Nonisotopic Detection Methods for Strand Displacement Assays of Nucleic Acids

Calvin P. H. Vary, Frank J. McMahon, Francis P. Barbone, and Steven E. Diamond

Using the enzymes terminal deoxyribonucleotidyltransferase (EC 2.7.7.31) and polynucleotide phosphorylase (EC 2.7.7.8), we constructed polyriboadenylated acid tracts, approximately 8000 AMP residues long, attached to the 3'-terminus of a synthetic deoxy-nucleotide. The polyadenylated DNA, termed the "signal strand," was used in a displacement-type nucleic acid probe assay (see pp 1631–6, this issue). A probe–signal strand complex was made by hybridizing the signal strand to a deoxyribotidylate-terminal probe DNA. The probe–signal strand complex was immobilized on an oligo (dG)-cellulose support and subsequently displaced from the immobilized hybrid complex with various amounts of analyte DNA. After the displacement procedure, the polyadenylate tracts were converted to ATP by the combined action of polynucleotide phosphorylase and pyruvate kinase. ATP was quantified by a bioluminescence assay with luciferase from Photinus pyralis. Displacement events were also quantified with biotinylated signal strand bound to avidin-conjugated horseradish peroxidase. Such enzyme-amplified assays offer considerable versatility: they may be coupled to a variety of detection systems including colorimetric, fluorimetry, and luminometry.

Additional Keyphrases: enzymatic assay • DNA • bioluminescent assay • polynucleotidyl acid • probes

The detection of specific nucleic acid sequences through the use of hybridization probes has been established for some time (1). These methods typically involve the immobilization of the analyte on a solid support (e.g., nitrocellulose, diazobenzylomethyl cellulose, nylon, etc.). The immobilized nucleic acid is then denatured, if it is double stranded, and subsequently hybridized to a complementary probe. The probe DNA is commonly labeled with 32P, or nonisotopically with a biotin–avidin–enzyme system (2). In contrast to radiosotopically labeled probes, nonisotopic systems offer advantages of safety, relatively low cost, and ease of use. However, enzymatic detection of adsorbed nucleic acid analytes suffers from high background values (3), e.g., from nonspecific adsorption of biotinylated probe and avidin–enzyme complexes to the solid support. Such nonspecific adsorption necessitates multiple washing steps, which add to the length and difficulty of the procedure.

A novel method for detecting a specific polynucleotide sequence involves the displacement of a labeled nucleic acid. A labeled polynucleotide "signal strand" is hybridized within a larger DNA sequence (the "probe strand"), which is, in turn, complementary to the analyte of interest. Interaction of the adsorb complex with a nucleic acid analyte displaces the signal strand from the immobilized complex. After separating the bound from the displaced signal strands, one quantifies the latter by measuring the isotopic or nonisotopic labels in the signal strands. Such assays are potentially more sensitive because of the reduction in background noise, with the nonspecific adsorption not giving rise to signal.

Here we describe a preliminary evaluation of colorimetric and bioluminescence detection systems involving model probe and analyte DNAs in conjunction with the polynucleotide displacement approach (4). The bioluminescence detection system involves a polycrboadenylated signal strand. Displacement events are detected by the bioluminescence of firefly luciferase (EC 1.13.12.7) after the displaced polyriboadenylate tracts are converted first to ADP and then to ATP by the sequential action of polynucleotide phosphorylase (PNP; polynucleotide nucleotidyltransferase, EC 2.7.7.8) and pyruvate kinase (EC 2.7.1.40). The resulting ATP is then converted to AMP by firefly luciferase with the accompanying emission of light. Using this assay system and the displacement procedure, we can currently detect as little as 10-15 mol of model analyte DNA.

The colorimetric assay involves a 27-meric signal sequence derivatized with a short chain (one to seven residues) of biotinylated deoxyuridine monophosphate residues. Biotinylated signal strands are subsequently conjugated with avidin–enzyme complexes. After exposure of the probe–signal complexes to analyte DNA, the displaced, free signal strands bearing bound avidin–enzyme conjugates are collected. Signal strand avidin–enzyme complexes are subsequently detected by using the chromogenic substrate o-phenylenediamine. The enzyme-amplified detection system currently provides a limit of detection of about 10-12 to 10-13 mol of analyte DNA, which corresponds to the limit of detection expected for a single enzyme label per signal strand.

Materials and Methods

Materials

Primer-dependent PNP (140 kU/L), ADP (disodium salt), and oligo(dG)-cellulose (Type 7) were obtained from Pharmacia, Inc., Piscataway, NJ; ATP (disodium salt), pyruvate kinase (200 kU/g), phosphoenolpyruvate, and HS™ and CLS™ bioluminescence reagent kits were from Boehringer Mannheim Biochemicals, Indianapolis, IN. Terminal deoxyribonucleotidyl transferase (TdT; DNA nucleotidylxotransferase, EC 2.7.7.31) was purchased from International Biotechnologies Inc., New Haven, CT. TdT buffer was prepared as described elsewhere (5). Biotinylated-dUTP and T4 polynucleotide kinase (polynucleotide 5'-hydroxyl kinase; EC 2.7.1.78) were obtained from Bethesda Research Laboratories, Gaithersburg, MD; NENSORB™ columns, GENESCREEN™, and γ-[32P]ATP (8000 kCi/mol) from

Allied-Signal Inc., Corporate Research, P.O. Box 1021R, Morris-town, NJ 07960.

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New England Nuclear, Boston, MA; and avidin–horseradish peroxidase (EC 1.11.1.7) conjugate was obtained from Vector Laboratories, Burlingame, CA. LuminoDex cuvettes were purchased from LKB Wallac, Gaithersburg, MD.

Procedures

Construction of the DNA probes. Synthetic 50-meric probe DNA and its complement were produced by a Model 380A DNA Synthesizer (Applied Biosystems, Foster City, CA). The oligonucleotides were purified by electrophoresis on preparative 10% denaturing polyacrylamide gels; the bands corresponding to full-length products were excised and eluted with an electro-elution apparatus (International Biotechnologies). The DNA was quantified by absorbance at 260 nm. Using TdT and dCTP, we derivatized the 50-mer probe DNA at its 3'-terminus with a poly(dC) tract. The poly(dC)-terminal probe DNA was further purified by chromatography on an oligo(dG)-cellulose column. After incubating the TdT reaction mixture for 120 min at 37 °C, we added the sample to the oligo(dG)-cellulose column material (approximately 15–20 mg dry weight), which had been equilibrated with Buffer A (per liter, 0.2 mol of NaCl and 10 mmol of Tris·HCl, pH 8.0), and placed the columns on ice for 30 min. The columns containing the poly(dC)-terminal probe DNA were washed repeatedly with Buffer A, and the product was eluted with water.

Riboadenylation of DNA. We reacted 10 pmol of a 27-meric-base synthetic oligonucleotid, designated PM27, with 15 U of TdT and 2 mmol of ATP (final volume, 20 μL) in TdT buffer for 60 min at 37 °C. The resulting riboadenylated product (PM27tA) was purified by chromatography on a NENSOR™ 20 column to remove unreacted nucleotides. After being eluted with an equivalent mixture of methanol and water, the solvent was evaporated and the residue containing the product was dissolved in water to give a final concentration of 1 pmol per 20 μL. We then added 1–2 pmol of the riboadenylated 27-mer to 20 μL of a solution containing 0.14 U of PNP in a pH 8.5 buffer containing, per liter, 5 mmol of ADP, 10 mmol of Tris·HCl, 10 mmol of 2-mercaptoethanol, and 5 mmol of MgCl₂. After incubation at 37 °C for 1 h, the polyriboadenylated DNA, termed PM27tAₙ, was purified by NENSOR column chromatography. We then removed the water and methanol by evaporation, redissolved the product in 0.5 mol/L NaCl solution to give a concentration of approximately 0.5 μmol/L, and precipitated the DNA by adding three volumes of ethanol. The precipitates were collected by centrifugation for 30 min and redissolved in water to give a final concentration of 0.1 μmol/L.

Labeling signal strands with ³²P. The 27-mer signal strand was labeled at its 5'-terminus with T₄ polynucleotide kinase and γ[³²P]ATP. Unincorporated ATP was removed by chromatography on either DE-52 cellulose (Whatman) or NENSOR columns.

Probe–signal strand hybridization. Polyadenylated signal strand, 5'-[³²P]PM27tAₙ, was hybridized to the (dC)-terminal 50-mer DNA in Buffer A at 50 °C for 1 h. The total strand concentration was approximately 0.2 μmol/L.

Displacement. We added a small aliquot of the probe–signal complex to each individual oligo(dG)-cellulose column (15 mg dry weight), then placed the columns on ice for 30 min, after which we washed them repeatedly with Buffer A. Competitor DNA was then added to individual columns in Buffer A, in a volume comparable with that occupied by the probe–signal complex. The columns were then inserted into an aluminum heating block and heated at 50 °C for 30 min, after which the displaced signal strands were eluted with a 0.2 mL wash of Buffer A.

Phosphorolysis of PM27tAₙ and conversion to ATP. Signal strand polyadenylate tracts were converted to ATP by incubation for 60 min at 37 °C in a solution containing, per liter, 0.1 mol of Tris·HCl (pH 8.5), 10 mmol of 2-mercapto-ethanol, 10 mmol of phosphate, 24 mmol of phosphoenolpyruvate, 5 mmol of MgCl₂, 2 U of pyruvate kinase, and 0.14 U of PNP in a final volume of 20 μL.

Bioluminescence measurement. After adjusting the sample volumes to 250 μL with de-ionized distilled water, we measured their bioluminescence, using either CLS or HS Bioradiolization ATP reagent and a Model 1251 luminometer (LKB, Bromma, Sweden). The composition of the CLS reagent is as follows: 4-(2-hydroxyethyl)-1-piperazineethanesulfonate buffer, pH 7.75, 40 mmol/L; luciferase from Pho/itus pyralis, 1.6 μg/mL; d-luciferin, 700 μmol/L; magnesium chloride, 20 mmol/L; EDTA, 4 mmol/L; dithiothreitol, 0.36 mmol/L; AMP, 0.3 mmol/L. The composition of the HS reagent is as follows: 4-(2-hydroxyethyl)-1-piperazineethanesulfonate buffer, pH 7.75, 25 mmol/L, luciferase (P. pyralis), 2.5 μg/mL; d-luciferin, 450 μg/mL; magnesium chloride, 6.25 mmol/L; EDTA, 0.625 mmol/L; dithiothreitol, 75 μmol/L; ATPase, Mg²⁺-activated, traces.

Biotinylation of signal strands. We added 5'-[³²P]PM27 at its 3'-terminus one to seven biotinylated-dUMP residues, using TdT as described above for the poly(dC) reaction.

Displacement. Hybridization of biotinylated 5'-[³²P]PM27 signal strand with the (dC)-terminal 50-mer DNA, its adsorption to the oligo(dG)-cellulose, and subsequent displacements were performed as described for polyadenylates signal strands.

Colorimetric determination of horseradish peroxidase. o-Phenylenediamine was dissolved in 0.1 mol/L sodium citrate buffer, pH 5.0, at a concentration of 1 g/L. We then added 0.3 mL of the o-phenylenediamine solution to a glass test tube plus an equal volume of H₂O₂, 0.12 g/L, in citrate buffer, then added 0.2 mL of the sample, and shook the tubes at room temperature for 30 min. The incubation reaction was stopped by adding 0.2 mL of H₂SO₄, 2 mol/L. Absorbance was measured at 490 nm with a spectrophotometer (Lambda 5; Beckman Instruments, Brea, CA).

Standard curves of the peroxidase–avidin concentration vs A₄₉₀ were linear in the range of 0 to 2, corresponding to 0 to 100 pmol of enzyme–avidin conjugate. For greater absorbance values, we made a small correction for nonlinearity, to avoid underestimating enzyme concentration.

Results

Bioluminescent Detection of Polyadenylated Signal Strands

For riboadenylation of synthetic DNAs (Figure 1) we used TdT, followed by primer-dependent PNP in the presence of ADP. TdT has only marginal ability to transfer ribonucleoside phosphate residues to a DNA terminus (6). The reaction reaches completion after one to two residues are transferred to the oligodeoxyribonucleotide. Reactions in which riboadenylation occurred were present with riboadenylation DNAs indicate that only ribonucleotides-amplified DNA results as substrate for primer-dependent PNP (6). Polyadenylate polymerase (EC 2.7.7.19) may also be used with riboadenylation primers, but this results in much shorter polyadeny-
Polyadenylate constant probe activity phosphate signal amounts strand DNA (data exonucleolytic late 1698 FIg. Ag. VP-DNA P-DNA---f 2. Reaction scheme for production of 3'-polyadenylated signal strand DNA late tracts, possibly the result of an associated 3' to 5' exonuclease activity (7).

We used probe strands consisting of synthetic 50-mer DNA extended with approximately 100 deoxycytidyl monophosphate residues. Shorter poly(dC) tracts gave rise to unacceptable background from dissociation of the probe DNA from the oligo(dG)-cellulose support. Maximum sensitivity was achieved by using the least poly(dC)-terminal probe DNA needed to completely bind all of the signal strand DNA. We determined this amount by titrating a constant amount of signal strand DNA with increasing amounts of probe DNA, then determining the fraction of signal strand bound to probe strand by gel electrophoresis (data not shown).

Figure 2 illustrates the reaction sequence leading to the bioluminescent detection of a target DNA sequence by on-column strand displacement. Hybridization of target DNA sequences to the probe--signal strand complex displaces the signal strands. After being eluted from the solid support, the poly(A) tracts associated with the displaced signal strands are converted to ATP and subsequently detected by bioluminescence. The bioluminescent yield obtained, expressed as a function of the numbers of moles of 5'-[32P]PM27rAe and decarboxyadenylie acid standard, (Ap)6A, is shown in Figure 3. The curve for PM27rAe is displaced from that for (Ap)6A by approximately 2.9 log units. This indicates a molar bioluminescent yield nearly 800-fold that obtained for (Ap)6A and, therefore, corresponds to a poly(A) tract length of approximately 8000 for PM27rAe. This value has been independently confirmed by comparing the electrophoretic mobilities of 5'-[32P]PM27rAe vs single-stranded nucleotides 8000 residues long (data not shown). The inset of Figure 3 is an autoradiogram demonstrating the complete degradation of the poly(A) tract. The last ribonucleotide residue cannot be phosphorolyzed, given the chemical nature of that particular phosphodiester linkage.

Figure 4 shows the dose-dependent displacement of 5'-[32P]PM27rAe by competitor 50-mer DNA as measured by both radioactivity and bioluminescence. Increasing the concentration of competitor results in a corresponding displacement of PM27rAe, as evidenced by the increasing signal in the effluent eluted with 0.2 mol/L NaCl. Note that the bioluminescence yielded by a given quantity of signal strand reacted with the HS reagent is not strictly comparable with that obtained with the CLS reagent.

![Fig. 1. Reaction scheme for production of 3'-polyadenylated signal strand DNA](image1)

![Fig. 2. Polyadenylate-driven bioluminescent detection of displacement](image2)

![Fig. 3. Bioluminescent detection of polyadenylated DNA and decarboxyadenylic acid](image3)
Colorimetric Determination of Peroxidase–Avidin-Labeled Signal Strands

Analyte DNAs can be detected by using enzymatically labeled signal strands. Figure 5 illustrates the sequence of reactions leading to the colorimetric detection of a target DNA sequence. The constituents of the ternary complex, probe/biotinylated signal/avidin–enzyme, were assembled on small oligo(dG)-cellulose columns. After displacement of the enzyme-labeled signal strand by competitor DNA, and elution from the support, the enzymatic activity associated with the displaced signal strands was determined by addition of the appropriate substrates. Figure 6 shows displacement of signal strand labeled with 32P as well as that labeled with biotin–avidin–peroxidase. In both cases the curves represent the displaced signal strand as a percentage of the total signal eluted (color or radioactivity) in the Buffer A wash and the water washes. For a given quantity of input competitor DNA, slightly less color than radioactivity was eluted for the peroxidase–avidin-labeled signal strands. This may result from a loss of enzyme activity with time or, alternatively, from the adsorption of a small amount of enzyme to the cellulose during column preparation, which was subsequently eluted from the column during the water wash used to obtain an estimate of total enzyme bound.

A detection limit of approximately 1 pmol of competitor DNA correlates well with data expected for a single enzyme–avidin conjugate bound per signal strand, and suggests that the displacement efficiency of the on-column assay approaches 100%. The colorimetric measurements involved a background signal of approximately 2–3%. Calibration assays with peroxidase alone indicate a lower limit of detection for the colorimetric assay in the range of picomoles of enzyme (data not shown).

Discussion

The nucleic acid displacement assay geometry is easily adapted to several nonisotopic methods for the detection of
specific DNA sequences, and may be conducted in either of two formats, on-column or in solution. The on-column displacement occurs within the solid support, and displaced signal strands are collected by elution from the support. The solution format requires a post-displacement separation to isolate displaced signal strands from those still bound to the probe DNA. The on-column approach, used here, has several advantages over the in-solution approach, including speed, simplicity, and the elimination of a reagent purification step (a principal source of background, unhybridized signal strand, is removed from the column by a wash step before analysis). In terms of the time needed to execute a single assay, isotopic readout is the fastest. For bioluminescent as well as isotopic readouts, the serial processing of samples during the automated stage of luminescence and scintillation spectrometry, respectively, would be the time-consuming stage of a large batch operation. In contrast, the enzyme-amplified analysis of a large number of samples can be conducted in parallel on one solid-support, resulting in a much shorter turnaround time per sample. Therefore, these different assays offer considerable versatility, depending on an individual's needs for sensitivity or speed of processing.

Labeling the 3'-terminus of the probe DNA with a polycl(C) tract provides a means for adsorbing the probe–signal complex to the solid support, oligo(dG)-cellulose. A limitation, however, is the difficulty of terminating large, naturally occurring DNAs with polydeoxyribodiplate tracts of sufficient length to provide efficient adsorption to the support. An alternative approach would involve direct covalent coupling of the probe DNA to a solid support—or the enzyme could be directly coupled to the signal strand to be used with the biotinylated probe strands. In the latter case, solution displacement would be followed by trapping the probe signal complexes on an avidin–agarose column.

The sensitivity achieved in this study reflects the fact that no more than one enzyme molecule is associated with each signal strand. The colorimetric assay, which involves a biotin–avidin linkage, has the potential for increased sensitivity through the addition of multiple avidin–enzyme molecules per signal strand and the use of polynzymes (8). In addition, long homopolymeric or mixed heteropolymers may be. An advantage associated with enzyme-amplified assays involving avidin–peroxidase and avidin–alkaline phosphatase is the adaptability of these enzymes to alternative, more sensitive readout systems. For example, alkaline phosphatase may be measured with fluorescence detection of methyleneumbelliferone phosphate after hydrolysis (9) or with bioluminescence detection via the conversion of NADP+ to NAD+ followed by quantification with bacterial bioluminescence (10, and F. McMahon and C. Vary, unpublished results). Horseradish peroxidase is adaptable to enhanced chemiluminescent detection with luminol and hydrogen peroxide as substrates (11).

Another way to increase sensitivity and ease of use would be to trap displaced signal strands on a membrane. Experiments with avidin–alkaline phosphatase or avidin–horseradish peroxidase, one enzyme molecule per signal strand, followed by capture and visualization of signal strands on supports of nitrocellulose or Octascreen, yielded detection limits in the attomole (10^-18) range (data not shown). A noticeable background from a slight dissociation of bound complexes is a persistent problem. As indicated above, direct coupling of the probe DNA to the support should minimize this.

In contrast to enzyme-amplified detection systems, the poly(A)-driven readout is protein free during the probe reagent construction, column assembly, and displacement reactions. Therefore, one need exercise no special care to protect labile components such as those present in the enzyme-amplified assay. Consequently, displacements may be conducted over a wider range of solution compositions, including those that would be harmful to enzymes—e.g., in the presence of chaotropic agents such as guanidinium chloride, sodium dodecyl sulfate, and high salt concentrations, as well as exposure to high temperatures. As we have thus far demonstrated, the poly(A)-driven assay coupled with bioluminescence is more sensitive than the enzyme-amplified assay because of the presence of only one enzyme per signal strand in the amplified assay and the inherent sensitivity of the bioluminescent determination (12). The HS-bioluminescent reagent can detect as little as 10^-16 mole of ATP in a 200-μL reaction mixture (13); therefore, the theoretical lower limit of detection of signal strands with 8000 adenylyle residues per strand should be 10^-19 to 10^-20 mol.

So far we have not reached this limit of sensitivity, primarily because of the luminescent background from the reagents used. Assays of ATP must take into account the presence of endogenous ATP and ADP, which can contribute to background signals. One approach we have used to remove luminescent background is to treat the reagents with apyrase (EC 3.6.1.5) or nucleotide pyrophosphatase (EC 3.6.1.9) linked to cyanogen bromide-activated Sepharose (F. McMahon and C. Vary, unpublished results). Another potential source of background is de novo synthesis of polyadenylate tracts during polyadenylation of the signal-strand DNA, given that the enzyme preparation contains a small amount of contaminating primer-independent PNP activity. However, unprimed poly(A) synthesis is slight and poly(A) tracts without associated DNA sequences are removed during preparation of the oligo(dG)-cellulose bound complex.

Another factor potentially limiting the sensitivity of the poly(A)-driven assay is inefficient conversion of poly(A) tracts to ATP at very low concentrations of poly(A). Work is proceeding to link the poly(A)-dependent production of ATP to various amplification systems, including the ATP-driven production of NADH or NADPH, which is detectable by bacterial luciferase (12). Such cascade systems may allow a closer approach to the sensitivity potentially achievable with this system.

References


