Journal of Chromatography, 444 (1988) 67–77
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1301

PREPARATION AND CHROMATOGRAPHIC USE OF 5′-FLUORESCENT-LABELLED DNA PROBES

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SUMMARY

A convenient procedure for synthesizing and purifying fluorescently-labelled short DNA probes is reported. DNA probes were chemically synthesized on an automated instrument using the "Aminolink" reagent in the final cycle to attach a primary amino group at the 5′-terminus in the final step. The synthetic oligonucleotides were purified by polyacrylamide urea gel electrophoresis, followed by reversed-phase high-performance liquid chromatography (HPLC). The oligomers were then allowed to react with a fluorescent compound, and the products were separated by HPLC with consecutive detection by UV absorption and fluorescence. Gel permeation chromatography demonstrated that the fluorescent probes were able to form stable hybrids with complementary oligodeoxynucleotides. Furthermore, essentially 100% of the purified fluorescent probe was capable of hybridizing to its complementary strand. Special precautions in handling the fluorescent probes, such as stability, were investigated.

INTRODUCTION

DNA probes are most commonly radiolabelled with ^{32}P by using a phosphokinase[1]. Non-radioactive labelling procedures are preferable from the safety viewpoint, but any new procedure must also offer the sensitivity and ease of preparation of radiolabelling. One such approach involves the enzymatic incorporation of a biotin-labelled nucleotide[2]. The low-molecular-weight biotin moiety interacts tightly with the protein, avidin. Avidin, in turn, may be covalently coupled to a variety of indicator molecules, such as a fluorescent dye, an electron-dense protein, an antibody or an enzyme. Since there are numerous potential applications for non-radiolabelled DNA, it is desirable to have a convenient process that could be used in

0021-9673/88/$03.50

individual research laboratories. This would allow investigators to choose the reporter group most suitable for a particular research project. Incorporation of a primary amino group into the oligonucleotide probe should provide such a convenient site of attachment for the addition of a variety of reporter substances. A recent example of a modified base, having a primary amine functional group, is N^4-(6-aminohexyl)deoxycytidine[3]. The nucleoside triphosphate of this substance can be incorporated at the 3′-terminus of DNA with the use of the enzyme terminal deoxynucleotidyl transferase. Another approach utilizes a protected amino-derivatized thymidine phosphoramidite[4]. Our report focuses on the incorporation of a primary amino group at the 5′-terminus of an oligonucleotide by using the reagent 2-methoxy-3-trifluoroacetyl-1,1,3,2-oxazaphosphacyclopentane ("Aminolink"). This reaction can be automated in the DNA synthesizer. Aminolink is used in place of a phosphoramidite for the final cycle of synthesis at the 5′-terminus. Since at the present time probes are commonly prepared by chemical synthesis, this new reagent provides the investigator with a convenient site of attachment. Furthermore, attachment of the fluorescent moiety on the deoxyribose group at the 5′-terminus should result in minimal interference with duplex formation; it may even provide greater stability of the duplex through an intercalation mechanism[5].

Aminolink has recently become commercially available, and, to our knowledge, there is no publication in which it has been evaluated. Our laboratory has used this reagent in the synthesis of fluorescent DNA probes, and details of these procedures are presented here. The specificity of fluorescent labelling only at the 5′-terminus amino group is demonstrated. Some experimental data are presented as examples of proper storage and use of these fluorescent DNA probes. High-performance liquid chromatographic (HPLC) techniques play a central role in all aspects of this study, including purification and analysis of the intermediates and final products. The potential use of HPLC procedures for fluorescent probe assays is explored.

EXPERIMENTAL

Materials

Fluram (fluorescamine) was supplied by Roche Diagnostics (Hoffmann-La Roche, Nutley, NJ, U.S.A.). Fluorescein isothiocyanate (FITC) and rhodamine B isothiocyanate were purchased from Sigma (St. Louis, MO, U.S.A.); triethylamine from Matheson, Coleman & Bell (Norwood, OH, U.S.A.); glacial acetic acid, HPLC-grade acetonitrile, HPLC-grade methanol, HPLC-grade water, and sodium borate from J. T. Baker (Phillipsburg, NJ, U.S.A.); ammonium hydroxide from Fisher Scientific (Fair Lawn, NJ, U.S.A.); ethylenediaminetetraacetic acid, tetrasodium salt, trihydrate (EDTA) and ammonium acetate from Aldrich (Milwaukee, WI, U.S.A.); sodium dodecyl sulfate (SDS), xylene cyanol FF and Bromophenol Blue were from Bio-Rad Labs. (Richmond, CA, U.S.A.); Sephadex G-25 medium was from Pharmacia (Piscataway, NJ, U.S.A.); sodium chloride from Mallinckrodt (Paris, KY, U.S.A.), and sodium citrate from Specialty Chemicals Division (Morristown, NJ, U.S.A.). Water was purified with a CDOF 01205 system from Millipore (Ridgefield, NJ, U.S.A.).

Equipment

The HPLC system consisted of a Varian (Sunnyvale, CA, U.S.A.) 5000 liquid chromatograph ternary system, a Varian 9060 polychrom photodiode-array detector, a McPherson (Acton, MA, U.S.A.) FL-750 spectrofluorescent detector, a Kipp & Zonen (Bohemia, NY, U.S.A.) two-channel recorder, and a Varian 4290 integrator. Chromatographic separations were performed on a Supelco (Bellefonte, PA, U.S.A.) 300-Å, 5-μm, C_4 column (2 × 0.46 cm I.D.). Oligonucleotides were synthesized on Applied Biosystems (Foster City, CA, U.S.A.) 380A and 380B DNA synthesizers. The gel electrophoresis system consisted of a Bio-Rad Labs. Protean slab cell and an LKB (Gaithersburg, MD, U.S.A.) 2197 electrofocusing constant-power supply. A Beckman (San Ramon, CA, U.S.A.) Du-7 spectrophotometer was used for measuring absorption.

Aminolink-DNA probes

All Aminolink-oligonucleotides were synthesized on an Applied Bioystems Model 380B DNA synthesizer, using Applied Biosystems β-cyanoethyl phosphoramidites and reagents and wash solvents from J. T. Baker according to the manufacturer's directions. Thiophenol was not used for phosphate deprotection. Details on the Aminolink reagent may be found in ref. 6.

Polyacrylamide gel electrophoresis (PAGE) separation and extraction of Aminolink-DNA probes

All Aminolink-oligonucleotides were deprotected by heating 5 mg of the crude reaction mixture in 3 ml of ammonium hydroxide at 55°C for 6 h. The samples were concentrated on a Savant (Farmingdale, NY, U.S.A.) speed-vacuum overnight at room temperature. Each sample was then aliquoted and loaded onto two 20% acrylamide gels (16 × 20 cm) containing 8 *M* urea. A constant power of 20 W was applied to the gels until the Bromophenol Blue dye (equivalent to twelve bases) reached the bottom of the gel. The bands were excised and extracted at 37°C overnight with 3 ml of 0.5 *M* ammonium acetate buffer (pH 7.0) containing 1 m*M* EDTA and 0.1% SDS. Each extract was passed through a Gelman Sciences, (Ann Arbor, MI, U.S.A.) Acrodisc 0.22-μm filter, and then concentrated overnight on the Savant speed-vacuum.

HPLC purification of Aminolink-DNA probes

The aqueous mobile phase (A) for the HPLC purification was 0.1 *M* triethylammonium acetate (TEAA) (pH 7.0). This buffer was prepared by dilution from a stock solution which was 1 *M* in triethylamine, 1 *M* in acetic acid, and adjusted to pH 7.0 with sodium hydroxide. The organic mobile phase (B) was 0.1 *M* TEAA in 60% aqueous acetonitrile. Chromatography was carried out with the following gradient: 100% mobile phase A for 3 min, then a shallow linear gradient from 0 to 35% mobile phase B (21% acetonitrile) in 25 min and then a sharper linear gradient from 35 to 90% mobile phase B (54% acetonitrile) in 25 min. The flow-rate was 1 ml/min, and the effluent was monitored at 258 nm.

Synthesis of fluorescent probes

Fluorescamine-DNA probes. Aliquots containing 10 nmol (as determined by

A_{260}) of each of the Aminolink-oligonucleotides was reconstituted in 100 µl of 0.1 *M* sodium borate buffer (pH 8.5). A volume of 20 µl of fluorescamine (1 mg/ml of anhydrous acetonitrile) was then added, while vortexing the oligomer solution. The reaction products were then purified by HPLC.

Fluorescein-DNA probes. An aliquot of an Aminolink-oligonucleotide containing 39 nmol was dissolved in 100 µl of water. Triethylamine (5 µl) was added to bring the pH to between 10 and 11. Then, 500 µl of fluorescein isothiocyanate (4 mg/ml of dry methanol) was added. The mixture was vortexed and incubated at 55°C for 10 min and then diluted with 1.5 ml of 0.1 *M* TEAA buffer (pH 7.0).

Rhodamine B-DNA probes. An aliquot of an Aminolink-oligonucleotide, containing 12 nmol, was dissolved in 1 ml of water. A volume of 5 µl of triethylamine was added to bring the pH to between 10 and 11. A volume of 200 µl of rhodamine B isothiocyanate (4 mg/ml of methanol) was added, vortexed and incubated for 10 min at 55°C. Another 200 µl of rhodamine B isothiocyanate solution was added, vortexed and incubated at 55°C for 10 min. The resulting mixture was then diluted with 1.5 ml of 0.1 *M* TEAA buffer (pH 7.0).

o-Phthalaldehyde (OPA)-DNA probes. Varying amounts of 1–10 nmol of an Aminolink-oligonucleotide in 10 ml of water were allowed to react with 400 µl of OPA at 37°C for 30 min. The preparation of the OPA reagent has been previously described[7]. The reagent was made by adding 8 mg of OPA in 0.1 ml of 95% ethanol and 20 µl of reagent-grade 2-mercaptoethanol to a 10-ml volumetric flask. The mixture was diluted to 10 ml with 0.2 *M* potassium borate buffer (pH 10.2) (Brij-35 was not used in the preparation). The product was injected into the reversed-phase column without further purification.

Calibration of the fluorometer with quinine sulfate

A 10 mg/ml stock solution of quinine hydrochloride was prepared with 0.05 *M* sulfuric acid. An aliquot was taken and diluted by a factor of 10^4 in 0.05 *M* sulfuric acid to give a final concentration of 1 µg/ml. The fluorometer flow cell was filled with this solution and the excitation and emission maxima were optimized at 350 and 450 nm, respectively. The fluorometer gain was then set to give 1.0 relative fluorescence unit. The HPLC column was then connected, and samples for the recovery experiment were analyzed.

Chromatographic purification of fluorescent probes

The reaction mixtures of Aminolink-oligomer with rhodamine B isothiocyanate and with fluorescein isothiocyanate were freed of excess fluorescent reagent by passage through a Sephadex G-25M column (100 × 2 cm I.D.), equilibrated with 0.1 *M* TEAA (pH 7.0) and then purified by reversed-phase HPLC. The fluorescamine and OPA reaction mixtures were directly purified by reversed-phase HPLC, which was carried out with the same mobile phases described above. All samples were lyophilized and reconstituted in 0.5 ml of mobile phase A. A linear gradient from 0 to 35% mobile phase B in 60 min, followed by a 5-min linear gradient to 90% mobile phase B was used to separate the labelled from the unlabelled oligomer. The flow-rate was 1 ml/min. The effluent was monitored by absorbance at 258 nm and by the fluorescence excitation (ex) and emission (em) wavelengths, corresponding to each fluorescent reagent. These wavelengths are as follows: fluorescamine ex/em, 390/475

nm; fluorescein ex/em, 475/540 nm; rhodamine B ex/em, 556/583 nm; and OPA ex/em, 340/440 nm.

Hybridization of fluorescent probes

A mixture of a 22-mer fluorescent probe (10 μl containing 400 pmol) and a complementary 46-mer target (5 μl containing 1.0 nmol) in the commonly used 6 × SSC buffer (1.2 *M* sodium chloride–0.1 *M* sodium citrate in water) was heated to 70°C [(5°C over the calculated melting temperature (T_M)] for 10 min. The mixture was allowed to cool to room temperature for 30 min. Hybridization was monitored by gel permeation chromatography with 0.3 *M* TEAA (pH 7.0) at room temperature on an Altex Spherogel TSK column 3000 SW (300 × 7.5 mm I.D.). In another experiment, 0.8 nmol of the fluorescent probe was hybridized with 1.0 nmol of the 46-mer complementary oligomer.

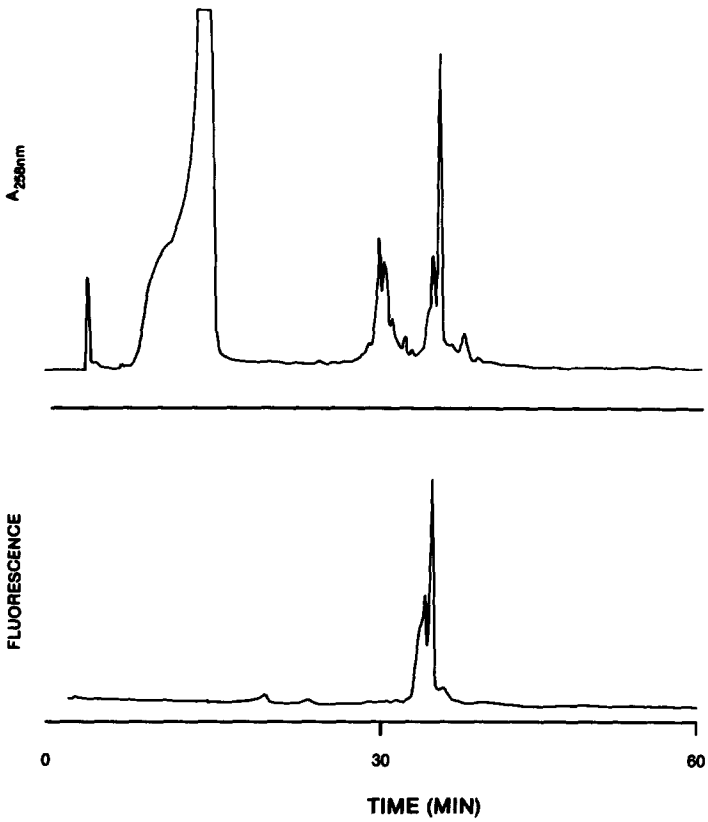

Fig. 1. Reversed-phase HPLC analysis of the reaction mixture of fluorescamine with an Aminolink-oligomer (16-mer). The column effluent was monitored, consecutively, by absorption (258 nm) (top tracing), followed by fluorescence (390 nm excitation, 475 nm emission) (bottom tracing). The major peak at about 13 min is hydrolyzed fluorescamine, which is non-fluorescent. The jagged peak at about 28 min corresponds to unreacted Aminolink-oligomer. The jagged peak at about 35 min is the fluorescamine derivative of the Aminolink oligomer. It represents the only fluorescent substance in the reaction mixture.

RESULTS AND DISCUSSION

Synthesis and purification of fluorescent probes

Aminolink is a reagent specifically designed to react with the 5′-hydroxyl of oligonucleotides when used in place of a phosphoramidite in the automated DNA synthesizer. It was observed that a jagged peak (see Figs. 1 and 2) is obtained on reversed-phase HPLC with an Aminolink-oligomer, rather than the smooth peak, typically seen with an oligomer that terminates in a 5′-hydroxyl group. Only about half of the Aminolink-oligomer was converted to a fluorescent derivative upon reaction with fluorescamine. This derivative was eluted later in the gradient (Fig. 1). The fluorescent derivative peak was also jagged in appearance. The unreacted Aminolink-oligomer peak was collected, taken to dryness, and then again allowed to react with fluorescamine. The mixture was analyzed by reversed-phase HPLC. This revealed that there was no further conversion to the fluorescent oligonucleotide (not

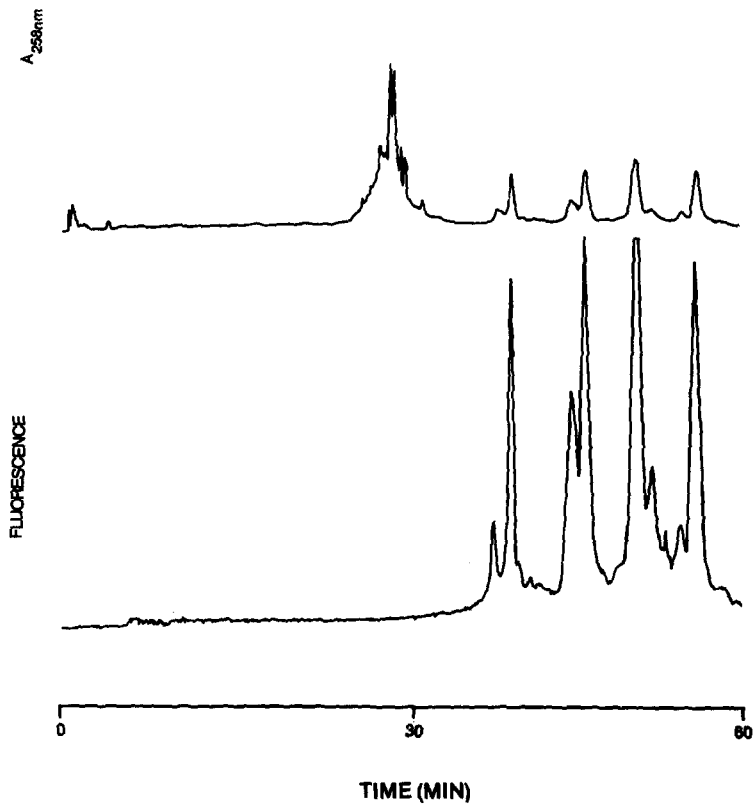

Fig. 2. Reversed-phase HPLC analysis of prepurified rhodamine-oligomer (22-mer). The products of reaction of rhodamine B isothiocyanate and an Aminolink-oligomer were first separated by gel permeation chromatography. The high-molecular-weight fractions, freed of excess rhodamine reagent, were concentrated and then applied to the HPLC column. The jagged peak at about 27 min is unreacted Aminolink-oligomer. The four groups of peaks between 30 and 60 min represent fluorescent derivatives of the Aminolink-oligomer. Excess rhodamine was found to be eluted as four separated peaks at a concentration of acetonitrile higher than that required for elution of the oligomer derivatives (not shown).

shown). This indicated that only about half of the starting Aminolink-oligomer was in a form that contained a reactive primary amino group. It should be noted that even if fluorescamine is not desired as the fluorescent dye for the final product, this reagent is especially useful for the rapid analysis of the quality of the synthetic Aminolink-oligonucleotide.

Reversed-phase HPLC of the rhodamine-oligomer, after prepurification by gel permeation chromatography, is shown in Fig. 2. Again, about half of the Aminolink-oligomer did not react with rhodamine B isothiocyanate. The fluorescent oligomer was resolved into four jagged peaks. This multiplicity may be explained by the presence of four isomers of rhodamine, as revealed by reversed-phase HPLC of a control reaction of rhodamine isothiocyanate in the absence of oligomer (not shown). Fluorescein isothiocyanate yielded two jagged fluorescent oligomer peaks, corresponding to two isomers of this fluorescent reagent (not shown). The product of reaction with OPA produced multiple oligomer derivatives, but none of them was fluorescent (not shown). Absorption spectra of the rhodamine and fluorescein oligomers (Fig. 3) revealed maxima at the expected wavelengths for the oligonucleotide and the respective fluorescent dye.

Control experiments were carried out to ensure that only the primary amino group and not the aromatic amino groups reacted with the fluorescent reagents. Reactions and purifications were performed with the usual non-Aminolink oligomers, using either fluorescein isothiocyanate or fluorescamine, as described in the Experimental section. Fluorescent oligonucleotides were not observed in these reaction mixtures by HPLC analysis, indicating that the derivatization reactions are specific for the primary amino group, which had been added at the 5'-terminus.

Recovery from polypropylene tubes

A study of the recovery of picomol amounts of fluorescent oligomers from polypropylene tubes was performed. This study was undertaken in anticipation of the use of picomol quantities of fluorescent oligomer probes in particular assays. The following amounts of 5'-fluorescein-labelled 17-mer oligonucleotide were added to ten polypropylene tubes, containing 100 μl of 6 × SSC buffer: 1.9, 3.8, 9.5, 13, 19, 19, 38, 95, 133 and 190 pmol. Each sample was diluted with 300 μl of water and

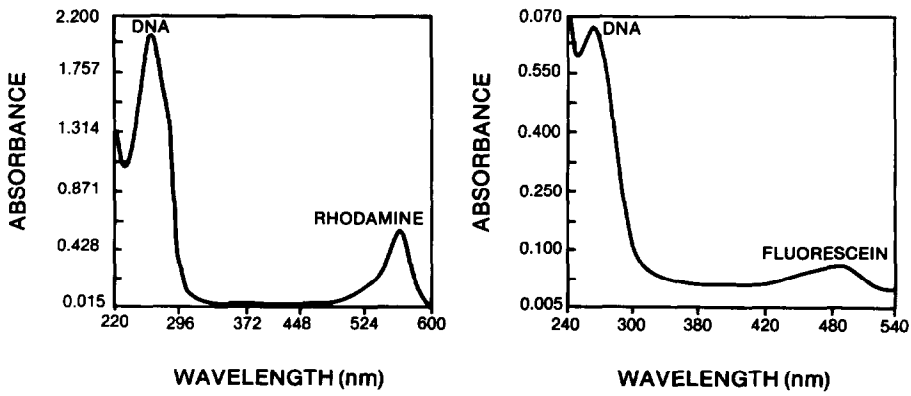

Fig. 3. Absorption spectra of purified fluorescent oligodeoxynucleotides.

injected into the Supelco C_4 column. The same set of experiments was performed with the addition of 5 nmol of carrier DNA to each sample. The recovery of picomole amounts of fluorescent DNA probes from polypropylene tubes was close to 3% when only high salt concentrations were used (6 × SSC), but when 5 nmol of an unrelated oligomer was added, the recovery of the fluorescent probes was above 80%. The fluorescent response of the fluorescein-labelled DNA proved to be linear in the 19–200 pmol range. This experiment illustrates the importance of adding carrier DNA when working with picomole amounts of fluorescent oligonucleotide probes.

Stability of fluorescent-labelled DNA probes

This study was undertaken to determine appropriate storage conditions for the fluorescent derivatives. From a stock solution of a 16-mer Aminolink-oligonucleotide, nine aliquots containing 3 nmol each were pipetted into polypropylene tubes (see Table I). On day 1, six aliquots in 100 μl of 0.1 *M* sodium borate buffer (pH 8.5) were allowed to react with 20 μl of fluorescamine (1 mg/ml of dry acetonitrile); three of these were dried and stored at −20°C, while the other three were analyzed that day by HPLC. The remaining three tubes of unreacted Aminolink-oligomer were stored in the frozen state until day 7. On day 7, the dried and stored fluorescent-labelled oligomers were reconstituted in 0.1 *M* borate buffer (pH 8.5) and analyzed by HPLC. The final three aliquots of unreacted Aminolink-oligomer, which had been stored frozen in 100 μl of 0.1 *M* sodium borate buffer (pH 8.5) were allowed to react with 20 μl of fluorescamine (1 mg/ml of dry acetonitrile) on day 7 and also analyzed by HPLC. The experiment was repeated with the addition of 4 nmol of carrier DNA to each of the nine tubes. The results of these analyses are given in Table I.

When the Aminolink-oligomer was stored for seven days and then allowed to react with fluorescamine and injected into the HPLC system, the recovery was found to be 86%, in comparison with the samples that had been allowed to react and

TABLE I

RECOVERY OF FLUORESCENT PROBES

Day of reaction	*Day of chromatography*	*Average* area*	*Recovery (%)*
Fluorescamine without carrier DNA			
1	1	8.6 (0.06)	
1	7	6.2 (0.40)	71
7	7	7.4 (0.81)	86
Fluorescamine with carrier DNA			
1	1	8.3 (0.64)	
1	7	5.8 (0.06)	70
7	7	8.5 (0.30)	102
Rhodamine B without carrier DNA			
1	1	7.7 (0.95)	
1 (dried)	6	7.7 (0.37)	100
1 (solution)	6	6.8 (0.43)	88

* Each value is the average of triplicates, the standard deviation is given in parentheses.

analyzed on day 1. When the fluorescamine-oligomer was stored for seven days, recovery of the fluorescence averaged 71%. The addition of the carrier DNA did not appear to improve the recovery of fluorescent-labelled oligomer that was dried and stored for seven days, but it did improve the recovery of the Aminolink-oligomers that were stored for seven days before reaction and injection into the HPLC column. The recoveries were 70 and 102%, respectively. It may be concluded that the fluorescamine oligomer is somewhat unstable. Aminolink-oligomers may also suffer some losses due to adsorption on surfaces, such as the walls of polypropylene tubes, but this can be minimized by adding carrier DNA.

A similar study was performed with a rhodamine-labelled oligomer. Nine aliquots, each containing 1 nmol in 6 × SSC buffer, were pipetted into polypropylene tubes. A sample (300 pmol) from each of the first three aliquots was analyzed by HPLC on the first day. Another three aliquots were dried and stored at −20°C, and the remaining three aliquots were stored in solution at −20°C. On day 6, the three tubes of dried rhodamine-oligomer were reconstituted with water, and a sample (300 pmol) from each was analyzed by HPLC. A sample (300 pmol) from each of the final three aliquots, stored frozen in 6 × SSC buffer, was also analyzed on day 6. In each chromatographic run, the areas of the component peaks were added up to determine the total fluorescence of the rhodamine-oligomer (see Fig. 2). The data are summarized in Table I. The average recovery of fluorescence was 100% for the dried samples and 88% for the samples kept frozen in solution, indicating that storage in the dried state is preferable. The values reported in Table I should be considered only as approximations, since they are based in a limited number of data points.

Hybridization analysis

The hybridization of a fluorescent, rhodamine-labelled, 22-mer to a non-fluorescent complementary 46-mer was monitored by gel permeation chromatography, as illustrated in Fig. 4. Panel 1 shows the 46-mer (peak B), which is non-fluorescent; peak D represents low-molecular-weight contaminants. Panel 2 shows the fluorescent 22-mer (peak C), which is eluted later than the 46-mer, as expected. Completeness of hybridization of the probe is illustrated in panel 3 by the appearance of a new peak (A), eluted earlier than the 46-mer. This 68-nucleotide duplex is fluorescent. When hybridization of the probe is incomplete (panel 4), both the fluorescent duplex (peak A) and unhybridized, fluorescent 22-mer (peak C) are observed. It is to be noted that the presence of multiple isomers of this fluorescent rhodamine B-probe (see Fig. 2) does not have a deleterious effect on hybridizing capability or elution position by gel permeation chromatography.

In an actual assay, an excess of fluorescent probe over the target to be measured would be added. The fluorescence area or height of peak A would be proportional to the concentration of the target nucleic acid. The specificity of fluorescence monitoring at the optimal wavelengths of the probe should allow the detection of a complementary target in a complex mixture. This brief experiment only illustrates the potential application of HPLC in DNA-probe assays. Gel permeation chromatography, which is employed in this study, may not be sufficient for resolving multiple species of complementary targets that may be present in a test sample. Ion-exchange HPLC[8] of the target-fluorescent probe duplexes may be more suitable in such instances. This approach may be considered as a chromatographic version

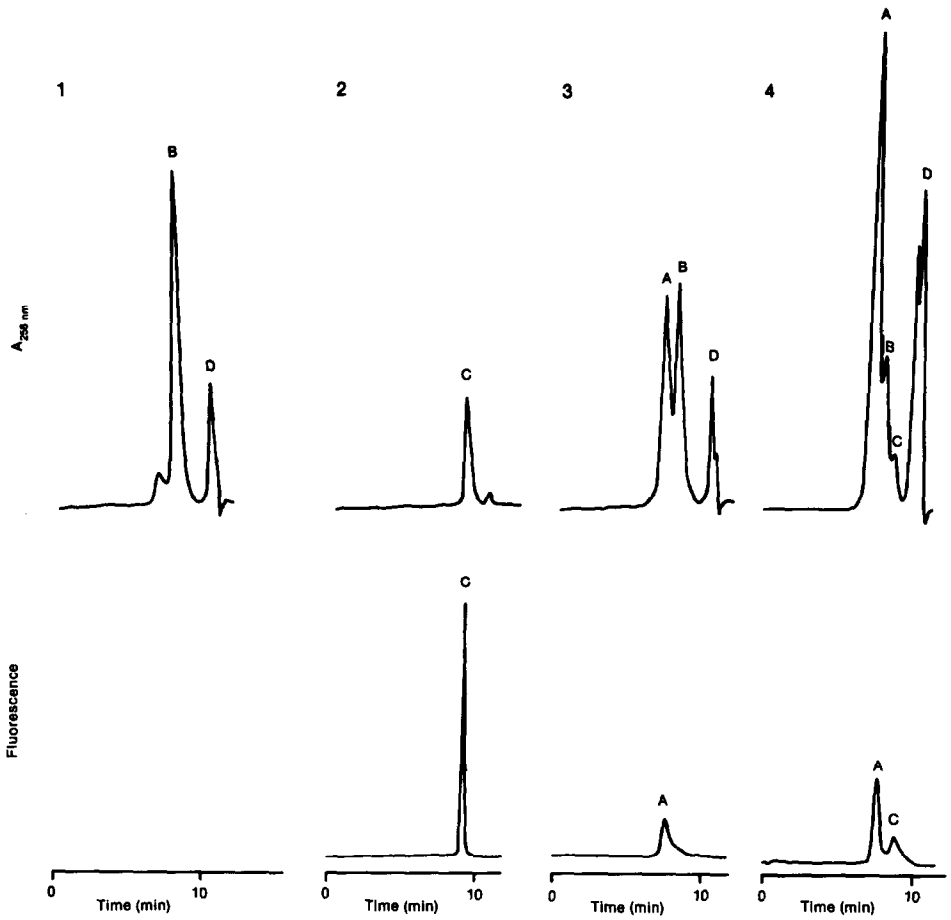

Fig. 4. Gel permeation chromatographic analysis of hybridization between an oligomer and a complementary fluorescent oligomer. Panel 1, chromatogram of a non-fluorescent 46-mer, designated as peak B. Panel 2, chromatogram of the fluorescent 22-mer, designated as peak C. Panel 3, chromatogram of a hybridization mixture, containing 1 nmol of 46-mer and 0.4 nmol of 22-mer. Peak A is the hybridized, duplex product, peak B is the excess 46-mer, and peak C (the 22-mer) is no longer present. Peak D represents low-molecular-weight contaminants. Panel 4, chromatogram of a hybridization mixture, containing 1 nmol of 46-mer and 0.8 nmol of 22-mer. The hybridization is incomplete under these conditions, as judged by the presence of peak C.

of Southern blotting[9], which is based on resolution of a DNA mixture by PAGE and the use of a radioactive DNA-probe.

ACKNOWLEDGEMENT

This project was partially supported under SBIR contract DAMD17-87-C-7052.

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