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Preparation of oligodeoxynucleotide–alkaline phosphatase conjugates and their use as hybridization probes

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ABSTRACT

Short synthetic oligonucleotides have been covalently cross-linked to alkaline phosphatase using the homobifunctional reagent disuccinimidyl suberate. The oligomers, twenty-one to twenty-six bases in length, are complementary to unique sequences found in herpes simplex virus, hepatitis B virus, Campylobacter jejuni and enterotoxigenic Escherichia coli. Each oligomer contains a single modified base with a 12-atom "linker arm" terminating in a reactive primary amine. Cross-linking through this amine results in oligomer-enzyme conjugates composed of one oligomer per enzyme molecule that have full alkaline phosphatase activity and can hybridize to target DNA fixed to nitrocellulose within 15 minutes. The hybrids are detected directly with a dye precipitation assay at a sensitivity of 10^6 molecules (2×10^{-18} mol) of target DNA in 4 hours development time. The enzyme has no apparent effect on selectivity or kinetics of oligonucleotide hybridization and the conjugates can be hybridized and melted off in a conventional manner.

INTRODUCTION

The most common method for the detection of nucleic acid hybrids is autoradiography of probes radiolabeled with ^{32}P. This method can reproducibily visualize 0.5–5 pg of target DNA in an overnight exposure (1). Recently, nonradiometric detection methods for cloned, double-stranded nucleic acids have been reported. These are predominantly "indirect" methods which require the detection of biotinylated (2–7) or hapten-labeled (8–10) probes by protein complexes. Although other methods have been reported (11–14), the most successful approach uses complexes of alkaline phosphatase, which catalyze dye precipitation (4), to detect biotinylated nucleic acids. Sensitivities have generally been 0.5–5 pg target DNA.

Direct, nonradioactive detection systems have seen little use in DNA hybridization studies. One group reported coupling of microperoxidase to DNA as a label (15). Renz and Kurz (16) have described a direct detection system based on enzymes covalently linked to long DNA. An enzyme complex

was first prepared by attaching peroxidase or alkaline phosphatase to a polyethyleneimine core followed by coupling the residual free amines to DNA via glutaraldehyde. The resulting complex had a large protein to DNA mass ratio (about 30) and a detection sensitivity similar to nick-translated biotinylated probes. Nonradiometric detection of oligonucleotide probes have been limited to enzymatic end-labeling with biotin (17) or chemical end-labeling with either biotin (18,19) or fluorescent moieties (20,21). A dodecamer containing an internal fluorescent base has also been described (22). Ruth (23) has reported a method for chemically incorporating modified bases with functionalized " linker arms" into synthetic oligomers and has described the synthesis of biotinylated and fluorescent oligomers. This approach is more general and offers control over location and number of labeling sites.

We have used this latter method to prepare several active covalent complexes composed of alkaline phosphatase and synthetic oligonucleotides. The usefulness of these conjugates in the detection of complementary target DNA is demonstrated by hybridization and visualization of target DNA fixed to membranes in both dot-blot and Southern blot formats. The sensitivity of colorimetric detection of these conjugates (2 attomol) is better than optimum overnight exposure with ^{32}P-labeled oligomers (5 attomol) and 5-100 times more sensitive than either end-labeled [400-2000 attomol (18,19)] or internally-labeled [10 attomol (23)] biotinylated oligomers. In addition, processing steps and time are significantly reduced. Total hybridization and assay times are 2-5 hours.

MATERIALS AND METHODS

Calf intestine alkaline phosphatase (E.C. 3.1.3.1), enzyme immunoassay grade, was obtained from Boehringer Mannheim. Disuccinimidyl suberate (DSS) was from the Pierce Chemical Company. Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP) were gifts from American Research Products Company. Bovine serum albumin fraction V (BSA), was purchased from Miles Laboratories. Polyvinylpyrrolidone (PVP), p-nitrophenyl phosphate and 4-methylumbelliferyl phosphate came from Sigma Chemical Company. Acrylamide, catalysts, sodium dodecyl sulfate (SDS) and Bio-Gel P-100 were obtained from Bio-Rad Laboratories. Sephadex G-25 was from Pharmacia Fine Chemicals and DEAE cellulose (DE-52) came from Whatman. Collodion bags with a molecular weight exclusion of 25,000 and nitrocellulose filter material were purchased from Schleicher and Schuell. Gene Screen Plus

nylon membranes were obtained from New England Nuclear. Oligomers were radiolabeled with ^{32}P-ATP from ICN using T4 polynucleotide kinase from New England Biolabs. BamHI was supplied by Bethesda Research Laboratories.

Preparation of linker arm oligonucleotides

Protected linker arm nucleoside 3'-phosphoramidite [5'-O-dimethoxytrityl-5-[N(7-trifluoroacetylaminoheptyl)-3-acrylamido]-2'-deoxyuridine 3'-O-(methyl N,N-diisopropyl) phosphoramidite] was prepared by the method of Ruth (24). The linker arm monomer was incorporated directly into automated oligonucleotide synthesis (23) using an Applied Biosystems Model 380A DNA synthesizer. Crude oligomers were analyzed and purified to electrophoretic purity by reverse phase HPLC on a Unimetrics 5u RP-8 column (0.46 cm x 25 cm), eluting with a 60 min linear gradient of 7% to 35% acetonitrile in 100 mM ammonium acetate, pH 7.1. Isolated oligomers were analyzed by 20% polyacrylamide gel electrophoresis, HPLC, and by hybridization of ^{32}P-labeled oligomers against target and control DNA.

A total of five specific oligomers were designed for four different infectious agents and extensively tested using a radioisotopic label. For conjugation to enzymes, the oligomers were constructed with one amine linker arm nucleoside replacing a thymine base. The linker arm oligomers were tested against known targets, and have the characteristics shown in Table 1. Hybridization and radioisotopic detection results for some of the oligomers have been described elsewhere [HSV (25); ETEC (26)].

Preparation of target and control DNA

The following DNA targets were used: for HSV, pHSV106 (a 7.7Kb plasmid containing a 3.4 Kb insert of HSV1 thymidine kinase gene in pBR322, from Bethesda Research Laboratories); for HBV, pAM6 (a 7.6 Kb plasmid containing the entire 3.2 Kb HBV virus in pBR322 (27); for *C. jejuni*, pPC10X (a 1.9 Kb insert in pBR322 purchased from Stanford University). Bacterial strains of *E. coli* (LT^+ ST^-) and *E. coli* (LT^- ST^+) were obtained from The American Type Culture Collection and the Centers for Disease Control, respectively. DNA from human placenta (Boehringer Mannheim), herring sperm and *E. coli* (Sigma) were used as control DNAs. The latter were extracted with buffer saturated phenol and chloroform by standard methods to remove contaminating protein. Plasmid DNA was isolated by the method of Just *et. al.* (28).

Preparation of nitrocellulose filters

Nitrocellulose filters containing a dilution series of target DNA were prepared as previously described (29). Both target and control DNAs were

TABLE I
"LINKER ARM" OLIGONUCLEOTIDES

Length of Oligomer	Target	%GC	Tm of Linker Arm Oligomer
21mer	Hepatitis B virus (HBV)	48	58°C
22mer	Herpes simplex virus (HSV) [core antigen specific]	64	64°C
26mer	C. jejuni	19	61°C
26mer	Enterotoxigenic E.coli [heat stable toxin (ST) specific]	38	63°C
26mer	Enterotoxigenic E.coli [heat labile toxin (LT) specific]	35	55°C

The melting temperature (Tm) of ^{32}P-labeled oligomers was determined by stringent hybridization against nitrocellulose-bound plasmid target, then melting by increasing temperature in 2°C increments. (Tm = temperature at which 50% of hybrids have melted.)

heated to 100°C for five minutes in 0.3 M NaOH, then chilled on ice. The samples were neutralized to a final concentration of 1 M ammonium acetate and applied to a wetted nitrocellulose filter in a "Hybridot" apparatus (Bethesda Research Laboratories) according to the manufacturer's instructions. The filter was air dried for one hour followed by baking in a vacuum oven for two hours at 80°C to fix the DNA. The filters were stored at room temperature in sealed plastic bags. All samples, with the exception of solution blanks, contained 1 ug control DNA.

Hybridization conditions

Target DNA fixed to nitrocellulose filters was prehybridized for 15 min in 5X SSC (1X SSC = 0.15 M sodium citrate, 0.15 M NaCl, pH 7.0), 0.5% BSA, 0.5% PVP and 1% SDS at 50°C, then hybridized with the equivalent of 50 ng/ml oligomer (1 ug/ml conjugate protein) in fresh prehybridization solution for a period of 15-30 min at 50°C. Loss of enzyme activity of the conjugates under these conditions was negligible. The filters were washed twice with 10 ml of 1X SSC, 1 % SDS at 50°C followed by a final rinse with 1X SSC to remove detergent, which can interfere with the dye precipitation assay of alkaline phosphatase. The filters were developed in 0.33 mg/ml NBT and 0.17 mg/ml BCIP in 0.1 M tris, pH 8.5, containing 0.1 M NaCl and 50 mM $MgCl_2$, for up to 4 h. Strongly positive samples show dye precipitation

within 30 min. The reaction was terminated by replacing the substrate solution with water. For nylon membranes, prehybridization was performed for 10 min at 50°C. Hybridization was performed for 30 min at 50°C in 1 ml of fresh prehybridization buffer, as described previously for nitrocellulose target filters. Washes were 5 min in duration and performed as follows: Twice in 1X SSC with 1% SDS at 50°C, twice in 1X SSC containing 1% Triton X-100 at 50°C, twice in 1X SSC at room temperature. These conditions have been shown to give optimum results with ^{32}P-oligomers.

Alkaline phosphatase assays

Alkaline phosphatase was assayed spectrophotometrically at 23° C by following the hydrolysis of 0.6 mM p-nitrophenyl phosphate in 0.1 M tris, 0.1 M NaCl and 50 mM $MgCl_2$, pH 8.5, at 405 nm (30). The enzyme was also measured fluorometrically with 0.1 mM 4-methylumbelliferyl phosphate in the same buffer. The excitation and emission wave lengths were 363 nm and 447 nm, respectively.

Protein determination

Protein measurements were performed according to the method of Bradford (31) using Bio-Rad Protein Assay dye reagent concentrate. The assay was calibrated with known concentrations of alkaline phosphatase, $A^{1\%}_{1cm} = 7.6$ at 278 nm (32), and standardized with known concentrations of BSA.

Electrophoresis

Nondenaturing polyacrylamide gel electrophoresis was performed according to Ornstein (33) and Davis (34) in a Bio-Rad Protean Slab Cell apparatus. Oligonucleotides were analyzed by polyacrylamide gel electrophoresis in 8 M urea according to Maxam and Gilbert (35).

Instrumentation

Spectrophotometric measurements were obtained on a Bausch and Lomb Spectronic 2000. Fluorescence assays were performed on a Farrand System 3 scanning spectrofluorometer.

RESULTS

Preparation of oligonucleotide-alkaline phosphatase conjugates

Linker arm oligomer was first derivatized with the homobifunctional cross-linking reagent, DSS (36), in order to provide a mechanism for covalent coupling to alkaline phosphatase. Twenty-five microliters of linker arm oligomer (14 nMol) at a concentration of 4 mg/ml in 0.1 M sodium bicarbonate and 2mM EDTA was combined with 50 ul solution of DSS

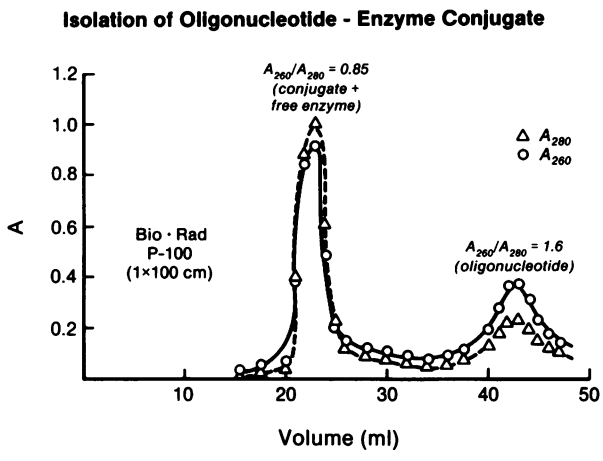

Figure 1. Gel filtration chromatography of the HSV specific oligomer-alkaline phosphatase conjugation reaction mixture. The reaction solution was applied to a Bio-Rad P-100 column (1 cm x 100 cm) equilibrated and eluted with 50 mM tris, pH 8.5, at 4°C.

(1.4 uMol) at a concentration of 10 mg/ml in dimethyl sulfoxide. The reaction was allowed to proceed for 5 minutes at room temperature in the dark, then immediately applied to a Sephadex G-25 column, 1 cm x 40 cm, and eluted at 4°C with water. The eluted fractions (0.5 ml) were monitored by absorbance at 260 nm. The first peak fractions, containing activated linker arm oligomer, were pooled and frozen for lyophilization as quickly as possible to minimize hydrolysis of reactive succinimidyl groups. Unreacted DSS and products were well resolved from the modified oligomer fractions.

The amine group of the linker arm oligomer appears to react quantitatively with DSS. An aliquot of the cross-linking reaction mixture, quenched after 5 minutes with excess ethanolamine, migrated as a single band well resolved from starting oligomer on 20% polyacrylamide-8 M urea gels. Cross-linked oligomer was not detected.

The lyophilized and modified linker arm oligomer was rehydrated with a two-fold stoichiometric excess of alkaline phosphatase (4 mg) in 200 ul of 0.1 M sodium bicarbonate, 3 M NaCl, 0.05% sodium azide, pH 8.25. The conjugation reaction mixture was maintained at room temperature for 16 h.

Isolation of oligonucleotide - alkaline phosphatase conjugates

The products of the conjugation reaction were separated by gel filtration chromatography (Fig. 1). This step separates protein from non

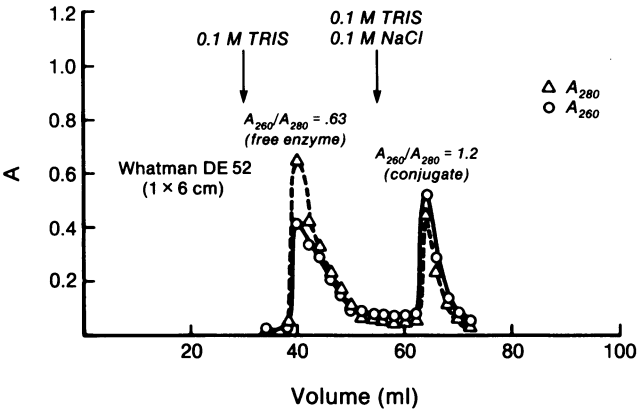

Figure 2. Isolation of the HSV specific oligomer-alkaline phosphatase conjugate. The pooled protein-containing fractions from the P-100 column were applied to a 1 cm x 6 cm DEAE cellulose column equilibrated with 50 mM tris, pH 8.5, at room temperature. Unconjugated enzyme was eluted stepwise with 0.1 M tris, pH 8.5. The oligomer-enzyme conjugate was eluted as a distinct second peak with 0.1 M tris containing 0.1 M NaCl, pH 8.5.

protein components and results in a mixture of conjugated and free alkaline phosphatase in the first eluted peak fractions. Unreacted oligomer was well resolved from the protein components. The protein containing fractions were pooled and dialyzed against 50 mM tris, pH 8.5, in the cold.

Pure oligomer-alkaline phosphatase conjugate was obtained by chromatography on DEAE cellulose (Fig.2). The peak fractions were pooled and concentrated to approximately 1 mg/ml protein by vacuum dialysis against 50 mM tris, pH 8.5, and stored at 4°C in the presence of 0.05% sodium azide.

Alkaline phosphatase retained full enzymatic activity, 400-500 U/mg protein, as determined by the spectrophotometric assay, throughout the conjugation and purification processes. The overall yield with respect to oligomer was approximately 30-50%. All of the oligomer-enzyme conjugates were prepared and purified by the same procedures.

Figure 3 shows the protein electrophoresis pattern in a nondenaturing 7% polyacrylamide gel at various stages in the preparation of the HSV specific conjugate. The purified conjugate migrates as a single band, ahead of free alkaline phosphatase. This material has a 260 nm/280 nm absorption ratio of 1.2, equal to the theoretical value for a conjugate composed of one oligomer per enzyme. Unconjugated alkaline phosphatase and oligomer have absorption ratios of 0.59 and 1.8, respectively.

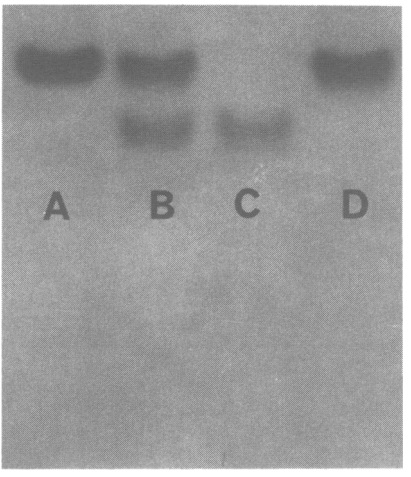

Figure 3. Polyacrylamide gel electrophoresis of alkaline phosphatase and HSV specific oligomer-enzyme conjugate. (A) Alkaline phosphatase starting material, (B) conjugation reaction mixture, (C) oligomer-enzyme conjugate eluted from DEAE cellulose (peak 2), (D) unreacted enzyme eluted from DEAE cellulose (peak 1).

Hybridization of Conjugate to Target DNA

Figure 4 shows the results of oligomer-enzyme conjugates hybridized to plasmid and genomic target DNA. Control filter targets and solution blanks were without detectable color background. Free alkaline phosphatase did not bind to any of the target filters. All conjugates hybridized specifically to their designated targets and did not cross-hybridize with heterologous DNAs tested (human placenta, herring sperm, *E. coli*), using the appropriate stringency. Heterologous DNA was present at up to a 10^6 weight excess over target.

The melting temperature of the conjugates was determined by incubating 100 ng hybridized plasmid target filters in 100 ul volumes of 5X SSC, 0.5% BSA and 0.5% PVP for 15 min at increasing temperatures and assaying the supernatant for alkaline phosphatase activity by the fluorescence assay. The melting temperature was determined to be approximately 10° C less than that found for linker arm oligomer labeled with ^{32}P (fig. 5), although this value could be artificially low if enzyme activity is significantly reduced at the higher temperatures.

The percent of sites hybridized by a conjugate was estimated by quantitative removal of that conjugate from hybridized plasmid target by

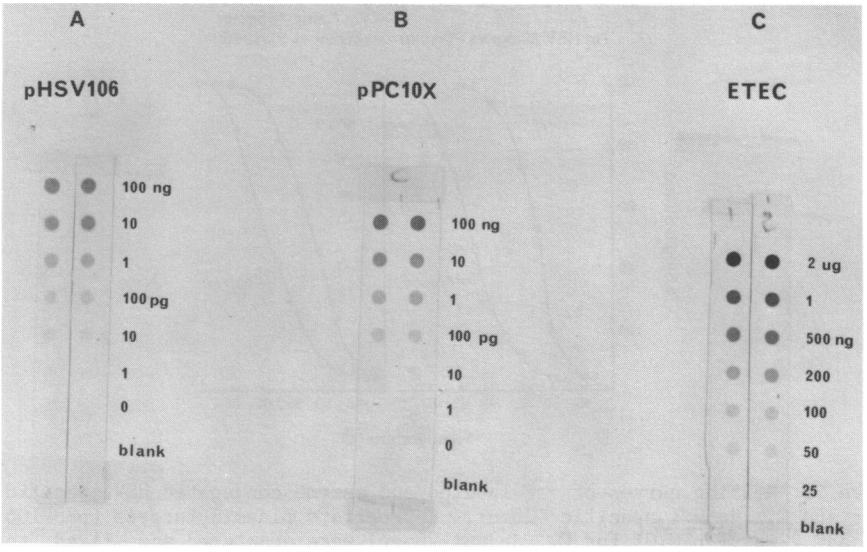

Figure 4. Hybridization of oligomer-alkaline phosphatase conjugates to nitrocellulose bound target DNA. The target filters were prepared as described in Materials and Methods. Each target area contains 1 ug human placental DNA and the indicated quantities of target. The blank areas are solution controls. Alkaline phosphatase conjugate, specific for HSV, was capable of colorimetrically detecting 10-20 pg of 7.7 Kb pHSV106 plasmid target (A), or down to 2 x 10^{-18} moles (10^6 copies). Conjugate of HBV specific 21mer against 7.6 Kb pAM6 targets had equivalent sensitivity (not shown). Conjugate of C. jejuni specific 26mer was capable of detecting 1-5 pg of 6 Kb pPC10X plasmid target (B), or 3 x 10^{-19} moles (10^5 copies). Conjugate specific for enterotoxigenic E. coli toxin genes can clearly detect 50 ng of genomic bacterial DNA from cultured cells (C), or 2.5 x 10^{-17} moles (10^7 copies).

incubation in a low ionic strength solution. Filters were incubated twice in 100 ul of 1 mM tris, pH 7.0, at 30°C for 30 min. The supernatant was removed and quantitated for alkaline phosphatase by the fluoresence assay. It was assumed that nitrocellulose filters quantitatively bound plasmid target DNA (MW = 5 x 10^6) and that 100 ng and 10 ng target filters contained 20 fMol and 2 fMol of hybridization sites, respectively. The alkaline phosphatase activity recovered from both pHSV106 and pAM6 filter targets hybridized with the appropriate conjugate represented average enzyme concentrations of 3.0 ng/ml and 0.25 ng/ml, respectively, for the 100 ng and 10 ng target filters. This corresponds to a site coverage of approximately 10%. Enzyme activity recovered from pPC10X target filters hybridized with C. jejuni specific conjugate was approximately ten-fold

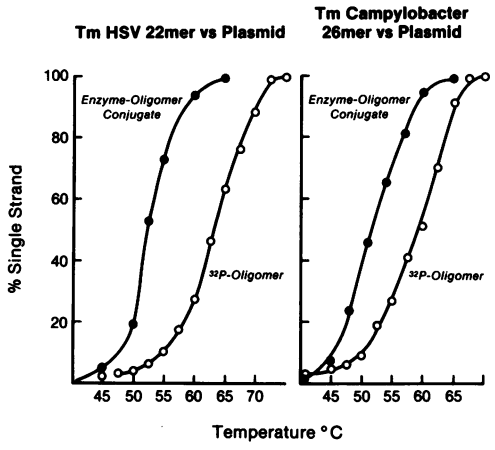

Figure 5. Melting curves of ^{32}P-labeled and enzyme conjugated HSV specific 22mer and C. jejuni specific 26mer. Appropriate plasmid targets (pHSV106 for HSV 22mer, pPC10X for C. jejuni 26mer) were denatured and fixed to nitrocellulose. The labeled oligomers were hybridized at high stringency. The filters were washed and the hybridized conjugate eluted with increasing temperature. The 22mer melted with Tm = 64°C, the 26mer Tm = 61°C. The respective alkaline phosphatase conjugated 22mer had a Tm of 52°C, while the conjugated 26mer dissociated at 51°C. The conjugated HBV specific 21mer (not shown) had a Tm of 50°C.

greater. This result is consistent with the higher sensitivity of this conjugate and could be interpreted as a more efficient site coverage, but may also represent multiple target sites on the pPC10X plasmid, a difference in the efficiency of capture of the plasmid DNA by the filter material, or both.

To assess the removal of conjugate from the filters in these experiments, filter targets were assayed by dye precipitation after conjugate elution. Color developed at a level less than ten fold that of the target quantity, indicating a removal of greater than 90% of the conjugate by the incubation in low ionic strength solution. Hybridized filter targets were also assayed for fluorogenic enzyme activity before conjugate removal by suspending the target area in 200 ul of fluorogenic substrate solution and mixing continuously for several minutes. The rate of production of fluorescent 4-methylumbelliferone agreed with that produced by the eluted conjugate, indicating that hybridized and eluted conjugate are fully active. In a separate study, it was determined that conjugate hybridized to plasmid target in solution did not result in a loss of enzymatic activity.

Figure 6 shows a comparison for the detection of target DNA between

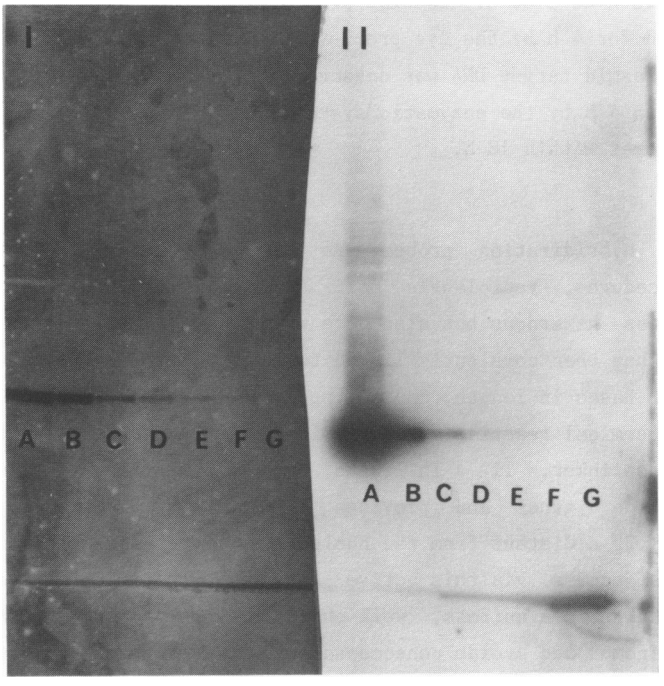

Figure 6. A comparison of the detection of pAM6 target DNA by ^{32}P-labeled or enzyme conjugated HBV specific 21mer. The identical dilution series was run on 0.8% agarose and transferred to Gene Screen Plus. The filters were probed with (I) the oligomer-alkaline phosphatase conjugate. or (II) oligomer labeled with ^{32}P-ATP (7000 uCi/pmol) by T4 polynucleotide kinase (38) to 1.25x10 cpm/ng The following amounts of pAM6 were used: A, 100 ng, B, 10 ng; C, 1 ng;D, 250 pg; E, 100 pg; F, 50 pg; G, 10 pg.

^{32}P-labeled and enzyme conjugated oligomer specific for HBV (core Ag). Plasmid pAM6 was digested to completion with BamHI, giving three restriction fragments; 4.4 Kb (pBR322), 1.8 Kb containing the core antigen sequences of HBV, and 1.4 Kb containing the surface antigen sequences of HBV. Two identical dilution series of the restricted plasmid DNA were applied to an agarose gel. After electrophoresis, the DNA was denatured and transferred by capillary blotting to a nylon membrane filter (37). Following neutralization and drying, the membrane was divided into two identical sections, each containing a complete dilution series. One series was probed with a 10 ng/ml solution of ^{32}P-labeled oligomer. The other half was hybridized in 50 ng/ml (oligomer weight) enzyme conjugated oligomer. The blot hybridized with the ^{32}P-labeled 21mer was air dried on Whatman 3mm paper, then placed in a film cassette with Kodak XAR5 film and 2 Cromex quanta III intensifying screens. Autoradiography was carried out

for 4 h and 18 h at -80°C. The blot probed with enzyme conjugated oligomer was developed for 4 h by the dye precipitation assay. The results indicate that the plasmid target DNA was detected at a 5-fold greater sensitivity (10pg) within 4 h by the enzymatic system than by autoradiography of ^{32}P-labeled oligomer within 18 h.

DISCUSSION

If DNA hybridization probes are to evolve into routinely used clinical procedures, radiolabels must be replaced by detection methods not only less hazardous but also more rapid and sensitive. Alkaline phosphatase has been covalently linked to short synthetic oligonucleotide probes, 21-26 bases in length, containing a single 12 atom (CA 14 Å) linker arm with a terminal reactive primary amine function. The disuccinimidyl suberate crosslinker, 11 Å in length, reacts quantitatively with the oligomer amine group and provides a reactive succinimidyl group, approximately 25 Å distant from the nucleotide base. Acylation of enzyme amino groups occurs via this active N-hydroxysuccinimidyl ester. This procedure results in a uniform, well characterized product, of one enzyme label per oligomer and avoids unneccessary modifications of the protein.

The coupling reaction between oligonucleotide and alkaline phosphatase is inefficient. This may be due to the electronegative repulsion between oligomer and protein and can be overcome by using relatively high concentrations of reactants in a high salt medium. This was accomplished by lyophilizing the activated oligomer to dryness, which also helped preserve the reactive succinimidyl group, then rehydrating with at least 20 mg/ml enzyme in slightly alkaline buffer. The resulting conjugate is composed of one oligomer per enzyme with a mass ratio of protein to DNA of about 20. The catalytic function of the enzyme portion of the conjugate is equivalent to that of the starting material.

The conjugates are capable of hybridizing to specific complementary target sequences found in plasmid or genomic DNA fixed to filter membranes. Surprisingly, the efficiency of hybridization appears to be unaltered from unmodified oligomer, and filter background nonexistent. The concentration of conjugate DNA and hybridization times are consistent with those reported for ^{32}P-labeled synthetic short probe. The Tm may be decreased by approximately 10°C, indicating a slightly lowered binding free-energy of hybridization for the conjugate. However, hybridization at temperatures well above the apparent Tm (i.e., 60°C for a conjugate with an apparent Tm of 53°C) does not reduce signal.

Nonspecific binding to control DNA, or to nonspecific plasmid target is not detectable at the levels tested, much improved over that observed for biotin-avidin enzyme systems or ^{32}P-labeled probes. The enzyme component of the conjugate appears to enhance the overall specificity of the oligomer for the complementary sequence in the target by limiting random association with DNA or filter material in either direct dot-blot or in Southern blot format.

The combination of alkaline phosphatase and short oligomer for detection of DNA hybridization results in a markedly diminished time for the hybridization and detection process over that of long nick-translated probes. Inputs of conjugate at concentrations equivalent to 50 ng/ml oligomer allows hybridization times of 15-30 minutes, and less nonspecific interaction allows total washing times after hybridization to be only a few minutes. Plasmid target DNA at a level of 1 ng or more is visualized in several minutes, with detection as low as 10 pg (2 attomol) within 4 hours. Overnight development further enhances visualization, but without increased sensitivity.

Alkaline phosphatase is a stable protein, remarkably resistant to elevated temperature and SDS. The conjugate is also remarkably stable and has been stored for up to 6 months in solution at 4°C with 0.05% sodium azide. The conjugates are stable in the frozen state and can be lyophilized in the presence of PVP and BSA from hybridization concentrations.

The conjugate is capable of catalyzing the removal of phosphate from a host of substrates including fluorogenic compounds such as 4-methylumbelliferyl phosphate and fluorescein phosphate. The exploration of such systems should lead to increased sensitivity and reduced assay time with the potential for automation.

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