A homogeneous nucleic acid hybridization assay based on strand displacement

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ABSTRACT

A homogeneous nucleic acid hybridization assay which is conducted in solution and requires no separation steps is described. The assay is based on the concept of strand displacement. In the strand displacement assay, an RNA "signal strand" is hybridized within a larger DNA strand termed the "probe strand", which is, in turn, complementary to the target nucleic acid of interest. Hybridization of the target nucleic acid with the probe strand ultimately results in displacement of the RNA signal strand. Strand displacement, therefore, causes conversion of the RNA from double to single-stranded form. The single-strand specificity of polynucleotide phosphorylase (EC 2.7.7.8) allows discrimination between double-helical and single-stranded forms of the RNA signal strand. As displacement proceeds, free RNA signal strands are preferentially phosphorylated to component nucleoside diphosphates, including adenosine diphosphate. The latter nucleotide is converted to ATP by pyruvate kinase (EC 2.7.1.40). Luciferase catalyzed bioluminescence is employed to measure the ATP generated as a result of strand displacement.

INTRODUCTION

Currently, there exists a need for sensitive, rapid and convenient methods for the detection of nucleic acid sequences. The importance of nucleic acid based diagnostic assays is especially evident in cases where pathogens such as Epstein-Barr virus(1,2) or cytomegalovirus(3) are latent or difficult to culture. An additional advantage of nucleic acid based diagnostic assays arises in cases where the presence of a viral nucleic acid may be a more accurate indication of potential infectivity than the presence of antigen, as appears to be the case with hepatitis B virus presence in serum(4).

One limitation to the widespread use of nucleic acid assays in clinical diagnosis has been the reliance on isotopic readout systems. Isotopic labels are, in many applications, to be avoided since they pose a health risk to the individuals performing the assay and require special containment procedures. A frequently employed approach to nonisotopic detection takes advantage of the strong affinity of avidin for biotin which may be incorporated into nucleic

acid probes via biotinylated nucleoside analogs (5). Hybridization assays are often conducted following adsorption of the sample nucleic acid on solid supports such as nitrocellulose. Hybridization events using biotinylated probe nucleic acids are detected through the use of avidin-enzyme conjugates such as avidin-horseradish peroxidase(6) and avidin-alkaline phosphatase(7). These methods, however, frequently suffer from elevated background signals due to non-specific adsorption of the enzyme and/or probe nucleic acids to the solid support. Non-specific adsorption necessitates extensive and laborious blocking and washing steps in order to minimize this problem (8). In addition, partial homology of the probe nucleic acid to nucleic acid sequences other than those of primary interest, can be a source of false positive results(9). Blot procedures generally require prior adsorption of sample DNAs to a membrane support prior to analysis. This practice results in lower inherent sensitivity since adsorbed nucleic acids can become partially inaccessible to hybridization (10). To circumvent these problems, an alternative approach based on strand displacement has been described (11, 12). This analytical geometry utilizes a signal strand DNA homologous to the target DNA sequence which is in turn hybridized to a larger single-stranded probe DNA. The probe DNA contains a region complementary to the signal strand as well as a contiguous region of sequence complementary to the target DNA. The probe-signal hybrid complex is contacted with a sample containing denatured DNA. If the target DNA sequence is present, hybridization will occur initially within the single-stranded region of the probe DNA. Branched fork migration subsequently causes the target nucleic acid to anneal through the region in which the signal strand is bound resulting in release of the signal strand from the probe-signal reagent complex. False positive signals arising from partial homology between probe DNAs and contaminating nucleic acids are suppressed since displacement of the signal strand occurs only if the hybridized sample nucleic acid possesses sufficient complementarity to the probe DNA to effect branch migration through the signal binding region.

Quantitation of the amount of displacement takes place after separation of displaced signal strands from those signal strands still bound to the probe DNA. Separation of displaced signal strands from probe-signal reagent complexes may be accomplished by capture of an affinity label present on the probe DNA strand. For instance, poly-(dC) tailed probe DNAs may be captured on oligo-(dG) cellulose supports whereas displaced signal strands are not retained by the oligo-(dG) cellulose. Further simplification is possible since displacement reactions can be conducted within the solid support itself(12).

In this case the displaced signal strands are simply spun out of the support prior to detection.

Since the released signal strand is present in solution, a variety of analytical methods may be used to measure displacement. Displacement assays have been coupled to colorimetric readout systems by binding avidin-horse radish peroxidase and avidin-alkaline phosphatase conjugates to biotinyl-dUTP tailed signal strands (12, F.P. Barbone and C. Vary, unpublished results). A different approach to nonisotopic detection involves tailing of DNA signal strands with long polyriboadenylic acid tracts. Phosphorolysis of the poly-(rA) tracts associated with displaced signal strands liberates as many as 8000 ADP molecules per signal strand (12). Pyruvate kinase is used to convert the ADP residues to ATP for detection by bioluminescence(13) or other ATP dependent assays (14). RNA signal strands can serve as a source of ATP, thereby eliminating the need for covalent attachment of poly-A tracts to the signal strand.

These methods, as well as other heterogeneous systems including sandwich hybridization (15) and strand capture techniques (16) require one or more separation steps to remove excess unbound probe nucleic acids. Since these manipulations all require time and are labor intensive, elimination of separation steps would be advantageous. Homogeneous assays, in which all signal generating reactions occur in a single phase, eliminate the need for separation steps. Such assays have found application in certain immunological diagnostic tests (17). In contrast to the situation with immunological procedures, no simple homogeneous assay for nucleic acids has been reported. This paper describes a homogeneous nucleic acid assay in which the strand displacement nucleic acid assay geometry is combined with specific degradation of released RNA signal strands, yielding products which are ultimately detected by firefly luciferase catalyzed bioluminescence.

MATERIALS AND METHODS

Primer-dependent PNP (140 kU/L) and ADP (disodium salt) were obtained from Pharmacia Inc., Piscataway, NJ; ATP (disodium salt), pyruvate kinase (200 kU/g), phosphoenolpyruvate and CLS bioluminescence reagent kits were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Apyrase, grade VIII was obtained from Sigma Chemical Co., St. Louis, Mo. Alpha-[³²P] ATP (3000 Ci/mmol) was obtained from New England Nuclear, Boston, MA. Luminometer cuvettes were purchased from LKB Wallac, Gaithersburg, MD. *In vitro* transcription reagents were purchased in kit form from Promega Biotech Inc.,

Madison, WI. All other chemicals were reagent grade.

Cloning and preparation of DNA

A 1.1 kilobase pair (Kbp) DNA fragment resulting from the cleavage of plasmid pBR-322 DNA with restriction endonucleases PstI and BamHI was subcloned into PstI and BamHI cut double-stranded replicative forms of M13mp8 and M13mp19 viral DNAs, providing constructs, designated M13mp8-20 and M13mp19-3/2 respectively, in which the insert exists in both orientations. This procedure allows production of single-stranded DNAs which contain either orientation of the 1.1 Kbp insert(11). Clones containing the 1.1 kilobase fragment were selected and phage carrying the plus strand were prepared from culture supernatants of infected *Escherichia coli* strain JM101. A 0.375 Kbp DNA fragment contained within the aforementioned 1.1 kbp DNA was obtained by digestion of plasmid pBR-322 DNA with restriction endonucleases EcoRI and BamHI. This fragment was subcloned into the transcription competent plasmid pSP65 following digestion of the latter with the restriction endonucleases EcoRI and BamHI (L. Laughlin and M. Ellwood, unpublished results). Following transformation of *E. coli* strain HB101 with the pSP65 derivative, termed pSP65-15, colonies were selected and plasmid DNA isolated by a standard alkaline lysis procedure (18). DNAs used as model samples for bioluminescence analyses were rendered free of contaminating nucleoside di- and triphosphates and RNA by sequential treatment with apyrase and sodium hydroxide, respectively. DNA (10-100 ug) was incubated with 1 unit/ml apyrase at 37°C for 1 hour in the presence of 0.05 moles/liter Tris HCl (8.0) and 0.01 moles/l MgCl₂. The enzyme was inactivated and any contaminating RNA was removed by the addition of 0.2 moles/liter NaOH followed by incubation at 90°C for 30 minutes. The DNA was precipitated by addition of 0.3 moles/l of sodium acetate (final pH 6.0) and 3 volumes of cold ethanol.

Preparation of RNA signal strands

Plasmid pSP65-15 template DNA was linearized by cleavage with the restriction endonuclease EcoRV. A 195 nucleotide (nt) RNA signal strand was obtained by *in vitro* transcription of the linearized template DNA directed by the viral SP6 transcription promoter (19). This transcript is complimentary to the Bam HI proximal portion of the 1.1 Kbp insert present in M13mp8-20. 1 ug of EcoRV linearized DNA was incubated for 90 minutes in a 50 ul reaction containing 40 mmol/liter Tris HCl (pH 7.5), 6 mmol/liter MgCl₂, 2 mmol/liter spermidine, 10 mmol/liter NaCl, 10 mmol/liter dithiothreitol, 0.5 mmol/liter of the nucleotides GTP, CTP and UTP, 0.1 mmol/liter ATP, 250 uCi of alpha[³²P]ATP and 80 units of RNasin. One unit of RNasin activity is defined

as that amount required to inhibit by 50% the activity of 5 nanograms (ng) of ribonuclease A. The reaction was initiated by the addition of 45 units of SP6 RNA polymerase. One unit of polymerase is defined as that amount of enzyme required to catalyze the incorporation of 1 nmole of labeled nucleoside triphosphate into acid insoluble product in one hour at 37°C. Following the incubation period, the reaction mixture was treated with 1 unit of RNase free DNase per microgram of template DNA for 15 minutes at 37°C. The reaction mixture was adjusted to a concentration of 5 moles/liter urea and 0.01% (w/v) bromphenol blue and xylene cyanol blue. This sample was loaded onto a 10% semi-preparative polyacrylamide gel containing 8.3 moles/liter urea, 89 mmol/liter boric acid, 89 mmol/liter Tris base (pH 8.3), and 2 mmol/liter disodium ethylenediaminetetraacetic acid. Electrophoresis was conducted at 200 volts for sufficient time to allow the xylene cyanol to migrate 10 cm. The band corresponding to the 195 nucleotide RNA product was located by autoradiography of the gel and excised. RNA was extracted from gel slices by electroelution, precipitated overnight at 4°C in the presence of 66% (v/v) ethanol and 1 mole/liter sodium acetate (pH 5.6).

Preparation of RNA:DNA hybrids

The concentration of each preparation of RNA was estimated by hybridization with known amounts of complementary M13mp8-20 single-stranded DNA. Hybridization was carried out for 60 minutes at 50°C in the presence of 0.4 moles/liter NaCl, and 0.04 moles/liter Tris HCl (pH 8.0). Following electrophoresis of hybrids on 0.8% agarose gels, quantification of the binding of the RNA was achieved by excision and counting of the gel slices corresponding to the positions of bound and free RNA.

Phosphorolysis reactions

Quantitation of phosphorolysis was achieved by counting resolved nucleoside di- and triphosphate fractions following chromatography of reaction aliquots (1-5 ul) on strips of polyethyleneimine cellulose (PEI). Samples were spotted near the bottom of the PEI strip, along with 1 ul aliquots of 20 mmol/liter authentic ADP and ATP standards. Chromatograms were developed in a solution of 0.8 moles/liter acetic acid and 0.8 moles/liter lithium chloride. After the strips were dried, the positions corresponding to ADP and ATP were visualized with short wave ultraviolet light, excised and quantitated by scintillation counting.

Displacement reactions

Displacement of the RNA from the RNA:DNA complex was achieved by incubation of the complex with samples composed of single-stranded M13mp19-3/2

DNA and heat denatured calf thymus DNA as carrier. Displacement was found to be complete in 60 minutes when conducted at 50°C in the phosphorolysis/kinase signal strand conversion buffer (data not shown). In addition to sample nucleic acid, each reaction mixture contained per liter, 0.1 mol of Tris-HCl (pH 8.5), 10 mmol of 2-mercaptoethanol, 10 mmol orthophosphate, 24 mmol of phosphoenolpyruvate, 5 mmol of MgCl₂, 2 U of pyruvate kinase, and 0.04 U of polynucleotide phosphorylase in a final volume of 20 µL.

Bioluminescence measurements

Following displacement, sample volumes were adjusted to 250 µL with de-ionized distilled water. The assay was initiated by addition of an equal volume of bioluminescence monitoring reagent. Following a three minute delay period, steady state bioluminescence was monitored for one minute in a Model 1251 luminometer (LKB, Bromma, Sweden). The composition of the CLS bioluminescence monitoring reagent is as follows: Hepes buffer, pH 7.75, 40 mmol/L, luciferase from *Photinus 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.

RESULTS

The scheme for a homogeneous DNA hybridization assay is portrayed in figure 1. This method is based on the displacement approach to detection of hybridization of a target nucleic acid (11, 12) and is modified here to incorporate an RNA signal strand. Single-stranded M13 phage derived DNA probe molecules are constructed so as to contain a region of complementarity with the target nucleic acid. The region of complementarity is co-extensive with the RNA binding region and extends past it in single-stranded form on one side of the RNA. This method relies on the protection from phosphorolysis afforded the RNA signal strand by the DNA to which it is annealed. Initial hybridization of a target DNA sequence to the single-stranded portion of the probe DNA is followed by "zipping up" of the helix, resulting in displacement of the RNA. Only the displaced RNA is single-stranded and therefore susceptible to phosphorolysis and is converted to nucleoside diphosphates by the action of polynucleotide phosphorylase. Pyruvate kinase subsequently converts the ADP to ATP which can be detected by a number of methods including luciferase catalyzed bioluminescence in the readout stage of the assay.

In figure 2, denaturing polyacrylamide gel electrophoresis and non-denaturing agarose gel electrophoresis of phosphorolysis reactions was used to estimate the extent to which the 195mer RNA signal strand is protected

Figure 1. Displacement and detection of DNA sequences using a homogeneous format.

from phosphorolysis by hybridization to the single-stranded complementary probe DNA (195mer RNA, fragments and nucleotide comigrate on the agarose gel). As shown in panel A, no detectable reduction in radiolabel corresponding to RNA-DNA hybrid was observed when the concentration of polynucleotide phosphorylase was increased to a level 175 fold in excess of that sufficient to cause extensive phosphorolysis of free RNA.

Denaturing polyacrylamide gel electrophoresis allowed further examination of the RNA signal strand for cryptic nicking or removal of 3' terminal nucleotide residues following exposure to high levels of phosphorylase and kinase. Panel B of figure 2 shows the results of gel electrophoresis of the 195mer following exposure to the assay reagents and subsequent removal of RNA from the hybrid by denaturation with 8M urea. Lane 2 shows that in the absence of a complementary DNA strand, 195mer RNA (lane 1) was extensively degraded and the fragments electrophoresed off the gel. However, incubation

Figure 2. Analysis of the integrity of 195mer RNA in RNA-DNA hybrids. Panel A, 1 pmol of [32 P]-AMP labeled 195mer RNA was hybridized to approximately 1.2 pmol of M13mp8-20 single-stranded phage DNA at 50°C for 1 hour in the presence of 0.4M NaCl, 40mM Tris HCl (pH 8.0), and 1mM Na₂ EDTA, in a final reaction volume of 20 ul. Phosphorolysis reactions were conducted at 50°C for 40 minutes with 2 ul hybrid (0.1 pmol) and 5 ul of 2x phosphorolysis buffer in a final reaction volume of 10 ul. Reactions were combined with 10 ul sample loading buffer containing 0.05% Xylene cyanol, 0.05% bromophenol blue, 5% glycerol, 10 mM Na₂ EDTA, and 20 mM tris borate (pH 8.0) and loaded onto a 1.5% agarose gel. Electrophoresis was conducted for 30 minutes at 200 volts. From the left, lane 1, 195mer RNA; Lane 2, hybrid incubated in the absence of enzyme; Lanes 3-7, hybrid incubated in the presence of 0.04 U/ml PNP for 3, 10, 60, 90 and 300 minutes respectively.

Panel B. Following phosphorolysis, as described above, the hybrids were denatured by heating to 90°C in the presence of 5M urea and electrophoresed on a 15% polyacrylamide gel containing 8M urea. The positions of the origin and the intact 195mer RNA are indicated to the left of the figure. Lane 1, 195mer hybrid incubated in the absence of PNP; Lane 2, unhybridized 195mer incubated for 0.1 hour in the presence of 0.04 units/ml PNP; Lanes 3-4, hybrid incubated in the presence of 0.04 u/ml PNP for 0.5 and 1 hour, respectively; Lanes 5-7, hybrid incubated in the presence of 7 u/ml PNP and for 0.5, 1.0 and 5 hours respectively.

of an equal amount of RNA present as an RNA-DNA hybrid with as much as 7 units/ml of PNP at 50°C for times up to 5 hours had no noticeable effect on the electrophoretic mobility of the 195mer RNA shown in lanes 3-7.

Polyethyleneimine cellulose chromatography was used in conjunction with autoradiography to monitor the formation of ADP in the displacement assay. As shown in figure 3, lane C, ADP was detected only when the assay system was contacted with sample containing the target sequence. Incubations carried out either in the absence of DNA, or in the presence of nonhomologous denatured calf thymus DNA, showed no significant formation of ADP.

Figure 3. PEI-cellulose chromatography of the phosphorolysis products of 195meric RNA. 0.1 pmol of [32 P-AMP] labeled 195mer RNA hybridized to M13mp8-20 single-stranded DNA was incubated in the presence of 0.04 units/ml polynucleotide phosphorylase and standard phosphorolysis buffer components at 50°C for 40 minutes. Reactions were terminated by addition of Na₂ EDTA to a final concentration of 10 mM. 1 μ l of each reaction was spotted onto PEI cellulose sheets. Following development of the chromatogram in 0.8M acetic acid, 0.8M LiCl for 120 minutes, the PEI sheet was dried and autoradiographed. The positions of intact nucleic acids and authentic adenosine diphosphate are indicated to the right of the panel. Lane A, hybrid incubated in the absence of competitor M13mp19-3/2 DNA; Lane B, hybrid incubated in the presence of 10 μ g denatured calf thymus DNA; Lane C, hybrid incubated in the presence of 1 fmol of competitor M13mp19-3/2 DNA.

PEI cellulose chromatography was also used to characterize the time course of phosphorolytic release of alpha- 32 P-ADP from the RNA signal strand. The upper panel of figure 4 reveals that the progress of phosphorolysis of the 195mer RNA, in the absence of pyruvate kinase, is poor, reaching only 50% phosphorolysis after 60 minutes incubation at 50°C. However, coupling of the PNP and pyruvate kinase reactions resulted in more efficient conversion of the adenosine residues present in the RNA to ADP and ATP as shown in the lower panel of figure 4. In the presence of pyruvate kinase the RNA was converted to nucleoside di- and triphosphates to an extent of approximately 80%. The residual 20% of the RNA left intact after 60 minutes may reflect some stable secondary structure probably near the 5' end of the RNA. This

Figure 4. Kinetics of phosphorolysis of 195mer RNA. Reactions containing approximately 5 fmol of [32 P-AMP] labeled 195mer RNA were phosphorolyzed in the presence of the standard buffer components including either 0.04 units of PNP or 0.04 units of PNP plus 2 units of pyruvate kinase. 1 μ l aliquots were taken at 2, 10, 20, 30 and 60 minutes and immediately applied to PEI-cellulose sheets which had been pre-loaded with 20 nmols each of ATP and ADP. Following chromatography, spots corresponding to RNA (origin) ADP and ATP were located by UV shadowing, excised and quantitated by scintillation counting. The amount of adenosine released from RNA and converted to ADP and ATP is expressed in terms of the percent total cpm loaded onto the chromatogram. Upper panel, PNP only; Lower panel, PNP and pyruvate kinase combined. The curves correspond to the compounds indicated. Dashed lines in the lower panel correspond to reactions conducted following preincubation of the reaction mixture containing the enzymes PNP and PK in the absence of RNA substrate for 60 minutes at 50°C followed by the addition of 100 fmol of 195mer RNA.

Figure 5. Isotopic and bioluminescent detection of displacement. Eight 8 fmol of hybrid in the standard phosphorolysis/kinase conversion buffer were combined with varying amounts of a sample containing $1\mu\text{g}/\mu\text{l}$ heat denatured calf thymus DNA and either 1 fmol/ μl non-complementary M13mp11 DNA or 1 fmol/ μl M13mp19-3/2 DNA containing sequences complementary to the DNA strand of the 195mer-M13mp8-20 RNA-DNA hybrid. Individual reactions were incubated at 50°C for 30 minutes and then analyzed by: Panel A, PEI-cellulose chromatography; Panel B, agarose gel electrophoresis followed by cutting and counting of bands corresponding to hybrid and to free RNA plus nucleotides; Panel C, Bioluminescence analysis.

possibility is supported by the observation that addition of more enzyme to the reaction results in no further conversion of RNA to nucleoside phosphates, while addition of more 195mer RNA results in efficient conversion of the newly added RNA to nucleoside phosphates (data not shown). Preincubation of the

enzymes at 50°C for 60 minutes, followed by addition of 195mer RNA, also results in an identical initial velocity of conversion as shown by the dashed lines in the lower panel of figure 4. This result indicates that the enzymes and substrates are sufficiently stable to the elevated temperature used in this assay. Despite the thermal instability of phosphoenolpyruvate, pyruvate kinase was used instead of creatine phosphokinase for ADP phosphorylation because of its slightly lower K_m for ADP (20,21). Contaminating RNase activity provides an alternative explanation for incomplete conversion of this RNA to nucleoside diphosphates. An RNase which leaves a 3' phosphate upon hydrolysis of the RNA could render the RNA resistant to phosphorolysis (22). This interpretation seems unlikely since no such behavior was observed for either a 57mer or a 375mer RNA in this system (data not shown).

Figure 5 shows the results of displacement caused by the presence of target DNA sequences complementary to the probe DNA throughout the RNA binding region. Varying amounts of model samples containing heat denatured calf thymus DNA plus either target DNA (M13mp19/3-2) or control M13mp11 DNA lacking the target sequence were used in this assay. Following the addition of samples to the detection reagent, displacement and nucleoside phosphate formation were measured. Panel A shows that addition of the sample containing target DNA results in liberation of radiolabel migrating with ATP and ADP markers as assayed by PEI-cellulose chromatography. Panel B shows displacement quantified by agarose gel electrophoresis followed by scintillation counting of excised gel slices corresponding to hybrid and to free RNA plus nucleoside phosphates. Background levels of less than 0.01% of the total input counts were released by addition of non-homologous heat denatured calf thymus DNA. However, RNA is removed from the hybrid in a dose dependent fashion by addition of DNA samples containing the target sequences. Panel C shows a dose dependent relationship between bioluminescence and the levels of input target nucleic acids. Consistent with the other assays, control samples show no response indicating the absence of the target sequence. These data taken together indicate that nucleoside phosphate generation, as monitored by radioactivity and bioluminescence, accurately reflects the presence of the target DNA sequences.

DISCUSSION

This paper describes studies using model target and probe nucleic acids designed to demonstrate a novel homogeneous assay for the detection of specific DNA sequences. The assay is conducted in solution and, as such, does not require prior adsorption of the target nucleic acid to a solid support.

Separation steps required to distinguish bound from displaced signal strands are unnecessary. Instead, the conversion of the signal strand RNA from double helical to partially single-stranded form provides a structural switch which allows the single-strand specific polynucleotide phosphorylase to monitor the extent of displacement of the RNA. Thus, only displaced RNA is converted to nucleoside diphosphates and ultimately, to ATP. In this paper, 2 fmol of M13mp19-3/2 DNA was easily detected using a bioluminescence readout. This number represents an upper limit since uncertainty exists as to the concentration of target sequence present in the DNA preparations as a result of contamination of the M13mp19-3/2 DNA with extraneous nucleic acids. The sensitivity potentially achievable is indicated by the detectability of the 195mer RNA signal strand. Bioluminescence analysis of gel purified 195mer RNA indicates that as little as 50-100 cpm of [32 P]-JAMP labeled RNA is sufficient to give a detectable bioluminescent signal (data not shown). At an approximate specific activity of 10^8 cpm/ μ g, this corresponds to a lower limit of detection of approximately 10^{-12} g of the 195mer RNA or about 15 attomoles of 195mer RNA. In principle, RNA molecules of any size may be used as signal strands provided a substantially longer single-stranded DNA is available for use as a probe DNA.

Sample and reagent preparation is crucial to the sensitivity of the assay. The sensitivity of this assay was limited by endogeneous background bioluminescence present in the enzyme reagents. This method of analysis, therefore, requires removal of endogeneous RNA and nucleoside phosphate contaminants present in the sample and analytical reagents. Removal of unhybridized RNA signal strands is most easily accomplished by gel electrophoretic purification of probe-signal hybrids following pretreatment of the hybrids with excess PNP. Endogeneous background signal due to nucleoside di- and triphosphates can be removed from samples by treatment of the latter with apyrase, which hydrolyzes nucleoside di- and triphosphates to the corresponding monophosphates, followed by base treatment to remove the RNA and to inactivate the enzyme. Similarly, backgrounds due to endogeneous nucleotides present in labile reagents may be reduced by pretreatment with a slurry of apyrase linked to Sepharose. This treatment, followed by removal of the immobilized enzyme by centrifugation, results in a dramatic reduction in bioluminescent backgrounds present in the phosphorylase-kinase enzymes and reagents (McMahon and Vary, manuscript in preparation).

Cycling colorimetric assays for ADP-ATP measurement have been developed and possess sensitivities comparable to that obtained with bioluminescent analysis

(14). These assays have the advantage of being instrument-free and suggest the possibility of fabrication of a single reagent assay containing all the necessary components to "detect" a specific nucleic acid sequence, which if present, would result in color development.

Since an RNA is used as the signal strand, nonisotopic detection of an RNA sample is not practicable using ATP evolution for nonisotopic detection. This limitation does not apply to readouts based on isotopic or non-naturally occurring substituents present in the RNA signal strand. A pseudo-homogeneous assay may be envisioned which would combine displacement and structure specific degradation of free signal strands followed by rapid separation of the liberated label from high molecular weight material. For instance, hydroxylapatite can be used to achieve rapid separation of displaced RNA bound avidin-enzyme conjugates following release of the latter from much larger double-stranded structures (C. Vary, unpublished results). Though no longer strictly homogenous, such a format would allow utilization of enzyme labeled signal strands and provide a means for detection of RNA as well as DNA samples.

In conclusion, a model system has been employed to demonstrate the feasibility of a homogeneous nucleic acid diagnostic assay based on strand displacement. The system allows the detection of DNA through the sequence specific evolution of ATP. Homogeneous assay protocols should facilitate the development of simple and convenient DNA based diagnostic tests.

REFERENCES

1. Ambinder, R.F., Wingard, J.R., Burns, W.H., Hayward, S.D., Saral, R., Perry, H.R., Santos, G.W., Hayward, G.S. (1984) *J. Clin. Microbiol.* 21:353-6.
2. Saemundsen, A.K., Purtilo, D.T., Sakemoto, K., Sullivan, J.L., Synerholm, A.C., Hanpo, D., Simmons, R., Anvret, M., Collins, R., Klein, G. (1981) *Cancer Res.* 41:4237-42.
3. Spector, S.A., Rua, J.A., Spector, D.H., McMillan, R. (1984) *J. Infect. Dis.* 150:121-6.
4. Scotto, J., Hadchouel, M., Hery, C., Yvart, J., Tiollais, P., Brechot, C. (1983) *Hepatology*, 3:279-84.
5. Langer, P.R., Waldrop, A.A., Ward, D.C. (1981) *Proc. Natl. Acad. Sci. USA* 78:6633-7.
6. Molden, D.P., Nakamura, R.M., Suzuki, H., Greer, S., Pergolizzi, R.G., Brakel, C.L. (1985) *Clin. Physiol. Biochem.* 3:174-183.
7. Leary, J.J., Brigati, D.J., Ward, D.C. (1983) *Proc. Natl. Acad. Sci. USA* 80:4045-9.
8. Chan V.T.W., Fleming, K.A., McGee, J.O.D. (1985) *Nucl. Acids Res.* 13:8083-91.
9. Ambinder, R.F., Charache, P., Staal, S., Wright, P., Forman, M., Hayward, S.D., Hayward, G.S. (1986) *J. Clin. Microbiol.* 24:16-20.
10. Bunemann, H. (1982) *Nucl. Acids Res.* 10:7181-96.

11. Ellwood, M.S., Collins, M., Fritsch, E.F., Williams, J.I., Diamond, S.E., Brewen, J.G. (1986) Clin. Chem. 32:1631-6.
12. Vary, C.P.H., McMahon, F.J., Barbone, F.P., Diamond, S.E. (1986) Clin. Chem. 32:1696-1701.
13. Schram, E., Gorus, F. (1979) Clin. Chem. 25:512-9.
14. Lowry, O.H. (1980) Molecular and Cellular Biochemistry 32:135-46.
15. Ranki, M., Palva, A., Virtanen, M., Laaksonen, M., Soderlund, H. (1983) Gene 21:77-85.
16. Syvanen, A.C., Laaksonen, M., Soderlund, H. (1986) Nucl. Acids Res. 14:5037-47.
17. Ullman, E.F., Maggio, E.T. (1980) in: Enzyme Immunoassay, Maggio E.T., ed., CRC Press, Boca Raton, FL., pp. 106-134.
18. Maniatis, T., Fritsch, E.F., Sambrook, J. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
19. Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., and Green, M.R. (1984) Nucl. Acids Res. 12:7035-56.
20. McQuate, J.T. and Utter, M.F. (1959) J. Biol. Chem. 234:2151-56.
21. Kuby, S.A. and Noltman, E.A. (1962) in: The Enzymes, Boyer, P.D., Lardy, H. and Myrback, K., eds., vol. 6: pp. 515-603.
22. Haver, L., Soreq, H. (1983) in: The enzymes, Boyer, P.D., ed., vol. 15: pp. 517-53.