzyme is measured. Furthermore, it was estimated that 12−14 luciferase molecules were synthesized from each DNA template molecule. In the present work, we extend our investigation in the area of hybridization assays.

EXPERIMENTAL SECTION

Instrumentation. Luminescence measurements were carried out using a liquid scintillation counter (Model LS-6500, Beckman Instruments Inc., Fullerton, CA) in the single photon monitoring mode. Fluorescence measurements were performed with the Fluoroscan II microplate fluorometer (Labsystems, Needham Heights, MA) along with the transmit software 4.0. The excitation and emission wavelengths were set at 355 and 460 nm, respectively. The Amerlite shaker/incubator was from Amersham Canada Ltd. (Oakville, ON, Canada). The microtiter well washer, Model EAW II, was from SLT Lab Instruments (Salzburg, Austria). The G24 environmental incubator shaker was from New Brun-

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swick Scientific (Edison, NJ). An imaging densitometer (Model GS-670, Bio-Rad Laboratories Ltd., Mississauga, ON, Canada), along with the molecular analyst version 1.1 software, was used for quantitation of DNA fragments following electrophoresis.

Reagents. The TNT T7 wheat germ extract, used for the in vitro coupled transcription/translation reactions, was purchased from Promega Corp. (Madison, WI). The complete reaction mixture consisted of wheat germ extract (containing ribosomes, tRNA, and other translation factors), T7 RNA polymerase, and amino acids in the appropriate buffer. The mixture was prepared according to the manufacturer's instructions. The Wizard PCR preps DNA purification system and beetle luciferin were also from Promega. Ultrapure 2′-deoxyribonucleoside 5′-triphosphates (dNTP), FPLCpure terminal deoxynucleotidyl transferase, coenzyme A (CoA), and NAP-5 size exclusion columns were purchased from Pharmacia Biotech. (Montreal, PQ, Canada). Linear DNA markers (pUC18 DNA digested with *Hae*III), streptavidin, magnesium carbonate pentahydrate, and tricine were from Sigma (St. Louis, MO). Adenosine triphosphate (ATP), digoxigenin-11−2′-deoxyuridine 5′-triphosphate (Dig-dUTP), polyclonal antidigoxigenin antibody (from sheep), bovine serum albumin, streptavidin−alkaline phosphatase conjugate, 4-methylumbelliferyl phosphate, and blocking reagent for hybridization and detection (Catalog No. 1096 176) were obtained from Boehringer (Laval, PQ, Canada). The Klenow fragment of DNA polymerase I (exonuclease-free) was purchased from United States Biochemical (Cleveland, OH). Diethanolamine was from BDH (VWR Scientific, Toronto, ON, Canada). The chemiluminescent substrate for alkaline phosphatase (CSPD) and the chemiluminescence enhancer Sapphire II were gifts from Tropix (Bedford, MA). Biotin-dATP (biotin attached at the 6-position of adenine by a 14-atom linker) was obtained from Life Technologies (Burlington, ON, Canada). "U" bottom and flat-bottom, transparent polystyrene microtiter wells (Nunc, Maxisorp) were also from Life Technologies. White, flat bottom microtiter wells were from Dynatech Laboratories Inc (Chantilly, VA).

The oligonucleotide probes used in this work were synthesized by Biosynthesis Inc. (Lewisville, TX). The sequence of the "capture" probe (a 26-mer) was as follows: 5′-GCTGAA-GGGCTTTTGAACTCTGCTTA-3′. The "detection" probe (a 22-mer) had the following sequence: 5′-TCAGACCCTGAGGC TCAAAGTC-3′.

The "blocking solution" contained 1% (w/v) blocking reagent in 0.1 mol/L maleate and 0.15 mol/L NaCl, pH 7.5. The "wash solution" consisted of 50 mmol/L Tris, pH 7.4, 0.15 mol/L NaCl, and 0.1% (v/v) Tween-20. The "hybridization solution" contained 60 mmol/L sodium citrate and 0.6 mol/L NaCl in blocking solution (pH 7.5). The Tris-EDTA (TE) buffer consisted of 10 mmol/L Tris and 1 mmol/L EDTA, pH 7.6.

Preparation of the DNA Target. A 200 bp DNA fragment was used as a target. It was prepared by amplifying (using nested polymerase chain reaction) the BCR-ABL mRNA from K562 cells. The mRNA isolation, reverse transcription, and amplification were performed as previously described[10] but using unlabeled primers. The amplification product (200 bp) was purified from the excess of primers and dNTPs with the Wizard PCR preps DNA purification system and stored at 4 °C. The concentration of the stock solution of target DNA was determined by imaging densitometry as follows. The DNA target was electrophoresed in a 2% agarose gel and stained with ethidium bromide (0.6 mg/L in the running buffer). A lane containing linear DNA markers with known concentrations was also included for construction of a calibration curve. The gel was photographed, under UV exitation, by using a Polaroid 665 film, and the negative was scanned by the densitometer. Solutions of target DNA at various concentrations were prepared by diluting the stock with a diluent containing 1% (w/w) blocking reagent in distilled autoclaved water.

Labeling of the Oligonucleotide Probes. The capture probe was labeled at the 3′ end with Dig-dUTP by using terminal deoxynucleotidyl transferase. The labeling reaction was carried out in a final volume of 20 μL consisting of 0.2 mol/L potassium cacodylate, 25 mmol/L Tris-HCl (pH 6.6), 0.25 g/L bovine serum albumin, 5 mmol/L $CoCl_2$, 42 μmol/L of each dNTP, 16.7 μmol/L Dig-dUTP, 100 pmol of probe, and 24 units of terminal transferase. The reaction mixture was incubated at 37 °C for 45 min, after which the labeled probe was purified two times by size exclusion chromatography on Sephadex G-25 disposable columns (Nap-5) equilibrated with 10 mmol/L sodium phosphate buffer, pH 6.8. The detection probe was labeled at the 3′ end with biotin-dATP. The reaction was carried out exactly as above but 0.5 mmol/L dATP and 0.05 mmol/L biotin-dATP were included in the reaction mixture instead of dNTP and Dig-dUTP, respectively. After labeling and purification, the probes were stored at −20 °C.

Preparation of Streptavidin−Luciferase Coding DNA Complex. An expressible DNA fragment containing the T7 RNA polymerase promoter and a luciferase-coding sequence was prepared as previously described[9] with some modifications. Briefly, a suitable plasmid was digested with Alw 44 I, which produced three fragments with recessed 3′ ends. The ends were filled-in with dGTP, dCTP, dTTP, and biotin-dATP by using the Klenow DNA polymerase. As a result, all the fragments were biotinylated at both termini. The DNA was purified from the excess of biotin-dATP by precipitation with 2 volumes of cold (4 °C) ethanol and 0.1 volume of 3 mol/L sodium acetate, pH 5.2. A small segment (0.49 kbp) was then removed from one end of the luciferase-coding DNA fragment just upstream of the T7 promoter, thus leaving a 2.1 kbp fragment labeled with biotin only at one terminus. After electrophoretic separation, the band of interest was excised, the DNA was purified, and its concentration was determined by densitometry. The biotinylated DNA was then complexed with a 30-fold molar excess of streptavidin in 1 mol/L NaCl. The complex was purified by HPLC (using a size exclusion column), concentrated, and stored at 4 °C in a solution containing 1 mol/L NaCl and 1 mmol/L EDTA, pH 8.0.

Hybridization Assays Using a Luciferase-Coding DNA Fragment as Label. "U" bottom polystyrene microtiter wells were coated overnight at 4 °C, by physical adsorption, with 25 μL of 5 mg/L anti-digoxigenin antibody solution in 0.1 mol/L sodium carbonate, pH 9.6. The wells were washed twice with wash solution, and the remaining binding sites were blocked with 100 μL blocking solution for 60 min at room temperature. The wells were washed twice as above and 25 μL of 2.4 nmol/L Dig-labeled capture probe, diluted in blocking solution, was pipeted into each well and incubated for 60 min. The unbound probe was then removed by washing the wells three times with wash solution. Subsequently, 10 μL of 10 nmol/L biotinylated detection probe, diluted in hybridization solution and preheated at 42 °C, was added into each well. The target DNA was denatured by heating at 95 °C for 7 min and kept on ice. A 15 μL aliquot of the denatured

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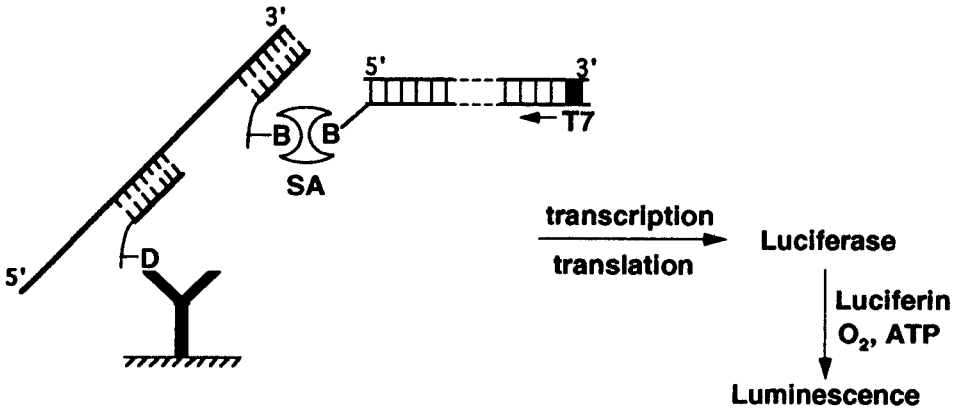

Figure 1. Principle of the hybridization assay using a luciferase-coding DNA fragment as a label. The target DNA is 200 bp and the expressible DNA label is 2.1 kbp. The relative positions of the capture and detection probes are shown: D = digoxigenin; B = biotin; SA = streptavidin; T7 = T7 RNA polymerase promoter. The arrow shows the direction of transcription and translation.

DNA target was then pipeted into each well. The hybridization of the target with the immobilized capture probe and the detection probe was allowed to proceed for 60 min at 42 °C with shaking. At the end of this incubation period, the wells were washed three times, to remove the excess of detection probe. Then, 25 μL of 1.7 mg/L (with respect to DNA) streptavidin−luciferase DNA complex, diluted in hybridization solution, was added into each well and incubated for 20 min. The wells were washed four times with wash solution and one time with cold (4 °C) TE buffer. Afterward, 25 μL of the transcription/translation mixture was added into each well and incubated at 30 °C for 90 min (in the G24 incubator) to allow for the expression of the solid-phase bound luciferase-coding DNA. The synthesized luciferase was then measured by pipeting 10 μL of the reaction mixture into 50 μL of substrate solution[11] (20 mmol/L tricine, 1.1 mmol/L magnesium carbonate pentahydrate, 2.7 mmol/L $MgSO_4$, 0.1 mmol/L EDTA, 33 mmol/L dithiothreitol, 270 μmol/L CoA, 530 μmol/L ATP, and 470 μmol/L luciferin, pH 7.8) in a microcentrifuge tube. The tube was placed in a glass scintillation vial, and the luminescence was measured for 1 min using a liquid scintillation counter in the single photon monitoring mode.

Fluorescence Hybridization Assays. The coating of the wells, the immobilization of the capture probe, and the hybridization reaction were carried out as described above (see Hybridization Assays Using a Luciferase-Coding DNA Fragment as Label) except that flat-bottom wells were used and the reagent volumes were 50 μL instead of 25 μL. After completion of the hybridization reaction, the wells were washed three times and 50 μL of 100 units/L streptavidin−alkaline phosphatase conjugate, diluted in hybridization solution, was pipeted into each well and incubated for 15 min. The wells were washed three times with wash solution, and 50 μL of substrate solution (0.1 mmol/L 4-methylumbelliferyl phosphate, 0.1 mol/L Tris, 0.1 mol/L NaCl, and 1 mmol/L $MgCl_2$, pH 9.1) was added into each well. After a 60 min incubation period, the solution was transferred into white wells and the fluorescence was measured with the microplate fluorometer.

Chemiluminescence Hybridization Assays. The reactions involved in the chemiluminescence hybridization assays, up to the addition of streptavidin−alkaline phosphatase conjugate, were as described above (see Fluorescence Hybridization Assays). Next, 50 μL of substrate solution (0.4 mmol/L CSPD, 1 g/L sapphire II, 0.1 mol/L diethanolamine, and 1 mmol/L $MgCl_2$, pH 9.5) was added into each well and incubated for 30 min. The wells were then placed in glass scintillation vials, and the luminescence was measured for 0.15 min with the liquid scintillation counter in the single photon monitoring mode.

RESULTS AND DISCUSSION

The principle of the hybridization assay is illustrated in Figure 1. The capture probe was tailed with Dig-dUTP, and after purification, it was bound to polystyrene wells coated with anti-digoxigenin antibody. Denatured DNA target was hybridized simultaneously with the immobilized capture probe and the detection probe which was tailed with biotin-dATP. The hybrids were reacted with a preformed streptavidin−DNA complex. The complex was prepared by mixing biotinylated luciferase-coding DNA with a large excess of streptavidin. This ensures that the complex has at least one site available for binding to the biotinylated detection probe. The assay was completed by expressing the solid-phase bound DNA label and measuring the activity of synthesized luciferase. Luciferase catalyzes the luminescent reaction of luciferin, O_2, and ATP to produce oxyluciferin, AMP, pyrophosphate, and CO_2.[12,13] Because the T7 promoter sequence is present only in the DNA label, the transcription/translation process uses the luciferase-coding DNA exclusively, and not the probe−target hybrids.

The time required for completion of the hybridization reaction was studied in the range of 15−120 min, and the results are presented in Figure 2. The signals reported are corrected for the background. The background is the luminescence obtained in the absence of target DNA and is a measure of nonspecific binding of the detection probe and the streptavidin−DNA complex to the solid phase. A rapid increase of the luminescence in the first 60 min of incubation is observed and then a plateau is reached where the signal increases by only 15% in the second hour of incubation. With the increase in hybridization time, the background did not change. The average value for the background was 29 477 counts/min. Thus, the hybridization time was kept constant at 1 h for all subsequent experiments.

The effect of the streptavidin−DNA concentration was studied by preparing various dilutions of the complex in hybridization

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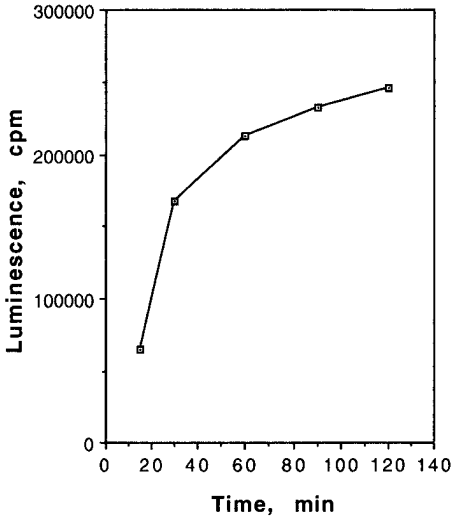

Figure 2. Time dependence of the hybridization reaction. The assays were carried out as described in the Experimental Section with 500 amol of target DNA. The concentration of streptavidin–luciferase DNA complex was 0.86 mg/L.

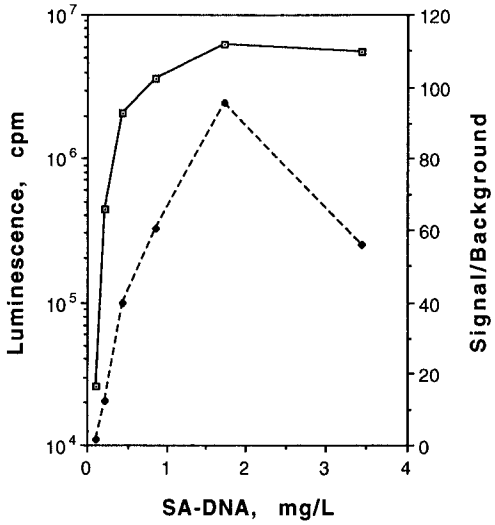

Figure 3. Optimization of the concentration of streptavidin–luciferase DNA complex. The solid and dashed lines represent the luminescence and the signal/background ratio, respectively. The hybridization assays were carried out as described in the Experimental Section with 500 amol of target DNA. The concentrations refer to the DNA label.

buffer and using them in the assay of 500 amol of target DNA (Figure 3). As the complex concentration is increased, a continuous increase of the luminescence signal is observed. A plateau is reached at 1.7 mg/L (30 fmol/well). At a higher concentration there is a significant increase of the nonspecific binding of the complex and the signal-to-background ratio drops.

In order to assess the sensitivity and linear range of the optimized assay, target DNA was serially diluted in a solution containing 1% (w/v) blocking reagent in water and aliquots were analyzed. In Figure 4A, the luminescence is plotted vs the amount of target DNA. As low as 5 amol of target can be detected with a signal-to-background ratio of 2. Also, the luminescence is linearly related to the amount of target DNA up to about 5000 amol. It is therefore proved that although the transcription/translation process consists of a series of complex (and not fully understood) reactions, which require the concerted action of numerous factors (such as RNA polymerase, translation initiation,

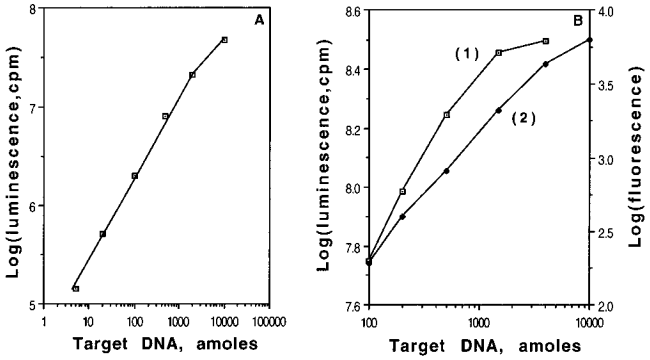

Figure 4. (A) Quantitation of DNA target by hybridization assay using the luciferase-coding DNA fragment as a label. The assays were carried out as described in the Experimental Section, and the luminescence was plotted against the amount of target DNA present in the well. (B) Quantitation of the target DNA by (1) a chemiluminescence and (2) a fluorescence hybridization assay. The assays were performed as described in the Experimental Section, and the luminescence or the fluorescence were plotted vs the amount of target DNA present in the well.

elongation and termination factors, ribosomal subunits, and aminoacyl-tRNA synthetases), the final outcome is a simple linear relationship between the target DNA (analyte) and the activity of the in vitro synthesized luciferase. In order to enhance the practicality of the assay, we performed the transcription/translation reaction in one step (addition of a single reagent).

The reproducibility of the proposed system was studied by analyzing 20 and 100 amol of target DNA in quadruplicate, in the same run. The CVs obtained were 6.5 and 10.8%, respectively ($n = 4$).

The proposed methodology was compared directly with a fluorescence and a chemiluminescence hybridization assay. In these assays a streptavidin–alkaline phosphatase conjugate was used, instead of the streptavidin–DNA complex, for detection of the hybrids. The activity of solid-phase bound alkaline phosphatase was then measured by using a fluorescent (4-methylumbelliferyl phosphate) or a chemiluminescent substrate (CSPD). The results are presented in Figure 4B. The fluorescence assay can detect 200 amol of target DNA with a signal-to-background ratio of 2.4. The chemiluminescence assay gave a signal-to-background ratio of 3.2 for 100 amol of target DNA. The curvature observed at high levels of target DNA is due to saturation of the liquid scintillation counter from the light produced.

The total time required for completion of the proposed assay is 230 min. The fluorescence and chemiluminescence hybridization assays are completed in 195 and 165 min, respectively. Nevertheless, the lower sample throughput of the expression-based assay is compensated by its higher sensitivity.

Generally, any DNA fragment that contains an enzyme-coding sequence positioned downstream from a potent RNA polymerase promoter can be used as a label. The luciferase-coding DNA was chosen in this work because luciferase is a single polypeptide chain (550 amino acids), it requires no posttranslational modification, and its activity can be readily measured in the transcription/translation mixture without prior purification. Because of these attractive properties, the luciferase cDNA has been used extensively as a reporter gene to monitor gene expression in various tissues.[14] However, to our knowledge, luciferase has not been

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used as a reporter molecule in hybridization assays. This is probably due to the significant loss of enzymatic activity upon conjugation.[15] Geiger et al. have used the firefly luciferase-catalyzed reaction in hybridization assays as an indicator reaction.[2] The probes were labeled with alkaline phosphatase, and luciferin phosphate was used as a substrate. The luciferin released was measured with high sensitivity using excess of luciferase. A distinct advantage of the system proposed here is that a luciferase-coding DNA fragment is used as a label instead of the enzyme itself, thus avoiding the problem of inactivation. The synthesized luciferase remains free in the solution.

Because enzymes are the most widely used nonradioactive labels in nucleic acid hybridization assays, recent efforts have focused on the design of substrates that allow monitoring of enzymatic activity with greater sensitivity. The highest sensitivities are achieved with substrates that are either converted to chemiluminescent molecules (e.g., CSPD for alkaline phosphatase) or give products that form fluorescent chelates with lanthanide ions allowing determination by time-resolved fluorometry (e.g., fluorosalicyl phosphate for alkaline phosphatase).[2,3] The assay described here is complementary to these efforts because, in principle, any enzyme-coding DNA may be used as a label in combination with the best substrate for that particular enzyme.

An alternative approach for further enhancing the sensitivity of hybridization assays is to attach multiple enzyme molecules to the probe−target hybrids, thus introducing an additional amplification step. This has been accomplished by using sequential hybridization with three types of probes.[16] The primary probe contains two parts, a region complementary to the target and a segment that allows binding of a polymerized secondary probe (amplification multimer). Then, each secondary probe reacts with an enzyme-labeled probe. Thus, branches of DNA are formed which carry several enzyme molecules. The present work represents a novel approach for introducing multiple enzyme molecules in the system, because from a single expressible DNA fragment several enzyme molecules (12−14 luciferase molecules) can be synthesized by in vitro transcription and translation.[9]

From our perspective, the following directions of ongoing research hold the potential for dramatically improving the sensitivity and versatility of the proposed system: (a) Efforts aiming at increasing the yield of the transcription/translation reaction. This can be accomplished by improving the DNA label and/or by optimizing the transcription/translation mixture. The design of labels containing appropriate enhancer and termination sequences will ensure the highest yield.[17,18] Also, the synthesis of capped RNA has been reported to increase the translation efficiency.[19] (b) Another area of investigation involves the preparation of expressible DNA fragments that code for other enzymes, the activity of which can be monitored with high sensitivity (e.g., alkaline phosphatase). (c) Finally, the sensitivity could be improved by conjugating, directly, the DNA label with the specific probe, thus eliminating the need for biotinylated reagents and streptavidin.

In conclusion, we have designed, for the first time, a hybridization assay that utilizes as a label an expressible DNA fragment coding for an enzyme. The assay is automatable because it is performed in microtiter wells and avoids blotting procedures and membrane hybridization. The high sensitivity achieved is a result of the combined amplification due to transcription/translation and the substrate turnover. In our opinion, the proposed system may have a significant impact on hybridization technology and clinical diagnostics.

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