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Chemical and enzymatic biotin-labeling of oligodeoxyribonucleotides

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ABSTRACT

Biotin has been converted to 2-(biotinylamido)ethanol and condensed to phosphorylated oligonucleotides in a solid phase synthesis. The 5'-biotinylated oligonucleotides were enzymatically coupled to other DNA fragments by T4 DNA ligase or T4 RNA ligase. The hybridization properties of such biotin-labeled oligonucleotide probes were studied.

INTRODUCTION

Oligonucleotide probes are useful diagnostic tools with applications in human, animal and plant research areas.[1] Two criteria for these diagnostic aids are availability and ease of detection. The use of biotin to earmark compounds has been readily accepted due to its safety and sensitivity to detection. The growing interest in making non-radioactive probes makes it desirable to have a chemical synthesis for biotinylated nucleotides; this accomplishment is facilitated by solid phase synthesis. A synthetic biotin-labeled oligonucleotide can be used directly as a hybridization probe or used to prepare longer probes by enzymatic reactions.

Biotinylation of nucleotides was first studied on RNA.[2] RNA molecules were converted to 3'-aldehydes by oxidation and condensed with alkyldiamines or polyamines to provide reactive sites for a biotin condensation.[3,4] In some cases the reactive site for the attachment of biotin molecules was the protein attached to the 5'-end of an RNA.[5] These biotin-labeled RNA molecules were used as probes in the identification of gene segments and in the selection of specific DNA or RNA molecules.[6] An alternative method which attached biotin to the 3'-end of an RNA by RNA ligase has recently been reported.[7]

Methods for biotin-labeling DNA has recently been developed.[8-10] The incorporation of biotin was accomplished by a DNA polymerase reaction using TTP and UTP analogues which contained biotinylated molecules at the C-5 position of the pyrimidine rings. Similar compounds of biotin-labeled DNA

at the C-5 position have also been made by solid phase synthesis.[11] As in the study of biotin-labeled RNA molecules, these DNA probes have been used in locating genes and specific sequences of nucleotides.[12-16]

This paper describes a chemical synthesis of biotinylated oligonucleotides on solid support of silica. An aminoethanol derivative of biotin was condensed to the 5'-hydroxyl group of the ribose ring to give a stable phosphodiester bond between the biotin molecule and the nucleotide upon deprotection. The biotinylated nucleotides were isolated using conditions similar to procedures for DNA synthesized on silica solid support.[17,18] The chemically synthesized biotinylated nucleotides were ligated to a pentadecamer to give 5'-biotin-labeled oligomers 18 and 20-nucleotides long. The effect of 5'-biotinylation on the melting temperature of oligonucleotide probes was studied in hybridization experiments with filter-immobilized DNA. A decrease of 5-7^{o}C in melting temperature was observed for a biotinylated 20-mer DNA probe. However, a biotinylated octadecamer of mixed DNA/RNA composition did not show a detectable decrease.

RESULTS AND DISCUSSION

2-(Biotinylamido)ethanol was prepared from biotin-N-hydroxysuccinimide ester[19] and aminoethanol. It was condensed to a phosphorylated polymer-supported nucleotide (Figure 1). Phosphorylation of the nucleotide on silica had been accomplished following the procedure reported by Itakura[20] and van Boom[21] for

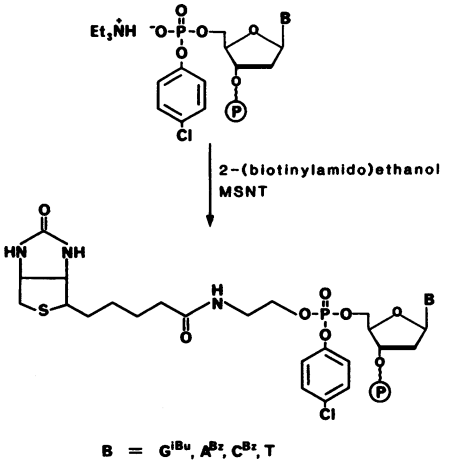

Figure 1.

HPLC Purification of 5'-Biotinylated Oligonucleotides and Intermediates

Nucleotides	Yield (%)	Retention time min (% CH_3CN)	Gradient of % CH_3CN-0.1 M Et_3NHOAc
rC		5.0 (1.7)	5-25%, 60 min
5'-p-chlorophenylphosphate	62	56.0 (18.7)	
5'-(2-biotinylamido)ethylphosphate	60	40.5 (13.5)	
dCCrC		19.0 (11.3)	0-20%, 60 min
5'-p-chlorophenylphosphate	94	36.0 (17.0)	
5'-(2-biotinylamido)ethylphosphate	85	27.0 (14.0)	
dTTCCC		20.0 (14.6)	8-18%, 30 min
5'-p-chlorophenylphosphate	99	30.0 (18.0)	
5'-(2-biotinylamido)ethylphosphate	72	24.5 (16.1)	

Analysis on Altex Ultrasphere ODS, 5μ column (4.6 x 250 mm), flow rate of 1 mL/min.

solution chemistry. The fully protected nucleotide was detritylated with zinc bromide and the 5'-hydroxyl group treated with p-chlorophenylphosphodi-triazolide at room temperature. The remaining phosphorous-triazole bond was hydrolyzed with 1 M triethylammonium acetate. The phosphorylated nucleotide was treated with 2-(biotinylamido)ethanol in the presence of the condensing reagent 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT)[22,23]. Removal from the support and deprotection afforded the biotinylated oligonucleotides.

Ammonia was used in the deprotection scheme instead of pyridinealdoximate[24] for removal of the p-chlorophenyl protecting group since the aldoximate interfered with HPLC analysis of the product. Samples from each individual steps were deprotected, isolated and analyzed on HPLC. The intermediates in the biotin-labeling reaction, e.g., the parent nucleotide, the corresponding 5'-p-chlorophenylphosphate and the 5'-2-(biotinylamido)ethyl phosphate were all separable by HPLC (see Table). The yield of phosphorylation was nearly quantitative for the trimer and the pentamer; the monomer showed a lower yield. The overall yields of these reactions after isolation were in the range of 60-85%. (See Table.)

The synthetic biotinylated oligonucleotides were sized and analyzed for purity on a 20% polyacrylamide gel. They were labeled with [5'-^{32}P]-cytidine 3',5'-bis(phosphate) at the 3'-end.[25,26] Analysis showed a homogeneous band for each compound. A comparision between dCCrC and biotin-dCCrC showed that the addition of a biotin at the 5'-end caused it to appear about 1.5 nucleotides longer. A similar trend was seen for the biotinylated dTTCCC compared with the parent compound. (See Figure 2.)

As further evidence for the presence of a biotin moiety the compounds assigned to be biotin-labeled DNA were found to be completely retained when passed through an avidin-agarose column.[27] The parent compound and the

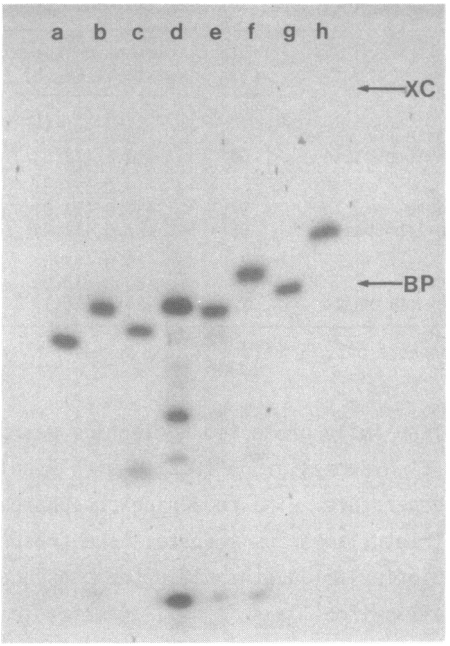

Figure 2. Autoradiogram showing the relative mobilities of synthetic biotinylated oligonucleotides on 20% polyacrylamide-TBE gel under denaturing conditions. Positions for the dye markers xylene-cyanol FF (XC) and bromophenol blue (BP) are indicated. a) dCCrC-^{32}prCp, b) biotin-dCCrC-^{32}prCp, c) ^{32}pdTTCCC, d) dTTCCC-^{32}prCp, e) p-chlorophenyl-pdTTCCC-^{32}prCp, f) biotin-dTTCCC-^{32}prCp, g) ^{32}pdGTTACC, h) ^{32}pdT$_8$

5'-phosphorylated derivative could be recovered as shown by HPLC analysis of the eluent. Treatment of the avidin-agarose support with excess biotin in water at 90^{o}C for 5 minutes displaced greater than 70% of the bound biotinylated oligonucleotides from the support. This technique thus allowed for the enrichment of short biotin-labeled compounds. Recovery of the biotin-labeled trimer and the pentamer were 100% and 70% respectively.

Biotinylated oligonucleotide probes of 20 and 18-nucleotides long were prepared by two methods. The synthetic biotinylated pentamer was ligated[28] to a pentadecamer (5'dAGTCACGACGTTGTA)[29] over a template using T4 DNA ligase; the biotinylated trimer was ligated to the same pentadecamer using the template-independent T4 RNA ligase[25]. The DNA ligase reaction was essentially quantitative (Figure 3). The product mixture was desalted and deprotein-

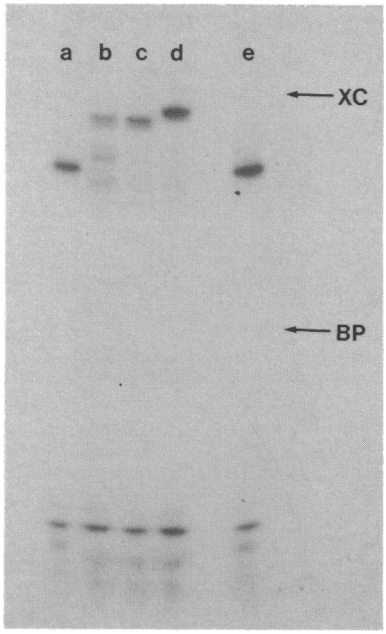

Figure 3. Autoradiogram showing relative mobility of biotinylated 20-mer on 20% polyacrylamide-TBE under denaturing conditions. a) 15-mer, ^{32}pAGTCACGACGTTGTA; b) 20-mer, dTTCCC-^{32}pAGTCACGACGTTGTA; c) 20-mer intermediate, p-chlorophenyl-pdTTCCC-^{32}pAGTCACGACGTTGAT; d) biotinylated 20-mer, biotin-dTTCCC-^{32}pAGTCACGACGTTGTA; e) 15-mer, ^{32}pAGTCACGACGTTGTA.

ized before use in the hybridization studies. The RNA ligation, however, yielded only about 20% of the desired product and required isolation from a polyacrylamide gel prior to use (Figure 4).

The effect of a 5'-biotin moiety on the hybridization properties of oligonucleotides was studied. Plasmid pUC9 DNA was linearized, immobilized on nitrocellulose filter and hybridized to radiolabeled oligonucleotides at 30^{o}C in 1 M sodium chloride for 24-28 hours. The filters were washed at various temperatures for 10 minutes over 3 changes of buffers containing 1 M sodium chloride. They were dried and the oligonucleotides retained were quantitated by liquid scintillation counter. The amounts of radioactivity retained at various temperatures were normalized to that obtained at 30^{o}C (Figure 5). Results from this study showed the melting temperatures for the 20-mer (5'dTTCCCAGTCACGACGTTGTA) and the 15-mer (5'dAGTCACGACGTTGTA) to be 60^{o}C and 47^{o}C respectively. These values closely approximate the melting

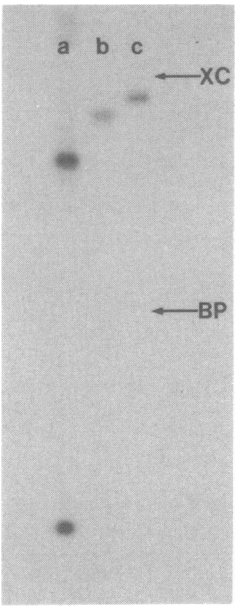

Figure 4. Autoradiogram showing relative mobilities of oligonucleotide probes on 20% polyacrylamide-TBE gel under denaturing conditions. a) 15-mer, dAGTCACGACGTTGTA; b) biotinylated 18-mer, biotin-dCC(rC)AGTCACGACGTTGTA; c) biotinylated 20-mer, biotin-dTTCCCAGTCACGACGTTGTA.

temperatures for oligonucleotides predicted by the equation proposed by Suggs et al.[30] for Tm values in solution. Introduction of a biotin molecule at the 5'-position as the 2-(biotinylamido)ethanol derivative of the nucleotide was found to produce a slight destabilization of the duplex. The 5'-biotinylated 20-mer showed a decrease of 5-7°C in melting temperature. A similar decrease in temperature was observed by Langer et al.[8] when 12.5% of the total nucleotides of minute virus of mouse (MVM) DNA was substituted by biotinylated dUMP. Therefore, biotinylation appeared to have a more pronounced effect on the lowering of melting temperatures of short oligonucleotides than on DNA molecules of several thousand base pairs. On the other hand, the 5'-biotinylated 18-mer with one rC residue (5'-biotin-dCC(rC)AGTCACGACGTTGTA) showed a melting temperature expected for a non-biotinylated deoxynucleotide 18-mer; this indicated a stabilization of the duplex by the ribonucleotide substitution.

Experiments were conducted to show the utility of such 5'-biotin-labeled oligonucleotides as hybridization probes. The biotinylated 20-mer was used in a standard Southern blot analysis[31]. Fragments of plasmid pUC9 DNA

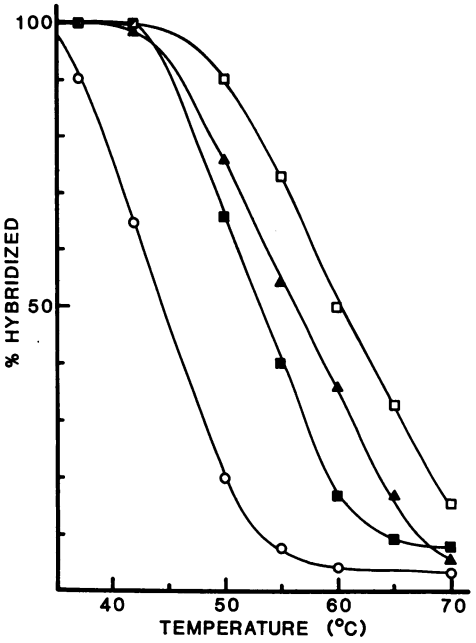

Figure 5. Determination of the melting temperature of biotinylated oligonucleotides by hybridization to DNA immobilized on nitrocellulose filters. Oligonucleotide probes used were: 20-mer (5'dTTCCCAGTCACGACGTTGTA, □ ; 15-mer (5'dAGTCACGACGTTGTA), ○ ; biotinylated 20-mer (5'-biotin-dTTCCCAGTCACGACGTTGTA), ■ ; biotinylated 18-mer (5'-biotin-dCC(rC)AGTCACGACGTTGTA), ▲ .

digested by restriction enzyme TaqI were hybridized to this 20-mer probe as well as probes made by labeling with biotinylated UTP in a nick-translation. Conditions for hybridization and rinses were similar to the previous experiment. As shown in Figure 6, specific hybridization between the biotinylated probe and the DNA fragment containing the complementary sequences could easily be detected by the activity of horseradish peroxidase conjugated with avidin. The hybridization signal was about 40 times less intense for the oligonucleotide probe than that detected in the procedure using nick-translation. This difference could be explained by the difference in the lengths of the hybridization probes (800 vs. 20 nucleotides). These results showed that 5'-biotin-labeled oligonucleotides would be useful probes for the detection of specific sequences and possibly also useful in the isolation of such sequences by immobilizing the probe on an avidine-agarose column.

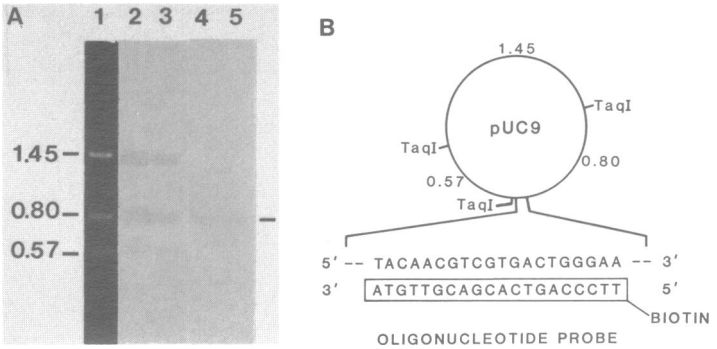

Figure 6. Southern blot analysis using biotinylated oligonucleotides as hybridization probes. (A) TaqI digest fragments of plasmid pUC9 DNA were resolved on a 1.4% agarose gel and the contents transferred to nitrocellulose filter. The amounts of DNA immobilized on the filters were 0.1 μg, 0.02 μg, 1.0 μg and 0.1 μg for lanes 2 to 5. The probes used were: lanes 2 and 3, biotinylated pUC9 DNA made by nick-translation, lanes 4 and 5, biotinylated 20-mer. (B) Diagram of plasmid pUC9 DNA. The positions of TaqI cleavage sites and the sequence complementary to the biotinylated oligonucleotide probes are as indicated. The sizes of TaqI digest fragments are indicated in kilobase pairs.

EXPERIMENTAL SECTION

Materials

All chemicals were commercially available. Nucleosides were purchased from the following sources: cytidine (Sigma), deoxycytidine and deoxyguanosine (P-L Biochemicals), deoxyadenosine and thymidine (Calbiochem). Porasil C silica (80-100μ, Waters) was used as the solid support. Reversed phase chromatography was performed on Altex Ultrasphere ODS, 5μ analytical column (Beckman). C-18 disposable extraction columns (J. T. Baker) were used for removing buffer and proteins from DNA preparations. The nucleotides [5'-^{32}P]-cytidine 3',5'-bis(phosphate) and [γ-^{32}P]-adenosine 5'-triphosphate were purchased from New England Nuclear. The following enzymes were used: T4 polynucleotide kinase (New England Nuclear), T4 DNA ligase (Promega Biotec) and T4 RNA ligase (the laboratories of R. I. Gumport, University of Illinois).

Preparation of 2-(Biotinylamido)ethanol

To an ice-cooled solution of biotin-N-hydroxysuccinimide ester[19] (3.75 g, 11 mMol) in DMF (40 mL) was added aminoethanol (1.0 mL, 16.4 mMol). The mixture was stirred for 30 min. The reaction was monitored by TLC (Merck Silica 60, HF 254, 20:80 methanol-dichloromethane v/v). The biotin-N-hydroxysuccinimide ester (R_f 0.76) was consumed within 15 min and the alcohol

product appeared (R_f 0.47). The reaction mixture was concentrated at reduced pressure and the residue coevaporated with DMF to remove excess amounts of aminoethanol. The white residue was recrystallized from water to yield 1.7 g (38%). An analytical sample was obtained from a second recrystallization, m.p. 192°C, Anal. Calc. for $C_{12}H_{21}N_3O_3S$: C, 50.15; H, 7.37; N, 14.62; O, 16.70 and S, 11.16. Found: C, 50.06; H, 7.82: N, 14.29 and 11.14.

Phosphorylation of Oligonucleotides on Solid Support

The phosphorylating reagent, p-chlorophenylphosphoditriazolide, was prepared according to the method described by Itakura et al.[20] To an ice-cooled solution of THF (5 mL), 1,2,4-triazole (0.28 g, 4 mMol) and triethylamine (0.49 mL, 3.5 mMol) was added p-chlorophenylphosphodichloridate (0.37 g, 1.5 mMol). The reaction was stirred at RT for 25 min. The triethylammonium hydrochloride that formed was sedimented through centrifugation.

A nucleotide (or oligonucleotide), which had been previously synthesized on the silica support (100 mg) and still covalently bound to the support, was detritylated with zinc bromide-H_2O-nitromethane and washed with THF.[17] The support was dried and transferred to a 3.5-mL vial. To the silica was added the phosphorylating reagent in THF (1 mL) and the mixture gently shaken for 25 min. Excess phosphorylating reagent was decanted and 1 mL of 1 M triethylammonium acetate pH 7.0 was added. Hydrolysis of the triazolide was completed after 20 min at RT. The polymer support was transferred to a funnel constructed of a pasteur pipette equipped with a glass wool plug. The silica was washed with water and THF. It was recovered from the funnel and dried under nitrogen.

Biotin Condensation to DNA

The phosphorylated polymer bound oligonucleotide (50 mg) was combined with 2-(biotinylamido)ethanol (12 mg, 0.042 mMol) and pyridine (1 mL) in a 3.5-mL vial. To the mixture was added MSNT (250 mg, 0.84 mMol) in pyridine (0.5 mL) Condensation proceeded for 40 min at RT. The support was then transferred to a pasteur funnel with a glass wool plug and washed with pyridine (3 mL) and THF (5 mL).

Deprotection of Biotin-labeled DNA

The support was treated with concentrated ammonium hydroxide (2 mL) in a 3.5-mL septum sealed vial for 2 h at RT. The ammonia solution containing the oligonucleotide was removed from the silica and placed in another vial where deprotection of the bases and the phosphate groups were continued at RT for 40 h followed by 3 h at 50°C. The mixture was concentrated at reduced pressure on a Speed Vac (Savant). The residue was dissolved in water and

isolated by HPLC.

3'-End Labeling by the Addition of Cytidine 3',5'-bis(phosphate)

The compounds dTTCCC, p-chlorophenyl-pdTTCCC and biotin-pdTTCCC were treated with [5'-^{32}P]-prCp and T4 RNA ligase in the manner described by Gumport[25] and Uhlenbeck[26]. The oligonucleotide (20 pMol) was labeled in a 10 μL reaction mixture containing [5'-^{32}P]-prCp (10 pMol), ATP (40 pMol), RNA ligase (1.6 μg), 50 mM HEPES pH 8.0, 10 mM $MnCl_2$, 10 mM DTT and 10% DMSO. The reaction proceeded at 17°C for 16 h. The product mixture was analyzed on a 20% polyacrylamide gel.

Biotinylated Oligonucleotide by DNA Ligation

The oligonucleotide, biotin-dTTCCCAGTCACGACGTTGTA, was prepared from a ligation of biotin-dTTCCC and pdAGTCACGACGTTGTA over the template dTGACTGGGAA. The pentadecamer (40 pMol) was phosphorylated with 1.5 equiv. of [γ-^{32}P]-ATP and 1 U of T4 polynucleotide kinase according to established procedures.[32,33] After the reaction was completed, kinase was denatured by heating the reaction mixture for 5 min at 100°C. The mixture was cooled and the biotinylated pentamer (40 pMol) and the decamer (40 pMol) were added. The entire mixture was heated at 100°C for 2 min and cooled slowly to 4°C over a 5-h period. The mixture was adjusted to a volume of 20 μL containing 50 mM Tris pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 100 μM ATP and 10 U T4 DNA ligase. The ligation proceeded at 4°C and was essentially completed after 16 h. The ligation mixture was diluted with water to a 100 μL volume and passed through a short C-18 column to remove salt and proteins following the procedure described below. The eluted fractions were checked by scintillation counting and the desired ones were pooled together and lyophilized. (The final material also contained the template which was carried along into the hybridization studies.)

Biotinylated Oligonucleotide by RNA Ligation

The oligonucleotide, biotin-dCC(rC)AGTCACGACGTTGTA, was prepared from a ligation of biotin-dCCrC and pdAGTCACGACGTTGTA. The pentadecamer donor (200 pMol) was phosphorylated with 1.5 equiv. of [γ-^{32}P]-ATP and 2 U of T4 polynucleotide kinase according to established procedures.[32,33] After 30 min at 37°C, the kinase was denatured by heating 5 min at 100°C. To the reaction mixture was added 10 equiv. of the biotinylated acceptor and 3 equiv. of ATP. The ligation was performed in a 40 μL reaction mixture containing 50 mM HEPES pH 8.0, 10 mM $MnCl_2$, 10 mM DTT and 10% DMSO. (The reaction also contained 25 mM Tris pH 7.8, 5 mM $MgCl_2$ and 5 mM β-mercaptoethanol from the previous reaction.) T4 RNA ligase (5 μg) was added to the reaction mixture. After 48 h at 17°C, the reaction was made 20 mM DTT and another 5 μg of RNA ligase was added.

After an additional 48 h, the reaction mixture was loaded onto a 20% polyacrylamide gel for isolation. The product mixture showed 20% starting material, 20% 18-mer and 60% the circular self-ligated product. The band containing the biotinylated octadecamer was excised from the gel and the compound recovered by the crush-and-soak method.[34] After elution from the gel, the mixture (500 µL) was loaded onto a C-18 column and desalted according to the procedure outlined below. The yield after isolation was 10% as determined from scintillation counting.

Desalting on Reversed-phase Silica

This procedure was used for the removal of salts and proteins from preparations of small DNA. A 1-mL C-18 disposable extraction column was conditioned by a 4-mL wash of methanol and a 6-mL wash of 50 mM triethylammonium acetate pH 7.0. The solvent was flushed through the C-18 silica by applying positive pressure to the rubber septum sealed column. The sample to be desalted and the eluting buffers were passed through using this method; the flow was sufficiently slowed to see individual droplets of eluent.

The sample in 100 µL (or greater) of buffer was loaded onto the column by positive pressure. The column was eluted with 3 mL of 50 mM triethylammonium acetate. All salts were removed by the first 1.5-mL wash. The column was then eluted with 2 mL of an 8% acetonitrile-50 mM triethylammonium acetate; this removed ATP and other small DNA. The compound was eluted off with 6 x 100 µL of 20% acetonitrile-50 mM triethylammonium acetate. The fractions containing the desired material as determined by scintilliation counting were combined and lyophilized.

Determination of the Melting Temperatures of Biotinylated Oligonucleotides

Plasmid pUC9 DNA[35] (1 µg) was linearized by digestion with restriction enzyme BamHI. After deproteinization by phenol extraction and ethanol precipitation, the DNA was redissolved in 20 µL of 10 mM Tris pH 7.9 and 1 mM EDTA. Denaturation was done by heating the sample at 100°C for 3 min followed by rapid cooling in liquid nitrogen. Aliquots (2 µL) containing 0.1 µg of the linearized denaturated pUC9 DNA was spotted onto nitrocellulose filters. The filters were washed extensively in 6xssc (1xssc = 0.15 M NaCl + 0.015 Na citrate), air-dried and then heated for 1 h at 80°C under vacuum. Prehybridization was carried out for 2-4 h at 30°C in 6xssc + 0.2% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone. Hybridization was done at the same temperature for 24-28 h in the same buffer with the addition of 0.5% SDS, 1 mM EDTA and 5 pMol of ^{32}P-labeled oligonucleotides. The preparation of these oligonucleotides was described in previous sections. Hybridized filters

were washed in 3 changes of 100 mL of 6xssc at various temperatures. Radioactivity retained on these filters was assayed by liquid scintillation counting. The values obtained at various temperatures were normalized to that obtained at 30°C. The temperature at which 50% of the hybridization probe was retained was determined as the melting temperature for the oligonucleotide.

Hybridization of Biotinylated Oligonucleotides to Specific DNA Fragments Immobilized on Nitrocellulose Filters

Plasmid pUC9 DNA was digested to completion by restriction enzyme TaqI. The resulting fragments were separated by electrophoresis through a 1.4% agarose gel. The contents of the gel were transferred to a nitrocellulose filter by the methods of Southern[31]. Conditions for prehybridization and hybridization were identical to that described in the previous section except that the temperature used for the hybridization and the washings was 65°C for the pUC9 probe. This probe was labeled with biotinylated UTP (Enzo Biochem, Inc., New York) by nick-translation.[8] The detection of biotinylated probes hybridized on the filters was made by a complex of biotinylated horseradish peroxidase and streptavidin (Detek II, Enzo Biochem, Inc., New York) under conditions specified by the manufacturer.

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