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Novel Chemical Method for the Preparation of Nucleic Acids for Nonisotopic Hybridization

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A novel chemical method was used to prepare biotin-labeled nucleic acids for nonisotopic hybridization. The method involves the transamination of unpaired cytosine residues in polynucleotides with sodium bisulfite and ethylenediamine. Primary amino groups on the cytosine derivatives are then reacted with biotinyl-*e*-aminocaproic acid *N*-hydroxysuccinimide ester. Biotinylated probes hybridized with 1 to 2 pg of nitrocellulose filter-bound DNA and were visualized with a colorimetric detection technique. This method is simpler and less expensive than other methods for the preparation of nonisotopic probes. In addition, it is more versatile since the chemically modified bases can potentially react with other "indicator" molecules or proteins such as an enzyme. The specificity for unpaired cytosine residues is another advantage which could allow for the selective labeling of a specific region of a double-stranded nucleic acid. This improved labeling method should lead to the wider application of hybridization techniques in diagnostic microbiology and basic research in infectious diseases.

Sensitive, specific, and rapid diagnostic techniques for the detection of microbial agents in body fluids are important for the prevention and management of infectious diseases in clinical medicine and for the large-scale study of the epidemiology of infectious agents. The traditional method of diagnosing infectious diseases involves the growth of an infectious agent in an in vitro cultivation system. While potentially accurate, cultivation is often insufficiently rapid to be clinically useful and may be inadequate in identifying fastidious microbial pathogens. Immunoassay techniques have also been utilized for the detection of microbial antigens in clinical specimens. However, the sensitivity of immunoassay systems is limited by the kinetics of the antigen-antibody reaction, the limiting quantities of immunoreactants present in the reaction, and the rate of nonspecific immunological reactions (21). Thus, immunoassays have limited applicability for the direct detection of microbial pathogens in human body fluids.

One nonimmunological technique which offers great promise for the detection of microbial agents in body fluids is the detection of microbial nucleic acids by hybridization techniques. Nucleic acid hybridization is a kinetically efficient reaction due to the large number of hydrogen bonds involved in base pairing. This allows for the possibility of detecting very small amounts of microbial nucleic acids in clinical samples. Another important benefit of nucleic acid hybridization is the ability to detect a wide range of organisms by constructing nucleic acid probes containing genetic sequences that are highly conserved in antigenically diverse strains of pathogenic microorganisms. An additional advantage of hybridization assays derives from the chemical stability of nucleic acids. This allows for the easy handling and storage of clinical specimens under diverse environmental conditions. Furthermore, hybridization assays can detect microbial pathogens in the presence of coexisting antibody, since the chemically more stable nucleic acid in the immune complex can be released by the proteolytic treatments which are used in the assay procedure. In addition to these advantages, recent advances in recombinant DNA technology make it feasible to produce large quantities of nucleic acids with defined sequences, thus allowing for the widespread usage of standardized reagents.

The major obstacle to the wider application of nucleic acid hybridization reactions in diagnostic microbiology is the fact that current techniques routinely use nucleic acid probes labeled with radioisotopes such as ^{32}P or ^{35}S. These probes have a short functional half-life and must be prepared and standardized frequently. The expense, radiation exposure, and isotope disposal problems associated with radioactive probes are additional disadvantages. As an alternative, enzymatic detection methods overcome many of the problems associated with isotopic methods, and furthermore, they provide the possibility for greater sensitivity through the magnifying nature of the enzyme-substrate reaction. Several nonisotopic nucleic acid hybridization techniques which utilize an enzymatic detection method have recently been developed (2, 5, 9, 10, 14, 20). The most extensively applied method involves the incorporation of nucleotide analogs containing biotin into nucleic acid probes by means of a standard nick translation reaction. However, the modified nucleotides are expensive and the incorporation reaction is difficult to standardize. In addition, a comparison of biotinylated probes and radioactive probes for the detection of viral sequences in clinical specimens has shown that this nonisotopic technique is less sensitive by a factor of 10 (8).

We report here a chemical method for labeling nucleic acid with affinity ligands such as biotin. Probes labeled with biotin can be detected by a variety of enzymatic methods exploiting the specific and tenacious interaction of biotin with avidin. The chemistry of the labeling procedure involves the modification of cytosine residues in single-stranded regions of nucleic acids with a bifunctional amine in the presence of sodium bisulfite (7, 17, 18). This results in the formation of a derivative which has a side chain terminating in a reactive amino group (3, 16). The formation of such

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modified cytosine residues in polynucleotides allows the attachment of amine-specific reagents to nucleic acids.

MATERIALS AND METHODS

Materials. Lambda phage DNA, strepavidin, and a nick translation kit were purchased from Bethesda Research Laboratories. Sodium bisulfite, ethylenediamine, hydroquinone, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue tetrazolium (NBT; grade III), polyethylene glycol compound (molecular weight, 15,000 to 20,000), and agarose (type I) were obtained from Sigma Chemical Co. All other chemicals were reagent grade. Deoxycytidine 5-[α-^{32}P]triphosphate was purchased from Amersham Corp. Biotinyl-*e*-aminocaproic acid *N*-hydroxysuccinimide ester was obtained from Enzo Biochemicals. Calf intestinal alkaline phosphatase labeled with biotinyl-*e*-aminocaproic acid was obtained from Calbiochem-Behring, snake venom phosphodiesterase I and mung bean nuclease were obtained from P-L Biochemicals, Inc., and DNase I was from Sigma Chemical Co. Thin-layer chromatography was performed with polyethyleneimine-cellulose on polyester plates. Low-melting agarose was purchased from International Biotechnologies Inc. Econoflour was obtained from New England Nuclear. Autoradiography was performed with Kodak XAR-5 film and Cronex quanta III intensifying screens. Pure nitrocellulose (BA-85) was purchased from Schleicher & Schuell. Adenovirus type 2-adenoid (ATCC VR#1079) and type 5-adenoid 75 (ATCC VR#1082) were obtained from the American Type Culture Collection. The Toronto 2 strains of an adenovirus type 5 was a gift from F. R. Bishai, Toronto, Ont., Canada.

Modification of nucleic acid with sodium bisulfite and ethylenediamine. Sodium bisulfite (1M)–ethylenediamine (3M) solutions were freshly prepared by adding, slowly on ice, 1 ml of concentrated HCl to 1 ml of water, 1 ml of ethylenediamine, and 0.475 g of sodium bisulfite. The pH of the solutions were adjusted to 6, 6.5, or 7 by titration with concentrated HCl, and the final volumes were brought to 5 ml with distilled water. A 2 M sodium bisulfite-ethylene diamine solution with a pH of 5.5 was also prepared. A 1-mg/ml portion of hydroquinone (dissolved in 95% ethanol) was added to the bisulfite solutions to scavenge free radicals. Since the bisulfite-catalyzed transamination of cytosine residues is single strand specific, nucleic acid samples (5 to 25 μg) in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA were heat denatured and cooled on ice. The reaction was initiated by adding 9 volumes of a bisulfite-amine solution to 1 volume of single-stranded nucleic acid. The reaction mixture was incubated for 3 h at 42°C. After an overnight dialysis against three exchanges of 5 mM sodium phosphate buffer, pH 8.5, the samples were concentrated to 100 μl by ultrafiltration (YM30 membrane) in an Amicon 8MC stirred microcell. Alternatively, samples were concentrated by covering the dialysis bag with polyethylene glycol compound (molecular weight, 15,000 to 20,000) in a plastic dish at room temperature until the sample volume was reduced to approximately 200 μl.

Labeling of modified nucleic acids with biotin. Modified nucleic acids (1 to 10 μg) were diluted in 100 μl of a 0.1 M sodium phosphate buffer, pH 8.5. Biotinyl-*e*-aminocaproic acid *N*-hydroxysuccinimide ester was freshly prepared as a 0.2 M stock solution in *N'*,*N'*-dimethylformamide. Five microliters of the ester was added to the reaction mixture, which was incubated at room temperature for 1 h. Labeled nucleic acid probes were dialyzed against three exchanges of 150 mM NaCl–10 mM sodium phosphate buffer (pH 7)–1 mM EDTA and stored at 4°C until used.

Analysis of dCMP composition. Lambda phage DNA labeled with deoxycytidine 5'-[α-^{32}P]triphosphate by nick translation was modified with each of the bisulfite-amine solutions described above, and the modified nucleic acids were biotinylated. A portion of each preparation was digested with 20 μg of DNase I per ml for 30 min at room temperature in 50 mM Tris hydrochloride (pH 7.5)–5 mM $MgCl_2$. The reaction mixture was adjusted to 100 mM Tris hydrochloride (pH 9.3)–20 mM $MgCl_2$–100 mM NaCl and incubated for an additional 4 h at 37°C with 0.25 mg of snake venom phosphodiesterase I per ml. One microliter of the resulting mixture of nucleotide 5'-monophosphates was spotted on polyethyleneimine-cellulose thin-layer chromatography plates. The plates were developed with a 65% isopropranol–2 N HCl solvent. The R_f values of the nucleotides were determined from autoradiograms of the plates, and the percentages of modified cytosine residues were calculated by counting in a Beckman scintillation counter the appropriate spots dissolved in Econofluor.

Reassociation of DNA. Lambda phage DNA for reassociation experiments was sonicated in 1 M NaCl–10 mM Tris hydrochloride–1 mM EDTA, pH 7.8, for repeated 30-s pulses to an average double-strand length of 500 nucleotides. Unmodified lambda phage DNA and biotinylated lambda phage DNA labeled with ^{32}P were mixed with 12 μg of nonradiolabeled lambda phage DNA in 100 μl of 50% formamide–2× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7). The mixture was heat denatured by boiling and allowed to renature at 37°C. Five-microliter samples were removed at various times and digested for 20 min at 42°C with 10 U of mung bean nuclease in 100 μl of 50 mM sodium acetate buffer–50 mM NaCl–1 mM $ZnCl_2$, pH 5.0. The amount of ^{32}P-labeled DNA resistant to this single-strand-specific nuclease was determined by acid preciptation onto a glass-fiber filter. The proportion of reannealed nucleic acid at each time point was plotted against the log of initial concentration in moles of nucleotide per liter multiplied by time in seconds.

Preparation of nitrocellulose blot. Serial twofold dilutions of lambda phage DNA were denatured by boiling in 0.2 N NaOH–6× SSC, and 1-μl samples were spotted onto nitrocellulose filters. The filters were neutralized in 2× SSC, air dried, and baked overnight at 65°C. Tissue culture fluid suspensions were boiled in 0.5 M NaCl–0.2 N NaOH and dot blots were prepared as described above. For Southern blots, DNA samples were electrophoresed in 1% agarose gels as described by Maniatis et al. (12). DNA was transferred bidirectionally to nitrocellulose filters by the method of Smith and Summers (19). DNA filters were air dried and baked overnight at 65°C.

Hybridization conditions. Nitrocellulose filters were prehybridized for 4 h at 37°C in 45% formamide–3× SSC–10× Denhardt solution–0.1% Sodium dodecylsulfate–0.1 mM EDTA–1 mM sodium pyrophosphate–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7, containing 100 μg of salmon sperm DNA per ml. The hybridization reaction was performed in the same solution containing 10% dextran sulfate and a heat-denatured biotinylated probe. After an overnight incubation at 37°C, the filters were washed three times each with 2× SSC–0.1% sodium dodecyl sulfate (10 min at room temperature), 0.1X SSC–0.1% sodium dodecyl sulfate (30 min at 45°C), and 0.1× SSC (10 min at room temperature).

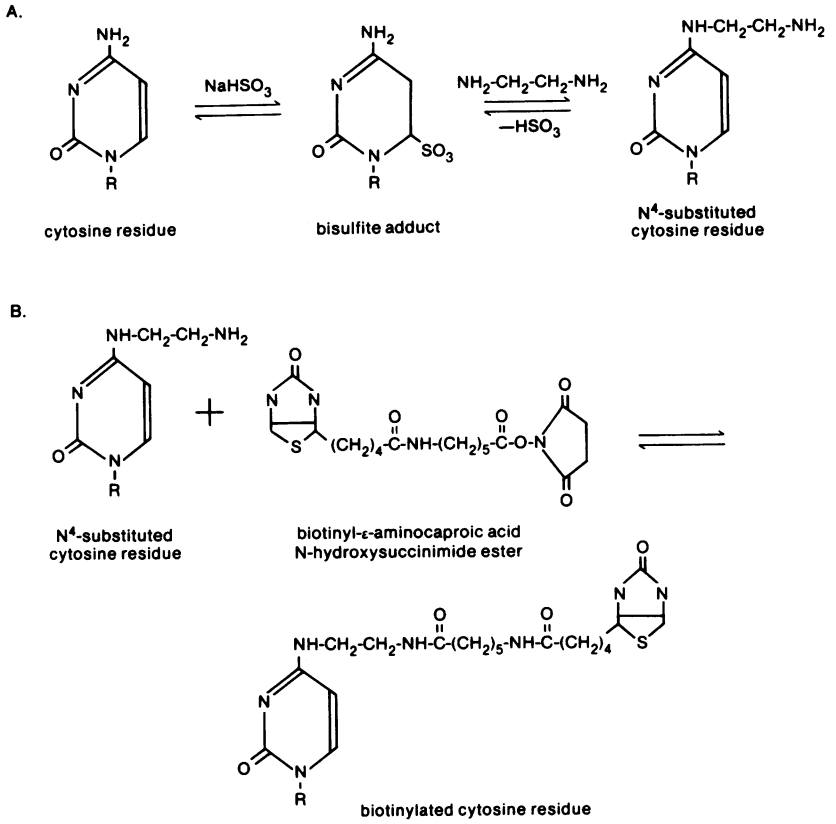

FIG. 1. Scheme for labeling nucleic acid with biotin. (A) Bisulfite-catalyzed transamination reaction with ethylenediamine. (B) Biotinylation reaction. R = RNA or DNA.

Colorimetric detection. For the enzymatic detection of biotinylated probes, the filters were incubated for 1 h at 37°C with a mixture of 0.25 μg of strepavidin per ml and 0.2 U of calf intestine alkaline phosphatase per ml labeled with biotinyl-*e*-aminocaproic acid diluted in phosphate buffered saline-0.5% fetal calf serum. After washing the filters three times with 150 mm NaCl–10 mm sodium phosphate buffer, pH 7.2, containing 0.05% Tween 20, the filters were developed with a precipitatable, colorimetric substrate composed of 0.33 mg of NBT and 0.17 mg of BCIP per ml diluted in 100 mM Tris hydrochloride (pH 9.3)–0.1 M NaCl–5 mm $MgCl_2$ (11). Filters were incubated with the substrate solution for 1 h in the dark at room temperature. The reaction was terminated by washing the filters with tap water and blotting them dry.

RESULTS

The chemistry of the labeling procedure is outlined in Fig. 1. Cytosine residues in single-stranded regions of nucleic acids were modified by the addition of sodium bisulfite to the 5,6-double bond of the pyrimidine base. These cytosine-bisulfite adducts were subsequently converted to N^4-substituted cytosine derivatives by transamination with ethylenediamine. The cytosine derivatives which have a side chain terminating in a primary amino group were then labeled with biotinyl-*e*-aminocaproic acid *N*-hydroxysuccinimide ester.

The optimum conditions for the labeling procedure were determined by utilizing lambda phage DNA as the nucleic acid probe. Lambda phage DNA labeled with deoxycytidine 5′-[α-^{32}P]triphosphate by nick translation was reacted with various solutions of sodium bisulfite and ethylenediamine. Bisulfite-amine-modified and biotin-labeled nucleic acids were digested with DNase and snake venom phosphodiesterase. One microliter of the resulting mixture of nucleotide 5′-monophosphates was analyzed by thin-layer chromatography on polyethyleneimine-cellulose thin-layer chromatography plates. The approximate R_f values of unmodified dCMP, the N^4-substituted deoxycytidine derivative, and the biotinylated nucleotide were 0.71, 0.33, and 0.94, respectively. Table 1 shows the percentage of modified cytosine residues. By adjusting the pH and bisulfite concentration of the reaction mixture, the percentage of N^4-substituted cytosine residues could be varied from 12 to 56%. Under the conditions used, 77 to 100% of the bisulfite-ethylenediamine-modified nucleotides reacted with the biotinyl-*e*-aminocaproic acid *N*-hydroxysuccinimide ester.

TABLE 1. Deoxycytidine composition of lambda phage DNA modified by bisulfite and ethylenediamine and extent of reaction of cytosine derivatives with biotin

Bisulfite concn mol/liter)	pH	dCMP (%)	N^4-substituted dCMP (%)	Extent of reaction with biotin ester (%)	Total nucleotides biotinylated (%)[a]
2	5.5	44	56	77	10.3
1	6.0	67	33	79	6.2
1	6.5	80	20	90	4.3
1	7.0	88	12	100	2.9

[a] Calculation based on a 24% cytosine content for lambda phage DNA.

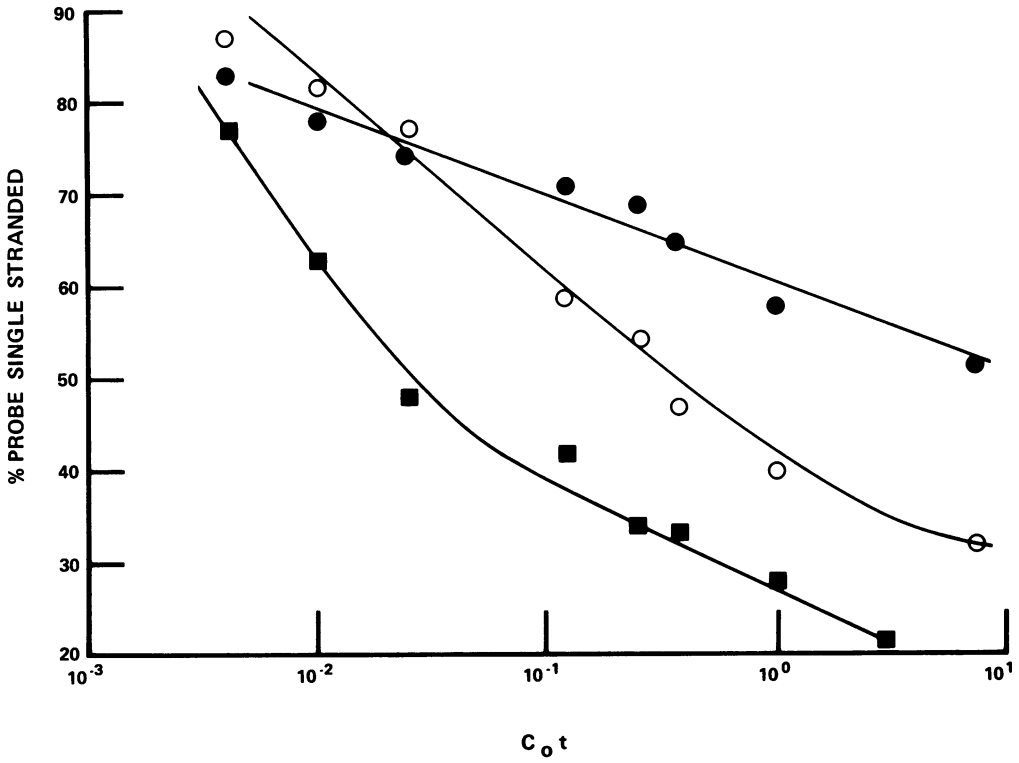

FIG. 2. Reassociation rate of biotinylated lambda phage DNA. Symbols: (■) unmodified lambda phage DNA; (●) lambda phage DNA with 56% of its cytosine residues modified (10.3% of the total nucleotides bioitinylated); (○) lambda phage DNA with 20% of its cytosine residues modified (4.3% of the total nucleotides biotinylated). C_0t = Initial concentration of DNA (moles of nucleotide per liter) × time (seconds).

Since the cytosine content of lambda phage DNA is 24%, the percentage of biotinylated bases ranged from 2.9 to 10.3%. Lambda phage DNA which was unmodified and DNA which was not heat denatured before the modification reaction could not be labeled with biotin.

Since the bisulfite-catalyzed transamination of cytosine affects the N^4 position, which is involved in base pairing, the influence of this modification on the reassociation kinetics of biotinylated probes was assessed. The liquid phase reassociation of unmodified lambda phage DNA and biotinylated lambda phage DNA was determined by a single-strand-specific nuclease method. The experimental data were used to construct C_0t curves which relate the proportion of reannealed probe to the initial nucleotide concentration and time (Fig. 2). The hybridization rate of a probe with 56% of its cytosine residues modified was significantly reduced compared with that of unmodified DNA. The $C_0t_{1/2}$ or point at which 50% of the nucleic acid had reannealed was never reached under the experimental conditions used. Probes containing 20% of their cytosine residues modified exhibited a reassociation rate that was moderately reduced; the $C_0t_{1/2}$ was about 1 log greater than that of unmodified DNA.

Probes differing in their content of biotin-labeled cytosine residues were evaluated in a routine dot hybridization procedure. Nitrocellulose filters spotted with serial twofold dilutions of lambda phage DNA were hybridized with 1 μg of a heat-denatured biotinylated lambda phage probe per ml. For the enzymatic detection of biotinylated probes, the filters were first incubated with a mixture of streptavidin and biotinylated alkaline phosphatase and then developed with a precipitable, colorimetric substrate composed of NBT and BCIP. These experiments revealed that biotinylated probes could readily detect 1 to 2 pg of filter-bound DNA (Fig. 3). Since all probes provided equivalent sensitivity, it was not necessary to biotinylate more than 3 to 4% of the bases. The detection limit was the same with probe concentrations of 0.1 μg/ml. To assess the sensitivity of the labeling method with a shorter nucleic acid sequence, lambda phage DNA sonicated to a mean size of 500 base pairs was biotinylated and used as a probe. Nitrocellulose filters spotted with serial 10-fold dilutions of lambda phage DNA were hybridized with 0.1 μg of a lambda phage probe per ml with a 2.6 or 13% biotin content. A spot containing 1 pg of complementary

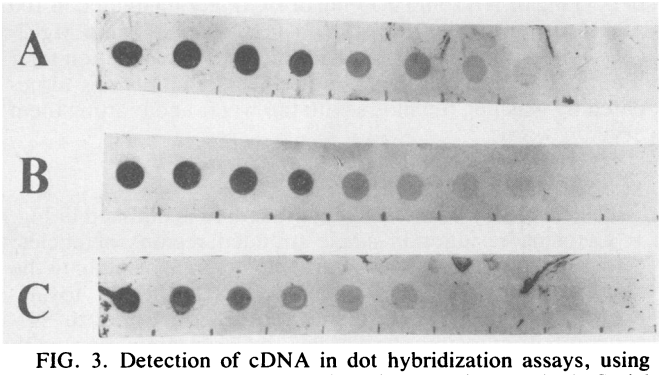

FIG. 3. Detection of cDNA in dot hybridization assays, using biotinylated probes and a colorimetric detection method. Serial twofold dilutions of lambda phage DNA (from 256 to 1 pg) were spotted on nitrocellulose filters. The last dot on the right represents 10 ng of salmon sperm DNA. Dot blots were hybridized with a lambda probe containing 10.3% of the total nucleotides biotinylated at a concentration of 1 (A) or 0.1 (B) μg/ml or with 1 μg of a lambda probe per ml containing 2.9% of the total nucleotides biotinylated (C).

nucleic acid was visualized with both probes (Fig. 4). Experiments revealed that filters developed with alkaline phosphatase and the NBT-BCIP substrate gave a 10-fold greater sensitivity compared with filters developed with horseradish peroxidase and the precipitatable substrate 3,3'-diaminobenzidine. The sensitivity of dot hybridization assays was comparable with probes prepared by incorporation of biotinylated nucleotides by nick translation (the Enzo Bioprobe system, Enzo Biochemicals Inc.).

Experiments were also performed to determine the ability of biotinylated probes to visualize nucleotide sequences in a Southern blot format. Various amounts of *Hin*dIII-digested lambda phage DNA were electrophoresed on 1% agarose and then transfered bidirectionally to nitrocellulose filters. The filters were hybridized with 1 μg of a lambda phage probe per ml with a 4% biotin content. Bands containing as little as 2.5 pg of DNA could be detected by the enzymatic method (Fig. 5). *Hin*dIII-digested lambda phage DNA was also electrophoresed in low-melting agarose. The DNA fragments were heat denatured in the agarose, modified with a bisulfite-amine solution, and labeled with biotin. Nucleic acids biotinylated under these conditions were suitable as probes in a dot hybridization assay. Thus the presence of low-melting agarose did not adversely effect the chemical labeling procedure. Biotinylated probes could be stored for up to 4 months at 4°C without loss of activity and hybridized filters could be stored dry at room temperature for up to 3 months before they were assayed by the enzymatic detection method.

After defining some of the parameters of the labeling procedure, we extended its application to a second nucleic acid probe. Nucleic acid from the Toronto 2 strain of adenovirus type 5 was biotinylated and the probe was used in a hybridization assay for the detection of viral DNA from infected tissue culture cells. Three strains of adenovirus, type 2-adenoid 6, type 5-adenoid 75, and the Toronto 2 strain of type 5, were inoculated onto HeLa cells and samples were obtained after 1, 3, and 7 days of incubation. One-microliter portions of undiluted and a 10^{-1} dilution of tissue culture fluid suspensions were spotted on a nitrocellulose filter. The filter was hybridized with 0.5 μg of the adenovirus probe per ml with a 4% biotin content and developed with the strepavidin-biotinylated alkaline phosphatase complex and the NBT-BCIP substrate solution. The biotinylated probe and enzymatic detection method was capable of detecting adenovirus-specific DNA from all three strains after 1 day of incubation (Fig. 6).

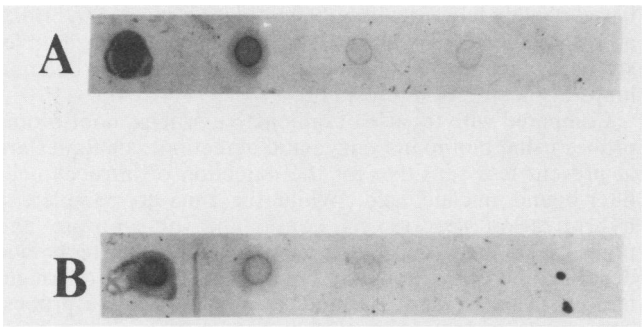

FIG. 4. Detection of cDNA in dot hybridization assays, using biotinylated probes and a colorimeteric detection method. Serial 10-fold dilutions of lambda phage DNA (from 1,000 to 1 pg) were spotted on nitrocellulose filters. The last dot on the right represents 10 ng of salmon sperm DNA. Dot blots were hybridized with 0.1 μg of a sonicated lambda probe (size = 0.5 kilobase) per ml containing (A) 13% or 26% (B) of the total nucleotides biotinylated.

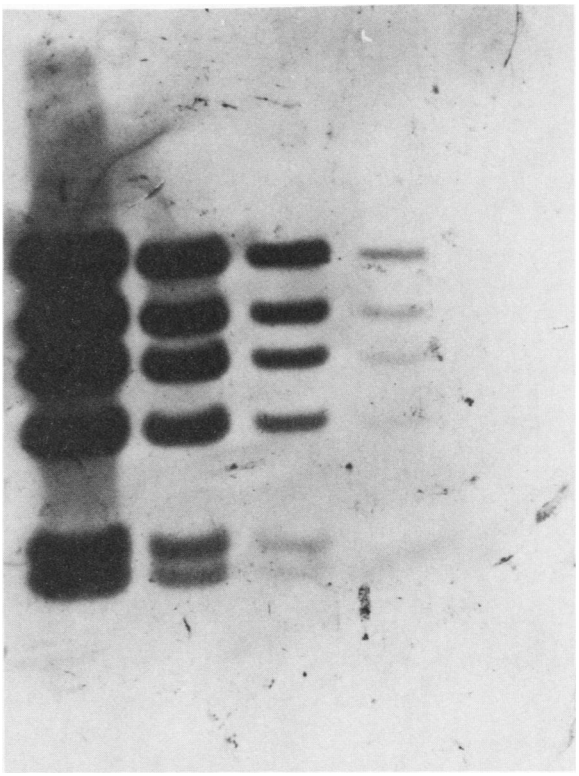

FIG. 5. Detection of cDNA in Southern blots, using biotinylated probes and a colorimetric detection system. A bidirectional Southern transfer was made from serial 10-fold dilutions of a *Hin*dIII digest of lambda phage DNA. The agarose gel electrophoresis lanes contained from 100 to 0.01 ng of nucleic acid, and thus the lanes of the blot show from 50 to 0.005 ng. The blots were hybridized with 1 μg of a lambda probe per ml containing 4.3% of the total nucleotides biotinylated.

DISCUSSION

The bisulfite-catalyzed transamination reaction of polynucleotides with biotinylation of the cytosine derivatives is a simple and practical means of preparing nonisotopic hybridization probes. The reagents necessary for the labeling procedure are inexpensive and commercially available. The method uses basic laboratory procedures which can be readily performed in a reproducible fashion. Biotinylated probes prepared by this method can be used for both dot hybridization assays and Southern blot analyses following standard hybridization protocols. Nucleic acid probes with 2.9 to 10.3% of their bases labeled with biotin functioned efficiently as hybridization probes. The endpoint sensitivity for nitrocellulose filter bound DNA (1 to 2 pg) was equivalent irrespective of biotin content; however, the more heavily modified probes gave a slightly more intense signal. The optimum biotin content may vary with other probes and the conditions for the labeling procedure should be determined on an individual basis. The extent of modification of a nucleic acid can be easily controlled by adjusting the pH and bisulfite concentration of the reaction. Sodium bisulfite can also catalyze the deamination of cytosine to uracil. However, this reaction has a more acidic pH optimum than the

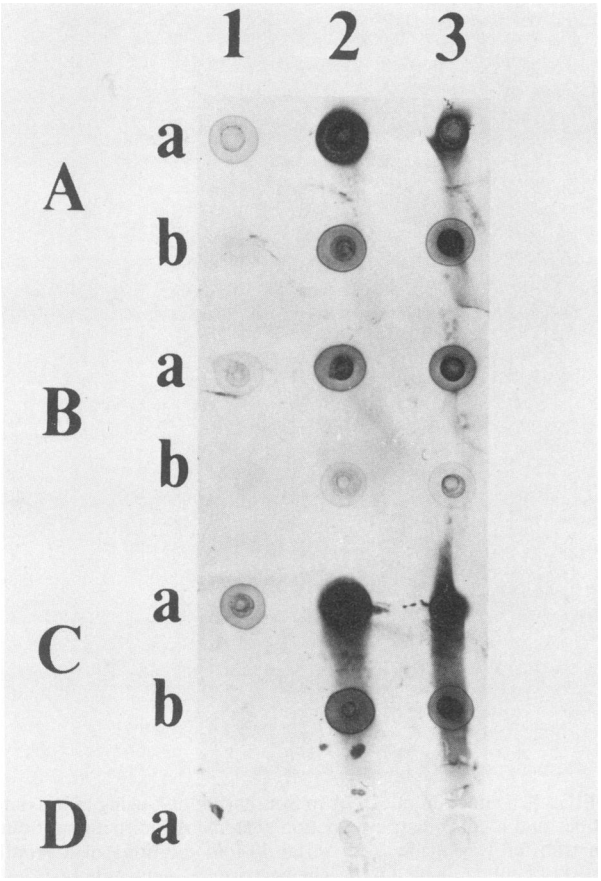

FIG. 6. Detection of adenovirus DNA in HeLa cells. A type 2 adenovirus (A), a type 5 adenovirus (B), the Toronto 2 strain of type 5 adenovirus (C), and uninfected HeLa cells (D) were incubated for 1, 3, or 7 days (lanes 1 to 3, respectively). One-microliter samples of a tissue culture fluid suspension, undiluted (a) or at a 10^{-1} dilution (b), were spotted on a nitrocellulose filter. The filter was hybridized with 0.5 μg of a biotinylated Toronto 2 adenovirus DNA probe per ml.

transamination reaction and it proceeds at a negligible rate at a neutral pH (3). In addition, the transamination reaction is highly favored over deamination in the presence of a high molar concentration of a compound with a low amine pK such as ethylenediamine (pK = 7.6). Under the conditions used here polycytidylic acid has been shown to be >95% converted to the transamination product (3). Nucleic acid probes with 4.3 to 10.3% of their bases labeled with biotin showed a reduced rate of reassociation; however, even the most extensively modified nucleic acid performed well as a hybridization probe. Thus, the greater number of biotin molecules on the probe appear to compensate for the reduced rate of reassociation. Alternatively, the differences in liquid-phase reassociation kinetics may not be reflected by similar differences in solid-phase reassociation rates under standard hybridization conditions. The sensitivity for nitrocellulose filter-bound DNA was equivalent with probe concentrations in the 0.1- to 1-μg/ml range. We observed acceptable levels of nonspecific binding to nitrocellulose even with probe concentrations up to 4 μg/ml. Background reactivity was best reduced by simply reusing hybridization mixtures. The ability to use higher probe concentrations should make it possible to complete hybridization reactions in a shorter period of time. This will be important if hybridization procedures are used for clinical diagnosis. The parameters of the labeling method and the characteristics of the biolinylated probes were initially determined with an approximately 50-kilobase probe. However, the sensitivity of biotin-labeled probes for nitrocellulose filter-bound DNA was equivalent with probes which are only 0.5 kilobase in size. Biotinylated polynucleotides can be optimally visualized by enzymatic techniques that use complexes of strepavidin and biotinyl-*e*-aminocaproic acid-labeled alkaline phosphatase. The greater sensitivity achieved with alkaline phosphatase compared with horseradish peroxidase may reflect enhanced accessibility of the precipitable substrate for immobilized alkaline phosphatase or decreased binding to biotinylated nucleic acids of the more acidic peroxidase protein due to electrostatic repulsion.

Compared with other methods for the preparation of nonisotopic hybridization probes, the present method is simpler, less expensive, and equally sensitive. Unlike methods that use nick translation protocols, a nonenzymatic labeling procedure has the advantage of being less influenced by impurities such as phenol, salts, or agarose present in some nucleic acid preparations. Compared with other chemical methods for labeling nucleic acids, the reagents for this method are inexpensive, noncarcinogenic, and readily available. Another important advantage of the method reported here is its versatility. The selective introduction of a reactive amino group on a polynucleotide allows the attachment of a variety of amine-specific reagents including other haptens such as dinitrophenol or "indicator" molecules such as fluorescein and electron-dense particles (4). By labeling probes with different ligands, each detectable by a separate enzyme-substrate reaction, the recently described technique of double-label hybridization in situ could be performed nonisotopically (6). In addition, the primary amino group can be coupled to succinimide esters of protein affinity-labeling reagents which are capable of forming covalent bonds between nucleic acids and a variety of proteins (15). A DNA-protein complex in which the protein molecule is an enzyme could be used as a hybridization probe. Where the protein is a ligand for a specific cellular receptor, the construction of DNA-proteins complexes could be utilized as a method for ligand-directed gene transfer studies (1). The specificity for cytosine residues in single-stranded regions of nucleic acids is another advantage of the bisulfite-catalyzed transamination reaction. Thus single-stranded, cytosine-containing homo- or heteropolymeric tails on a nucleic acid probe could be selectively labeled without effecting the specific hybridizable sequences. In addition, specific regions of a double-stranded nucleic acid could be labeled by creating deletion loops at the site of interest (13).

Compared with the use of radioactive probes, nonisotopic probes using biotin and enzymatic detection techniques are at present less sensitive for the detection of nitrocellulose filter-bound nucleic acid. While the time to complete a hybridization reaction is equivalent for isotopic and nonisotopic methods, the enzymatic detection technique described here requires only 2 h versus a typical overnight film exposure time for the detection of radioactive probes. The improvement of this method for labeling nucleic acids and for the enzymatic detection of nucleic acid hybridization reactions will lead to the wider application of this technology in diagnostic microbiology and basic research in infectious diseases. The successful development of practical techniques for the detection of microbial nucleic acids in clinical specimens might markedly improve the care of patients with

infectious diseases and allow for a greater understanding of the epidemiology and pathophysiology of human infections.

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