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The estimation of distances between specific backbone-labeled sites in DNA using fluorescence resonance energy transfer

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ABSTRACT

A series DNA helices of twenty-four base pairs has been prepared for the study of fluorescence resonance energy transfer. Each of the DNA helices contains two phosphorothioate diesters (one in each strand) at pre-selected sites for introduction of the desired donor and acceptor fluorophores. The phosphorothioate-containing oligodeoxynucleotides have been prepared as pure Rp or Sp derivatives or as deastereomeric mixtures. Fluorescein and eosin are employed as the respective donor and acceptor fluorophores. A series of donor-acceptor pairs was generated by labeling of the appropriate phosphorothioate diester with the desired fluorophore and annealing the two complementary DNA strands (one containing the acceptor and one containing the donor fluorophore) to form the double-stranded helix. The 24-mer helices containing two covalently attached fluorophores exhibited some thermal destabilization and the extent of this destabilization was dependent upon the stereochemical orientation of the fluorophore. The Sp derivatives direct the fluorophore out, away from the the DNA helix, while the Rp derivatives direct the fluorophore toward the major groove. As expected, the Sp labeled duplexes were more stable than the corresponding Rp labeled sequences. However, all of the duplex structures formed were stable under the conditions used to measure energy transfer. Energy transfer could be observed with these complexes from the quenching of the donor fluorescence in the presence of the acceptor fluorophore. Using Förster's theories, distances separating the fluorophores could be calculated that were generally in reasonable agreement with the distances expected in an idealized B-form DNA helix. However anomalous results were obtained for one donor/acceptor pair where the expected distance was less than 20 Å. Fluorescence anisotropy values determined in solutions of varying viscosity were quite high suggesting that the fluorophores did not experience complete freedom of movement when attached to the DNA helix.

INTRODUCTION

The ability to use spectroscopic methods in solution to measure distances between selected sites within a biomolecule permits the rapid assimilation of relevant structural information without the time-consuming efforts necessary for x-ray crystallographic analysis. Such spectroscopic analyses have been most successful in proton NMR techniques, particularly when employing the dipole-dipole relaxation methods available with the nuclear overhauser enhancement (NOE) effect. Protein (1) and nucleic acid (2) structures have been partially or completely resolved using such spectroscopic tools, but one difficulty remains, the distances obtained from NOE experiments are limited to a few angstroms (3). NOE techniques have been very useful for structural analyses of relatively small biomolecules, but the sizes of many proteins, nucleic acids, or multisubunit complexes, typically range from tens to hundreds of angstroms and there are few techniques that allow the measurement of distances of this magnitude.

Fluorescence spectroscopy has been widely used in a number of applications where changes in structure or dynamics of a biomolecule are of interest. The excitation and emission spectra as well as the quantum yield of an individual fluorophore are all sensitive to the surrounding microenvironment such that small changes in this environment arising from structural changes or complexation with other molecules can often be monitored with high sensitivity. The decrease in fluorescence quantum yield (quenching) can be caused by a number of environmental factors, but quenching as the result of energy transfer to a second chromophore can be exploited for structural analysis since the transfer process (a dipole-dipole coupling event) is a distance-dependent phenomena. Fluorescence resonance energy transfer is based upon Förster's theories (4) which suggest that transfer occurs through weak dipole-dipole interactions between a 'donor' and an 'acceptor' chromophore, and that the efficiency of energy transfer is proportional to the inverse sixth power of the distance between the two chromophores.

Stryer and Haugland suggested that the Förster resonance energy transfer could be used as a basis for distance measurements and demonstrated this concept using a series of end-labeled oligopeptides (5). Since this pioneering work, resonance energy transfer techniques have been used in a number

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of studies involving both proteins (6) and nucleic acids (7). However, the number of distances that can be obtained for a given macromolecule is often dependent upon the ability to place the donor and acceptor chromophore at preselected sites. For example, the labeling of a specific cysteine residue within a protein, followed by introduction of a second chromophore by site-specific ligand binding, provides one set of the desired donor and acceptor molecules (6a). However, the production of additional sets of donor acceptor pairs may be limited, and in some cases require the generation of modified proteins that contain a cysteine residue at an alternate location.

The studies reporting the use of resonance energy transfer with nucleic acids also are somewhat limited. Early reports (7a) by Cantor described the 3′-terminal labeling of tRNAs and exploited as the second fluorophore the naturally occurring wyosine base. The fluorescent wyosine base has also been used to probe the tRNA-ribosome complex. More recently, a series of end-labeled DNA sequences have been used to study the structure of recombination intermediates (7d,e). In these latter reports, a series of DNA fragments of different sizes have been prepared such that the relative position of the end-label varies proportionately with the length of the DNA fragment (7e). Resonance energy transfer has also been employed in the study of DNA protein complexes (8). However, the limitations in studies with nucleic acids or protein nucleic acid complexes, as with proteins alone, is typically the difficulty in generating a series of donor acceptor pairs.

We have developed a simple approach for the site-specific introduction of fluorophores into short DNA sequences (9). This procedure exploits standard DNA synthesis methodology with an altered oxidation step at a pre-selected site in order to incorporate a phosphorothioate residue (10), which is then amenable to alkylation by a variety of fluorophores. Labeling the internucleotide phosphorus in this manner places the fluorophore on the outer surface of the DNA where it is less likely to interact with the duplex DNA, yet remains available for resonance energy transfer processes. The use of this labeling procedures permits the generation of a series of donor-acceptor pairs in an expedient manner. In the present paper we describe studies on the estimation of distances between a series of donor-acceptor fluorophores for a simple DNA duplex.

EXPERIMENTAL

Materials

High performance liquid chromatography (HPLC) was carried out on ODS-Hypersil (4.6×250 mm) or MOS-Hypersil (9.4×250 mm) columns, (Shandon Southern products Limited, England) using a system consisting of two Beckman 114M pumps, 163 variable wavelength detector and 421A controller. Chromatograms were recorded on a Shimadzu C-R3A Chromatopac. ^{31}P NMR spectra were obtained with Varian XL-300 multinuclear spectrometer (^{31}P NMR 121.421 MHz). Absorption spectra were recorded by Perkin-Elmer Lambda 3B UV/Vis spectrophotometer. Cell temperature was controlled by digital temperature controller. Oligodeoxynucleotides were synthesized by a phosphoramidite method on an Applied Biosystems 381A DNA synthesizer. Protected nucleoside 3′-O-(diisopropylamino methoxy phosphine) and protected nucleoside 3′-O-(diisopropylamino β-cyanoethoxy phosphine) were purchased from ABN or Cruachem. N^6-Benzoyl-3′-methoxyacetyl-2′-deoxyadenosine was prepared by the previously described method (11) except that methoxyacetic anhydride was prepared in dichloromethane and used for the preparation of N^6-Benzoyl-3′-methoxyacetyl-2′-deoxyadenosine without purification. Nuclease P1 was purchased from GIBCO BRL. Snake venom phosphodiesterase and alkaline phosphatase were purchased from Boehringer Manheim Biochemicals 5-Iodoacetamidofluorescein (5-IAF) and 5-iodoacetamidoeosin (5-IAE) were obtained from Molecular Probes Inc.. All other reagents were purchased from Aldrich or Sigma. The Rp and Sp diastereoisomer of 5′-O-(N^6-Benzoyl-2′-deoxyadenosyl) 3′-O-[5′-O-(dimethoxytrityl)-thymidine] O-methyl phosphorothioate were prepared and converted to the corresponding phosphoramidites as described elsewhere (9,12).

Methods

Synthesis of oligonucleotides containing a single phosphorothioate diester

Method A. An oligodeoxynucleotide containing a diastereomeric mixture of phosphorothioate linkages was prepared by altering the oxidation step of the synthetic cycle to use 2.5 M sulfur in carbon disulfide/2,6-lutidine (1 : 1) as previously described (9,10). After completion of the sulfur oxidation step, synthesis of the sequence continued using normal phosphoramidite coupling procedures.

Method B. Fragments containing a single phosphorothioate diester (Rp or Sp) were prepared using normal phosphoramidite coupling procedures. At the appropriate position in the sequence, the Rp or Sp phosphorothioate dimer phosphoramidite building block was incorporated into the sequence by using the same coupling cycle and reaction time as employed for the 'common' nucleoside phosphoramidite derivatives.

Purification of the phosphorothioate diester-containing oligodeoxynucleotides

Isolation of modified oligodeoxynucleotides was performed with the 5′-terminal dimethoxytrityl group by reversed-phase HPLC on MOS-Hypersil (9.4×250 mm) using 50 mM triethylammonium acetate (pH 7.0) and gradient of 14.0−45.5% acetonitrile in 40 min.(13). Isolated strands were treated with 80% acetic acid solution for 20 min to remove a dimethoxytrityl group, evaporated a number of times from an ethanol/water mixture, and desalted over a column of Sephadex G-10.

Fluorescence labeling of the phosphorothioate diester-containing oligodeoxynucleotides

Labeled strands were synthesized by the reaction of phosphorothioate-containing 24-mers with 5-iodoacetamidofluorescein (5-IAF) or 5-iodoacetamidoeosin (5-IAE) in potassium phosphate buffer (pH 8.0) at 50°C for 12−17 h.(9). Yields based on HPLC were 58−88%. The resulting labeled strands were isolated by reversed-phase HPLC on a column of ODS-Hypersil (4.6×250 mm) using 50 mM triethylammonium acetate (pH 7.0) with a gradient of acetonitrile. Isolated compounds were desalted on a Sephadex G-10 column and lyophilized to dryness. The extent of fluorophore incorporation into the DNA fragments was determined spectrophotometerically from the ratio of the extinction coefficient of the fluorophore to that of the 24-mer. For these analyses, the following extinction coefficients were employed: 5-IAF, $\epsilon_{490} = 6.8\times10^4\ M^{-1}cm^{-1}$; 5-IAE, $\epsilon_{520} = 1.08\times10^5\ M^{-1}cm^{-1}$ (14) The extinction coefficients for the 24-mers were calculated (15) to be $2.44\times10^5\ M^{-1}cm^{-1}$ for

5'-d[CGAACTAGTTAACTAGTACGCAAG] and 2.27×10^5 $M^{-1}cm^{-1}$ for 5'-d[CTTGCGTACTAGTTAACTAGTTCG]. The extinction coefficients of the labeled 24-mers at 260 nm was corrected for the contribution by the fluorophore using correction terms estimated from the absorption spectra of free fluorophores. The extinction coefficient for eosin-labeled sequence 5'-d[CGAACTAGTTAACTAGTACGCAAG] (sites of labeling not shown) was calculated to be 2.86×10^5 $M^{-1}cm^{-1}$ and that of fluorescein-labeled sequence 5'-d[CTTGCGTACTAGTTAACTAGTTCG] (sites of labeling not shown) was calculated to be 2.51×10^5 $M^{-1}cm^{-1}$. The ratios of incorporated fluorophore to 24-mer were calculated from the absorption ratio at 490 or 520 nm for the fluorophore vs. absorption at 260 nm for the 24-mer.

Tm measurements

Tm's were measured in 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride at DNA (duplex) concentration of 2×10^{-7} M. The rate of heating was 0.5°C/min. Tm values were determined from the first and second differentials of the absorbance *vs.* temperature plots.

Fluorescence resonance energy transfer measurements

Fluorescence measurements were performed on a Shimadzu RF5000U controlled by a Shimadzu DR-15. The cell temperature was maintained at 20°C by a Lauda RM6 water bath. A sample volume of 350 μL was used in a micro cell (minimum sample volume: 300 μL). All samples were prepared in 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride. Before measurements, the samples were kept at 80°C for 1 min, and then slowly cooled down to 20°C. An excitation wavelength of 490 nm and excitation/emission band width of 5 nm were employed. The distances between donor and acceptor were calculated by the following equation (16):

$$R = R_0\left(\frac{1}{E}-1\right)^{1/6} \quad (1)$$

where R_0 is the distance for 50% efficiency of resonance energy transfer. R_0 was calculated according to:

$$R_0 = (9.79\times10^3)(J\ \kappa^2\ n^{-4}\ \theta_D)^{1/6}\ \text{Å} \quad (2)$$

where κ^2 is an orientation factor of the relative orientation in space of the transition dipoles of the donor and acceptor chromophores. For the donor-acceptor pairs that have isotropic rotation on the time scale of the fluorescence lifetime, a value of 2/3 can be used for κ^2. The refractive index in medium is n and was taken to be 1.4. J is the spectral overlap integral and was calculated from equation 3:

$$J = \frac{\Sigma F_D(\lambda)\epsilon_A(\lambda)\lambda^4\Delta\lambda}{\Sigma F_D(\lambda)\Delta\lambda} \quad (3)$$

for every 5 nm over the range of wavelengths from 470 to 600 nm. Emission spectra were corrected by comparison with the known spectral distribution emission of quinine sulfate (17). The quantum yield (θ_D) of the donor in the absence of acceptor was calculated by comparison to a reference solution of fluorescein in 0.1 N sodium hydroxide, using the reported quantum yield for the latter of 0.90 (18).

The efficiency of energy transfer from the donor to the acceptor fluorophore was determined from the quenching of the steady-state donor intensity. For each set of experiments, fluorescence spectra of the donor-labeled DNA, accepter-labeled DNA, and donor- and acceptor-labeled DNA were obtained. Efficiency (E) was calculated from the following equation:

$$E = 1-\frac{F_{DA}-F_A}{F_D} \quad (4)$$

where F_{DA}, F_A and F_D are the integration values for the fluorescence intensity of the donor- and acceptor-labeled DNA, acceptor-labeled DNA, and donor-labeled DNA, respectively. F_{DA}, F_A and F_D were obtained from the emission values between 500 nm and 515 nm.

Fluorescence anisotropy

Fluorescence anisotropy measurements of both donor-labeled DNA and acceptor-labeled DNA were determined by integration of the emission spectra over the wavelength range 510 to 530 nm for donor-labeled DNA and over the wavelength range 540 to 550 nm for acceptor-labeled DNA using a set of polarizing filters and a sharp cut-off filter GG495 (Melles Griot). Anisotropy, r, was calculated by the following eq.:

$$r = \frac{I_{VV}(I_{HH}/I_{HV})-I_{VH}}{I_{VV}(I_{HH}/I_{HV})+2I_{VH}} \quad (5)$$

where I represents the integration of fluorescence intensity. The subscripts refer to the vertical (V) or horizontal (H) settings of the excitation and emission polarizer, respectively. Anisotropy measurements were performed on samples containing various concentrations of sucrose at 20°C to increase solvent viscosity (η). Literature values for viscosity of sucrose were used (19). Limiting anisotropies (r_0) were calculated from Perrin plot of 1/r *vs* T/η as defined by following eq. (20):

$$\frac{1}{r} = \frac{1}{r_o}\left(1+\frac{\tau_F kT}{V_h\eta}\right) \quad (6)$$

where τ_F is the fluorescence lifetime, V_h is a molecular volume, and k is a Boltzmann's constant.

RESULTS

A number of reports have described the incorporation of fluorescent derivatives into DNA (for a review see 21). One of the most common techniques involves the use of groups attached to the 5'- or 3'-terminus of the sequence (see for example 22). A tether is typically incorporated at one terminus carrying a protected functional group that can be unmasked after deprotection and purification of the synthesized sequence (for examples see 23). Tethered amino and thiol functionalities have been used most commonly in this respect (23). For some applications it may be more desirable to incorporate the fluorescent labels at internal sites within the sequence, and such labeling was first accomplished using suitably modifed base residues. Although in some cases the fluorophore has been incorporated during the assembly of the DNA sequence (24), it is generally more versatile to use a post-synthetic modification strategy. Interest in this general approach continues with recent procedures describing the attachment of protected, tethered functional groups at the C_5 of thymine (25,26) or the N^4 of cytosine (26). In addition to base modification, the carbohydrate portion of the nucleoside residue can also be used to incorporate

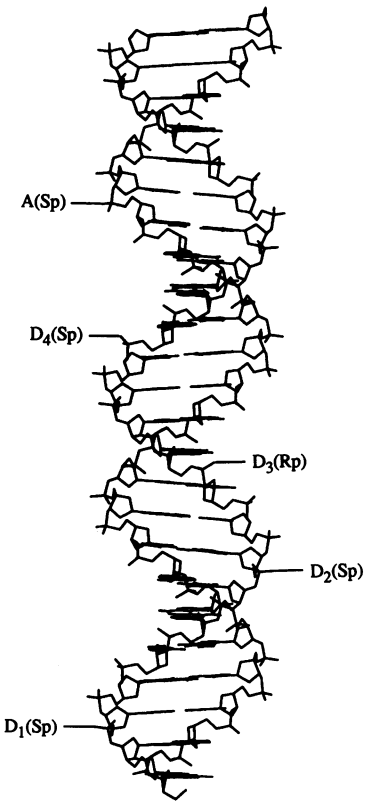

Figure 1. A B-form DNA duplex containing the twenty four base pairs used in the present study. 'A' marks the location of the acceptor fluorophore (eosin); D_1, D_2, D_3 and D_4 mark the four locations of the donor fluorophores (fluorescein). The parentheses show the absolute configuration at the phosphotriester linkage marked.

reporter groups (27). Two reports have described the incorporation of a single ribonucleoside into a DNA fragment such that the 2'-hydroxyl can be employed as a site for tethering fluorophores. In one case the fluorophore was attached directly to the 2'-hydroxyl prior to assembly of the DNA/RNA sequence (27a), while in the second example, the 2'-hydroxyl has been used as a site to tether an appropriately protected functional group that can be unmasked for post-synthetic modification (27b).

The internucleotide linkage offers a third general site that can be used for the internal attachment of reporter groups. The most common approach employed for backbone modification involves the incorporation of a single phosphoramidite linkage (the nitrogen is placed in a non-bridging position) with the reporter group or the tether attached to the nitrogen atom. These phosphoramidate likages are most commonly formed from the corresponding H-phosphonate as described originally by Todd and coworkers (28) but oxidation of phosphite triesters in the presence of an amine offers a second procedure (29). Phosphoramidates have been used in place of phosphotriesters largely due to their enhanced stability to standard deprotection conditions during DNA synthesis protocols. Phosphoramidate chemistry has been exploited recently for the incorporation of a single fluorophore, or a suitable tether, at a pre-selected site within a DNA sequence (30). Single phosphorothioate diesters can also be incorporated at pre-selected sites within a DNA sequence (10) and this functional group is also amenable to alkylation by a variety of fluorophores (9).

In the present study, the introduction of the donor and acceptor fluorophores needed for the fluorescence resonate energy transfer studies relied upon the alkylation of a single phosphorothioate diester placed at a pre-selected site within the sequence. We have used this approach since the incorporation of a phosphorothioate diester employs essentially standard synthesis protocols, and the procedure is simple enough that the position of the phosphorothioate can be easily moved within the sequence by the synthesis of additional 24-mers. Backbone labeling techniques that employ a phosphorus derivative as the site of attachment of the fluorophore generally result in a pair of diastereomers (Rp and Sp) about phosphorus. Since the efficiency of the resonance energy transfer will be dependent upon the location of the fluorophore on the DNA helix, it is possible that sequences that differ in the diastereomeric placement of the donor and acceptor fluorophores will result in varying efficiencies of energy transfer. We have employed the phosphorothioate labeling procedure in the present work since a number of reports have described the synthesis and purification of individual phosphorothioate diasteromers and their incorporation into DNA sequences. The preparation DNA sequences containing individual phosphorothioate diastereomers can be accomplished by the synthesis of a dimer building block that contains the phosphorothioate derivative followed by resolution of the two phosphorus diastereomers, and finally conversion of each diastereomer into the corresponding phosphoramidite building block as has been described previously (9b,12). With this approach, four diastereomeric duplexes (Sp-Sp, Sp-Rp, Rp-Sp and Rp-Rp) can be formed each of which contains the donor and acceptor molecule in either the Sp or Rp configuration at phosphorus. The results from these duplexes could then be compared with those of similar sequences prepared as a diastereomeric mixture.

Synthesis of diastereomerically pure 24-mers containing a single phosphorothioate diester

Eight oligodeoxynucleotides of twenty four residues were prepared for the described studies. The 18 base pair operator sequence for the tryptophan repressor was placed within the twenty four residues. This was done such that the eight sequences could be used for further studies involving protein-nucleic acid interactions with this repressor-operator system. Six of the derivatives synthesized contained a single diastereomeric phosphorothioate linkage 3' to a 2'-deoxythymidine residue and 5' to a 2'-deoxyadenosine residue (three Sp derivatives and three Rp derivatives). Two of these pairs of sequences were regioisomers, differing in the location of the phosphorothioate diester, Sp & Rp 5'-d[CTTGCGT(s)ACTAGTTAACTAGTTCG] and Sp & Rp 5'-d[CTTGCGTACT(s)AGTTAACTAGTTCG]. The remaining pair of sequences were the two diastereomers of the complementary 24-mer, 5'-d[CGAACT(s)AGTTAACTAGTACGCAAG]. For comparison, two sequences 5'-d[CT(s)GCGTACTAGTTAACTAGTTCG] and 5'-d[CTTGCGTACTAGTT(s)AACTAGTTCG] were prepared as diastereomeric mixtures with both the Sp and Rp phosphorothioates present.

The two diastereomers, Sp and Rp 5'-d[CGAACT(s)AGTTAACTAGTACGCAAG) were each labeled with the acceptor fluorophore (eosin), while the other six sequences were all labeled with the donor fluorophore (fluorescein) in order to vary the distance between (and the stereochemical content of) the donor and acceptor pair. The sites chosen permitted the construction of eleven duplex DNAs of identical sequence that

Table 1. Retention Times and Extents of Label Incorporation For Modified Oligodeoxyribonucleotides

Compound[1]	Abbreviation	Retention Time (min)[2] Unlabeled	Labeled	Ratio of 24-mer/Label[3]
5'CTs(AF)TGCGTACTAGTTAACTAGTTCG3'-Mix	D_1 (Mix)	18.8	22.5	0.93
5'CTTGCGTs(AF)ACTAGTTAACTAGTTCG3'-Sp	D_2 (Sp)	16.5	19.7	0.97
5'CTTGCGTs(AF)ACTAGTTAACTAGTTCG3'-Rp	D_2 (Rp)	16.3	19.5	0.97
5'CTTGCGTACTs(AF)AGTTAACTAGTTCG3'-Sp	D_3 (Sp)	16.4	19.7	1.01
5'CTTGCGTACTs(AF)AGTTAACTAGTTCG3'-Rp	D_3 (Rp)	16.2	19.6	1.06
5'CTTGCGTACTAGTTs(AF)AACTAGTTCG3'-Mix	D_4 (Mix)	17.1 & 17.6	21.0	0.92
5'CGAACTs(AE)AGTTAACTAGTACGCAAG3'-Sp	A(Sp)	16.3	22.9	1.00
5'CGAACTs(AE)AGTTAACTAGTACGCAAG3'-Rp	A(Rp)	15.8	22.8	1.00

1 s(AF) = indicates the location of the phosphorothioate diester labeled with 5-iodoacetamidofluorescein (5-IAF); s(AE) = indicates the location of the phosphorothioate diester labeled with 5-iodoacetamidoeosin (5-IAE); -Mix = the fluorescent label was present as a mixture of diastereoisomers at phosphorous; -Sp and -Rp = the fluorescent label was present as a stereochemcically pure phosphorus diastereoisomer having the Sp or Rp configuration, respectively.
2 HPLC conditions: column, ODS-Hypersil; gradient, 7% to 21% acetonitrile (28 min) in 50 mM triethylammonium acetate (pH 7.0); flow rate, 1 ml/min.
3 The quantity of fluorescent label and 24-mer were determined from absorption spectra (see Experimental).

varied only in the relative positions of the attached fluorophores (Fig. 1).

Oligodeoxynucleotides containing a single diastereomeric phosphorothioate linkage were synthesized by using either the Rp or Sp dinucleoside phosphorothioate. In this study, 5'-O-(N^6-Benzoyl-3'-O-methoxyacetyl-2'-deoxyadenosyl) 3'-O-[5'-O-(dimethoxytrityl)-thymidine] O-methyl phosphorothioate was prepared from the coupling of 5'-O-dimethoxytritylthymidine 3'-O-(diisopropylaminomethoxyphosphine) and N^6-Benzoyl-3'-methoxyacetyl-2'-deoxyadenosine by a previously reported method (9b,12). After separation by silica gel chromatography, the purity of each diastereomer was estimated to be greater than 98% based upon the ^{31}P-NMR data [the 'fast' isomer (Sp) resonated at 67.5 ppm, while the 'slow' isomer (Rp) resonated at 66.9 ppm]. The sensitivity of each dimer to hydrolysis by nuclease P1 or snake venom phosphodiesterase permitted unambiguous assignment of the absolute stereochemical configuration of each dimer (9b,12). After conversion to the corresponding phosphoramidite derivative, each dimer could be employed in a standard solid-phase based synthesis to produce 24-mers containing a single stereochemically pure phosphorothioate diester. After coupling each of the dimers to the growing oligodeoxynucleotide, the color of the solution containing dimethoxytrityl cation indicated that incorporation of the dimer had occurred with essentially the same efficiency as that observed with the common phosphoramidite derivatives. Oligodeoxynucleotide products were cleaved from support, deprotected by the normal method, and purified by reversed-phase HPLC. The chromatograms obtained at this point appeared normal, and no excess failed sequences were present as a result of the dimer coupling. Using these procedures we typically obtained approximately 90 A_{260} units of each of the twenty four residue sequences. After purification of the oligodeoxynucleotides, the absolute stereochemistries of the phosphorothioate linkages could be confirmed by their sensitivity to hydrolysis by snake venom phosphodiesterase.

Oligodeoxynucleotides prepared as a diastereomeric mixture at the phosphorothioate linkage were synthesized by simply modifying the oxidation step at a pre-selected site within the sequence. Oxidizing the intermediate phosphite triester with sulfur in carbon disulfide and lutidine generated the phosphorothioate derivative. After completion of the synthesis, the 24-mers were deprotected and purified as described above.

Fluorescence labeling of the 24-mers

The two 24-mers containing the acceptor fluorophore were labeled with 5-iodoacetamidoeosin (5-IAE) and the six sequences containing the donor fluorophore were labeled with 5-iodoacetamidofluorescein (5-IAF). The labeling reactions proceeded with yields greater than 79% after incubation times between 12 and 16 h at 50°C. The use of relatively high concentrations of oligonucleotides (0.7−1.0 mM) resulted in more efficient labeling than experiments using lower concentration (< 0.5 mM) . In the latter cases the yields were reduced to to 50−60% (after 17 h) and increasing quantities of impurities were present. The reactions were monitored by reversed-phase HPLC; each labeled oligodeoxynucleotide was purified twice by HPLC, desalted on a Sephadex G-10 column and lyophilized to dryness. The relative elution order for each set of diastereoisomers (Table 1) did not change after the labeling reaction (i.e., the labeled or unlabeled oligodeoxynucleotides with the Sp configuration were eluted from the C18 column with longer retention times that the corresponding derivatives with the Rp configuration).

The purified labeled materials were evaluated for the presence of both fluorophore and DNA by absorption spectra. The relative amount of fluorophore incorporated into each 24-mer was calculated from the absorbance ratio (see Table 1) at 260 nm (for the labeled DNA) and the characteristic absorption of fluorophore. The extinction coefficients for the two labeled 24-mers, 5'-d[CGAACTAGTTAACTAGTACGCAAG] and 5'-d[CTTGCGTACTAGTTAACTAGTTCG], were calculated to be 2.86×10^5 M^{-1} cm^{-1} and 2.51×10^5 M^{-1} cm^{-1}, respectively. The extinction coefficients, ϵ_{490} = 6.8×10^4 $M^{-1}cm^{-1}$ for fluorescein and ϵ_{520} = 1.08×10^5 $M^{-1}cm^{-1}$ for eosin , were used. The resulting ratios of absorbance (0.92−1.06, see Table 1) indicated that, within experimental error, each 24-mer contained a single covalently-bound fluorophore.

Thermal stability of the donor/acceptor-labeled DNA duplexes

Thermal melting temperatures (Tm values) of donor/acceptor-labeled DNA duplexes were measured in the same buffer as that used in the fluorescence resonance energy transfer experiments (10 mM sodium phosphate, pH 7.0, 150 mM sodium chloride). Seven donor/acceptor duplexes were prepared and analyzed in this manner (Table 2). In each case there was a slight decrease in the Tm of the donor/acceptor labeled 24-mer duplexes in

Table 2. Tm Values for Donor/Acceptor-Labeled DNA Duplexes

Donor Position	Complex[1]	Tm (°C)[2] unlabeled	labeled	ΔTm (°C)[3]
1	D_1(Mix)-A(Mix)	57.1	52.6	4.5
2	D_2(Sp)-A(Sp)	57.6	50.3	7.3
2	D_2(Rp)-A(Rp)	56.7	47.6	9.1
3	D_3(Sp)-A(Sp)	56.8	51.7	5.1
3	D_3(Rp)-A(Rp)	56.7	48.9	7.8
4	D_4(Mix)-A(Sp)	57.3	51.9	5.4
4	D_4(Mix)-A(Rp)	57.1	51.0	6.1

1 Each 24-mer duplex contained a single donor (D) and a single acceptor (A) fluorophore present as a diastereomeric mixture (Mix) or a pure phosphorus diastereomer (Sp or Rp).
2 Tm values were measured at 2.0×10^{-7} M DNA (duplex) concentration in 150 mM sodium chloride/10 mM sodium phosphate buffer (pH 7.0) using a 0.5°C/min temperature gradient. The error in Tm values is estimated to be ± 0.5°C
3 ΔTm is difference in Tm value between the unlabeled 24-mer duplex and donor/acceptor-labeled sequences.

comparison with the corresponding unlabeled DNA (but containing two phosphorothioate linkages). The introduction of a fluorophore into Rp configuration at the internucleotide residue resulted in a more significant reduction in Tm value than the corresponding substitution into the Sp configuration. However, there were no significant sequence-related effects on thermal stability for the 24-mers. In all cases, the helices exhibited Tm values well-above ambient temperature and indicated that the doubly-labeled DNA fragments were stable under experimental conditions for fluorescence resonance energy transfer measurements.

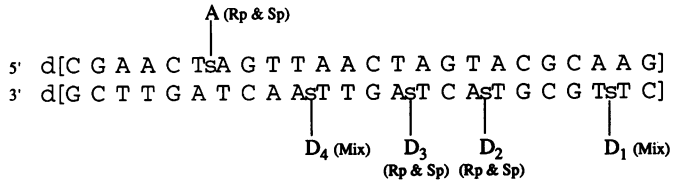

Figure 2. Linear illustration of the positions of the donor and acceptor molecules. Parentheses indicated whether the donor or acceptor was prepared as the Rp or Sp derivative or as a diastereomeric mixture (Mix).

Fluorescence resonance energy transfer between fluorophores site-specifically placed in a DNA duplex

Although it is possible to use a number of donor/acceptor fluorophore pairs for resonance energy transfer (16), the fluorescein/eosin pair have a number of advantages. Both fluorophores have been reported to have a high extinction coefficient and a correspondingly high quantum yield. This pair of fluorophores has a large spectral overlap between emission spectra of fluorescein (the donor) and absorption spectra of eosin (the acceptor). The iodoacetamide derivatives of both fluorophores are readily available and this functional group is compatible with labeling of internucleotidic phosphorothioate diesters (9,32). Additionally, we know of no reports that indicate that either fluorescein or eosin interact with duplex DNA by intercalative, groove binding, or other mechanisms although some quenching effectshave been reported (33). Non-covalent interactions of this type would be a disadvantage in the present study since they would reduce the population of the preferred random orientations for the fluorophores and complicate estimations for κ^2.

The eleven donor/acceptor duplexes employed in the present study are illustrated in a combined format in Figure 2. Two of the donor sites (D1 and D4) were prepared as a diastereomeric mixture (M) while the remaining two sites (D2 and D3) were prepared as pure diastereomeric compounds. A set of emission spectra obtained for a typical fluorescence resonance energy transfer measurement is shown in Figure 3. Each spectrum was corrected for Raman scatter by subtraction of the spectrum obtained for the unlabeled DNA. The observed quenching of donor emission in the presence of the acceptor fluorophore suggests the presence of a resonance energy transfer processes. The energy transfer efficiency was determined from the quenching of the donor emission by measuring the integral of

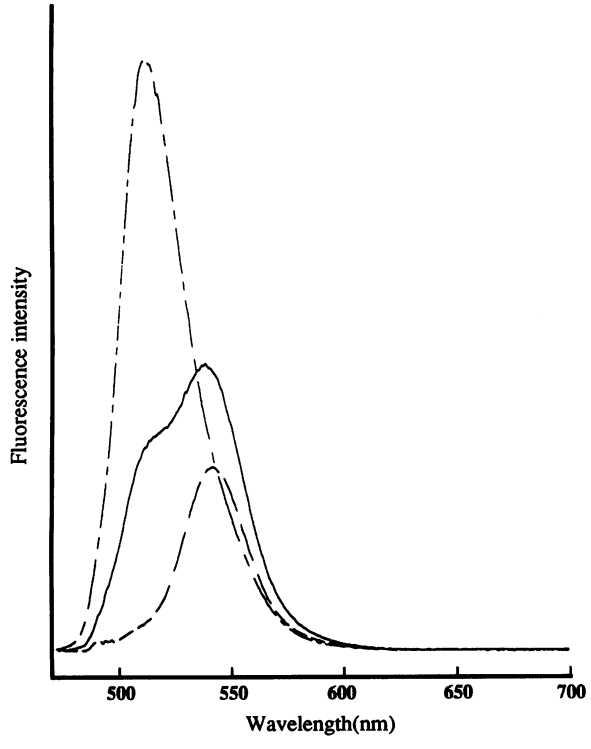

Figure 3. A set of emission spectra of labeled DNA (donor position D2, Rp isomers for both donor and acceptor sequences). Conditions are described in the Experimental. Donor- and acceptor-labeled DNA (– –); donor-labeled DNA (– · –) and acceptor-labeled DNA (—).

Table 3. Data from Fluorescence Resonance Energy Transfer Experiments

Donor Position	1				2			3		4	
Complex	D_1-A				D_2-A			D_3-A		D_4-A	
	Mix-Mix	Sp-Sp	Sp-Rp	Rp-Sp	Rp-Rp	Sp-Sp	Sp-Rp	R-pSp	Rp-Rp	Mix-Sp	Mix-Rp
$J \times 10^{13}(M^{-1}cm^3)$	4.09	4.15	4.19	4.00	4.19	4.12	4.27	4.15	4.20	4.04	4.22
θ_D	0.43	0.47	0.49	0.54	0.51	0.58	0.57	0.48	0.51	0.50	0.53
R_0(Å)	54.7	55.7	56.2	56.6	56.5	57.6	57.8	55.9	56.6	56.0	57.0
E	0.31	0.57	0.69	0.64	0.69	0.80	0.79	0.78	0.80	0.74	0.74
R(Å)	63	53	49	51	49	46	46	45	45	47	48
r_{0D}	0.26	0.31	0.30	0.29	0.32	0.29	0.34	0.30	0.30	0.30	0.29
r_{0A}	0.33	0.34	0.32	0.33	0.34	0.33	0.33	0.35	0.34	0.32	0.33
Rmax(Å)	81	70	65	67	65	60	62	60	60	61	63
Rmin(Å)	46	36	34	36	33	33	30	31	31	33	34

J, spectral overlap; θ_D, quantum yield of the donor-labeled DNA in the absence of the accepter; R_0, Förster critical distance; E, energy transfer efficiency; R, the derived donor-acceptor distance; r_{0D} and r_{0A}, fluorescence anisotropies of donor-labeled DNA and acceptor-labeled DNA, respectively; Rmax and Rmin, error limit calculated from fluorescence anisotropies.

fluorescence intensity over a narrow wavelength range (5 nm). The spectral data obtained for the eleven duplexes is listed in Table 3. Both the Förster critical distances, R_0 (calculated from spectral overlap), and the quantum yields of the donor in absence of acceptor , θ_D, for all donor/acceptor labeled duplexes are similar to previously reported values (7e). Assuming a value of 2/3 for the orientation factor κ^2, the distances between the donor and acceptor fluorophores for the four donor sites were determined for all eleven duplexes. Distances of 63 Å (an isomeric mixture), 50.5 Å (average of the values for the Rp and Sp isomers), 45.5 Å (average of the values for the Rp and Sp isomers), and 47.5 Å (average of the values for the Rp and Sp isomers) were derived for the separation of the acceptor fluorophore (eosin) and the donor fluorophore (fluorescein) at positions 1, 2, 3, and 4, respectively. The donor fluorophore at position 1 is removed from the position of the acceptor fluorophore by sixteen base pairs (see Fig. 2). Similarly, the donor at position 2 is eleven base pairs from the acceptor; the donor at position 3 is eight base pairs from the acceptor; and the donor at position 4 is four base pairs from the acceptor. In each case the acceptor is bound to one strand of the DNA duplex and the donor is bound at various positions to the complementary strand. We have estimated the distances between the various donor and acceptor pairs by using a computer graphics system that modeled the 24-mer sequence to correspond to an idealized B-form helix. Distance values have been estimated between centers of donor and acceptor fluorophores, assuming that both fluorophores are attached in a perpendicular orientation relative to the DNA helix axis. The distances obtained are 56, 54, 43, and 19 Å for the donor at positions 1, 2, 3, and 4, respectively. For comparison, we also estimated the distances between the phosphorus sites at which the fluorophores are covalently attached and determined values of 55, 41, 30, and 17 Å for positions 1, 2, 3, and 4, respectively. At positions 2 and 3, the average distance (R) measured from fluorescence resonance energy transfer experiments for a series of stereospecifically labeled 24-mer duplexes is roughly in agreement with the idealized distance. The distance value obtained with the donor at position 1 (63 Å) is somewhat larger than expected for a standard B-form helix (56 Å) and the average value obtained with the donor at position 4 (47.5 Å) is dramatically larger than the idealized value (19 Å).

In Förster's theories, an uncertainty arises in the calculation of the distance between a donor/acceptor pair (R_0) as a result of the value assigned to the orientation factor κ^2. κ^2 relates the relative orientation of the two transition dipoles of the donor and acceptor molecules. It is defined as: $\kappa^2 = (\cos\theta_T - 3\cos\theta_d \cos\theta_a)^2$; θ_T is the angle between the emission dipole of the donor and the absorption dipole of the acceptor, and θ_d and θ_a refer to the angles between these two dipoles and a vector between the donor and acceptor chromophores. The value for k^2 cannot be determined directly, and in principle can range in values from 0 to 4. However, using the method developed by Dale et. al. (34), the maximum and minimum values of κ^2 can be derived. In order to determine the limits of κ^2, the limiting anisotropy of donor-labeled DNA and acceptor-labeled DNA were measured using a Perrin plot (see Experimental). The values obtained from these experiments define the upper and lower limits for the distance measurements (R_{max} and R_{min}, Table 3). The results of anisotropy experiments suggest that the fluorophores do not experience complete freedom of mobility during the lifetime of the excited state, and this is reflected by the relatively high anisotropy values (Table 3). The relatively high anisotropy values increase the range of the calculated maximum and minimum distances between the donor and acceptor fluorophores. The expected distance values are within the limits of R for donor positions 1−3 and this suggests that measured values, based on the Förster theory, reflect the distances between two specific fluorophores covalently bound to the DNA. However, the value obtained for donor position 4, is much larger than the expected value and it is also beyond the minimum and maximum limits for R derived from the anisotropy data.

With the donors present at positions 2 and 3 we could examine the effects of chirality on the energy transfer process. Using the Sp and Rp diastereomers of both the acceptor-labeled sequence and the donor-labeled sequence, four duplexes were formed for the donor/acceptor pair at position 2 and an additional four duplexes were formed with the donor at position 3 (see Fig. 2 and Table 3). Generally the distances between the donor and acceptor chromophores obtained for the various diastereomeric duplexes were very similar with a range of less than 4Å. This result indicates that the stereoconfiguration at the phosphorothioate linkage may have a small affect on the derived distances, but it is unlikely to be a major source of discrepancy.

DISCUSSION

Phosphorothioate diesters are sites for covalent labeling of nucleic acids and they allow the generation of a series of sequences for the construction of a variety of duplexes containing donor/acceptor fluorophores located a pre-selected sites. The native phosphodiester internucleotide linkage is prochiral, with

two stereochemically identical non-bridging oxygens. The introduction of a reporter group to this prochiral site creates a pair of diastereomers. The diastereomeric character of the labeled sequences is a potential disadvantage that results with DNA sequences containing fluorophores bound to the phosphate backbone. However, with the phosphorothioate diesters, it is possible to prepare a dinucleoside building block containing a stereochemically pure phosphorus derivative (Sp or Rp). This approach to labeling in the present work permitted the incorporation of the fluorophore of interest at a pre-selected site and in a stereochemically pure fashion.

Fluorescent labeling of the oligodeoxynucleotides

The alkylation of internucleotidic phosphorothioate diesters provided a simple and rapid procedure for covalently binding the donor and acceptor fluorophores to the two twenty four residue sequences. The reactions proceeded in relatively high yield and could be highly purified by HPLC on reversed-phase columns. In order to assure the high purity of the labeled materials, each donor or acceptor-containing sequence was purified twice in this manner. Labeling of the 24-mers by alkylation of the negatively charged phosphorothioate diester converts the diester to a neutral species and reduces the overall negative charge of the labeled 24-mer by one (in comparison to the unlabeled sequence). The loss of one negative charge and the addition of a hydrophobic fluorescent label to the 24-mer in each case resulted in a species that eluted from the reversed-phase HPLC matrix significantly later that the unlabeled sequence (Table 1, see also Fig. 2). The base-line resolution available between the labeled and unlabeled sequences simplified the purification of the donor or acceptor labeled 24-mers. The purified labeled sequences all exhibited absorption characteristics typical of fluorescein or eosin as well as the DNA. The extinction coefficients for the labeled DNA at 260 nm and the fluorophore at 490 or 520 nm could be used to determine the ratio of fluorophore to 24-mer and confirm that each sequence contained a single fluorophore (Table 1).

The duplexes used in this study were designed such that each single strand contained either the donor or the acceptor fluorophore (see Fig. 3). By using this approach, it was never necessary to incorporate both the donor and the acceptor fluorophore into the same strand. Annealing of the complementary single strands, each containing a single donor or a single acceptor fluorophore then generated a series of 24-mer duplexes containing the donor/acceptor pairs separated by varying distances. The positions of individual donor/acceptor pairs were designed to span the shortest (~15Å) and longest (~70 Å) distances for which Förster type energy transfer processes are expected to be most efficient.

Duplex stability of the donor/acceptor labeled 24-mers

The introduction of two phosphorothioate diesters into the duplex 24-mers (one in each strand) did not appear to introduce any significant instability regardless if the phosphorothioates were of the Sp, Rp or racemic configuration (Table 2). However, the addition of the fluorescein and eosin donor and acceptor fluorophores, resulted in some destabilization of the helix structure in all cases examined. Placing the donor fluorophore at position 2 or 3 in the Sp configuration (with the acceptor also present in the Sp configuration) resulted in a more stable duplex than the corresponding substitutions in the Rp configuration (Table 2). This may reflect differences in the the orientation of the fluorophores with respect to the DNA helix. Labels in the Sp configuration in a B-form DNA duplex will be directed out away from the helix and should have less of a destabilizing influence. Fluorophores covalently bound in the Rp configuration will be directed somewhat toward the major groove and could impart a more significant destabilizing influence on the double-stranded helix. However, in all cases examined, the donor/acceptor labeled duplexes exhibited stability (Tm values $> 47°C$) sufficient for the fluorescence resonance energy transfer studies performed at 20°C.

Fluorescence resonance energy transfer studies

A comparison of the fluorescence emission spectra from the donor fluorophore in the donor-containing duplexes (in the absence of an acceptor) and of the donor in a donor/acceptor labeled duplexes clearly indicated that in the presence of the acceptor the quantum yield of the donor was diminished (Fig. 3). These initial experiments indicated that attachment of the fluorophores to the DNA backbone placed them in relative spatial orientations that facilitated dipole-dipole coupling and fluorescence resonance energy transfer.

Distance calculations using a κ^2 value of 2/3 resulted in distances between the donor and acceptor fluorophores that approximated the ideal distances expected for a B-form DNA helix when the donor was located at positions 2 and 3 (see Fig. 2 and Table 3). There was a slight discrepancy in the ideal (56 Å) and calculated (63 Å) distances with the donor at position 1. However, this difference could simply reflect the lack of agreement between the actual and the ideal distances between two points on a DNA helix separated by sixteen base pairs. Sequence-dependent structure modulation, such as changes in the base pair twist angles or individual distances for base pair steps over a series of base pairs could result in a distance somewhat altered from that expected for an ideal B-form helix. Additionally, the placement of the donor at position 1, three base pairs from the end of the duplex results in the label being subjected to end fraying effects that could result in some discrepancy between the expected and observed distances.

The discrepancy between the ideal (19 Å) and the observed (47.5 Å) values obtained when the donor is located at position 4 cannot be rationalized in such terms. However, a number of explanations could account for this large discrepancy between expected and observed values:

(i) The presence of the donor and acceptor labels results in some loss of helix stability and this may be exacerbated when the labels are placed nearer to one another. Although the overall stability of the 24-mer duplex containing the acceptor in one strand and the donor at position 4 is not dramatically altered in terms of the Tm value (see Table 2), there could still exist significant local structural modulation that results in poor relative spatial positioning of the donor and acceptor dipoles. Poor relative orientation of the dipoles could reduce transfer efficiency and result in a distance calculation much larger than expected.

(ii) Cooper and Hagerman (7e) have reported that base sequence can affect transfer efficiency, and a similar affect may contribute to the poor efficiency observed for this donor/acceptor pair.

(iii) The effective length of the extended fluorescein and eosin fluorophores is approximately 13 Å and they have an effective diameter of approximately 11 Å. The separation of the donor and acceptor pair by four base pairs and their location on complementary DNA strands places the two fluorophores on

opposite sides of the major groove. Although the estimated distance between the centers of the fluorophores (extended perpendicularly from the helix axis) is 19 Å, the effective length and diameter of the chromophores is such that direct interaction between the two moieties is possible and such interactions would be expected to drastically alter transfer efficiency.

The fluorescence anisotropy values determined for all donor- and acceptor-labeled sequences range from 0.26 to 0.35. The high anisotropy values broaden the range for the minimum and maximum distances (see Table 3) but the range of values obtained are consistent with previously reported work with tRNAs and holiday junctions (7a–e). The calculated distances with the donor at positions 1, 2 or 3 all easily fall within these limits while that calculated with the donor at position 4 does not. This latter observation reinforces the observation that this particular donor/acceptor pair results in extremely poor transfer processes. The relatively large anisotropy values suggest that the fluorophores do not have complete freedom of mobility during the lifetime of the excited state. It seems unlikely that the fluorophores bind or interact with the duplex in a specific manner since neither eosin nor fluorescein have been reported to interact with duplex DNA. Each fluorophore contains a short linker arm between the spectroscopically active chromophore and the site of alkylation, but ultimately the label is placed directly on to the DNA backbone by alkylation of the phosphorothioate diester. The high anisotropy values may result from some limitation in fluorophore mobility as a result of the length of the linker arm. End-labeling procedures have typically resulted in much lower anisotropy values (see for example 8a). It is perhaps better to compare the measured anisotropy values in this study with those obtained from a series of backbone-labeled sequences that incorporate a longer tether between the phosphorus residue and the site of labeling (7f). Tethering the fluorophores from an extended linker arm, bound through a phosphoramidate linkage (7f), results in much lower anisotropy values (0.017–0.056) suggesting more rotational freedom.

It is also noteworthy that the distances calculated for either the Rp or Sp donor/acceptor pairs (see positions 2 and 3 in Table 3) do not differ significantly. This suggests that future studies may use effectively the diastereomeric mixtures of phosphorothioate-containing sequences that can be easily and rapidly prepared using standard DNA synthesis methodology without the necessity of stereospecific dimer synthesis.

CONCLUSIONS

The fluorescent labeling of specific phosphorothioate diesters within a duplex DNA structure positions donor and acceptor fluorophores such that Förster type energy transfer processes can take place. Although some destabilization of the 24-mer duplexes containing both a donor and acceptor fluorophores was observed, in general the sequences labeled in this fashion could be used effectively for fluorescence resonance energy transfer. The chirality introduced to the sequence by the labeling of phosphorothioate diesters did not appear to induce significant errors in the distance calculations since a series of Rp-Rp, Rp-Sp, Sp-Rp and Sp-Sp donor acceptor pairs generated similar distance values. However, the measured anisotropy values were high and suggests some limitation in the mobility of the donor and acceptor fluorophores that could be corrected by lengthening the linker arm tethering the chromophores.

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