Direct Measurement of Thermodynamic and Kinetic Parameters of DNA Triple Helix Formation by Fluorescence Spectroscopy†

Mengsu Yang,‡ Soumitra S. Ghosh,§ and David P. Millar*,‡

Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037, and Applied Genetics, San Diego, California 92121

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ABSTRACT: Direct measurement of thermodynamic and kinetic parameters of oligonucleotide-directed DNA triple helix formation has been achieved by fluorescence spectroscopic methods. Fluorescence resonance energy transfer (FRET) was used to study the binding of an acceptor-labeled single-stranded oligonucleotide to a donor-labeled DNA duplex. Equilibrium binding constants and association rate constants for triplex formation between 5′-tetramethylrhodamine-labeled 11-mer, 13-mer, and 15-mer homopyrimidine oligonucleotides and a 5′-fluorescein-labeled, 25-bp DNA duplex containing a 15-bp homopurine site were determined by FRET measurements, and the values were in close agreement with those determined by established methods. The thermal dissociation profile of the triplex-to-duplex transition was also directly observed by FRET and was consistent with the triplex melting curves obtained by UV absorbance measurements. In addition, a homogeneous fluorescence anisotropy assay is described which enables determination of the binding constants between 5′-tetramethylrhodamine-labeled 11-mer and 13-mer homopyrimidine oligonucleotides and unlabeled 25-, 30-, and 50-bp double-stranded DNA containing a homopurine target site. These results demonstrate the utility of nonradioactive fluorescence measurements as an efficient method for studying triple helix formation under homogeneous solution conditions and highlight the uniqueness of the FRET method for obtaining equilibrium, kinetic, and thermal dissociation data in a straightforward manner.

Sequence-specific recognition of double-stranded DNA by oligonucleotide-directed triple helix formation has become an active area of research in recent years (Moser & Dervan, 1987; LeDoan et al., 1987; Lyamichev et al., 1988; Griffin & Dervan, 1989; Sun et al., 1989; Strobel et al., 1991; Roberts & Crothers, 1992; Giovannangéli et al., 1992; Sun & Hélène, 1993; Fossella et al., 1993). Recognition of the base sequence is primarily achieved through the formation of specific Hoogsteen-type hydrogen bonds when pyrimidine oligonucleotides bind to purine tracts in the major groove of DNA parallel to the purine Watson−Crick strand. Hoogsteen base pairing involves thymine (T) recognition of adenine−thymine (AT) base pairs and N3-protonated cytosine (C^+) recognition of guanine−cytosine (GC) base pairs. The stability of DNA triple helices depends on the nature of the DNA strands and the solution conditions. A variety of techniques have been employed to measure thermodynamic and kinetic parameters of triple helix formation, including UV melting (Xodo et al., 1990), differential scanning calorimetry (DSC) (Plum et al., 1990), gel electrophoresis (Roberts & Crothers, 1991), chromatography (Ito et al., 1992), DNase I footprinting (Brenowitz et al., 1986), affinity cleavage (Singleton & Dervan, 1992), and a filter binding assay (Shindo et al., 1993). Radiolabeled oligonucleotides or double-stranded DNA have been used in most of the techniques for quantitative measurements of triplex formation. Several of these techniques are indirect or have low time resolution, while others require large amounts of oligonucleotide samples. The filter binding assay suffers from low retention efficiency of single-stranded nucleic acids, and the efficiency is dependent on experimental conditions and the base sequence. On the other hand, UV melting and DSC are suitable only for thermodynamic studies near the triplex melting temperature. The present report assesses the utility of two fluorescence spectroscopic methods (fluorescence resonance energy transfer and fluorescence anisotropy) for analyzing thermodynamic and kinetic properties of triplex DNA. These fluorescence-based techniques overcome the limitations of the methods presently used to obtain quantitative data on triple helix formation.

Fluorescence resonance energy transfer (FRET) is the nonradiative transfer of excited-state energy from a fluorescent donor molecule to an unexcited acceptor molecule as a result of dipole−dipole coupling between the donor and the acceptor. The rate of energy transfer depends on the spectral properties of the donor and acceptor, the relative orientation of the donor and acceptor transition dipoles, and the distance between the donor and acceptor chromophores. FRET measurements have been widely used to determine proximity relationships in biological macromolecules (Stryer, 1978). This approach has been applied recently to both duplex (Cardullo et al., 1988; Cooper & Hagerman, 1990; Hochstrasser et al., 1992; Morrison & Stols, 1993; Sixou et al., 1994) and branched (Murchie et al., 1989; Clegg et al., 1992; Eis & Millar, 1993) nucleic acid species labeled at the 5′ termini with dyes. Earlier fluorescence studies involving triplex DNA have utilized nonspecifically bound fluorophores, such as intercalators or groove binders (Scaria &

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* Corresponding author.
‡ The Scripps Research Institute.
§ Applied Genetics.

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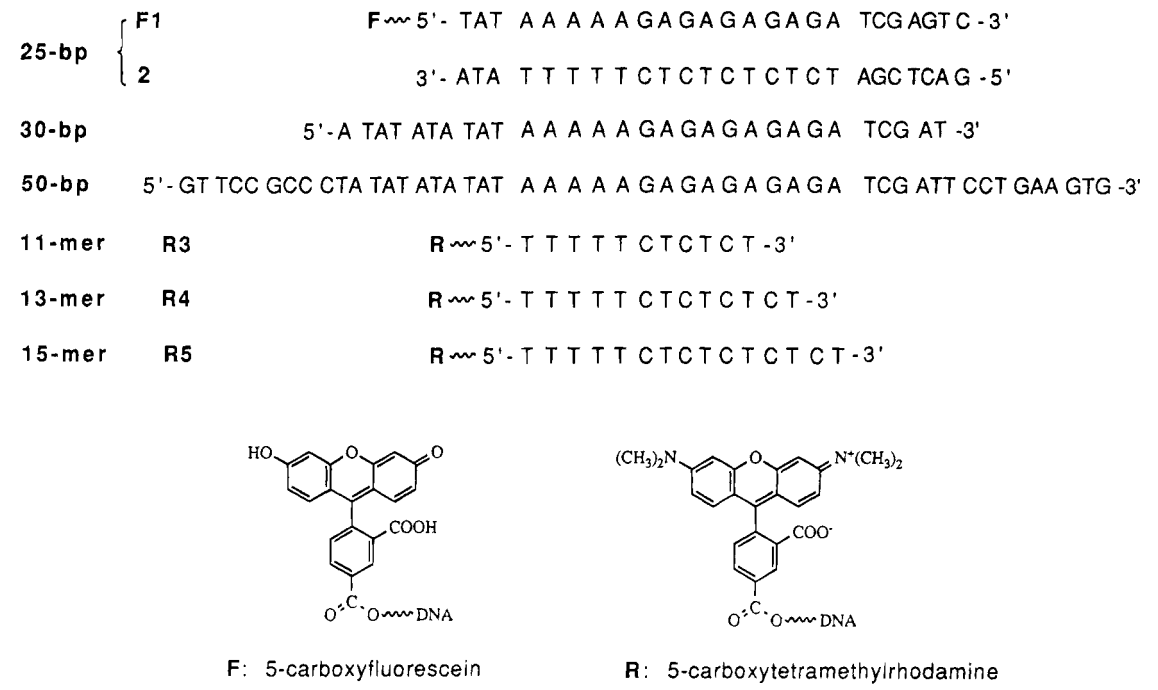

F: 5-carboxyfluorescein R: 5-carboxytetramethylrhodamine

FIGURE 1: Sequences of oligodeoxynucleotides used in this study. Each double-stranded DNA contains a 15-bp homopyrimidine–homopurine target site. The complementary strands for 30- and 50-bp double-stranded DNA are not shown. Oligonucleotides are 5′-end-labeled with a fluorescein donor (**F**) or a tetramethylrhodamine acceptor (**R**) where indicated.

Shafer, 1991; Mergny et al., 1991; Callahan et al., 1991). The information provided by these probes is limited by their lack of spatial localization at a unique position. In this study, we have prepared triple helices with fluorescent donor and acceptor groups attached to the 5′ termini of the duplex and the third strand, respectively. The efficiency of nonradiative energy transfer from the donor to the acceptor greatly increases as the acceptor-labeled oligonucleotide binds to the donor-labeled DNA duplex. By analyzing the fluorescence intensity of the donor upon triplex formation, we were able to determine the equilibrium binding constants and the association rates of DNA triplex formation. In addition, since thermal dissociation of triplex DNA to duplex DNA plus third strand usually occurs at a lower temperature than DNA duplex dissociation, the melting behavior of the triplex-to-duplex transition was directly monitored by FRET measurements.

We also describe a fluorescence anisotropy method that readily measures equilibrium binding constants for triple helix formation. Fluorescence anisotropy and polarization are two related parameters that describe the same phenomenon. Fluorescence anisotropy or polarization measurements reveal the time-average rotational motion of fluorescent molecules. When a fluorescently labeled, low molecular weight ligand binds to a large receptor molecule, its fluorescence polarization increases due to the large rotational volume of the ligand–receptor complex. While fluorescence polarization immunoassays based on this concept have been widely used for detection of antigens and other macromolecules (Cantor & Schimmel, 1980; Chan & Perlstein, 1987), few attempts have been made to apply this concept to affinity binding studies involving nucleic acids (Ellerton & Isenberg, 1969; Scaria & Shafer, 1991; Mergny et al., 1991; Callahan et al., 1991; Devlin et al., 1993). In this study, we demonstrate the use of the fluorescence anisotropy method as an alternate technique for measuring the equilibrium binding constants of 5′-tetramethylrhodamine-labeled 11-mer and 13-mer homopyrimidine oligonucleotides with unlabeled 25-, 30-, and 50-bp double-stranded DNA molecules containing a 15-bp homopurine target site.

MATERIALS AND METHODS

DNA Preparation. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer using β-cyanoethyl phosphoramidite chemistry. A 5′-aminohexyl phosphate linker was attached to each oligonucleotide during a final synthetic cycle using Aminolink2 reagent (Applied Biosystems). Oligonucleotides were cleaved and deprotected using standard methods.

Donor- and acceptor-labeled DNA strands were prepared by reacting the 5′-amino oligonucleotides with succinimidyl ester derivatives (Molecular Probe) of 5-carboxyfluorescein (**F**, Figure 1) or 5-carboxytetramethylrhodamine (**R**, Figure 1). A 30–50-fold molar excess of dye (0.2 mg of dye/mL of dimethylformamide) was added to a 2 mM solution of crude oligonucleotide in 0.1 M $NaHCO_3$, pH 9.0, and the reaction mixtures were kept in the dark at room temperature for 18 h. Unreacted dye was removed on a 10-mL Sephadex G-25 column. Dye-labeled and unlabeled 5′-amino oligonucleotides were purified by reverse-phase HPLC as previously described (Eis & Millar, 1993).

Double-stranded DNA was prepared by mixing the complementary oligonucleotide strands in an association buffer containing 100 mM NaCl, 50 mM Tris–acetate, and 1 mM spermine·4HCl at pH 6.8 (Singleton & Dervan, 1992). Concentrations of unlabeled and labeled DNA were estimated by the 260-, 496-, and 558-nm absorbances. The DNA strands were annealed by heating the solutions at 70 °C for 5 min and then slowly cooling them to room temperature.

Fluorescence Measurements. Steady-state fluorescence intensities of fluorescein-labeled DNA were measured with an SLM spectrofluorometer (SPF-500C) with a polarization accessory. Two methods were used to determine the

equilibrium binding constants of the oligonucleotide-directed DNA triplex formation. The first method was based on the FRET technique. A fluorescein-labeled 25-bp duplex DNA (1.2×10^{-7} M) and a tetramethylrhodamine-labeled 11-, 13-, or 15-mer oligodeoxynucleotide (1.0×10^{-9} M to 1.0×10^{-5} M) were allowed to equilibrate in the dark at room temperature over 18 h in the association buffer to ensure complete binding (Singleton & Dervan, 1992). Fluorescence emission intensity of the fluorescein-labeled, double-stranded DNA in the mixtures was measured at 518 nm (4-nm bandwidth) with an excitation wavelength of 485 nm at 20 °C and plotted against the concentrations of the single-stranded oligonucleotide to obtain a binding curve. The second method used to determine the equilibrium constants was based on measurements of fluorescence anisotropy. A tetramethylrhodamine-labeled 11- or 13-mer oligodeoxynucleotide (3.0×10^{-7} M) and an unlabeled 25-, 30-, or 50-bp duplex DNA (1.0×10^{-9} M to 1.0×10^{-5} M) were allowed to equilibrate for 18 h, and the fluorescence polarization of the tetramethylrhodamine-labeled oligonucleotide in the mixtures was measured at an emission wavelength of 585 nm with an excitation wavelength of 550 nm at 20 °C. The extent of binding was estimated from the fraction of fluorescence intensity due to the triplex DNA species and plotted against duplex concentrations to obtain a binding curve.

The association rate of the DNA triplex formation was determined by the FRET method. The kinetic measurements were performed by mixing an excess of tetramethylrhodamine-labeled oligonucleotide **R3** or **R4** with fluorescein-labeled duplex **F1·2** in the association buffer and immediately monitoring the fluorescence intensity change of the fluorescein moiety on **F1·2** at 20 °C. The fluorescence intensity value measured 20 h after the initial mixing was used as the value at equilibrium. (In the notation, a centered dot (·) represents Watson−Crick pairing between two complementary oligonucleotides and an asterisk (*) represents Hoogsteen pairing between the duplex DNA and a third strand homopyrimidine oligonucleotide.)

The melting behavior of the fluorescently labeled DNA triplex was also examined by the FRET method. Fluorescence melting curves were recorded on an SLM Aminco-Bowman Series 2 luminescence spectrometer with a NESLAB RTE-110 temperature-controlled water bath. The sample temperature was increased from 10 to 80 °C at a rate of 1 °C/min. For comparison, UV melting profiles were obtained by monitoring the absorbance at 260 nm with a Cary 1E spectrophotometer (Varian) equipped with a temperature controller. The melting curves of unlabeled and dye-labeled duplexes and mixtures of a duplex and a third strand oligonucleotide were measured from 10 to 80 °C at a heating rate of 1 °C/min. Temperatures at half-dissociation (T_m values) were estimated from fluorescence or UV absorbance melting profiles by drawing upper and lower baselines and then finding the temperature corresponding to the midpoint between baselines. The DNA concentration was 1×10^{-6} M for all the samples.

Data Analysis. The equilibrium constant for the binding of a duplex, D, by a single-stranded oligonucleotide, S, to form a triplex DNA, T (eq 1), is defined by eq 2,

$$D + S \rightleftarrows T \tag{1}$$

$$K_T = \frac{1}{K_D} = \frac{[T]_{eq}}{[D]_{eq}[S]_{eq}} \tag{2}$$

where K_T is the triplex association constant, K_D is the dissociation constant, and $[D]_{eq}$, $[S]_{eq}$, and $[T]_{eq}$ are the equilibrium concentrations of the unbound duplex DNA, the single-stranded oligonucleotide, and the triplex DNA, respectively.

In the FRET experiments, a given concentration of fluorescein-labeled DNA duplex was incubated with various concentrations of tetramethylrhodamine-labeled oligonucleotides, and the total observed fluorescence intensity of the fluorescein label (I_{obs}) is given by eq 3,

$$I_{obs} = I_D + I_T \tag{3}$$

where I_D and I_T are the fluorescence intensities of unbound duplex DNA and fully bound triplex DNA, respectively. The concentration of the triplex is estimated from eq 4,

$$[T] = \frac{I_D^0 - I_{obs}}{I_D^0 - I_T^\infty}[D_0] \tag{4}$$

where I_D^0 is the fluorescence intensity of the duplex in the absence of the oligonucleotide, I_T^∞ is the fluorescence intensity when all the duplex is completely bound with the oligonucleotide, and $[D_0]$ is the total duplex concentration. Combining eq 4 with eq 2 yields

$$I_{obs} = I_D^0 - \frac{(I_D^0 - I_T^\infty)}{2[D_0]}\left(b - \sqrt{b^2 - 4[D_0][S_0]}\right) \tag{5}$$

$$b = [D_0] + [S_0] + K_D \tag{6}$$

where $[S_0]$ is the total concentration of the single-stranded oligonucleotide. The equilibrium dissociation constant, K_D, was calculated by fitting the binding isotherm (eq 5) to the data of I_{obs} vs $[S_0]$ using a nonlinear least squares fitting procedure (*K*aleidaGraph; Abelbeck Software).

The fluorescence anisotropy, A, is defined in eq 7,

$$A = \frac{I_{||} - GI_\perp}{I_{||} + 2GI_\perp} \tag{7}$$

where $I_{||}$ is the fluorescence intensity measured with an emission polarizer aligned parallel to the vertical excitation polarization and $I_\perp$ is the fluorescence intensity measured with a perpendicular alignment of the emission polarizer. G is a factor that accounts for the polarization bias of the detection system:

$$G = \frac{I_{HV}}{I_{HH}} \tag{8}$$

where I_{HV} and I_{HH} are fluorescence intensities measured with horizontal excitation polarization and with the emission polarizer aligned either vertically or horizontally, respectively.

In a mixture of tetramethylrhodamine-labeled single-stranded oligonucleotides and unlabeled duplexes, the fraction of fluorescence, f_T, due to triplex species is given by eq 9,

$$f_T = \frac{A - A_S}{A_T - A_S} \quad (9)$$

where A, A_S, and A_T are the anisotropy values of the mixture, the totally free oligonucleotide, and the totally bound triplex DNA, respectively. If the binding of the duplex to the dye-labeled oligonucleotide does not alter the quantum yield of the dye, f_T also represents the molar fraction of the DNA triplex in the mixture. It can be shown that the equilibrium constant is related to the concentration of DNA duplex by the following relationship (eq 10):

$$[D_0] = \frac{f_T K_D}{1 - f_T} + f_T[S_0] \quad (10)$$

The K_D value can be obtained by a nonlinear regression fit of eq 10 to f_T vs $[D_0]$ data.

The FRET method was used for the kinetic study of triplex formation. In the presence of an excess amount of oligonucleotide, and assuming that the association rate is substantially faster than the dissociation rate, the concentration of duplex at time t after mixing is given by eq 11,

$$[D] = [D_0] \exp(-k_{obs}t) \quad (11)$$

$$k_{obs} = k_2[S_0] \quad (12)$$

where k_{obs} is the observed pseudo-first-order rate constant and k_2 is the second-order association rate constant. Similar to eq 4, it can be shown that

$$[D] = \frac{I_{obs} - I_T^\infty}{I_D^0 - I_T^\infty}[D_0] \quad (13)$$

where I_T^∞ is the fluorescence intensity value >20 h after the initial mixing. Substituting eq 13 into eq 11 provides an expression for the time course of the donor fluorescence:

$$I_{obs} = I_T^\infty + (I_D^0 - I_T^\infty) \exp(-k_{obs}t) \quad (14)$$

Equation 14 can be fitted to the experimental data of I_{obs} vs time to obtain the value of k_{obs}.

RESULTS

For the FRET study, 5-carboxyfluorescein and 5-carboxytetramethylrhodamine were chosen as the fluorescence donor−acceptor pair since their photophysical properties are well characterized (Cooper & Hagerman, 1990; Hochstrasser et al., 1992; Morrison & Stols, 1993) and covalent attachment of the dyes to the 5′ terminus of an aminohexyl-modified oligonucleotide is straightforward (Hochstrasser et al., 1992; Eis & Millar, 1993). The sequences of the oligodeoxyribonucleotides used in this study are shown in Figure 1. For experiments involving FRET measurements, two complementary oligonucleotides, **F1** and **2**, were annealed to form a 25-bp, double-stranded DNA target. The oligonucleotide **F1** contained a 15-base-long A-rich homopurine tract with a fluorescein donor group (**F**) attached to the 5′ terminus via an aminohexyl linker. Triple helix formation was mediated by Hoogsteen base pairing of the homopyrimidine oligonucleotides 11-mer (**R3**), 13-mer (**R4**), and 15-mer (**R5**) with the purine tract of **F1** within the **F1·2** duplex. The 5′ termini of the homopyrimidine strands were labeled with a tetramethylrhodamine acceptor group (**R**) via an aminohexyl linker. For triplex formation experiments involving fluorescence anisotropy measurements, unlabeled 25-, 30-, and 50-bp double-stranded DNAs containing the same 15-bp homopurine target site served as templates and the dye-labeled oligonucleotides 11-mer (**R3**) and 13-mer (**R4**) were used as the third strands. The sequences of the 11-mer, 13-mer, and 15-mer oligonucleotides as well as that of the 25-bp double-stranded DNA were adopted from a published study (Singleton & Dervan, 1992). A 5′-aminohexyl phosphate linker was attached to each oligonucleotide during DNA synthesis for subsequent conjugation of the fluorescent probes.

The association constants for the binding of the tetramethylrhodamine-labeled oligonucleotides **R3**, **R4**, and **R5** to the fluorescein-labeled, 25-bp DNA duplex **F1·2** were determined by the FRET method. Since homopyrimidine oligonucleotides bind to the homopurine target site of the DNA duplex in a parallel orientation, the 5′-fluorescein group on the DNA duplex and the 5′-tetramethylrhodamine group on the single-stranded oligonucleotide are brought into close proximity, thereby facilitating efficient fluorescence resonance energy transfer from the donor to the acceptor. As increasing the concentration of the oligonucleotide would drive more triplex formation in the solution, it was anticipated that the fluorescence intensity of the duplex would decrease as a result of increasing energy transfer. This was borne out by the results of the FRET titration shown in Figure 2a. The normalized emission intensity of the fluorescein-labeled, 25-bp DNA duplex was plotted as a function of the concentration of the tetramethylrhodamine-labeled oligonucleotides. The titration data points for three DNA triple helices, **F1·2*R3**, **F1·2*R4**, and **F1·2*R5**, were fitted with the theoretical binding isotherm (eq 5), and the equilibrium association constants for each triplex formation were determined from these curves (Table 1). For comparison, the equilibrium constants obtained from the affinity cleavage titration reported in a published study (Singleton & Dervan, 1992) for binding of EDTA·Fe-labeled 11-mer, 13-mer, and 15-mer to the same target site on a 339-bp 5′-^{32}P-labeled duplex are also listed in Table 1. To demonstrate that the changes in fluorescence intensity were caused by specific Hoogsteen-type binding of the oligonucleotide to the duplex, a nonspecific, 5′-tetramethylrhodamine-labeled 14-mer oligodeoxynucleotide, 5′-TGATCTCGAGTAAA-3′, was used as a control. The fluorescence intensities of **F1·2** (1.2×10^{-7} M) remained unchanged following the addition of the nonspecific oligonucleotide (1.0×10^{-9} M to 5.0×10^{-5} M).

Binding studies using the fluorescence anisotropy method were carried out by titrating a solution of tetramethylrhodamine-labeled 11-mer or 13-mer oligonucleotides (**R3** or **R4**) with various concentrations of unlabeled, double-stranded DNA (25, 30, or 50 bp in length). In order to calculate the mole fraction of the DNA triplex species in the mixtures, it was necessary to determine whether the fluorescence quantum yield of the dye-labeled oligonucleotide was altered upon triple helix formation. Fluorescence intensities of the mixtures, measured as a function of the increasing duplex concentration, showed no change in the emission intensity. Therefore, the mole fraction of bound species (triplex DNA) in the solution was the same as the

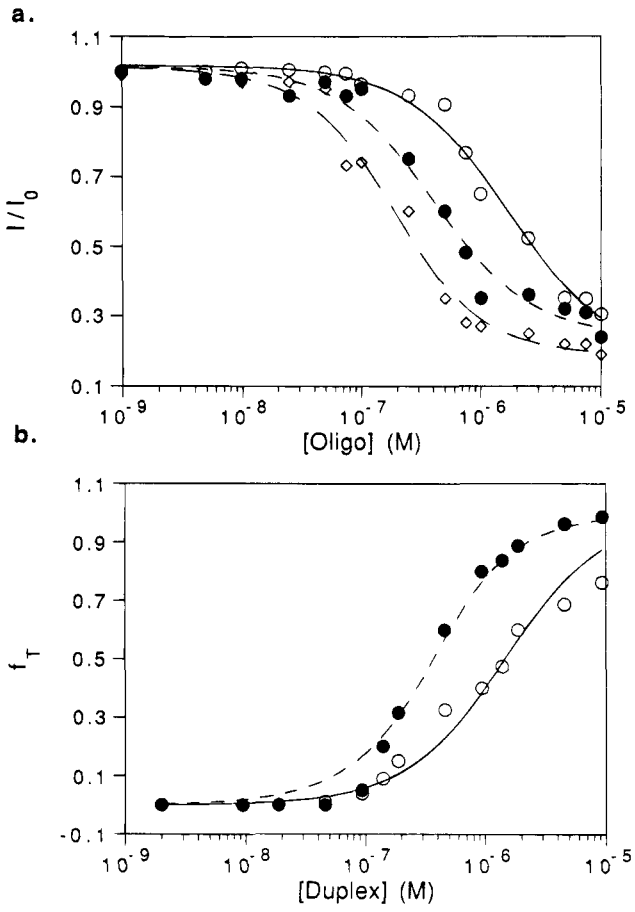

FIGURE 2: (a) Binding isotherms from FRET experiments involving the association of tetramethylrhodamine-labeled oligodeoxynucleotides **R3** (11-mer, open circles), **R4** (13-mer, closed circles), and **R5** (15-mer, diamonds) with fluorescein-labeled DNA duplex **F1·2** (1.2×10^{-7} M) in association buffer at 20 °C and pH 6.8. The data points were normalized using $I_0 = 1$. The solid curves are the nonlinear least squares best fit binding isotherms according to eq 5. (b) Binding isotherms from fluorescein anisotropy measurements involving the association of **R3** (11-mer, open) and **R4** (13-mer, closed) with unlabeled, 25-bp DNA duplex (**1·2**) in association buffer at 20 °C and pH 6.8. The fraction of binding was obtained from the fraction of fluorescence intensity due to the triplex DNA. The solid curves are the best fits to a theoretical isotherm based on eq 10.

Table 1: Comparison of FRET and Affinity Cleavage Titration Measurements of K_T

oligonucleotide[a]	K_T (M^{-1})	ΔG (kcal mol^{-1})[d]
R3[b]	$(6.0 \pm 1.0) \times 10^5$	−7.8 (±0.2)
R4[b]	$(2.9 \pm 0.6) \times 10^6$	−8.7 (±0.2)
R5[b]	$(7.7 \pm 1.6) \times 10^6$	−9.2 (±0.2)
EDTA·Fe·**3**[c]	$(6.2 \pm 3.8) \times 10^5$	−7.9 (±0.6)
EDTA·Fe·**4**[c]	$(1.8 \pm 0.4) \times 10^6$	−8.5 (±0.1)
EDTA·Fe·**5**[c]	$(3.7 \pm 1.1) \times 10^6$	−9.0 (±0.2)

[a] Tetramethylrhodamine-labeled oligonucleotides were mixed with fluorescein-labeled 25-bp DNA duplex (**F1·2**), and the binding curves were fitted with a theoretical binding isotherm (eq 5) to obtain the association constant, K_T. [b] FRET measurements were performed in association buffer at 20 °C and pH 6.8. [c] Affinity cleavage measurements in a published report (Singleton & Dervan, 1992) were performed in association buffer at 24 °C and pH 7.0. [d] The values for ΔG were calculated by taking into account the difference in temperature between the FRET and the affinity cleavage measurements.

fraction of fluorescence intensity due to the triplex DNA and proportional to the measured anisotropy. Fluorescence anisotropy of the tetramethylrhodamine-labeled oligonucleotides was measured at various duplex concentrations, and the corresponding mole fraction of DNA triplex in the mixtures was calculated according to eq 9. Figure 2b shows typical plots of the mole fraction of the triplex DNAs **1·2*R3** and **1·2*R4** as a function of the duplex concentration. The binding curves for the formation of six triplex DNAs consisting of combinations of tetramethylrhodamine-labeled oligonucleotides (11-mer or 13-mer) and unlabeled duplexes (25, 30, or 50 bp) were obtained, and the binding constant for each DNA triplex was calculated from a nonlinear least squares best fit of the data to eq 10 (Table 2). The K_T values determined by the anisotropy method (Table 2) were in good agreement with those obtained from the FRET method (Table 1). In addition, while the anisotropy of the 13-mer oligonucleotide **R4** increased from 0.094 to 0.172 upon specific binding to 25-bp DNA **1·2**, titration of **R4** with identical amounts of a nonspecific, unlabeled, 19-bp double-stranded DNA, 5′-TATTTACTCGAGATCTAGA-3′, did not produce any change in fluorescence anisotropy.

Table 2: Determination of K_T by Fluorescence Anisotropy Measurements

DNA duplex[a]	K_T (M^{-1}) 11-mer **R3**	K_T (M^{-1}) 13-mer **R4**
25 bp	$(7.3 \pm 0.8) \times 10^5$	$(4.3 \pm 0.6) \times 10^6$
30 bp	$(8.5 \pm 1.0) \times 10^5$	$(5.0 \pm 0.6) \times 10^6$
50 bp	$(6.3 \pm 0.7) \times 10^5$	$(3.8 \pm 0.6) \times 10^6$

[a] Tetramethylrhodamine-labeled oligonucleotides (3.0×10^{-6} M) were mixed with various concentrations of unlabeled DNA duplexes, and the binding curves were fitted with a theoretical binding isotherm (eq 10) to obtain the association constant, K_T.

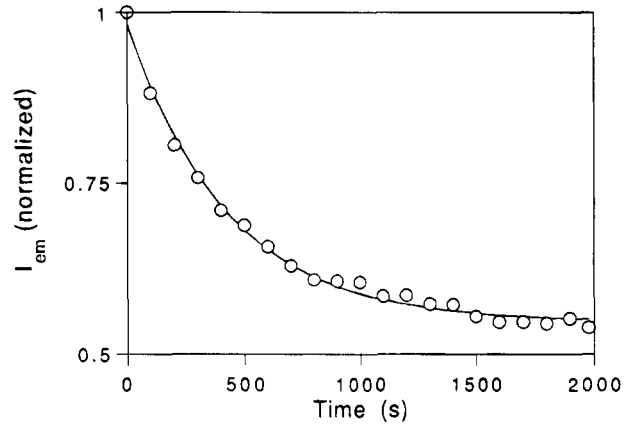

FIGURE 3: Time course of fluorescence intensity of the fluorescein-labeled duplex **F1·2** (1.2×10^{-8} M) upon the addition of the tetramethylrhodamine-labeled oligonucleotide **R4** (8.0×10^{-7} M) in association buffer at 20 °C and pH 6.8. The data points were normalized using $I_0 = 1$. Only selected data points are plotted for clarity. The solid curve is the single-exponential best fit for pseudo-first-order kinetics according to eq 14.

The kinetics of triple helix formation were also analyzed by the FRET method. To determine the association rate constants for the binding of an oligonucleotide to a double-stranded DNA target, an excess amount of tetramethylrhodamine-labeled oligonucleotide was mixed with a fluorescein-labeled duplex solution and the emission intensity of the fluorescein donor was monitored over time. The decay of the donor fluorescence was directly related to the association kinetics of the triplex formation. Figure 3 shows the changes in emission intensity of a 25-bp, fluorescein-labeled DNA duplex (**F1·2**, 1.2×10^{-8} M) upon the addition

of a 13-mer, tetramethylrhodamine-labeled oligonucleotide (**R4**, 8.0×10^{-7} M). In the presence of a greater than 10-fold excess of oligonucleotide relative to the target duplex DNA, the reaction kinetics of triplex formation are approximately pseudo-first-order in duplex concentration. The observed rate constant, k_{obs} (eq 11), can be obtained from the best fit of the single-exponential decay curves of the fluorescence intensity according to eq 14 (Figure 3). The values of k_{obs} were determined to be $(7.5 \pm 0.3) \times 10^{-4}$ and $(13.8 \pm 0.9) \times 10^{-4}$ s^{-1} for bindings of 4.0×10^{-7} and 8.0×10^{-7} M **R4** with 1.2×10^{-8} M **F1·2**, respectively. An average k_2 value of $(1.8 \pm 0.2) \times 10^3$ M^{-1} s^{-1} was calculated from eq 12. Similarly, an average k_2 value of $(2.7 \pm 0.3) \times 10^3$ M^{-1} s^{-1} was determined for binding of **R3** (11-mer) with **F1·2**.

Finally, the melting behavior of the DNA triplex was examined by the FRET method. Triple-helical DNA undergoes a triplex-to-duplex transition, followed by duplex-to-single-strand dissociation, as the temperature increases. For a triplex containing a fluorescein-labeled duplex and a tetramethylrhodamine-labeled oligonucleotide, the efficiency of nonradiative energy transfer decreases as the dissociation of triplex to duplex occurs, resulting in an increase in the fluorescence intensity of the donor group. Figure 4a shows the emission spectrum of a triplex DNA, **F1·2*R5** (25 bp + 15-mer, 1.0×10^{-6} M), at two different temperatures. With an excitation wavelength of 485 nm, the fluorescence intensity at 518 nm (fluorescein emission) increased while the emission intensity at 585 nm (tetramethylrhodamine emission) decreased as a result of thermal dissociation when the temperature was increased from 22 to 65 °C. Therefore, a fluorescence melting profile could be obtained by continuously monitoring the fluorescein emission at 518 nm while changing the temperature. The fluorescence melting profile of **F1·2*R5** was recorded for a solution containing equimolar concentrations (1.0×10^{-6} M) of a fluorescein-labeled, 25-bp duplex (**F1·2**) and a tetramethylrhodamine-labeled, 15-mer oligonucleotide (**R5**) in association buffer (Figure 4b). Under these conditions, approximately 70% of **F1·2** and **R5** will form the triplex, **F1·2*R5**, at 20 °C, based on the binding constant determined for the triplex by FRET measurements (Table 1). A significant transition in the fluorescence intensity at 518 nm was observed between 35 and 65 °C. The apparent melting temperature, T_m, defined as the temperature at half-dissociation, was determined as 52 °C. The fluorescence behavior of the fluorescein-labeled duplex, **F1·2** and the single-strand oligonucleotide **F1** were also recorded to examine the temperature dependence of the fluorescein probe. Since a change of less than 10% in the fluorescence intensity of the fluorescein probe was observed for both **F1·2** and **F1** as the temperature was increased from 10 to 80 °C, the fluorescence data presented in Figure 4b was not corrected for the temperature dependence of the fluorescein probe.

The T_m value obtained from the FRET measurement may reflect the triplex-to-duplex transition. On the other hand, it may represent the melting behavior including both the triplex-to-duplex transition and the duplex-to-single-strand transition. Thermal melting profiles for a series of unlabeled or dye-labeled duplex and triplex DNA samples were measured by UV hypochromicity at 260 nm in order to verify the corresponding melting temperatures for triplex and duplex dissociation. Table 3 summarizes the data from the melting

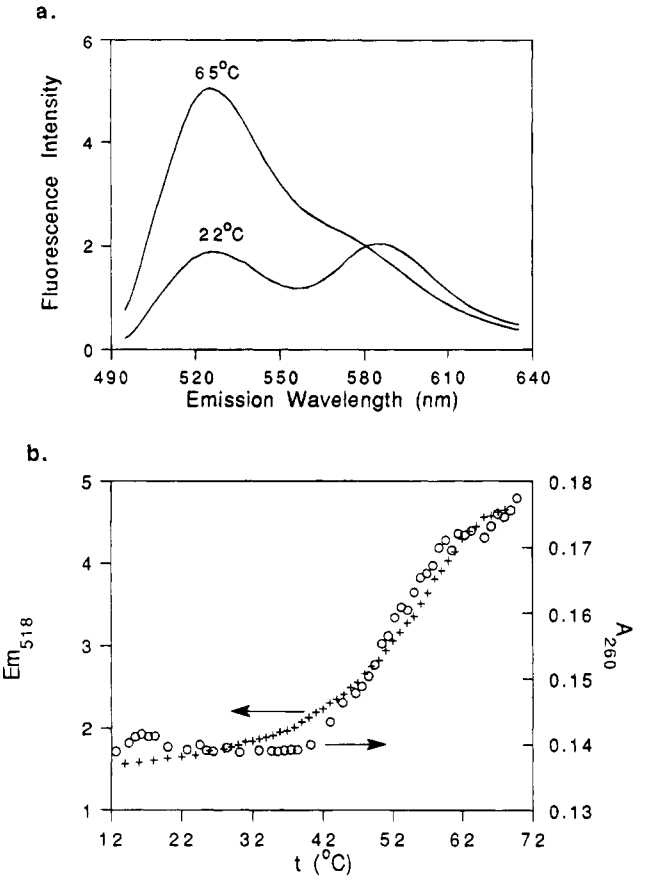

FIGURE 4: (a) Emission spectra for the mixture of the fluorescein-labeled, 25-bp DNA duplex **F1·2** (1.0×10^{-6} M) and the tetramethylrhodamine-labeled, single-stranded 15-mer oligonucleotide **R5** (1.0×10^{-6} M) in association buffer equilibrated at 22 and 65 °C. The excitation wavelength was 485 nm. (b) Dissociation profiles of the DNA triplex **F1·2*R5** (1.0×10^{-6} M) in association buffer as observed by FRET (crosses) and UV absorbance hypochromicity (circles) measurements. The FRET melting curve was directly recorded by monitoring the fluorescence intensity of the fluorescein moiety at 518 nm with an excitation wavelength of 485 nm. The UV melting curve was obtained by subtracting the 260-nm absorbance of the DNA duplex **F1·2** (1.0×10^{-6} M) from that of the mixture of **F1·2** + **R5**. The temperature was increased from 10 to 80 °C at a rate of 1 °C/min.

curves obtained for unlabeled (**1·2**) and fluorescein-labeled (**F1·2**) 25-bp double-stranded DNA, as well as triplex DNA species containing unlabeled 25-bp duplex with a 5′-hydroxyl 15-mer oligonucleotide [**1·2*5(N)**], unlabeled 25-bp duplex with a 5′-aminohexyl-modified 15-mer [**1·2*5(L)**], and fluorescein-labeled 25-bp duplex with a 5′-tetramethylrhodamine-labeled 15-mer (**F1·2*R5**). The UV melting profiles of both duplexes displayed a typical monophasic transition, and the T_m values (Table 3) determined at half-dissociation indicate that the fluorescein label does not affect the stability of the duplex. The melting profiles of all the triplex DNAs also showed monophasic transitions instead of the biphasic transitions expected for two distinct dissociation processes. The apparent T_m values of the transitions (Table 3) were very close to the values obtained for the duplex dissociation process, suggesting that the triplex-to-duplex transitions may overlap with the duplex-to-single-strand dissociation. In order to determine the exact T_m values for the triplex-to-duplex transitions, the triplex dissociation profiles were generated by subtracting the absorbances of the corresponding duplexes from those of the mixtures of

Table 3: Melting Temperatures of Duplex and Triplex DNA[a]

sample	apparent T_m (°C)[b]	recovered T_m (°C)[b]
duplex DNA		
1·2	66.6	
F1·2	66.3	
triplex DNA		
1·2*5(N)[c]	65.0	50.9
1·2*5(L)[c]	64.5	52.8
F1·2*R5	64.8	51.0
F1·2*R5 (FRET)[d]		52

[a] Melting behavior of duplex and triplex DNA was recorded by UV absorbance (260 nm) and fluorescence intensity (518 nm) measurements. The temperature was increased from 10 to 80 °C at 1 °C/min. Sample concentrations: 1.0×10^{-6} M. See Figure 1 for the sequences of the oligonucleotides. [b] T_m values were determined as the temperature at half-dissociation. Apparent T_m values were obtained directly from the original melting curves. Recovered T_m values were obtained from the melting curves due to triplex dissociation recovered by subtracting the absorbance of the duplex from that of the mixture of duplex and third strand. [c] **5(N)** represents the 15-mer oligonucleotide without a 5′-tetramethylrhodamine label or a 5′-aminohexyl linker. **5(L)** represents the 15-mer oligonucleotide without a 5′-tetramethylrhodamine label but with a 5′-aminohexyl linker. [d] The apparent and recovered T_m values are the same for T_m obtained from FRET measurement.

duplex and third strand (Escudé et al., 1993). A representative melting curve for thermal dissociation of triplex DNA **F1·2*R5** to a mixture of **F1·2** + **R5** is also shown in Figure 4b. A comparison between the two melting profiles shows that the melting curve of **F1·2*R5** directly observed by FRET is remarkably similar to the triplex-to-duplex melting curve obtained from UV absorbance. The T_m values for the triplex transition curves from UV hypochromicity (Table 3) are in excellent agreement with the T_m value determined by FRET measurement, indicating that the melting temperature determined by FRET measurement for **F1·2*R5** is the T_m value reflecting the triplex-to-duplex transition. We note that the T_m values for triplex dissociation determined by both the FRET and UV absorbance methods may depend on the rate at which the temperature is increased during measurement of the melting profiles (1 °C/min for both methods) (Rougée et al., 1992). Since the present results are sufficient to establish that the FRET-based method yields triplex dissociation profiles that are in accord with conventional UV absorbance profiles recorded under identical conditions, no attempt was made to assess the effect of heating rate on the observed profiles. Finally, in order to examine the effect of the fluorescent probes and the aminohexyl linker on the stability of the triplex, the melting curves were measured for triple helices containing unlabeled and fluorescein-labeled 25-bp DNA duplexes and 5′-hydroxyl (unmodified), 5′-aminohexyl-modified, and 5′-tetramethylrhodamine-labeled 15−mer oligonucleotides, and the triplex T_m values were determined by the UV absorbance measurements outlined above. No difference in T_m values for these triple helices was observed within experimental uncertainty (Table 3).

DISCUSSION

A number of techniques have been employed to study thermodynamic and kinetic properties of oligonucleotide-directed formation of triple-helical DNA (Xodo et al., 1990; Plum et al., 1990; Roberts & Crothers, 1991; Ito et al., 1992; Brenowitz et al., 1986; Singleton & Dervan, 1992; Shindo et al., 1993). However, due to limits of sensitivity and time resolution and the requirement for radiolabeling and separation for most of the methods, there is a need to develop a solution-phase measurement method which is capable of continuously monitoring triple helix formation with reasonable sensitivity. In the present study, we describe the use of fluorescence spectroscopic methods, based on fluorescence resonance energy transfer and fluorescence anisotropy, as viable approaches for direct monitoring of DNA triplex formation in solution with high sensitivity. Using these approaches, we have measured the equilibrium binding constants for a series of mixtures consisting of complementary double-stranded DNA and single-strand oligonucleotides. The binding constants between tetramethylrhodamine-labeled 11-mer, 13-mer, and 15-mer oligonucleotides and a fluorescein-labeled, 25-bp duplex containing a 15-bp target site were measured by FRET (Table 1). Both the K_T and the ΔG values, as well as the effect of oligonucleotide length on K_T, are in excellent agreement with those obtained from a method based on binding of EDTA·Fe-labeled oligonucleotides to duplex DNA containing the same target site under similar experimental conditions (Singleton & Dervan, 1992). No change in the fluorescence intensity of a fluorescein-labeled duplex was observed upon the addition of a nonspecific, tetramethylrhodamine-labeled 14-mer oligonucleotide, thereby confirming the specificity of the triplex formation.

For the alternate approach, the binding constants between tetramethylrhodamine-labeled 11-mer and 13-mer oligonucleotides and unlabeled 25-, 30-, and 50-bp DNA duplexes were determined by fluorescence anisotropy measurements (Table 2). The K_T values determined with this approach are consistent with those obtained from FRET (Table 1), although a fluorescein label was attached to the duplex in the latter approach. As expected, the sensitivity of the anisotropy method increased as the size of the target DNA duplex increased. A comparison of the measured K_T values for the 11-mer oligonucleotide with those for the 13-mer suggests that the association constant is dependent on the length of the single-stranded oligonucleotide but is not affected by the size of the DNA duplex. No change in fluorescence anisotropy was observed when the dye-labeled oligonucleotide was mixed with an unlabeled, nonspecific, 19-bp duplex. The agreement between the results obtained from these fluorescence spectroscopic methods and those from other studies (Singleton & Dervan, 1992) indicates that the spectroscopic approaches may be used in determining the equilibrium binding constants for triple helix formation.

To date, only a few attempts have been made to measure the association and dissociation kinetics of triple helix formation by methods such as restriction endonuclease protection (Maher et al., 1990), UV melting (Rougée et al., 1992), and filter binding (Shindo et al., 1993). In the present study, kinetic analysis of DNA triplex formation was accomplished by the FRET method. In the presence of a large excess amount of oligonucleotide, the observed pseudo-first-order rate constant and the derived second-order association rate constant for a tetramethylrhodamine-labeled oligonucleotide with a fluorescein-labeled duplex were obtained in a simple experiment. The k_2 values for triple helices **F1·2*R3** and **F1·2*R4** were determined to be $(2.7 \pm 0.3) \times 10^3$ and $(1.8 \pm 0.2) \times 10^3$ M^{-1} s^{-1}, respectively. While no literature value of k_2 for the same association reactions is available for quantitative comparison, we note that the association rate constants obtained from the FRET measurement are comparable in magnitude with those

determined by a restriction endonuclease assay (Maher et al., 1990) and a filter binding assay (Shindo et al., 1993) for triplex DNA species of similar sizes. Rougée et al. (1992) have determined association rate constants for triple helix formation by analysis of hysterisis in UV melting profiles. Their results show that the rate of triplex formation is strongly dependent on the salt concentration and the presence of multivalent cations. The k_2 values for triple helices **F1·2*R3** and **F1·2*R4** appear to be compatible with the results of Rougée et al. if differences in the solution conditions used in the present study are taken into account. It should be noted that the above assays are discontinuous or indirect, while the FRET measurement allows triple helix formation to be monitored continuously in real time. Greater accuracy in rate measurements may be achieved using stopped-flow methods to obtain rapid mixing for triplex formation with high association rates.

FRET was also used to measure the melting behavior of the DNA triplex-to-duplex transition. The melting profiles and T_m values determined by the FRET method for the dissociation of the triplex DNA **F1·2*R5** (1.0×10^{-6} M) were in excellent agreement with those for the triplex-to-duplex transitions obtained from UV hypochromicity (Table 3). It should be noted that only a single transition was observed from the absorbance melting curves of the triplex DNA, indicating the overlap of the triplex-to-duplex dissociation and the duplex-to-single-strand transition. The triplex-to-duplex transition can only be derived from absorbance measurements by a subtractive procedure (Escudé et al., 1993). On the other hand, the melting profile based on FRET shows the triplex-to-duplex transition without interference of the duplex-to-single-strand transition.

A previous study (Morrison & Stols, 1993) had reported that a 5′-fluorescein label has an overall stabilizing effect on hybridized duplex strands and had suggested that the effect was due to the binding interaction between the terminal fluorescein and the DNA double helix. Therefore, the presence of the fluorescent labels could also influence the binding characteristics of the triplex DNA. It should be noted that in that study the fluorescein was attached to the 5′-end via a C_2 linker, while in the present study a C_6 linker was used for dye attachment. To examine the effects of dye-labeled oligonucleotides on the stability of duplex and triplex DNA, UV absorbance melting curves for a series of unmodified and modified duplex and triplex DNAs were measured. As shown in Table 3, the T_m values for unlabeled and fluorescein-labeled 25-bp DNA duplexes are almost identical, indicating that the fluorescein label does not have a stabilizing or destabilizing effect on the duplex. Furthermore, a comparison of the recovered T_m values for the triplex-to-duplex transition for triple helices consisting of an unmodified, a 5′-aminohexyl-modified, and a 5′-tetramethylrhodamine-labeled 15-mer oligonucleotide indicates that the addition of a linker or a linker−dye conjugate to the 5′ end of the third strand has minimal effect on the stability of the triple helix. These observations corroborate the finding that covalent attachment of EDTA·Fe to the 5′ end of an oligonucleotide does not have a measurable effect on the binding constant for triple helix formation (Singleton & Dervan, 1992) and an earlier report that the flexibility of a 5′-aminohexyl linker imparts considerable freedom to a covalently attached fluorescein moiety (Hochstrasser et al., 1992).

In summary, the results demonstrate the feasibility of using fluorescence spectroscopic methods as a continuous, efficient, non-radioisotopic, and nondestructive technique for studying the thermodynamics and kinetics of oligonucleotide-directed DNA triple helix formation. The ease of dye labeling of nucleic acids ensures that these techniques are applicable for a wide range of conditions and DNA sequences. Moreover, since dyes can be readily attached to oligonucleotides containing modified bases or backbone groups, the fluorescence-based methods described here can be used to assess the effect of chemical modification of the single-stranded oligonucleotide on the stability of a DNA triple helix. Information from such studies should be useful in the design of antisense oligonucleotides. The FRET method can be used in a straightforward manner to determine the equilibrium association constants, the association rate constants, and the thermal melting behavior of triple-helical DNA. Additionally, since the donor group can be placed at the 5′ terminus of either strand of the duplex, this approach may be useful in determining the orientation of the third strand in triple helices that can form in either parallel or antiparallel configurations (Giovannangéli et al., 1992). For studies where the target DNA is relatively large, such that the distance between the 5′ termini becomes much longer than the critical distance of FRET, fluorescence anisotropy measurements provide an excellent alternative for determining the equilibrium binding constants of DNA triple helix formation. Association rate constants may also be determined by the anisotropy method using a T-format configuration in which both the parallel and the perpendicular emission components can be measured simultaneously.

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