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The synthesis of oligonucleotides containing an aliphatic amino group at the 5′ terminus: synthesis of fluorescent DNA primers for use in DNA sequence analysis

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ABSTRACT

A rapid and versatile method has been developed for the synthesis of oligonucleotides which contain an aliphatic amino group at their 5' terminus. This amino group reacts specifically with a variety of electrophiles, thereby allowing other chemical species to be attached to the oligonucleotide. This chemistry has been utilized to synthesize several fluorescent derivatives of an oligonucleotide primer used in DNA sequence analysis by the dideoxy (enzymatic) method. The modified primers are highly fluorescent and retain their ability to specifically prime DNA synthesis. The use of these fluorescent primers in DNA sequence analysis will enable DNA sequence analysis to be automated.

INTRODUCTION

DNA sequence determination is one of the most fundamental tools of modern molecular biology. Two methods for DNA sequence analysis exist and are in widespread use, the chemical degradation method of Maxam and Gilbert (1), and the enzymatic method of Sanger (2, 3). Although these methods are extremely powerful, they are also very laborious, time consuming, expensive, and require the use of hazardous and unstable radioisotopes. Any method for reducing or eliminating these problems would have a major impact upon molecular biology. For these reasons we have undertaken the automation of DNA sequence analysis, utilizing fluorescent chromophores in place of radioisotopes for detection. We are initially focusing on sequence analysis by the enzymatic method, as this is more immediately amenable to the use of fluorescent tags.

In conventional enzymatic DNA sequence analysis utilizing radioisotopic detection, a radiolabeled nucleoside triphosphate is included in the sequencing reactions and thereby is incorporated into the newly synthesized DNA strand. This permits the labeled DNA fragments to be detected by autoradiography subsequent to their separation by size on polyacrylamide sequencing gels. We have adopted an alternative strategy for the introduction of fluorescent molecules into the DNA strands. This strategy is to covalently attach fluorophores to chemically synthesized DNA primer oligonucleotides. When these fluorescent primers are used in sequencing reactions, the enzymatically synthesized polynucleotide products of the sequencing reactions are fluorescent. The

dye-primer conjugates must have the following characteristics. 1) They must have a free 3' hydroxyl group to allow chain extension by the polymerase. 2) The fluorophore must not interfere with the hybridization or prevent 3'-end extension by the polymerase. 3) The dye-oligonucleotide must be chemically homogeneous. 4) The fluorophore must retain its fluorescence when attached to the oligonucleotide. 5) The electrophoretic behavior of the polynucleotide products of DNA sequencing reactions must not be adversely affected by the presence of the dye on the primer oligonucleotide. A versatile approach to the synthesis is to prepare oligonucleotides which contain one or more aliphatic amino groups. Whereas the aromatic amino group present on the nucleoside bases of DNA are extremely unreactive (4), primary aliphatic amino groups are highly nucleophilic and hence are readily reacted with electrophilic functional groups. A wide variety of fluorescent dyes containing such electrophilic groups are commercially available. In this paper we describe the chemistry we have developed for the synthesis of oligonucleotides containing an aliphatic amino group at the 5' terminus. These amino oligonucleotides are synthesized using the solid phase synthetic methods developed by Caruthers and his coworkers (5-8). Suitably protected phosphoramidite derivatives of 5'-amino-5'-deoxythymidine are incorporated at the 5' terminus of a synthetic oligonucleotide as the last addition in oligonucleotide synthesis. The synthesis is readily performed either manually or in an automated fashion on an Applied Biosystems 380A DNA synthesizer. Cleavage from the support and deprotection yields the 5'-amino oligonucleotide. The synthesis and purification of fluorescent derivatives of such amino-oligonucleotides is also described. These fluorescent primers were tested by their use in sequence analysis with conventional radioisotope detection and found to be equally effective as the normal underivitized primers.

Two other reports of methods for amino-oligonucleotide synthesis similar to that we reported here have recently appeared. In one report (9), putrescinyl groups were introduced via derivitized thymidine monomers into synthetic DNA. The other report (10) described a method for the introduction of amino groups at the 5' terminus of oligonucleotides by reaction of diamines with a 5'-terminal phosphoroimidazolide.

EXPERIMENTAL PROCEDURES

Materials

Fluorescein isothiocyanate (FITC[1]) and Texas Red were obtained from Molecular Probes, Inc. (Junction City, OR). Tetramethyl rhodamine isothiocyanate (TMRITC) was obtained from Research Organics, Inc. (Cleveland, OH). NBD-fluoride was obtained from Sigma Chemical Co. (St. Louis, MO). S-ethyl trifluorothioacetate and 9-fluorenylmethyl-chloroformate were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Fluorescamine was obtained from Pierce Chemical Co. (Rockford, IL). Chloro-N,N-di-

isopropylaminomethoxyphosphine was obtained from American Bionuclear (Emeryville, CA) or synthesized according to published procedures (6). 5'-amino-5'-deoxythymidine was synthesized as described in the literature (11, 12). Absorption spectra were obtained on a H/P 8451 spectrophotometer. Fluorescence spectra were taken on an uncorrected Perkin-Elmer MPF-4 spectrofluorimeter. HPLC was performed on a system composed of two Altex 110A pumps, a dual chamber gradient mixer, Rheodyne injector, Kratos 757 UV detector, and an Axxiom 710 controller.

SYNTHETIC PROCEDURES

The synthetic schemes used are shown in Fig. 1, and the procedures are described below.

5'-(N-trifluoroacetyl)-5'-amino-5'-deoxythymidine (II)

5'-amino-5'-deoxythymidine (I, 1.25 gr, 5 mmoles) was dissolved in dry DMF (25 ml). S-ethyl trifluorothioacetate (1.3 ml, 10 mmoles) was added. The reaction was stirred gently at room temperature for 1 h. Thin layer chromatography (TLC) of the reaction mixture on silica gel F-254 plates run in methanol:acetone (1:1) showed a single product. The product has a high mobility in this solvent system in contrast to the starting compound which is immobile. The reaction mixture was evaporated to dryness under reduced pressure, transferred to an Erlenmeyer flask in 30 ml isopropanol, and recrystallized from isopropanol:methanol. Yield = 1.315 gr (3.9 mmoles, 80% yield), m.p. 261°-262° (dec); elemental anal calcd: C, 42.7%; H, 4.18%; N, 12.5%; obs. C, 42.7%, H, 4.16%; N, 12.4%. The structure of II was further confirmed by ^{1}H NMR.

5'-(N-trifluoroacetyl)-5'-amino-5'-deoxy-3'-N,N-diisopropylaminomethoxyphosphinyl thymidine (III)

All glassware, syringes and capillary tubes used in the reaction were baked overnight in a drying oven. DMF was stored over 4 Å molecular sieves. The protected amino thymidine II (63 mg, 0.19 mmoles) was added to a three-necked round bottom flask and dried several hours in a vacuum dessicator. The reaction vessel was placed under a gentle stream of dry nitrogen. DMF (2 ml) was added by syringe followed by 60 µl of dry DIPEA (0.34 mmoles). Chloro-N,N-diisopropylaminomethoxy phosphine (40 µl) was added and the reaction was stirred until all the starting material had dissolved. After 1 h at room temperature TLC on silica gel F-254 plates in chloroform:ethanol:triethylamine (88:10:2) showed a major spot of product of much higher mobility than the starting material II. Attempts to purify this product in a fashion similar to that described for the nucleoside phosphoramidites (6) were unsuccessful due to degradation of the product. Therefore, the crude reaction mixture was used directly for the coupling to the synthetic oligonucleotide on the solid phase support. The structure of III is inferred from its reactivity in the addition to the oligonucleotide, and from the expected product of the

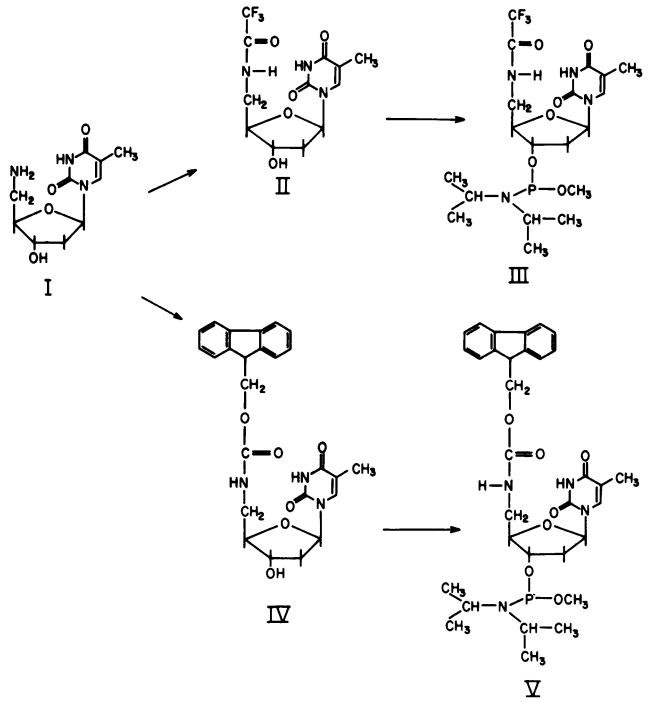

Figure 1. Synthetic route used for the preparation of protected amino-derivatized thymidine phosphoramidites III and V.

reaction based on literature results.

5'-(N-9-fluorenylmethyloxycarbonyl)-5'-amino-5'-deoxythymidine (IV)

Dry DIPEA (0.4 ml, 2.3 mmoles) was combined with dry DMF (3 ml) in a small round bottom flask. 5'-amino-5'-deoxythymidine (I, 0.5 g, 2.1 mmoles) was suspended in the mixture and 9-fluorenylmethylchloroformate (0.64 g, 2.5 mmoles) was added with stirring. The reaction rapidly became clear and thin layer chromatographic analysis (chloroform:ethanol:triethylamine, 88:10:2) showed a single major spot of product and only a trace of unreacted starting material. The product was precipitated by the addition of 25 ml of 1 M $NaHCO_3$. The resulting slurry was filtered and washed several times each with, successively, 1 M $NaHCO_3$, water, and a 1:1 mixture of hexanes and diethyl ether. The solid product was dried overnight in a vacuum dessicator to give 0.88 g (1.9 mmoles, 90% yield) of a white solid. The structure of the product was confirmed by proton NMR.

5'-(N-9-fluorenylmethyloxycarbonyl)-5'-amino-5'-deoxy-3'-N,N-diisopropylaminomethoxy phosphinyl thymidine (V)

This reaction was performed in the same manner as the synthesis of III. All

solvents and glassware were dry and an inert atmosphere was maintained. IV (0.88 g, 1.9 mmoles) was suspended in dry dichloromethane (14 ml, distilled over phosphorous pentoxide, then over potassium carbonate and calcium hydride, and stored over 4 Å molecular sieves). To this was added dry DIPEA (0.5 ml, 2.9 mmoles). The solution was stirred at room temperature, and chloro-N,N-diisopropylaminomethoxy phosphine (0.4 ml, 2.1 mmole) was added. The starting material gradually went into solution, and TLC in chloroform:ethanol:triethylamine (88:10:2) after 60 min showed that the reaction had gone to completion. Ethyl acetate (50 ml) was added, the organic phase was washed twice with cold saturated sodium bicarbonate and once with cold saturated sodium chloride, dried over magnesium sulfate and the solvent removed by evaporation under reduced pressure to yield a white foam (1.20 g, 100% crude yield). The structure of the crude product was confirmed by proton NMR. Phosphorous NMR showed singlets δ = 148.7 and 148.3 ppm corresponding to the phosphoramidite product, as well as a singlet at 13.9 ppm corresponding to hydrolyzed chloro-N,N-diisopropylaminomethoxy phosphine used in the phosphoramidite synthesis. This latter species has been shown not to interfere with the phosphoramidite coupling reaction (13). TLC of the phosphoramidite showed one major species (~95%) and two minor species of slightly lower mobility. Much of the hydrolyzed phosphine can be removed by precipitation in cold hexanes as described (6).

Coupling of 5'-amino-thymidine phosphoramidites to the 5' hydroxyl of an oligonucleotide

Both of the protected amino-thymidine phosphoramidites III and V have been used for the last addition of an oligonucleotide synthesis in solid phase DNA synthetic procedures as described elsewhere (5-8). The conditions used are identical to those used for conventional nucleoside phosphoramidites except that for compound III tetrazole activation is accomplished by combining equal volumes of 0.5 M tetrazole solution in acetonitrile with the crude reaction mixture containing III immediately prior to the coupling step. The protecting groups of III (trifluoroacetyl group) and V (Fmoc group) are base labile and consequently are removed during the cleavage and deprotection of the oligonucleotide with ammonium hydroxide. In some cases the presence of the aliphatic amino group on the oligonucleotide was measured by assay with the reagent fluorescamine (14). Interfering ammonium ions present from the deprotection steps were first removed by treatment of the DNA with a cation exchange resin, Dowex *AG50W-X4* (sodium form), for 30 min at room temperature. The procedure is as follows: 1 ml of water is added to a tube containing approximately 0.2 µmoles of oligonucleotide (dried down from the ammonium hydroxide solution in which it was deprotected). Freshly washed Dowex AG50W-X4 (sodium form) is added to give a volume of ~1.5 ml. This mixture is left at 4°C overnight, and the supernatant is removed by pipette. Quantitation by UV absorption of the amount of oligonucleotide present before and after

Table 1
Reverse Phase HPLC Conditions for
Dye-oligonucleotide Purification

Sample	Retention time
PLP-15[a]	18'
NH_2-T-PLP-15[b]	18'
FITC PLP-15[c]	27'
NBD PLP-15	25'
TMRITC PLP-15	32' and 34'[d]
Texas Red PLP-15	42'

Retention times shown are for HPLC gradients of 20% solvent B/80% solvent A to 60% solvent B/40% solvent A in 40 min, where solvent A is 0.1 M triethylammonium acetate pH 7.0 and solvent B is 50% acetonitrile, 50% 0.1 M triethylammonium acetate pH 7.0. The column was an Axxiom ODS 5 micron C18 column #555-102 available from Cole Scientific, Calabasas, CA. This gradient is not optimized for purification of PLP-15 and NH_2-T-PLP-15, but the retention times are included for comparison with the dye primer conjugates.

[a]PLP-15 is an oligonucleotide primer for DNA sequence analysis in the M13 vectors. Its sequence is 5' CCC AGT CAC GAC GTT 3'.

[b]NH_2-T-PLP-15 is the oligonucleotide PLP-15 to which a 5'-amino-5'-deoxythymidine base has been added at the 5' terminus.

[c]The nomenclature Dye PLP-15 signifies the conjugate of NH_2-T-PLP-15 and the dye molecule.

[d]Two fluorescent oligonucleotide products were obtained with TMRITC. Both were equally effective in sequencing. This is presumed to be due to the two isomers of TMRITC which are present in the commercially available material.

the resin treatment shows no losses. One hundred µl of oligonucleotide solution is combined with 500 µl of 0.1 M sodium carbonate/bicarbonate buffer, pH 9, and to this is added 500 µl of 0.03% fluorescamine in acetone with rapid vortexing. A standard curve is also constructed using 5'-amino-5'-deoxythymidine to permit quantitation of the amino groups. The resulting fluorescence is measured using an excitation wavelength of 390 nm and an emission wavelength of 487 nm.

Dye conjugation

The basic procedure used for the attachment of dye molecules to the amino oligonucleotides is to combine the amino oligonucleotide and the dye in aqueous solution buffered to pH 9, to allow the reaction to stand at room temperature for several hours, and then to purify the product in two stages. The first purification stage is to remove the bulk of the unreacted or hydrolyzed dye by gel filtration. The second purification stage is to separate the dye conjugate from unreacted oligonucleotide by reverse phase HPLC. Slight variations upon these conditions are employed for the different dyes, and the specific procedures and conditions used for four particular dyes are given below and in Table 1.

The following procedure is for use with fluorescein isothiocyanate or NBD-fluoride. Amino oligonucleotide (0.1 ml of ~1 mg/ml oligonucleotide in water) is combined with 1 M sodium carbonate/bicarbonate buffer pH 9 (50 µl), 10 mg/ml dye in DMF (20 µl) and H_2O (80 µl). This mixture is kept in the dark at room temperature for several hours. The mixture is applied to a 10 ml column of Sephadex G-25 (medium) and the colored band of material eluting in the excluded volume is collected. In control reactions with underivatized oligonucleotides, very little if any dye is associated with the oligonucleotide eluting in the void volume. The colored material is further purified by reverse phase HPLC on an Axxiom C_{18} column (#555-102, Cole Scientific, Calabasas, CA) in a linear gradient of acetonitrile:0.1 M triethylammonium acetate, pH 7.0. It is convenient for this separation to run the column eluant through both a UV detector (for detecting the DNA absorbance) and a fluorescence detector (for detecting the dye moiety). The desired product is a peak on the chromatogram which is both strongly UV absorbing and strongly fluorescent. The dye oligonucleotide conjugates elute at higher acetonitrile concentrations than the oligonucleotides alone, as shown in Table 1. The oligonucleotide is obtained from the HPLC in solution in a mixture of acetonitrile and 0.1 M triethylammonium acetate buffer. This is removed by lyophilization and the resulting material is redissolved by vortexing in 10 mM sodium hydroxide (for a minimum amount of time) followed by neutralization with a five-fold molar excess (to sodium hydroxide) of Tris buffer, pH 7.5.

The conjugation with Texas Red is identical to that described for fluorescein isothiocyanate and NBD-fluoride, except that a) prior to separation on Sephadex G-25 the reaction is made 1 M in ammonium acetate and kept at room temperature for 30 min, and b) the Sephadex G-25 column is run in 0.1 M ammonium acetate. This largely eliminates nonspecific binding of the dye molecule to the oligonucleotide.

The conjugation with TMRITC is identical to that for Texas Red except that the reaction is carried out in 10 mM sodium carbonate/bicarbonate buffer, pH 9.0, and 50% dioxane. This increases solubility of the TMRITC and a much higher yield of dye oligonucleotide conjugate is obtained.

In some cases, particularly with the rhodamine-like dyes, a substantial amount of non-specific binding of dye was observed, as manifested by an inappropriately large dye absorption present in the material eluted from the gel filtration column. In these cases the material was concentrated and reapplied to a second gel filtration column prior to HPLC purification. This generally removed the majority of the noncovalently associated dye. In all cases the second stage of purification on HPLC yielded a chemically homogeneous dye-oligonucleotide conjugate with no non-specifically associated dye (see Results).

DNA sequence analysis was performed by standard methods (2, 3) utilizing α-^{32}P-dCTP as a radiolabel.

RESULTS

Synthesis of 5'-aminothymidine phosphoramidites

Two phosphoramidite derivatives of 5'-amino-5'-deoxythymidine have been synthesized as described in Materials and Methods. These compounds, numbered III and V in Fig. 1, differ in the nature of their amino protecting group. The first compound synthesized was III, in which the amino group is protected by a base labile trifluoroacetyl moiety. We were unable to isolate this compound due to decomposition. This phosphoramidite was therefore coupled to oligonucleotides directly from the crude reaction mixture. As will be discussed below, the molecule coupled efficiently to the 5' hydroxyl group of an oligonucleotide to yield a 5' amino oligonucleotide. However, the considerable inconvenience of having to perform the synthesis of III every time it was to be used in oligonucleotide synthesis, as well as some problems encountered with the reproducibility of the coupling reaction, induced us to seek a more stable phosphoramidite which could be isolated as a relatively pure and stable solid. The lower hydrophobicity of the trifluoroacetyl group relative to the DMT group normally used in protected nucleoside phosphoramidites made the purification of III more difficult. Therefore, we prepared V, in which a less polar Fmoc protecting group is utilized. As we hoped, V proved to be stable to the purification procedures used for nucleoside phosphoramidites, was readily obtained as a solid and is effective in oligonucleotide synthesis (see below).

Addition of 5'-aminothymidine phosphoramidites to oligonucleotides

As described in Materials and Methods, the protected 5'-aminothymidine phosphoramidites III and V are coupled to the 5' hydroxyl of an oligonucleotide using well established DNA synthetic procedures. The solvents and reaction conditions used are identical to those used in oligonucleotide synthesis. The couplings have been performed both manually and on an Applied Biosystems Model 380A automated DNA synthesizer. The coupling of III or V to an oligonucleotide may be monitored by several methods. In normal oligonucleotide synthesis utilizing phosphoramidite chemistry the stepwise yield is monitored by colorimetric determination of the 5'-hydroxyl-protecting DMT group released by an acid wash step in every cycle of the synthesis. This approach may also be used to estimate the coupling efficiency of addition of the amino-derivatized nucleoside phosphoramidites. After the 5'-aminothymidine phosphoramidite coupling and associated washing steps, a coupling is performed with a normal dimethoxytrityl nucleoside phosphoramidite. Any remaining hydroxyl groups react with this phosphoramidite, and the colored DMT cation is released in the following detritylation step. Colorimetric

Table 2
Relative Dimethoxytrityl Absorbances at 498 nm Obtained Before and After Coupling of V in Four Independent Coupling Reactions

Prior addition	Subsequent addition	Coupling efficiency of V
1	0.16	85%
1	0.03	97%
1	0.07	93%
1	0.02	98%

determination of this ion allows estimation of the efficiency of the prior 5'-amino-thymidine phosphoramidite coupling. A second method for following the coupling reaction is to assay the oligonucleotide product for the presence of aliphatic amino groups after cleavage from the support and base deprotection. We have used the fluorescamine assay (14, 15) for this purpose. A third method is to react the putative amino oligonucleotide product with dye. A dye-oligonucleotide conjugate is formed with an amino-oligonucleotide but not with an underivatized oligonucleotide. This conjugate may be observed by a) the presence of a colored and fluorescent band traveling in the excluded volume of the gel filtration column (see Materials and Methods), b) the ratio of dye absorption to DNA absorption seen in an absorbance spectrum of the fluorescent product, and c) the presence of a major UV absorbing and fluorescent peak on an HPLC chromatogram. The relative sizes of the dye-oligonucleotide peak and the oligonucleotide peak on an HPLC chromatogram provide a lower limit for the amount of amino-oligonucleotide originally present in the amino-oligonucleotide preparation. These methods have been used to evaluate the coupling efficiency of III and V. For simplicity, data will be presented only for coupling with V. Similar results have been obtained with III.

Table 2 shows the results obtained in several independent coupling reactions from colorimetric determination of the dimethoxytrityl group released prior to and subsequent to coupling of V to the oligonucleotide. The absorbancies are scaled to the trityl absorbance obtained from the prior addition. No capping step was used after the addition of V so as to leave uncoupled hydroxyl groups available for assay. These values give a lower limit on the coupling efficiency inasmuch as a small trityl reading is obtained even when the coupling is performed on previously capped material.

The amino oligonucleotide is cleaved from the support and deprotected with concentrated ammonium hydroxide in the same manner as is normally used in oligonucleotide synthesis. The base labile TFA or Fmoc amino protecting group is removed during this treatment. This material may then be assayed using fluorescamine for the presence of primary amino groups as described in Materials and Methods. Control

experiments with underivitized oligonucleotide gave no significant fluorescence signal. An apparent value of 1.35 moles of amino group per mole of DNA was obtained in the case of a 15 mer which had been coupled to V. The DNA concentration was estimated from its absorption at 260 nm using a value of $E_{260} = 1.6 \times 10^5 M^{-1}$. This somewhat high value for the mole ratio of amino groups may be due to a higher fluorescence yield from the fluorophore when attached to the oligonucleotide than when attached only to 5'-amino-5'deoxythymidine. Such enhanced fluorescence is seen when polypeptides are compared with free amino acids by fluorescamine assay (15). The amino-oligonucleotide may be reacted with fluorescein isothiocyanate as described in Materials and Methods without prior ion exchange treatment and the conjugate separated from free dye by gel filtration. When this is done, a band of colored and fluorescent material traveling in the excluded volume of the column is observed. No such colored band is normally observed when underivitized oligonucleotide is treated in the same manner. An absorption spectrum of this material for an oligonucleotide of length 15 (excluding the 5'-terminal amino thymidine nucleoside) conjugated to FITC typically gives an absorption at 260 nm relative to 495 nm of 3:1. Using a value of $E_{495} = 7 \times 10^4$ for fluorescein (and $E_{260} = 1.6 \times 10^5$ for the oligonucleotide, as before) leads to a dye:DNA ratio of 0.65. This is an underestimate of the ratio since this crude material contains a substantial amount of shorter termination sequences which absorb at 260 nanometers but presumably do not contain an aliphatic amino group. When this crude dye:DNA conjugate is purified by HPLC, the fluorescent conjugate is well separated from the unreacted DNA (see Table 1). On the HPLC chromatogram, the fluorescent DNA conjugate gives an absorbance peak at 260 nm approximately threefold greater than the absorbance of unconjugated DNA peak, leading to an approximate lower limit of 75% for the efficiency of synthesis of the amino oligonucleotide.

Preparation and purification of dye-oligonucleotide conjugates

The conditions used for the preparation of four dye oligonucleotide conjugates are given in Materials and Methods. These four dyes make up a set of spectrally resolved fluorescent dyes suitable for use in automated DNA sequence analysis by the strategy described in the Discussion section (see below). Table 1 shows the relative retention times of the dye oligonucleotide conjugates on HPLC, and Table 3 gives some of their spectral properties. The HPLC retention times in Table 1 show that the dye-oligonucleotide conjugates are well resolved from underivatized oligonucleotide, and hence the separation on HPLC of the dye conjugate from unreacted oligonucleotide is extremely good. Spectrophotometry showed one molar equivalent of dye present in the purified dye-oligonucleotide conjugates, demonstrating that no non-specifically associated dye is present in the purified material.

Table 3
Spectral Properties of Dye-oligonucleotide Conjugates[a]

Dye	Excitation maximum (nm)	Emission maximum (nm)	Emission FWHM[b] (nm)
FITC	493	516	60
NBD	475	540	79
TMRITC	556	582	52
Texas Red	599	612	42

[a]These dyes are coupled to the oligonucleotide primer NH_2-T-PLP-15 (see Table 1).
[b]FWHM, full width at half maximum.

Properties of dye-oligonucleotide conjugates

As mentioned previously, our principal motivation for the development of chemistry for the synthesis of dye oligonucleotide conjugates is to allow their use as primers in DNA sequence analysis. Accordingly, we have tested the various dye primers by substituting them for the normal primer in DNA sequence analysis by the enzymatic method. Figure 2 shows the autoradiogram of a DNA sequencing gel in which these dye-conjugated primers were utilized in T reactions in place of the normal oligonucleotide primer. This autoradiogram was obtained by conventional methods employing α-^{32}-P-dCTP as a radiolabel. Several facts are evident from this autoradiogram. First, the underivitized primer, amino-derivitized primer, and dye conjugated primers all give the same pattern of bands (corresponding to the DNA sequence), indicating that the derivitized primers retain their ability to hybridize specifically to the complementary strand. Second, the bands generated using the different primers differ in their mobilities, showing that it is indeed the dye-primers which are responsible for the observed pattern, and not a contaminant of unreacted or underivitized oligonucleotide. Third, the intensity of the bands obtained with the different primers is comparable, indicating that the strength of hybridization is not significantly perturbed by the presence of the dye molecules. Recently, the fluorescein conjugated primer has also been used in sequencing reactions without radiolabel and the bands of DNA detected by their fluorescence using argon ion (488 nm) laser excitation in a tube polyacrylamide gel (data not shown). The apparatus and data from this experiment will be described elsewhere.

DISCUSSION

In this paper we have described a method for the synthesis of oligonucleotides which contain an aliphatic amino group at the 5' terminus. The attachment of other

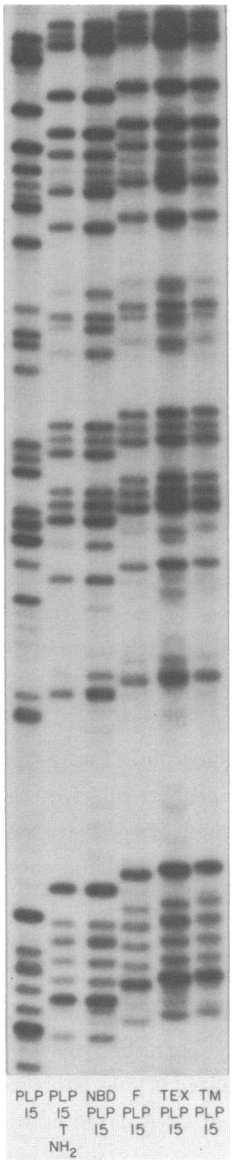

Figure 2. Autoradiogram of a 5% acrylamide sequencing gel. The samples are T reactions performed upon a M13 vector-derived (16) single stranded DNA template using the indicated primer. The legend to Table 1 defines the primers used, except that further abbreviations are used as follows: F, fluorescein isothiocyanate (FITC); TEX, TEXAS RED; TM, tetramethylrhodamine isothiocyanate (TMRITC). DNA fragments approximately 100 to 200 bases in length are shown in the figure.

compounds to the amino-oligonucleotide is readily accomplished, opening up many possible applications for this chemistry. These applications include their use in affinity chromatography for the isolation of sequence-specific DNA binding proteins, the attachment of DNA cleaving agents to produce synthetic restriction enzymes (17), the attachment of motional and/or environmental probes to study DNA structure and

dynamics, and the attachment of enzymatic or fluorescent tags for use in diagnostic assays as well as the general replacement of radioisotope detection in nucleic acid hybridization procedures.

Our primary motivation in developing this method was to synthesize fluorescent oligonucleotide primers for use in DNA sequencing by the enzymatic method. A fluorescent tag present on the 5' terminus of the primer oligonucleotide will also be present on the 5' terminus of the polynucleotide products of chain elongation by the Klenow fragment of DNA polymerase I, and therefore the bands of DNA present on the sequencing gel will be fluorescent. By using four different and spectrally resolved fluorescent chromophores, and using one of these in each of the four sequencing reactions, A, C, G and T, it should be possible to combine the products of the sequencing reactions and electrophorese the mixture on a single polyacrylamide tube gel. A detector near the bottom of the gel could then detect and identify the different colored bands as they pass by during electrophoresis, and the temporal sequence of the different colors would encode the DNA sequence. This approach should permit the automation of the electrophoresis, detection, and analysis portions of DNA sequence analysis.

We have therefore prepared a number of dye-primer conjugates. We have picked from these a preliminary set of four dyes which a) are spectrally resolved, b) are highly fluorescent, c) absorb and emit as far to the red end of the spectrum as possible (in order to minimize raman and fluorescence background), d) do not impair the hybridization of the dye-primer conjugate, and e) do not adversely affect the electrophoretic properties of DNA molecules to which they are attached. This set of four dyes is fluorescein isothiocyanate, NBD-fluoride, tetramethyl rhodamine isothiocyanate, and Texas Red. The synthesis and purification of these dye-primer conjugates is described in Materials and Methods, and some of their fluorescence properties are shown in Table 3. This set of four dyes is suitable for use in DNA sequencing by the approach described above. As mentioned briefly in Results, in preliminary measurements we have successfully detected the fluorescent bands of DNA generated in sequencing reactions. The detection limit for the fluorescent DNA primers is in the range of 10^{-16} moles (data not shown), well below the amount of DNA we are able to obtain in single bands on sequencing gels. These experiments will be presented in detail elsewhere.

In summary, we have developed a novel chemistry for the automated synthesis of 5' amino oligonucleotides. This chemistry has been applied to the synthesis of several fluorescent oligonucleotide primers for use in the automation of DNA sequence analysis.

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Abbreviations Used:

FITC, fluorescein isothiocyanate; NBD, 7-nitrobenzofurazan; TMRITC, tetramethylrhodamine isothiocyanate; DMF, dimethylformamide; Fmoc, 9-fluorenylmethyloxycarbonyl; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance spectroscopy; DIPEA, diisopropylethylamine; dCTP, 2' deoxy cytosine triphosphate.

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