"Post-Assay" Covalent Labeling of Phosphorothioate-Containing Nucleic Acids with Multiple Fluorescent Markers†

Robin R. Hodges, Nancy E. Conway, and Larry W. McLaughlin*
Department of Chemistry, Boston College, Chestnut Hill, Massachusetts 02167
Received January 28, 1988; Revised Manuscript Received July 15, 1988

ABSTRACT: A simple protocol has been developed which allows the covalent introduction of multiple fluorescent markers into DNA fragments after gel electrophoresis techniques, that is, while the nucleic acid is imbedded in the polyacrylamide gel matrix. "Post-assay" fluorescent labeling in this manner employs DNA fragments containing phosphorothioate diesters, which can be easily incorporated during chemical and enzymatic synthesis procedures, and can be alkylated with the fluorescent marker monobromobimane. Labeling the internucleotidic phosphorus residue in this manner allows the introduction of a fluorescent marker for each nucleotide residue present. Roughly a linear increase in emitted fluorescence, thus detection sensitivity, is observed with an increasing number of bimane markers. With this technique, oligodeoxynucleotides and DNA fragments can be observed in the gel matrix, without sophisticated electronic detection devices in the low femtomolar (10^{-15} mol) range.

The enormous utility of chemical and enzymatic DNA sequencing procedures results in part from the high sensitivity associated with the detection of radioisotopes. Such sensitivity typically allows one to locate femtomolar quantities of nucleic acid material but is rendered less than ideal because of the associated problems of safety and disposal. Fluorescent rather than radioisotopic labeling procedures are an attractive option which avoids these liabilities but have been generally compromised by greatly reduced sensitivity.

In addition to the detection of nucleic acids via the intercalative fluorescent markers such as ethidium bromide (LePecq et al., 1964; Fuller & Waring, 1964; LePecq & Paoletti, 1967; Morgan & Paetkau, 1972), many techniques have been developed to allow covalent modification with fluorescent dyes.

In some cases, a single fluorescent reporter group has been attached to one of the termini of RNA (Bauman et al., 1981a,b; Richardson & Gumport, 1983; Cosstick et al., 1984; Agarwal et al., 1986; Tyagi & Wu, 1987) or DNA (Connolly & Rider, 1985; Smith et al., 1985, 1986; Prober et al., 1987; Ansorge et al., 1987; Chu et al., 1983; Agarwal et al., 1986). This approach is analogous to end-labeled procedures employing radioactive phosphate. Such procedures have required the development of sophisticated instrumentation for detection of the single fluorescent marker at levels comparable to that observed with radioisotopes (Smith et al., 1986; Prober et al., 1987; Ansorge et al., 1987). Other covalent labeling techniques allow for the introduction of multiple fluorescent dyes into a nucleic acid. However, these procedures often involve the time-consuming preparation of a modified nucleotide which is then derivatized with the fluorescent molecule either before incorporation into the nucleic acid fragment or shortly thereafter (Eshaghpour et al., 1979; Langer et al., 1981; Brigati et al., 1983; Leary et al., 1983; Kempe et al., 1985; Forster et al., 1985; Chollet & Kawashima, 1985). To date, the covalent introduction of fluorescent labels into nucleic acids has been accomplished prior to the desired assay such as polyacrylamide gel electrophoresis. The labeled nucleic acids often exhibit anomalous electrophoretic mobility which can complicate subsequent analysis of the gel (Smith et al., 1986; Prober et al., 1987).

We now describe a fundamentally simple technique in which multiple fluorescent molecules can be introduced covalently into an oligodeoxynucleotide after analysis by gel electrophoresis, that is, while the fragment is still embedded in the polyacrylamide gel matrix. Such "post-assay" labeling employs oligodeoxynucleotides containing phosphorothioate diesters (Eckstein, 1985), which can be introduced into the nucleic acid fragment either enzymatically (Potter & Eckstein, 1984) or chemically (Connolly et al., 1984; Stec et al., 1984). We have used monobromobimane (Kosower et al., 1978, 1979; Kosower & Pazhenchevsky, 1980) to covalently label these internucleotidic phosphorothioate diesters. This approach complements previous work in which monobromobimane was used to label tRNA by reaction with a single terminal phosphorothioate monoester (Cosstick et al., 1984). However, by use of internucleotidic phosphorothioate diesters as the site for attachment of the fluorophore, each of the internucleotidic phosphorus residues can potentially be labeled with monobromobimane. Multiple labels, ideally one for every nucleoside residue present, can then be introduced into the fragment.

Experimental Procedures

Materials

Monobromobimane was obtained from Calbiochem (San Diego, CA) under the name Thiolyte reagent. Thin-layer chromatography was performed on Merck Kieslegel 60 F_{254} plates while silica gel column chromatography was performed on Kieselgel 60, both obtained from Merck (Darmstadt, FRG). [γ-^{32}P]ATP and [α-^{35}S]dATP were obtained from New England Nuclear (Billerica, MA) while Sep-pak cartridges were purchased from Millipore (Milford, MA). T_4 polynucleotide kinase, M13 DNA, DNA polymerase I, T4 DNA ligase, and the restriction endonucleases *Hpa*II and *Ava*I were products of New England Biolabs (Beverly, MA). The 2′-deoxynucleoside 5′-*O*-(1-thiotriphosphates) were the generous gift of Prof. F. Eckstein, Goettingen, FRG. The DEAE-cellulose (DE-81) circles were purchased from Whatman (Whatman, Great Britain). Sephadex G-10 is a product of Pharmacia (Uppsala, Sweden). HPLC analyses were performed on a Beckman (Berkeley, CA) liquid chromatograph with a two-pump solvent delivery system and a 421 controller. The system was fitted with a variable-wavelength detector.

† This work was supported by USPHS Grant GM 37065 to L.W.M.
* Author to whom correspondence should be addressed.

0006-2960/89/0428-0261$01.50/0

The ODS-Hypersil and MOS-Hypersil were products of Shandon Southern Products Lts. (Runcorn, England). Integration of the HPLC peaks was performed on a Shimadzu C-R3A Chromatopac integrator (Kyoto, Japan). Polyacrylamide gels were destained with a KS-10 shaker from BEA-Enprotech Corp. (Hyde Park, MA), and the fluorescent bands containing DNA were viewed on a transilluminator, Model TL-33 (λ_{max} = 366 nm), manufactured by UVP, Inc. (San Gabriel, CA). Fluorescence in solution was measured with a Perkin-Elmer (Norwalk, CT) fluorescence spectrophotometer, Model 650-10S. The radioactivity was measured with an LKB Wallac RackBeta liquid scintillation counter (Uppsala, Sweden). A Varian multinuclear FT-NMR at 121.5 MHz was used for the ^{31}P NMR studies. Oligodeoxynucleotides were synthesized on an Applied Biosystems (Foster City, CA) 381A DNA synthesizer.

Methods

Chemical Oligodeoxynucleotide Synthesis. Tp(s)T[1] and d[GC(s)CCGGGC] were gifts of Professor F. Eckstein (Max-Planck-Institut für experimentelle Medizin, Göttingen, FRG).

The $(dT)_{15}$ with phosphorothioate diesters 3′ to thymidine residues 7, 8, and 9 was synthesized by using the phosphite triester methodology (Beaucage & Caruthers, 1981) on a solid-phase CPG support. The synthesis was interrupted prior to the oxidation step when the incorporation of a phosphorothioate diester was desired. The normal oxidation step with 0.1 M I_2 in tetrahydrofuran/distilled water/lutidine (40:1:10) was replaced with a solution of 2.5 M sulfur in CS_2/lutidine (1:1). The sulfur oxidation solution was injected directly onto the column with a syringe. After a reaction time of 1 h at ambient temperature, the column was washed with a 1:1 solution of CS_2 and lutidine to remove the residual sulfur. The column was then replaced on the machine, and the synthesis cycle was resumed. The 21-mer d[GCTATCGAAA-GATCTCATAAG] was synthesized in an analogous manner. The synthesis was interrupted at every oxidation step to allow oxidation with the sulfur solution.

Both oligodeoxynucleotides were deprotected in ammonia at 50 °C for 18 h. Isolation was done by reverse-phase HPLC on a 9.4 × 250 mm column of MOS-Hypersil using a buffer of 50 mM triethylammonium acetate, pH 7.0, with a gradient of 20–65% acetonitrile in 40 min.

Solution Fluorescent Labeling Studies. Monobromobimane (MBB) was dissolved in acetonitrile, and stock solution (100 mM) was stored in the dark at −20 °C.

Typically, the oligodeoxynucleotides of interest were treated with an excess of monobromobimane, and the reaction was monitored by HPLC. Specifically, a solution of Tp(s)T (3.6 mM) in water was allowed to react overnight (18 h) with a 6-fold excess of monobromobimane (22 mM). The octamer (0.3 mM) in water was allowed to react with either a 5-fold excess of MBB (1.5 mM) or a 10-fold excess of MBB (3.0 mM). The fragment Tp(s)Tp(s)Tp(s)T (0.43 mM, a phosphorothioate diester concentration of 1.29 mM) was treated with an 8-fold excess (with respect to the phosphorothioate diesters) of MBB (10.5 mM). Covalent fluorescent labeling of the 15-mer in solution (0.8 mM) with MBB was achieved at 7.5 mM MBB (3-fold excess for 2.4 mM phosphorothioate diester).

The bimane-labeled Tp(s)T was isolated by reverse-phase HPLC on a 4.6 × 250 mm column of ODS-Hypersil with 50 mM triethylammonium acetate, pH 7.0, and a gradient of 0–70% acetonitrile in 1 h. The other labeling reactions were monitored by reverse-phase HPLC on a 4.6 × 250 mm column of ODS-Hypersil with either 20 mM KH_2PO_4, pH 5.5, and a gradient of 0–70% methanol in 30 min (the octamer and tetramer) or 50 mM triethylammonium acetate, pH 7.0, and a gradient of 0–35% acetonitrile in 1 h (15-mer).

Thin-layer chromatography studies were performed on silica gel thin-layer plates with a mobile phase of dichloromethane/methanol (9:1).

pH Stability Studies. Duplicate reaction mixtures of 6 nmol of bimane-labeled Tp(s)T were incubated at ambient temperature in 50 mM buffer at the appropriate pH values. The following buffers were used: pH values 3, 4, and 5, acetic acid/potassium acetate; pH values 6 and 7, KH_2PO_4/K_2HPO_4; pH values 8 and 9, Tris-HCl; pH values 10 and 11, CAPS. At various reaction times, the samples were analyzed by HPLC on a 4.6 × 250 mm column of ODS-Hypersil using 0.02 M potassium phosphate, pH 5.5, with a linear gradient of 0–70% methanol in 30 min. The bimane-labeled Tp(s)T eluted at 21 min, while the product TpT eluted at 16 min.

^{31}P NMR. The ^{31}P NMR studies were done at 121.5 MHz using a Varian multinuclear FT-NMR. Positive chemical shift values are reported in parts per million (ppm) downfield from the external standard of aqueous 85% phosphoric acid. NMR analysis was done on a sample containing 1.2 μmol of Tp(s)Tp(s)Tp(s)T (3.5 μmol of phosphorothioate diesters) and 20 mM Na_2EDTA. The sample was adjusted to a volume of 250 μL with D_2O. After NMR analysis of the tetramer, 10 μmol of monobromobimane (a 3-fold excess with respect to the diesters) in 100 μL of acetonitrile was added to the NMR tube with a final volume of 350 μL. The sample was allowed to react for 2.5 h at ambient temperature in the dark. NMR analysis was then repeated.

Radioisotopic Labeling. ^{32}P End Labeling. A reaction mixture in a final volume of 200 μL containing 40.1 μM 15-mer (1 A_{260} unit), 40.7 μM ATP, 10 mM $MgCl_2$, 10 mM dithiothreitol, 5 μg/mL bovine serum albumin, 40 mM Tris-HCl, pH 8.7, 0.127 μM (0.152 mCi) [γ-^{32}P]ATP, and 10 units of T_4 polynucleotide kinase was incubated at 37 °C for 18 h. After the addition of the reaction mixture to the Sep-pak cartridge (prewashed with 20 mL of methanol and 20 mL of distilled water), it was washed with 10 mL of 1% aqueous methanol to elute the unincorporated ATP and buffer salts. The oligodeoxynucleotide was eluted with 10 mL of 50% aqueous methanol. The solution containing the DNA fragment was evaporated to dryness and redissolved in 0.4 M distilled water. Isolated yields ranged from 60 to 80%.

The 21-mer, 23.3 μM (1 A_{260} unit), was end labeled in an analogous manner but could not be eluted with aqueous methanol. In this case, the Sep-pak cartridge was prewashed with acetonitrile and distilled water. The unincorporated ATP and salts were then eluted with 1% aqueous acetonitrile while the oligodeoxynucleotide was eluted with 50% aqueous acetonitrile. Isolated yields also ranged from 60 to 80%.

Fluorescence Studies. The fluorescence (excitation 385 nm, emission 465 nm) of varying solutions of bimane-labeled Tp(s)T in 5 mM KH_2PO_4, pH 4.5, was measured by using a fluorescence spectrophotometer, and a standard curve of fluorescence vs phosphorothioate diester concentration was fitted to the data employing a linear least-squares analysis.

[1] Abbreviations: MBB, monobromobimane; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; Na_2EDTA, disodium salt of ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; TBE, tris–borate/EDTA; TpT = thymidylyl(3′→5′)thymidine; Tp(s)T, phosphorothioate diester derivative of TpT; dNTPαS, 2′-deoxynucleoside 5′-*O*-(1-thiotriphosphate).

After post-assay fluorescent labeling (see below) with monobromobimane, the 5′-^{32}P end-labeled 15-mer was electroeluted for 2 h from a 20% polyacrylamide gel into dialysis tubing containing 0.5× TBE buffer. The solution was evaporated to dryness, redissolved in 1 mL of distilled water, and desalted using a column of Sephadex G-10. The DNA fragment was collected, evaporated to dryness, and redissolved in 3 mL of 5 mM KH_2PO_4, pH 4.5. The fluorescence of the solution was measured and the concentration of the 15-mer determined by scintillation counting. The fluorescence as a function of concentration of the phosphorothioate diesters was plotted on the standard bimane-labeled Tp(s)T curve.

In similar fashion, the 5′-^{32}P end-labeled 21-mer was electroeluted for 24 h from the polyacrylamide gel after post-assay labeling. The solution was evaporated to dryness and redissolved in 0.5 mL of distilled water. In this case, the solution containing the 21-mer was adjusted to 10 mM $MgCl_2$ and 2 M ammonium acetate, 1 volume of ice-cold acetonitrile was added, and the solution was kept at −70 °C for 18 h. The salt precipitated out of solution while essentially all of the DNA remained in the supernatent. The solubility of the labeled 21-mer in acetonitrile is largely a result of the increased hydrophobicity conferred upon the oligonucleotide due to the presence of the bimane residues. The supernatant was decanted, evaporated to dryness, and dissolved in 3 mL of 5 mM KH_2PO_4, pH 4.5. The fluorescence and radioactivity were measured and compared with the standard curve.

DNA Polymerase and Restriction Endonuclease Reactions. M13 mp18 DNA was converted to the replicative form (RF) as follows. The template DNA (2.5 μg) and universal primer (0.1 μg) were annealed in 25 μL of buffer containing 100 mM NaCl, 20 mM $MgCl_2$, and 100 mM Tris-HCl, pH 8.0, by heating the mixture to 56 °C for 15 min followed by slow cooling to ambient temperature. The final 50-μL reaction mixture containing dATP, dGTP, dCTP, dTTP (500 μM each), ATP (1 mM), DNA polymerase I (*Escherichia coli*, 10 units), and T4 DNA ligase (8 units) was incubated overnight at 16 °C. Substitution of the appropriate dNTPαS derivative(s) for the corresponding dNTP(s) essentially as described (Taylor et al., 1985) allowed the enzymatic incorporation of phosphorothioate diesters in place of phosphodiesters. In some cases for internal standardization, [α-^{35}S]-dATP (1.15 Ci/mmol) was employed in the elongation reaction.

Restriction digests with *Ava*I and *Hpa*II were performed as follows. The *Ava*I reaction mixture contained RF M13mp19 DNA, 100 mM NaCl, 20 mM $MgCl_2$, and 100 mM Tris-HCl, pH 8.0. The *Hpa*II reaction mixture contained RF M13mp18 DNA, 3 mM KCl, 5 mM $MgCl_2$, 100 μg/mL BSA, and 5 mM Tris-HCl, pH 7.4. The reactions were initiated by the addition of the enzyme and incubated at 37 °C for 2 h. The reaction mixture was loaded onto 6% acrylamide/0.6% bis(acrylamide) gels (20 × 20 × 0.04 cm or 34 × 42 × 0.04 cm) containing 3 mM Na_2EDTA, 7 M urea, and 50 mM Tris–borate, pH 8.3. Fluorescent labeling proceeded as described below.

Post-Assay Labeling. Gel electrophoresis was performed on 20 × 20 × 0.04 cm or 34 × 42 × 0.04 cm gels of 20% acrylamide, 2% bis(acrylamide) [or 6% acrylamide and 0.6% bis(acrylamide)], 50 mM Na_2EDTA, and 13 mM sodium persulfate. Post-assay labeling was performed both in the presence and in the absence of 7 M urea. The DNA was fixed in the gel by soaking it in 10% aqueous acetic acid for 5 min. The gel was then transferred to a 4 mM solution of monobromobimane in 50% aqueous acetonitrile and allowed to react overnight (18 h) in the dark. The gel was destained by shaking in 50% aqueous acetonitrile for 1 h. The short destaining appeared necessary because of minor reactions with the gel components and monobromobimane. Following a brief treatment (5 min) in 60 or 75% aqueous dimethylformamide, the DNA was viewed on a standard long-wavelength ultraviolet transilluminator at 366 nm. In some cases for internal standardization, the fluorescent bands of DNA were cut out of the gel and lyophilized before determination of the amount of DNA present in the gel via scintillation counting.

The effect of solvents on fluorescent intensity was also investigated. After post-assay labeling and destaining, the gels were treated with one of the following: 75% aqueous mixtures of methanol, ethanol, butanol, dimethylformamide, or concentrated glycerol. The gels were viewed using a long ultraviolet wavelength light transilluminator.

Results

Monobromobimane was chosen as the fluorescent marker in this study involving post-assay covalent labeling for two reasons. It is a relatively small molecule when compared with many fluorophores, and diffusion into the polyacrylamide gel matrix was anticipated to readily occur. Second, monobromobimane is essentially nonfluorescent until it reacts with a thiol group (Kosower et al., 1979). Phosphorothioate diesters provide a similar sulfur-containing functionality. Lengthy destaining procedures could then be avoided since fluorescent bands present in the gel would be solely the result of the bimane-labeled phosphorothioate diesters and the unreacted monobromobimane in the gel would be largely nonfluorescent.

The key step for the success of the post-assay labeling technique as described in this report involved the reaction of monobromobimane with a phosphorothioate diester. Previous work indicated that the corresponding monoester could be alkylated with monobromobimane (Cosstick et al., 1984). However, the phosphorothioate diester was expected to be less nucleophilic than the monoester. One of the first experiments involved the reaction of a simple phosphorothioate diester, Tp(s)T, with monobromobimane. This reaction was carried out in 65% aqueous acetonitrile and monitored by ^{31}P NMR, thin-layer chromatography, and HPLC. The ^{31}P resonances corresponding to the starting material were centered at +56 ppm. After a reaction with monobromobimane, the peaks had shifted upfield to resonate at +26 ppm. With TLC analysis (silica gel thin-layer plates), the starting diester remained at the origin while the product triester had an R_f value of 0.2 (dichloromethane/methanol, 9:1). The product was highly fluorescent when viewed under long-wave ultraviolet light. HPLC analysis indicated two peaks corresponding to the diastereoisomers of the unlabeled starting material with retention times centered at 16 min. After a reaction with excess monobromobimane, two new peaks were present at 18 min. The labeled dimer (Figure 1) could be isolated by silica gel or liquid chromatography. Bimane-labeled Tp(s)T exhibited an excitation maximum at 385 nm and an emission maximum at 465 nm in aqueous solution. This is similar to the excitation maximum of 390 nm and an emission maximum of 482 nm reported for bimane-labeled glutathione (Kosower et al., 1979). By spotting various quantities of the singly labeled dimer (Figure 1) on filter paper and employing long-wave ultraviolet light, the limit of detection with the naked eye was determined as approximately 25 pmol.

For efficient use of post-assay fluorescent labeling of phosphorothioate diesters in the polyacrylamide gel matrix, it was necessary to assay the stability of the labeled triester, especially with respect to the pH of the reaction mixture (or

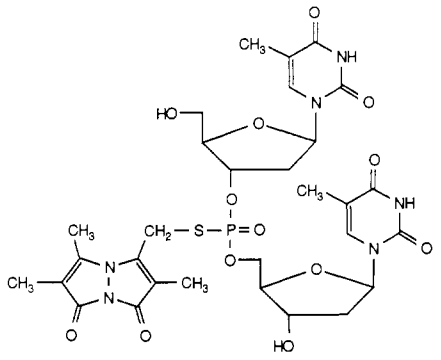

FIGURE 1: Structure of bimane-labeled Tp(s)T.

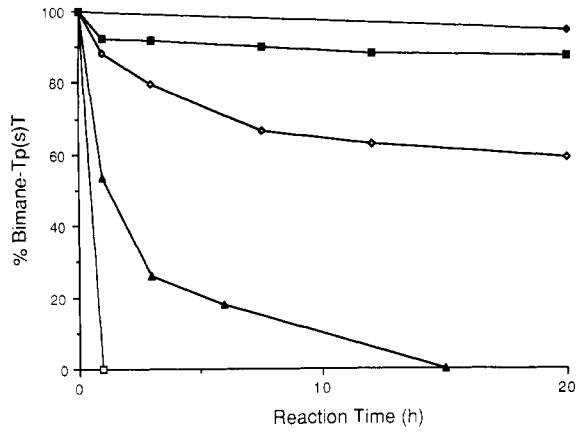

FIGURE 2: Stability of the bimane–Tp(s)T triester at ambient temperature during a total time period of 20 h at pH values 3–7 (♦), 8 (■), 9 (◇), 10 (▲), and 11 (□).

gel matrix). Phosphorothioate triesters are reported to be to be less stable than the corresponding diesters to base-catalyzed hydrolysis (Sammons & Frey, 1982). At low pH values (3–7), we observed less than 5% of the triester was hydrolyzed (Figure 2) after a 20-h incubation as determined by integration of the corresponding HPLC peaks. Upon incubation with Tris-HCl at pH 8 for 20 h, 11% of the triester was hydrolyzed. At pH 9, a 20-h incubation resulted in 40% of the hydrolysis product. The triester was completely hydrolyzed within 15 h at pH 10 and within 1 h at pH 11 (Figure 2). HPLC analysis confirmed that hydrolysis occurred by cleavage of the P–S bond and formation of TpT as expected.

To further characterize the reaction of monobromobimane with a phosphorothioate diester, the reaction was performed with an oligodeoxynucleotide which at ambient temperature exists largely in the double-stranded form. The reaction of the octamer d[GpCp(s)CpCpGpGpGpC] with a 10-fold excess of monobromobimane was performed in either distilled water or Tris-HCl pH 7, at ambient temperature. The HPLC analysis after a 5-h incubation (Figure 3) showed the starting material (14.88 min), a monobromobimane hydrolysis product (15.3 min), a product peak (17.75 min), and monobromobimane (25.21 min). The starting material was completely consumed within 23 h. With a 5-fold excess of monobromobimane, the reaction was complete within 48 h. The reacton proceeded equally well with either the R_P or the S_P diastereoisomer. A control reaction containing an oligodeoxynucleotide with only phosphodiesters failed to show any conversion to a labeled product.

Increasing the detection sensitivity of a bimane-labeled oligodeoxynucleotide beyond the 25 pmol achieved with the labeled Tp(s)T required the introduction of multiple fluorophores by alkylation of a number of phosphorothioate diesters with monobromobimane. Maximum detection sensitivity

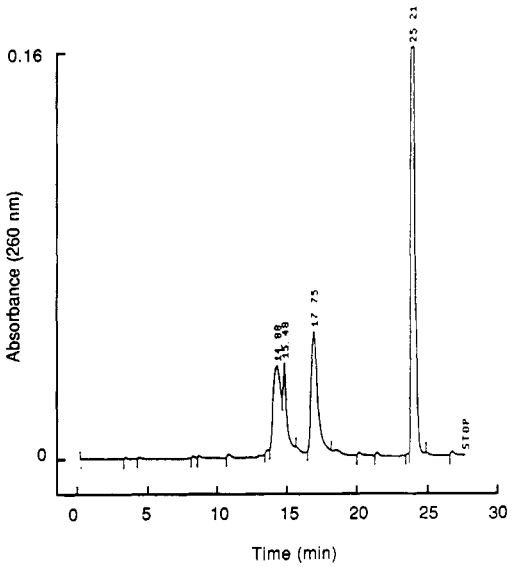

FIGURE 3: HPLC analysis of the reaction mixture containing the octamer d[GC(s)CCGGGC] (0.3 mM) and monobromobimane (3.0 mM) after reaction for 5 h at ambient temperature: 14.88 min (unlabeled octamer), 15.48 min (bimane hydrolysis product), 17.75 min (labeled octamer), and 25.21 min (monobromobimane).

necessitates that the reaction occur at adjacent residues. The labeling of the tetramer, Tp(s)Tp(s)Tp(s)T, containing three adjacent phosphorothioate diesters with monobromobimane was followed by ^{31}P NMR. The spectrum exhibited a number of resonances again centered around +56 ppm, corresponding to the phosphorothioate diesters. After reaction at ambient temperature for 2.5 h, these resonances had completely shifted upfield and were centered around +26 ppm, corresponding to the phosphorothioate triesters.

In a similar experiment, the reaction of the 15-mer containing three adjacent phosphorothioate diesters with monobromobimane was followed by HPLC. The starting material had a retention time of 29 min. Analysis of the reaction indicated the 15-mer was intially converted to a species which eluted with a retention time of 32 min. Subsequently, two additional peaks with retention times of 35 and 38 min were observed (data not shown). After a 3-h incubation time, only the peak eluting at 38 min was present. Electrophoretic and fluorescent studies performed on aliquots obtained from these three peaks suggested that the peaks corresponded to oligodeoxynucleotides containing one, two, and three bimane labels, respectively.

"Post-assay" fluorescent labeling was examined initially with a 21-mer containing 20 phosphorothioate diesters and the 15-mer containing 3 phosphorothioate diesters. Both fragments were end labeled with radioactive phosphate for internal standardization and then electrophoresed on a 20% polyacrylamide gel. After electrophoresis, the gel was fixed in aqueous acetic acid and soaked in a solution of 4 mM monobromobimane (see Experimental Procedures). After this post-assay labeling procedure, the fragments were electroeluted from the gel and desalted. Fluorescence as a function of this material was measured by using a fluorescence spectrophotometer, and the amount of the oligodeoxynucleotide, and thus the concentration of phosphorothioate diesters, was determined via scintillation counting. The fluorescence vs phosphorothioate diester concentration was compared with a standard curve obtained by measuring the fluorescence of varying concentrations of the bimane-labeled Tp(s)T. The fluorescence observed with the 15-mer was 3 times that observed with an equivalent amount of the labeled dimer, suggesting quantitative

labeling of the three phosphorothioate diesters. In contrast, similar measurements done with the 21-mer isolated by electroelution suggested that only about 20% of the phosphorothioate diesters were labeled with monobromobimane.

Some enhancement of fluorescence intensity in the gel could be observed by varying the polarity of the gel matrix. An aqueous solution of 75% butanol increased the intensity of fluorescence most significantly, followed by 75% aqueous ethanol, 75% aqueous methanol, 75% aqueous dimethylformamide, and concentrated glycerol. However, the gel became opaque and brittle, due to dehydration, in the presence of the first three solvents. An aqueous solution of 75% dimethylformamide was found to be most effective in enhancing the fluorescence properties while maintaining the integrity of the gel.

The 5′-^{32}P end-labeled 21-mer was viewed on a transilluminator (λ_{max} = 366 nm) after gel analysis and post-assay labeling. The bluish green bands were excised from the gel and lyophilized, and the amount of DNA present was determined by scintillation counting. The amount of the oligodeoxynucleotide visible as a result of the bimane fluorescence had decreased such that 500 fmol (500×10^{-15} mol) of the DNA fragments could be observed.

Longer DNA fragments containing phosphorothioates can be prepared by enzymatic synthesis if the dNTP substrates are substituted by the α-thio derivatives (Taylor et al., 1985). In order to generate fragments of defined length, we have extended an oligonucleotide primer using an M13mp18 or M13mp19 template and hydrolyzed the resulting material with a restriction endonuclease. It was possible to prepare M13 RF DNA containing phosphorothioates at each position. However, in the present studies, only one or two dNTPαS derivatives were employed since the incorporation of multiple phosphorothioate diesters appeared to interfere with the efficiency of the endonuclease reaction in agreement with previous observations (Potter & Eckstein, 1984). Cleavage of the elongated DNA with *Hpa*II produced fragments which migrated in the 6% polyacrylamide gel and could be visualized by post-assay fluorescent labeling (Figure 4A). A similar experiment with the *Ava*I restriction endonuclease produced a 444-nucleotide fragment which could be visualized by post-assay covalent labeling (Figure 4B). Some high molecular weight DNA could also be observed in this gel at the edge of the sample well (Figure 4B). With the 444-mer, the bands were excised, and the amount of DNA was determined by scintillation counting. Approximately 40 fmol (40×10^{-15} mol) of the 444-mer (containing a maximum of 104 bimane-labeled phosphorothioate diesters) could be visualized in this experiment.

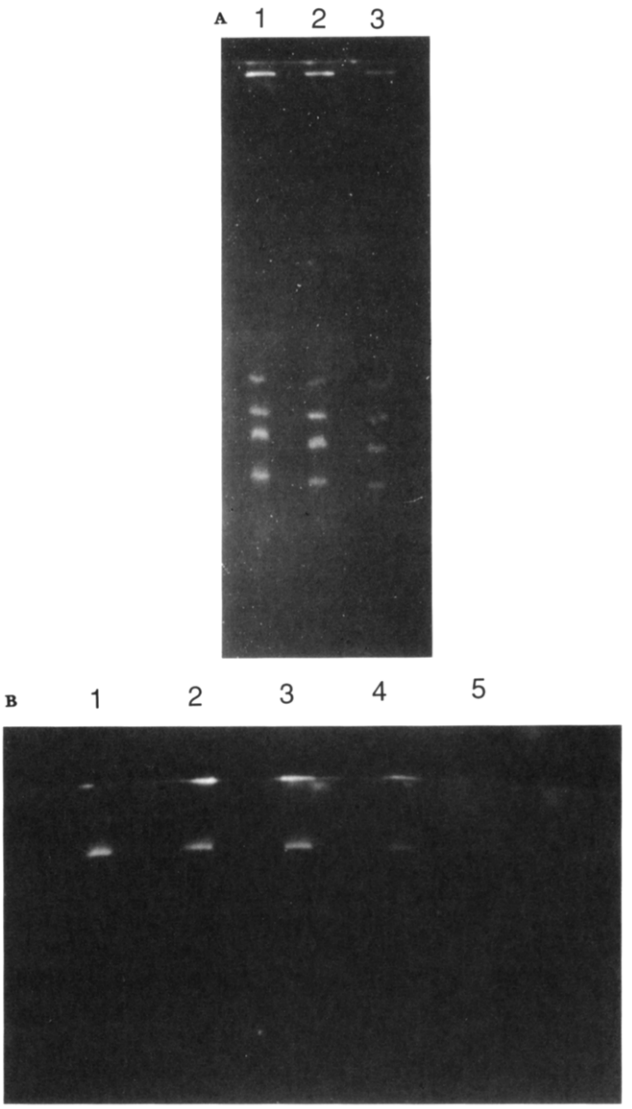

FIGURE 4: Post-assay labeling of DNA fragments (imbedded in 6% polyacrylamide gels) with monobromobimane. (*a*) *Hpa*II digest of an M13 mp18 DNA template which has been elongated with DNA polymerase I (*E. coli*) using dGTP, dCTP, dATPαS, and dTTPαS and then treated with the endonuclease: lane 1, 0.62 μg (260 fmol) of template; lane 2, 0.44 μg (183 fmol) of template; lane 3, 0.31 μg (130 fmol) of template. (B) *Ava*I digest of an M13 mp19 DNA template which was elongated with DNA polymerase I (*E. coli*) using dGTP, dCTP, dTTP, and dATPαS and then treated with the endonuclease. Only the 444-mer migrates into the gel (longer fragments are visible in the sample well). After visualization of the bands, the amount of DNA present was determined by scintillation counting: lane 1, 644 fmol; lane 2, 564 fmol; lane 3, 321 fmol; lane 4, 191 fmol; lane 5, 41 fmol.

Discussion

The substitution of a sulfur for a nonbridging oxygen creates a nucleophilic site in the nucleic acid fragment which allows for specific labeling of the phosphorus residue with monobromobimane without affecting the heterocyclic bases. This substitution at an internucleotidic linkage imparts chirality on the phosphorus molecule (Eckstein, 1985). At each phosphorothioate diester, the phosphorus–sulfur bond can be in either the S_P or the R_P diastereoisomeric form. The tetramer Tp(s)Tp(s)Tp(s)T and the 15-mer are both diastereoisomeric mixtures containing three phosphorothioate diesters (2^3 or eight compounds). In the case of the 21-mer, there are 2^{20} different species. After oligodeoxynucleotide synthesis, preparative HPLC analysis of the 21-mer, 15-mer, or tetramer performed with a 5′-hydroxyl protecting group resulted, despite the large number of species, in a single peak which could be easily collected and isolated. After the final deprotection, analytical HPLC analysis could resolve some of the individual diastereoisomers as has been previously reported in other cases (Bryant et al., 1981; Connolly et al., 1982).

The initial studies with Tp(s)T, the tetramer, the octamer, and the 15-mer indicate that the phosphorothioate diesters can be labeled with monobromobimane in solution. These reactions proceed smoothly and to completion as monitored by several analytical techniques. The monobromobimane labeling reaction with either the R_P or the S_P diastereoisomeric form of the octamer indicates that the reaction is not stereoselective. The time required for complete fluorescent labeling of the octamer was, however, considerably longer than the time necessary for complete fluorescent labeling of the other oligonucleotides. This suggests that the phosphorothioate diesters

in a double-stranded DNA helix are less accessible to reaction with monobromobimane.

To effectively employ a procedure involving the labeling of phosphorothioate diesters, it is necessary that the triesters have reasonable stability, especially with regard to pH. Although the bimane-labeled phosphorothioate triesters were more labile to base-catalyzed hydrolysis than the corresponding diesters, they were stable for a minimum of 20 h at pH values less than 8. Higher pH values resulted in increased hydrolysis of the bimane-labeled Tp(s)T. Similar results can be expected for the matrix-bound triesters and suggest that labeling procedures will be less efficient a pH values greater than 8.

The utility of fluorescent rather than radioisotopic labeling of nucleic acids requires high detection sensitivity, preferably without the use of sophisticated electronics. With the single labeled dimer Tp(s)T, the limit of detection to the naked eye was 25 pmol. With radioisotopic labeling, it is possible to detect low femtomolar quantities and in ideal situations subfemtomolar quantities. To increase the detection sensitivity of fluorescent labeling procedures to levels comparable with those of radioisotopic labeling, it is necessary to maximize the amount of label present in the nucleic acid. To achieve this goal with the present technique, it was necessary that adjacent phosphorothioate diesters be alkylated with monobromobimane. ^{31}P NMR of the Tp(s)Tp(s)Tp(s)T labeling reaction shows a complete shift in resonances indicating that adjacent diesters are being converted to the triesters. HPLC analysis of the solution labeling study done with the 15-mer also suggests that all three phosphorothioate diesters could be converted to the labeled triesters. These two studies indicate that a bimane derivative bound to a diester does not significantly hinder binding of a second bimane derivative at the adjacent diester. This is in contrast to the observation that bimane labeling of double-stranded DNA occurs at a much slower rate than it does with single-stranded DNA. Although the DNA helix may hinder binding of the fluorescent molecule, the presence of an adjacent bimane-labeled triester in a single-stranded fragment does not.

Post-assay labeling of the 15-mer appears to result in efficient reaction at the three adjacent phosphorothioate diesters within the polyacrylamide gel matrix on the basis of fluorescence measurements. The isolated 15-mer shows 3 times the fluorescence of an equivalent amount of the bimane Tp(s)T. This additionally suggests that fluorescent intensity is linear for multiple dyes and there is relatively little nonradiative quenching.

The evidence for the 21-mer is less conclusive. The fluorescence of the 21-mer electroeluted from the gel suggests that only 20% of the phosphorothioate diesters were labeled. However, this figure is unlikely to accurately represent the number of phosphorothioate diesters which had been labeled in the gel. The electroelution of this fragment in 50 mM Tris–borate, pH 8.0, was very slow. A substantial amount (approximately 50%) of the 21-mer remained in the gel in spite of electroelution for 24 h. This is consistent with a decrease in the electrophoretic mobility from the increase in molecular weight and conversion of the negatively charged diesters to neutral triesters. Additionally, we have been unsuccessful in using polyacrylamide gel electrophoresis for the analysis of prelabeled oligodeoxynucleotides (bimane-labeled prior to electrophoresis). The gels exhibit a fluorescent smear suggesting that the labeled triesters hydrolyze as they move through the gel matrix. This may reflect an increase in the rate of hydrolysis at pH 8.3 (Tris–borate buffer) as the result of steric strain on the bimane-labeled triester as it migrates through the gel matrix. During the 24-h electroelution of the 21-mer, it is also likely that many of the bimane-labeled triesters hydrolyzed and only those fragments containing relatively few bimane triesters were actually isolated. The observed figure of 20% of phosphorothioate diesters labeled is at best a minimum and likely not representative of the efficiency of the post-assay labeling procedure. However, we cannot exclude that in some cases quantitative alkylation of multiple phosphorothioate diesters may not occur during post-assay labeling.

The quantum yield of a fluorescent molecule generally increases with decreases in solvent polarity (LePecq & Paoletti, 1967; Kanaoka, 1977). With the DNA fragments embedded in the gel matrix, it was possible to qualitatively study the effects of solvent on fluorescence without nucleic acid solubility problems. A brief treatment with a solution of 60–75% aqueous dimethylformamide was most effective in increasing the observed fluorescence while maintaining the integrity of the gel.

The limit of detection with the post-assay labeling procedure for the 21-mer was reduced to 500 fmol. This represents a 20–30-fold increase in sensitivity over the singly labeled Tp(s)T derivative within the range of detection sensitivity expected if all 20 phosphorothioate diesters are labeled in the gel matrix and each label makes an equivalent contribution to the total emitted radiation. When a 444-mer generated by a restriction endonuclease was used, the limit of detection was reduced further to 40 fmol. In all cases examined to date, to increase in the number of bimane molecules bound to the DNA results in an approximately linear increase in detection sensitivity. On the basis of these observations, DNA fragments 300–500 nucleotides in length containing phosphorothioate diesters at every position should be visible after post-assay fluorescent labeling in the low femtomolar range [approximately (10–20) $\times 10^{-15}$ mol].

The post-assay fluorescent labeling technique of introducing multiple fluorescent molecules into the nucleic acid after gel electrophoresis is capable of detecting oligodeoxynucleotides and DNA fragments with high sensitivity. This procedure may be applicable to hybridization studies using membrane-bound nucleic acids (Southern, 1975). It is also possible that such techniques could be applied to enzymatic (Sanger) sequencing of DNA (Sanger et al., 1977). Current technology of Sanger sequencing often utilizes the [α-^{35}S]dNTP derivatives (Biggin et al., 1983). The fragments generated by this technique often contain hundreds of phosphorothioate diesters. Post-assay labeling would be directly applicable to the detection of these fragments. With hundreds of bimane-labeled phosphorothioate triesters, effective DNA sequencing in the absence of radioisotopes and sophisticated electronics may be possible.

ACKNOWLEDGMENTS

We thank Prof. F. Eckstein, Goettingen, FRG, for samples of the octamer and the dNTPαS derivatives as well as discussions concerning this approach to fluorescent labeling.

Registry No. MBB, 71418-44-5; (R_p)-Tp(s)T, 83199-36-4; (S_p)-Tp(s)T, 83199-37-5; (R_p)-d[GC(s)CCGGGC], 101141-83-7; (S_p)-d[GC(s)CCGGGC], 101020-72-8; $(dT)_{15}$ with phosphorothioate diesters 3′ to dT residues 7, 8, and 9, 117269-04-2; Tp(s)Tp(s)Tp(s)T, 117226-27-4; bimane labeled Tp(s)T, 117203-44-8; bimane labeled octamer, 117203-45-9; bimane labeled Tp(s)Tp(s)Tp(s)T, 117203-46-0; bimane labeled 15-mer, 117269-05-3.

REFERENCES

Agrawal, S., Christodoulou, C., & Gait, M. J. (1986) *Nucleic Acids Res. 14*, 6227–6245.

Ansorge, W., Sproat, B., Stegemann, J., Schwager, C., & Zenke, M. (1987) *Nucleic Acids Res. 15*, 4593–4602.
Bauman, J. G. J., Wiegant, J., & Van Duijn, P. (1981a) *J. Histochem. Cytochem. 29*, 227–237.
Bauman, J. G. J., Wiegant, J., & Van Duijn, P. (1981b) *J. Histochem. Cytochem. 29*, 238–246.
Beaucage, S. L., & Caruthers, M. H. (1981) *Tetrahedron Lett. 22*, 1859–1862.
Biggin, M. D., Gibson, T. J., & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3963–3965.
Brigati, D. J., Myerson, D., Leary, J. J., Spalholz, B., Travis, S. Z., Fong, C. K. Y., Hsiung, G. D., & Ward, D. C. (1983) *Virology 126*, 32–50.
Bryant, F. R., Benkovic, S. J., Sammons, D., & Frey, P. A. (1981) *J. Biol. Chem. 256*, 5965–5966.
Chollet, A., & Kawashima, E. H. (1985) *Nucleic Acids Res. 13*, 1529–1541.
Connolly, B. A., & Rider, P. (1985) *Nucleic Acids Res. 13*, 4485–4502.
Connolly, B. A., Romaniuk, P. J., & Eckstein, F. (1982) *Biochemistry 21*, 1983–1989.
Connolly, B. A., Potter, B. V. L., Eckstein, F., Pingoud, A., & Grotjahn, L. (1984) *Biochemistry 23*, 3443–3453.
Cosstick, R., McLaughlin, L. W., & Eckstein, F. (1984) *Nucleic Acids Res. 12*, 1791–1810.
Chu, B. C. F., Wahl, G. M., & Orgel, L. E. (1983) *Nucleic Acids Res. 11*, 6513–6529.
Eckstein, F. (1985) *Annu. Rev. Biochem. 54*, 367–402.
Eshaghpour, H., Söll, D., & Crothers, D. M. (1979) *Nucleic Acids Res. 7*, 1485–1495.
Forster, A. C., McInnes, J. L., Skingle, D. C., & Symons, R. H. (1985) *Nucleic Acids Res. 13*, 745–761.
Fuller, W., & Waring, M. J. (1964) *Ber. Bunsen-Ges. Phys. Chem. 54*, 805–815.
Kanaoka, Y. (1977) *Angew. Chem. Int. Ed. Engl. 16*, 137–147.
Kempe, T., Sundquist, W. I., Chow, F., & Hu, S.-L. (1985) *Nucleic Acids Res. 13*, 45–57.
Kosower, E. M., & Pazhenchevsky, B. (1980) *J. Am. Chem. Soc. 102*, 4983–4993.
Kosower, E. M., Pazhenchevsky, B., & Hershkowitz, E. (1978) *J. Am. Chem. Soc. 100*, 6516–6518.
Kosower, N. S., Kosower, E. M., Newton, G. L., & Ranney, H. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3382–3386.
Langer, P. R., Waldrop, A. A., & Ward, D. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6633–6637.
Leary, J. J., Brigati, D. J., & Ward, D. C. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4045–4059.
LePecq, J.-B., & Paoletti, C. (1967) *J. Mol. Biol. 27*, 87–106.
LePecq, J.-B., Yot, P., & Paoletti, C. (1964) *C. R. Acad. Sci. Paris 259*, 1786–1792.
Potter, B. V. L., & Eckstein, F. (1984) *J. Biol. Chem. 259*, 14243–14248.
Prober, J. M., Trainor, G. L., Dam, R. J., Hobbs, F. W., Robertson, C. W., Zagursky, R. J., Cocuzza, A. J., Jensen, M. A., & Baumeister, K. (1987) *Science (Washington, D.C.) 238*, 336–341.
Richardson, R. W., & Gumport, R. I. (1983) *Nucleic Acids Res. 11*, 6167–6184.
Sammons, R. D., & Frey, P. A. (1982) *J. Biol. Chem. 257*, 1138–1141.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Smith, L. M., Fung, S., Hunkapiller, M. W., Hunkapiller, T. J., & Hood, L. E. (1985) *Nucleic Acids Res. 13*, 2399–2412.
Smith, L. M., Sanders, J. Z., Kaiser, R. J., Hughes, P., Dodd, C., Connell, C. R., Heiner, C., Kent, S. B. H., & Hodd, L. E. (1986) *Nature (London) 321*, 674–679.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.
Stec, W. J., Zon, G., Egan, W., & Stec, B. (1984) *J. Am. Chem. Soc. 106*, 6077–6079.
Taylor, J. W., Schmidt, W., Cosstick, R., Okruszek, A., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8749–8764.
Tyagi, S. C., & Wu, F. Y.-H. (1987) *J. Biol. Chem. 262*, 10684–10688.