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Fluorescence energy transfer as a probe for nucleic acid structures and sequences

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ABSTRACT

The primary or secondary structure of single-stranded nucleic acids has been investigated with fluorescent oligonucleotides, i.e., oligonucleotides covalently linked to a fluorescent dye. Five different chromophores were used: 2-methoxy-6-chloro-9-aminoacridine, coumarin 500, fluorescein, rhodamine and ethidium. The chemical synthesis of derivatized oligonucleotides is described. Hybridization of two fluorescent oligonucleotides to adjacent nucleic acid sequences led to fluorescence excitation energy transfer between the donor and the acceptor dyes. This phenomenom was used to probe primary and secondary structures of DNA fragments and the orientation of oligodeoxynucleotides synthesized with the alpha-anomers of nucleoside units. Fluorescence energy transfer can be used to reveal the formation of hairpin structures and the translocation of genes between two chromosomes.

INTRODUCTION

Fluorescence excitation energy transfer, hereafter abbreviated as FET, is a dipole–dipole resonance interaction between two close molecules, where one molecule, called the 'donor', transfers its excitation energy to the other, called the 'acceptor'. FET has given valuable information on the fluidity of biological membranes (1–2), the conformation of proteins (3), the rate of enzymatic reactions (4), nucleic acid–drugs interaction (5–7), or the structure of nucleic acids (8–11) because of its distance and orientation dependence (12).

The binding of DNA ligands such as intercalators or minor groove binding ligands has been studied extensively. However, their binding specificity is somewhat limited, and no drug is able to recognize a long sequence. This observation led to the design of bifunctional nucleic acid ligands (13), i.e., molecules able to hybridize specifically to a single- or double-stranded sequence, e.g., oligonucleotides, and carrying a covalently linked group such as an acridine derivative (13–16), orthophenanthroline (17–18), EDTA (19–20), ellipticine (21), cholesterol (22), porphyrins (23–24), psoralens (25–26) [for a review of oligonucleotide conjugates, see (27)]. These groups play one or several roles: they can increase the binding affinity, chemically or photochemically induce an irreversible modification of the target sequence, or protect the oligonucleotide from degradation by nucleases. Many fluorescent dyes can now be covalently linked to an oligonucleotide, and the present study was undertaken to determine the potential use of derivatized oligonucleotides as probes of nucleic acid structures and sequences by the technique of fluorescence energy transfer.

We describe the chemical synthesis and some spectroscopic properties of new bifunctional agents, where dyes such as ethidium bromide, coumarin, fluorescein or rhodamine derivatives form the fluorescent part of the modified oligonucleotide. Hybridization of two fluorescent oligonucleotides on adjacent single-stranded sequences has been shown to lead to fluorescence energy transfer from a donor molecule to an acceptor (28–31), thus allowing detection of adjacent sequences. In the following experiments, we report that FET can also be used to probe the secondary structure of nucleic acid fragments, the orientation of [α]-oligonucleotides and the detection of a gene translocation event between two different chromosomes. Three different donor–acceptor couples were investigated: coumarin–ethidium; acridine–ethidium and fluorescein–rhodamine. The chemical structures and names of the fluorescent oligonucleotides used therafter are given in figure 1.

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EXPERIMENTAL PROCEDURES

Chemicals

Commercial products. All unmodified oligonucleotides were synthesized by the phosphoramidite method and used after purification by HPLC on reverse phase columns. All organic solvents from Merck were purified, redistilled and preserved from any trace of water in a dry dessicator at room temperature. Triethylamine was checked for absence of primary and secondary amines. Rhodamine B isothiocyanate was purchased from Fluka Biochemika. Fluorescein isothiocyanate, either on Celite™ or not, was from Sigma. All other reagents were commercial Aldrich products.

Obtention of modified chromophores. Since aromatic amines such as those present in ethidium bromide are not very reactive, the method of covalent linkage developed for aliphatic amines (24, 32) could not be directly applied, and activation of ethidium was necessary. As ethidium bromide contains two aromatic amino groups, doubly substituted ethidium derivatives were avoided by addition of trifluoroacetic anhydride: an aminoalkyl derivative of ethidium was obtained by mixing 55 mg of ethidium bromide, 3-(N-trifluoroacetyl) aminopropionyl chloride and trifluoroacetic anhydride (both in excess) in 5 ml DMF. The mixture was evaporated to dryness, the residue was dissolved in 5 ml of concentrated ammonium hydroxide and 2.5 ml of ethanol, then evaporated to dryness, redissolved in 50 ml of water and applied to an ion exchange Sephadex column from Pharmacia. Elution was performed with a gradient of ammonium bicarbonate (from 0 to 1 M). Fractions containing monosubstituted isomers were collected, evaporated to dryness, dissolved in 10 % ethanol in chloroform and applied to a column (1×50 cm, Silasorb 600, 5 μ 'Chemapol'). Elution was carried out with a gradient of ammonium trifluoroacetate in methanol. Fractions containing separated isomers were collected, evaporated, dissolved in water and purified by ion exchange chromatography on CM-Sephadex. The structure of the compounds were confirmed by proton magnetic resonance spectroscopy. Overall yield was 17 % for the monoacylated isomer selected for linkage (see figure 1).

4-trifluoromethyl, 7-ω-bromopropylaminocoumarin was obtained by heating together 4-trifluoromethyl-7-amino coumarin ('coumarin 500') with 1,3-dibromopropane for twelve hours at 140°C in presence of CuBr as a catalyst. The crude dye was then extracted from the solid product of the reaction by dissolving in CH_2Cl_2 in the cold. This solution was chromatographed on silica plates and eluted by a toluene:ethylacetate mixture 80:20 (rf = 0.55). The dye, extracted from silica, was crystallized in cyclohexane: needles, melting point = 148°C, λ max (in 96% EtOH) = 390 nm. The purity was checked by standard techniques, i.e. infrared spectroscopy, NMR, mass spectrum.

Modified oligonucleotides

The synthesis of oligonucleotides linked to 2-methoxy-6-chloro-9-aminoacridine (acridine) was previously described (33–34).

Linkage of coumarin to the 3'-end of TTTCCTCCTCT. Linkage of coumarin to the 3'-end of TTTCCTCCTCT involved coupling of the 3'-thiophosphate derivative of the oligonucleotide to 4-trifluoromethyl-7-ω-bromopropylaminocoumarin. The preparation of oligodeoxynucleotides carrying a phosphorothioate group at their 3'-end has already been described (45, 48). It requires the preparation of a modified derivatized support involving a disulfide bond, followed by the addition of the nucleoside 3'-phosphorothioate-triester (48). Sequential growth of the oligodeoxyribonucleotide chain was then performed using the phosphoramidite procedure. After detritylation, the protected oligonucleotide bound to the support was treated with 0.1 M dithiothreitol in concentrated aqueous ammonia at room temperature for 48 hours. The modified oligonucleotide was then purified by reverse phase HPLC. The reverse phase column was packed with 10 μM Lichrospher RP18 from Merck, and elution was performed with a linear gradient of acetonitrile (5–80%) in 0.1 M aqueous triethylammonium acetate, pH 7.

Synthesis of ethidium-dT_{11}, 13-mer-$^{3'}$ethidium and $^{5'}$ethidium-14-mer. Oligonucleotides were synthesised on the 1 micromole scale by solid support phosphoramidite chemistry, then purified by ion exchange chromatography. The sequences were confirmed by the Maxam–Gilbert sequencing method (50). Oligonucleotides were converted to their cetyltrimethylammonium salt using precipitation with a 8 % solution of cetyltrimethylammonium bromide in water. The precipitates were collected by centrifugation, washed with methanol and dried under vacuum. Thirty optical units of each oligonucleotide and excess of ethidium derivative, 6.6 mg of 2,2'-dipyridyl disulfide and 7.3 mg of triphenyl phosphine were dissolved in 60 μl of dimethylsulfoxide and kept for 2 hours at room temperature. The product was precipitated by addition of an excess of 2 % $LiClO_4$ in acetone. Samples were centrifuged and pellets were dried, then dissolved in water and reprecipitated. The oligonucleotides were then purified by reverse phase chromatography using a linear gradient of acetonitrile (from 0 to 30 %).

Synthesis of 18-mers linked to fluorescein- or rhodamine-isothiocyanates. For linkage to the 5'-end of an oligonucleotide, phosphorylation of the oligonucleotide was achieved by treating 0.5 mg of the oligonucleotide with 30–40 units of T4 polynucleotide kinase (Ozyme) in 100 μl of a 50 mM tris HCl pH 7.6 buffer, containing 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermine, 0.1 mM EDTA and 10 μCi of γ-^{32}P-ATP. Following a 30 min incubation at 37°C, 4 μl of a 0.1M 'cold' ATP solution were added. After a second incubation for 60 min at 37°C, the kinase was inactivated by addition of EDTA (10 mM final concentration) and heating (80°C; 2 min). Using radioactive γ-^{32}P-ATP was not mandatory (phosphorylation could be achieved with cold ATP only) but helped to estimate yields of furhter reactions. The oligonucleotide was separated from ATP by gel filtration on a G-50 column (Amersham). It was not necessary to separate the phosphorylated oligonucleotide from the initial 5'OH compound, which will not react with N-methylimidazole. The yield of phosphorylation was routinely above 90%. When a phosphate group was required at the 3'-end, the oligonucleotide was directly phophorylated at the 3'-end during solid-phase synthesis (48). Once phosphorylation was achieved, activation of the terminal phosphate via N-methylimidazolide derivative was carried out as previously described (24, 32). Covalent linkage of ethylenediamine to the activated phosphate was then obtained in pure DMSO during 30 min at room temperature with an excess of the diamine. Isothiocyanates are well known reagents for amino groups in proteins, and were used here to react with the primary amine linked to the oligonucleotide (46). After precipitation and resuspension in bidistilled water, reaction of RITC and FITC with

the terminal amine was performed overnight at 30°C (the oligonucleotide was dissolved in 0.4 M carbonate buffer, pH 10.5, and mixed with an equal volume of FITC dissolved in dimethyl formamide). The unreacted dye was first eliminated by extraction with 2-butanol (3 times) or by gel filtration on a G-50 column (Amersham). Purification of fluorescent oligonucleotides was achieved by denaturing gel electrophoresis using 20 % polyacrylamide and 7 M urea gels. After migration for two hours, the fluorescent band under U.V. illumination was cut out of the gel and the oligonucleotide was extracted by electroelution. The conjugated oligonucleotides migrated as single fluorescent bands retarded compared to the unreacted oligonucleotides (11, 24). The oligonucleotide was then precipitated by ethanol or acetone and resuspended in water. Its concentration was determined by UV absorption spectra. The global yield was 5−15% for rhodamine-substituted oligonucleotides and 40−60% for fluorescein-substituted oligonucleotides. This yield was calculated from the OD_{260} of the conjugated oligonucleotide (after gel purification of the fluorescent band and extraction from the gel) compared to the starting material (OD_{260} of unmodified oligonucleotide). Analysis of the absorption spectra of the conjugated oligonucleotides allowed us to determine the extinction coefficients of the dyes. This method allowed radioactive labeling of the oligonucleotide with γ ^{32}P-ATP at the 5′ end and variation in the length of the linker between the chromophore and the terminal phosphate, using a diamine NH_2-$(CH_2)_n$-NH_2 of appropriate length (24).

Spectroscopic studies

Unless otherwise specified, all the experients were performed in a 10 mM cacodylate buffer, pH 7, containing 100 mM NaCl, at 1 μM oligonucleotide strand concentration.

UV absorption studies. Thermal denaturation profiles were obtained with a Kontron Uvikon 820 spectophotometer, using Quartz cuvettes of 1 cm optical pathlength. The six-sample cell holder was thermostated by a circulating liquid (80% water; 20% glycerol). Temperature of the liquid was controlled by an Haake water bath, and temperature in one of the cells was measured by a thermoresistance. The temperature of the bath was increased or decreased at a rate of 0.11°C/min, thus allowing complete thermal equilibrum of the six cuvettes. All melting curves were reversible, giving the same profile with a high (70°C) or low (2°C) starting temperature. Reading of absorbance at 260 nm was performed every ten minutes.

Static fluorescence studies. All measurements were performed on a Spex Fluorolog DM1B instrument, using a bandwidth of 1.8 nm and 1 cm by 1 cm quartz cuvettes. For emission spectra, excitation wavelength was set at the wavelength where absorption of the donor was maximal or chosen so as to minimize absorption of the acceptor (i.e., 392 nm for coumarin, 424 nm for acridine and 444 nm for fluorescein). For excitation spectra, emission wavelength was chosen according to the donor-acceptor couple. Quantum yields were measured by comparison with fluorescence standards (fluorescein). Correction of the emission spectra for instrumental response was obtained by recording the spectra of sample and standards, and correcting for absorbance differences.

Fluorescence lifetime. Fluorescence lifetime were measured with a single photon counting system (35), an Edinburgh Instrument 199M, using a hydrogen-filled flash lamp, which gave an instrumental response profile width at half maximum height of 1.2 ns (36). 1 cm by 1 cm quartz cuvettes containing 1.5 ml of solution were placed in a cell holder, thermostated by a circulating liquid (80% water; 20% glycerol). All experimental decay curves were fitted with mono- or multi- exponential theoretical decays, using reduced χ^2 to check the quality of the fit.

Calculation of R_0. R_0 is the critical Förster distance, at which the transfer efficiency E could account for half of the deactivation processes of the donor. R_0 was calculated from the following formula:

$$R_0^6 = (8.79\times10^5)\cdot\kappa^2\cdot\eta^{-4}\cdot<\Phi_D\cdot J \text{ (in Å)} \qquad (1)$$

We used an average value of 2/3 for the orientation factor κ^2 (assuming random orientation for the two dyes; depending on the relative orientation of the emission dipole of the donor and the excitation dipole of the acceptor, κ^2 can take any value between 0 and 4), and 1.33 for the refractive index η. Φ_D is the quantum yield of fluorescence of the donor, and J is the overlap integral between the emission spectrum of the donor and the excitation spectrum of the acceptor:

$$J=\frac{\int f_D(\lambda).\varepsilon_D(\lambda).\lambda^4.d\lambda}{\int f_D(\lambda).d\lambda} \qquad (2)$$

where λ is the wavelength in nanometers, $\epsilon_A(\lambda)$ the extinction coefficient of the acceptor ($M^{-1}.\ cm^{-1}$) and $f_D(\lambda)$ the corrected emission spectrum of the donor in quanta per unit wavelength interval, As the covalent link between the chromophore and the oligonucleotide has an effect on its absorption and fluorescence properties, ϵ and Φ_D were calculated from the data obtained for the conjugated dye.

Determination of the transfer efficiency E. E can be calculated from the quenching of the donor emission in presence of the acceptor. This quenching was monitored at a wavelength where the emission of the acceptor was negligible (480 nm for the coumarin/ethidium and fluorescein/rhodamine couples).

$$E=\frac{I_D-I_{DA}}{I_D} \qquad (3)$$

where

I_D is the fluorescence intensity at the chosen wavelength with no acceptor. I_{DA} is the fluorescence intensity at this wavelength in the presence of the acceptor (I_D and I_{DA} are both measured in presence of the template) E is linked to R_0 by the following formula:

$$E\frac{R_0^6}{R_0^6+R^6} \qquad (4)$$

where R is the distance between donor and acceptor. Due to the 6th power dependence on R, E very quickly drops to zero when $R > R_0$ (12).

RESULTS

Spectroscopic studies of derivatized oligonucleotides

Ethidium-substituted oligonucleotides had an absorption maximum shifted to shorter wavelengths as compared to free ethidium (464 nm instead of 480 nm). These derivatives had a

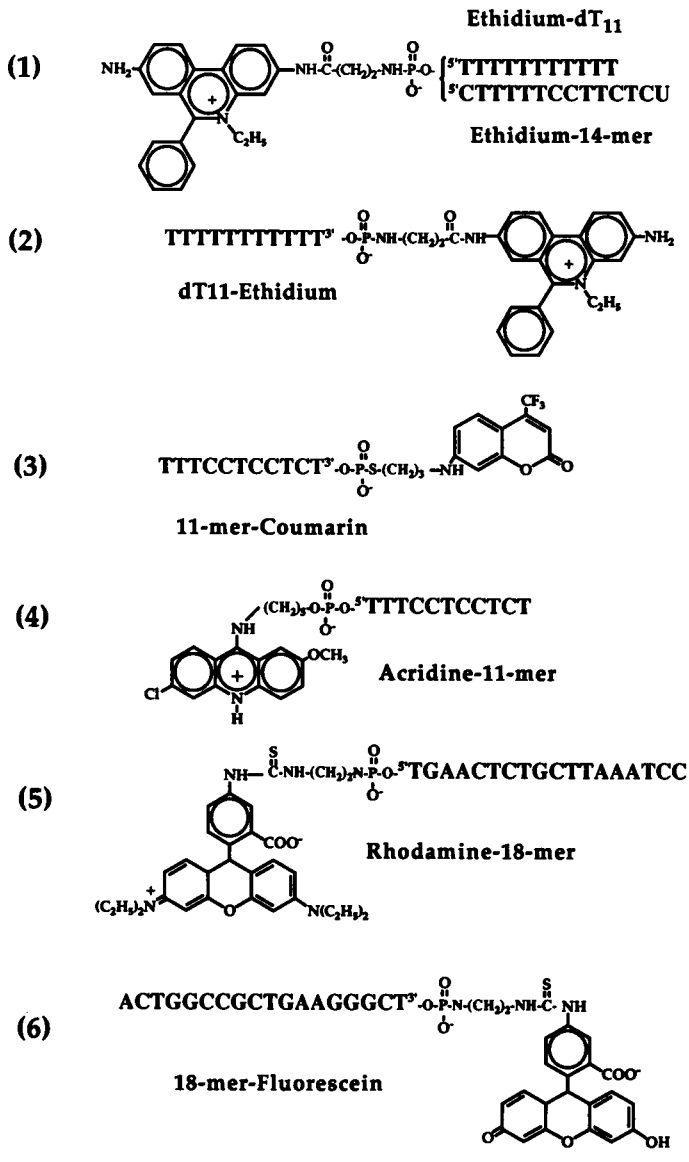

Figure 1. Chemical structure of the fluorescent oligonucleotides used in the present study. All oligonucleotides presented here have the β-anomeric nucleoside configuration. Abbreviations used for the fluorescent oligonucleotide are indicated, with the following convention: the dye is written first if it is linked to the 5′ end of the oligonucleotide; when linked to the 3′ end, it is written last. (1) 11 or 13-mers linked via their 5′-end to an ethidium derivative (ethidium−dT_{11} and ethidium−13-mer) (2) 11-mer (dT_{11}) with 3′ end linked to an ethidium derivative (dT_{11}−ethidium). (3) 11-mer with 3′ end linked to a trifluoro-methyl coumarin (11-mer−coumarin). (4) 11-mer with 3′ end linked to 2-methoxy-6-chloro-9-aminoacridine (acridine−11-mer). (5) 18-mer with 3′ end linked to rhodamine (rhodamine−18-mer). (6) 18-mer with 3′ end linked to fluorescein (18-mer−fluorescein).

higher fluorescence quantum yield than free ethidium in the same environment. The average fluorescence lifetime was between 18 ns and 33 ns at 0°C. The fluorescence decay was temperature and position (5′- or 3′-end)-dependent, droping to 3−4 ns at 60°C. As a control, free ethidium bromide, at the same concentrations and in the same conditions, gave a monoexponential decay, with a lifetime of 1.8 ns almost independent of temperature. None of the dye−oligonucleotides conjugates decays could be correctly fitted with a single exponential model, especially at low temperature, suggesting that the ethidium−oligonucleotide conjugate had several folded conformations, allowing partial protection of the phenanthridium group from solvent protonation. The fast decay of ethidium fluorescence has been previously ascribed to excited-state protonation which is abolished when ethidium intercalates into double-stranded DNA, hence a much longer lifetime of 24 ns for intercalated ethidium. Similar folding of a modified oligonucleotide covalently linked to acridine has been previously reported (37). At low temperature, the dT_{11} oligonucleotides linked to ethidium showed hypochromism at 260 nm, which was released by raising the temperature above 35°C, confirming the assumption of a folded conformation at low temperature.

The 11-mer−coumarin conjugate had a slightly temperature-dependent fluorescence quantum yield. Its emission maximum was also temperature dependent: a 15 nm red shift was observed between 0 and 60°C. The fluorescence decay of 11-mer−coumarin could be satisfactorily fitted with a mono-exponential decay for temperatures above 40°C ($\tau_{1/2}$ = 4 ns at 50°C), whereas, below 30°C, the decay was bi-exponential, suggesting that this oligonucleotide adopted a folded conformation at low temperature.

The spectroscopic properties of fluorescein and rhodamine were affected by covalent linkage to the oligonucleotide. Maximum absorption and excitation wavelength for the 18-mer−fluorescein was 490 nm, with an emission maximum at 520 nm, and a quantum yield (0.2) which was lower than that of free fluorescein. The two 18-mers linked to rhodamine had identical spectroscopic properties, with the same maximum excitation wavelength (560 nm) and emission wavelength (590 nm), very similar to that of free rhodamine. Note that the molar extinction coefficients of both fluorescein and rhodamine were decreased [45% for fluorescein ($\epsilon = 3.6\times10^4$ $M^{-1}.cm^{-1}$), and 15% for rhodamine ($\epsilon = 6\times10^4$ $M^{-1}.cm^{-1}$)] upon covalent linkage. Such a decrease has already been described for fluorescein conjugated to various molecules (11).

Excitation energy transfer studies

All the oligonucleotides were able to bind to their complementary sequence. Ethidium, when linked to the 5′ end, stabilized the binding of dT_{11} to poly (rA) or to dA_{14}. A temperature of half dissociation ('Tm') of 45°C was measured on a poly rA target and 38°C on dA_{14}, while the corresponding values for unsubstituted dT_{11} were 27 and 25°C, respectively. Duplexes formed with dT_{11} coupled to ethidium at its 3′ end were marginally less stable (Tm = 35°C on dA_{14}). Linking coumarin to the oligonucleotide did not stabilize the interaction of the 11-mer (5′TTTCCTCCTCT3′) with its target, giving a Tm of 30°C, the same Tm as that of the underivatized 11-mer. The fluorescence of ethidium was enhanced three fold at 20°C upon binding to its target, suggesting that the phenanthridium ring was at least partially intercalated. The folded conformations of the ethidium−oligonucleotide conjugates did not hamper binding to their target, suggesting that the energy required to unfold the oligonucleotide was low compared to the energy of complex formation.

A 30-mer target (see figure 2 for sequence) containing binding sites for two fluorescent oligonucleotides was designed. Simultaneous binding of the two 11-mers, one linked to ethidium at its 5′ end (ethidium−dT_{11}), the other linked to coumarin at its 3′ end (11-mer−coumarin) brings the two dyes in close proximity. When increasing amounts of ethidium−dT_{11} were added at 0°C to a solution containing the 11-mer−coumarin and their target, coumarin and ethidium−oligonucleotide fluorescence

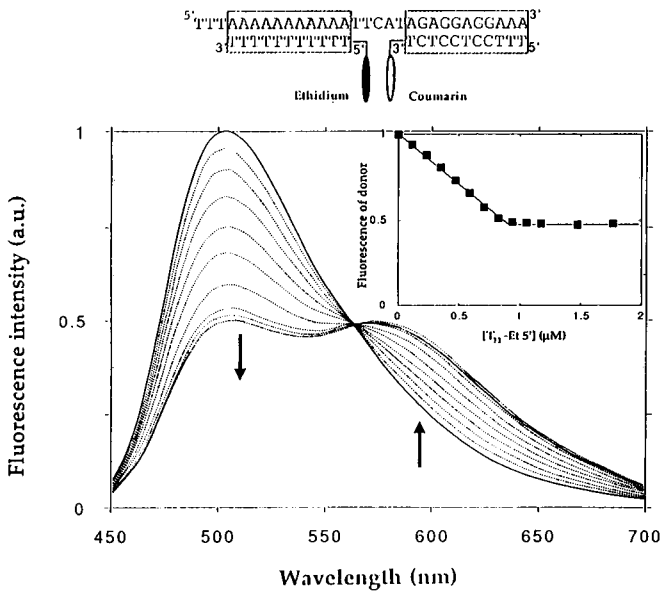

Figure 2. Upper part: Sequence of the two oligonucleotides bound to their single-stranded nucleic acid target. Coumarin chromophore is represented by an open oval and ethidium by a filled oval. Lower part: Titration of 11-mer–coumarin (0.8 μM) + 30-mer target (1 μM) by increasing amounts of ethidium–dT_{11}, from 0 to 2 μM. Temperature was set at 0°C. Excitation wavelength was 392 nm. Emission decreases at 480 nm and increases at 600 nm as symbolized by arrows. Note the presence of an isoemissive point at 565 nm, showing the presence of only two emitting species. Direct excitation of ethidium at 392 nm was negligible. *Inset:* Plot of fluorescence intensity at 480 nm (corrected for dilution) versus acceptor concentration.

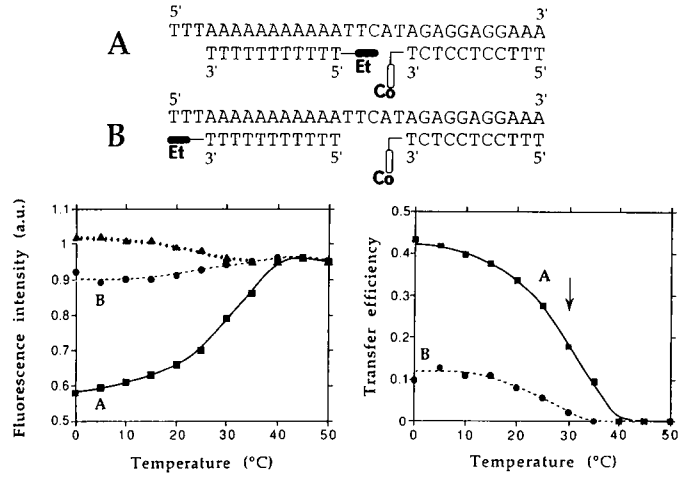

Figure 3. Upper panel: Schematic structures of the 30-mer in the presence of 11-mer–coumarin (donor) and either ethidium–dT_{11} (**A**) or dT_{11}–ethidium (**B**) as an acceptor. Et = Ethidium; Co = Coumarin. Lower panel, left: Temperature dependence of the intensity of fluorescence emitted by 11-mer–coumarin (0.8 μM) with no acceptor (triangles) or with ethidium–dT_{11} (A, squares; 1.5 μM) or dT_{11}–ethidium (B, circles; 1.5 μM) in presence of the 30-mer template (same conditions as in figure 2). Lower panel, right: Transfer efficiency for both acceptor oligonucleotides (same symbols as in the left part of this figure) was determined from the quenching of emission at 480 nm. The temperature of half dissociation of the 11-mer–coumarin is indicated by the arrow.

emission spectra were modified (figure 2). At the chosen excitation wavelength (392 nm), ethidium–dT_{11} has a very low extinction coefficient, (<500 M^{-1} cm^{-1}) which could not account for the observed enhancement of ethidium emission around 600 nm by direct excitation. The decrease in emission intensity in the coumarin emission range at short wavelengths and the increased intensity at wavelengths longer than 550 nm suggested that excitation energy transfer occurred from coumarin to ethidium. An isoemissive point was observed at 565 nm. The titration curve was clearly biphasic, showing that, once the 1:1 stŁchiometry was reached, further addition of acceptor did not change the shape of the emission spectrum, since all the acceptor sites were occuppied (figure 2, inset). The sensitized emission of ethidium was the result of absorption of photons by coumarin, since the excitation spectrum of the mixture of the 30-mer target with ethidium–dT_{11} and 11-mer–coumarin (1:1:1) measured at an emission wavelength of 610 nm exceeded that of the 30-mer target + 11-mer–coumarin (1:1) mixture in the coumarin absorption wavelength range (not shown).

In the absence of the single-stranded nucleic acid target, no transfer was observed, as the average distance of the two chromophores in solution was much higher than the Förster critical distance (calculated to be around 4 nm). Binding of the two oligonucleotides to two neighboring sequences on the same DNA fragment brought their chromophores in close enough proximity for energy transfer to occur. This transfer was temperature dependent. As shown in figure 3 A, thermal dissociation of one of the oligonucleotides from its target was sufficient to prevent transfer. Transfer efficiency was calculated from the decrease of donor (coumarin) fluorescence upon addition of the acceptor. The temperature at which the transfer efficiency was 50% of its maximum value was very similar to the temperature of half-dissociation of the 11-mer–coumarin conjugate, the least stable of the two oligonucleotides (Tm = 30°C and 38°C for 11-mer–coumarin and ethidium–dT_{11}, respectively, determined from UV melting curves under the same experimental conditions). Maximum transfer efficiency was greatly reduced (but not completely abolished), when the acceptor (ethidium) was covalently linked to the other end (3′) of the dT_{11} oligonucleotide (dT_{11}–ethidium; see '**B**' in figure 3) confirming that oligonucleotides were bound—as expected—in an antiparallel orientation with respect to their complementary target.

Evidence for hairpin formation and analysis of nucleic acid secondary structure

Single-stranded DNA or RNA may adopt hairpin structures which dramatically decrease the physical distance between two sequences which are, in terms of primary sequence, well separated. Two synthetic targets, 45 nucleotides in length were used. Both contained the complementary sequences for ethidium–13-mer and 11-mer–coumarin separated by the same number of bases. The first sequence '45 HP' was able to form a hairpin with a stem of 6 base pairs and a loop of four thymines. The second sequence '45 C' contained a 16 base-long sequence, which was not able to form a hairpin between the hybridization sites for the two oligonucleotides (see figure 4). As judged by thermal denaturation experiments, ethidium–14-mer, 13-mer–ethidium and 11-mer–coumarin were able to bind to both targets, with Tm of 42, 40 and 30°C respectively, in a 100 mM NaCl, 10 mM cacodylate, pH 7 buffer, and 27, 24 and 14°C in a 10 mM NaCl, 3 mM cacodylate, pH 7 buffer. FET occurred from 11-mer–coumarin to ethidium–14-mer when these two oligonucleotides were bound to '45 HP' but FET was very weak

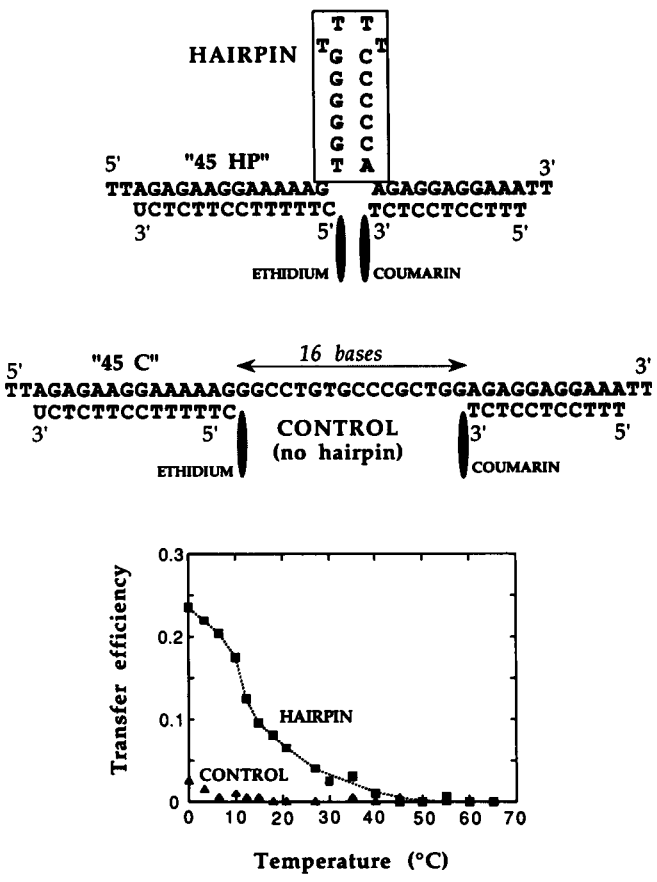

Figure 4. Upper panel: Sequences of the 45 mer containing a hairpin structure (45 HP) andcontrol 45 mer (45 C). Sites of hybridization with 11-mer−coumarin and ethidium−14-mer are shown. Lower panel: Plot of transfer efficiency versus temperature for 45 HP (squares) and 45 C (triangles). All strand concentrations were 1 μM. Measurements were performed in a pH 7 buffer containing 3 mM cacodylate, 10 mM NaCl.

even at 0°C when both oligonucleotides were bound to 45C (figure 4). This result is in good agreement with the existence of a hairpin structure in 45 HP. FET dropped to zero when the temperature increased, with half maximum efficiency reached at the temperature where the 11-mer−coumarin dissociated from its target: 14°C in the 10 mM NaCl buffer (figure 4); and 30°C in the 100 mM NaCl buffer (not shown). Maximum transfer efficiency was low (25%), but the spatial conformation of this particular three-way junction is only partially known [a related, but not identical, structure has been shown to be Y-shaped (10)], and transfer efficiency depends on the distance and the relative orientation of the donor and acceptor.

Determination of the orientation of [α]-oligonucleotides in [α]−[β] hybrids

Alpha-oligonucleotides (see figure 5A) are molecules of interest because they are very slowly degraded by nucleases and thus have a longer lifetime in biological systems (38). Their chemical synthesis and their terminal modifications are very similar to that of normal beta-oligonucleotides once the alpha-anomeric nucleosides are synthesized (33).

We used the same target sequence as in the experiments described in figures 2 and 3 was used. The donor was the [α]

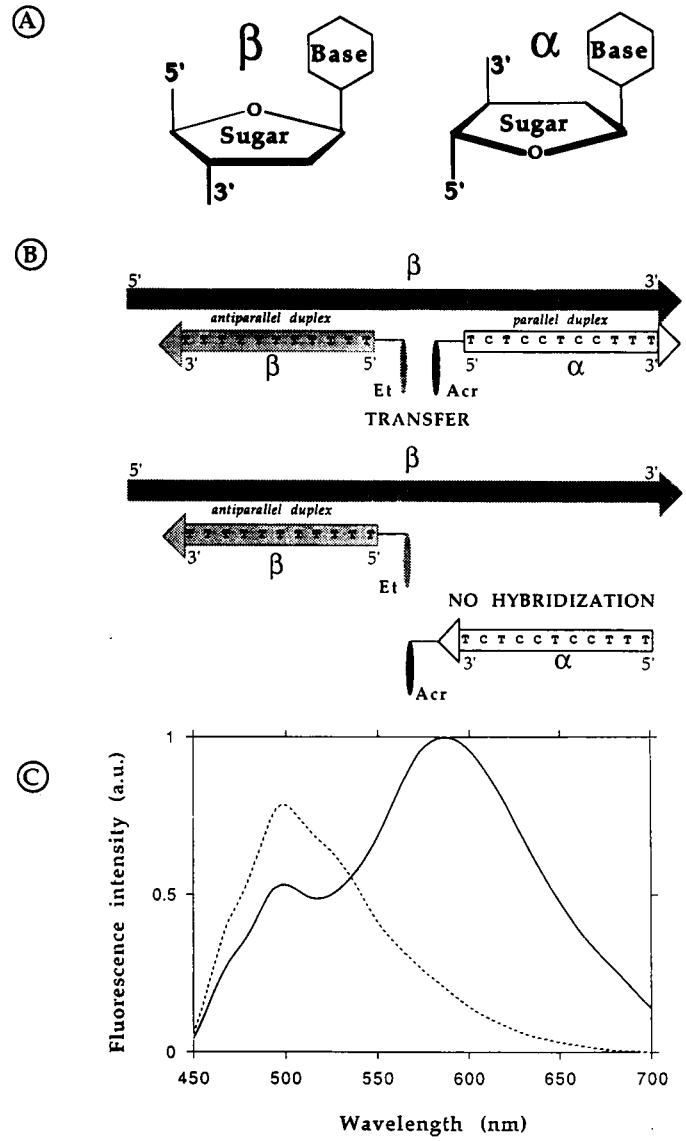

Figure 5. A. Structure of an [α]-deoxynucleotide, compared to a natural [β]-deoxynucleotide. **B.** Structure of the complex between [α] and [β] oligonucleotides. Transfer was evidenced on the upper target (the same as in figure 2) between [α] acridine-$^{5'}$TCTCCTCCTTT$^{3'}$ (1 μM) and [β] ethidium−dT_{11} (1 μM), but not with [α] $^{5'}$TCTCCTCCTTT$^{3'}$−acridine (1 μM), although both of them could bind their target. A control alpha-oligonucleotide, whose sequence was [α] $^{5'}$TTTCCTCCTCT$^{3'}$−acridine did not bind to its target, and no transfer was observed. Temperature was fixed at 15°C and excitation was set at 424 nm. **C:** Emission spectra of [α] acridine−$^{5'}$TCTCCTCCTTT$^{3'}$ in the absence (dotted line) and in the presence (full line) of [β] ethidium−dT_{11}. All strand concentrations were 1μM, in a 100 mM NaCl, 10 mM cacodylate buffer at pH 6.8. Temperature was fixed at 4°C.

11-mer $^{5'}$TCTCCTCCTTT$^{3'}$ linked via its 5'-end to an acridine derivative (39−40); and the acceptor was ethidium−dT_{11}. In the presence of the 30-mer target and ethidium−dT_{11}, no significant transfer ($\leq$ 5%) from acridine to ethidium was observed with the [α]-oligonucleotide linked to acridine at its 3'-end. This is in sharp contrast with the important transfer which was obtained when acridine was attached to the 5'-end, confirming that [α]-oligonucleotides are hybridized in a parallel orientation with respect to their single-stranded natural β

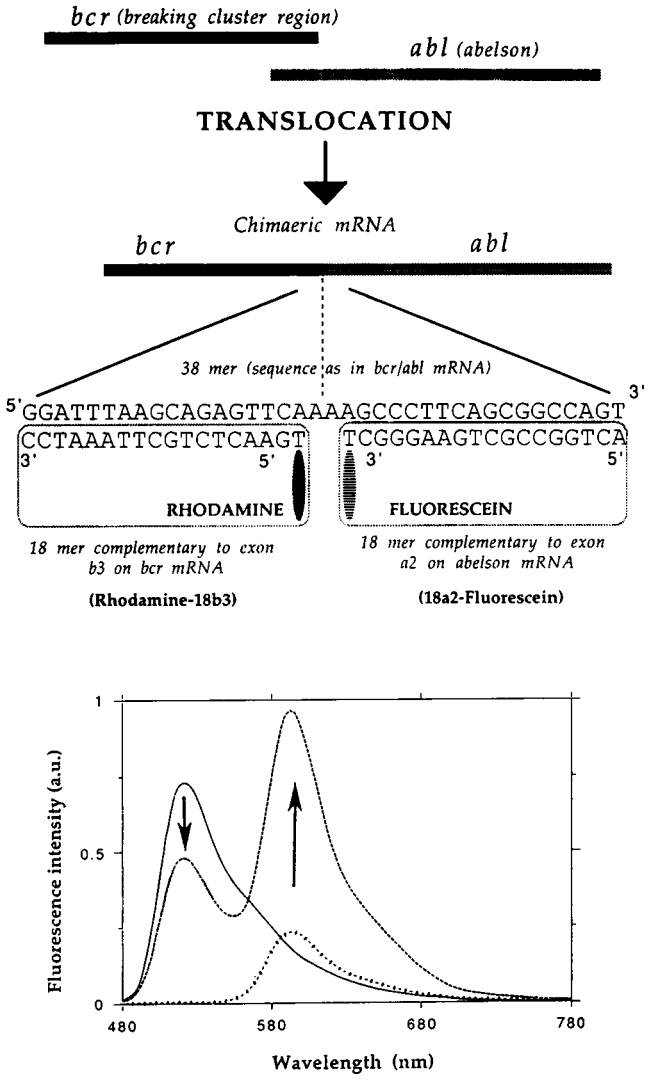

Figure 6. Upper Panel: Structure of the bcr/abl mRNA at the breakpoint region. The chimerical mRNA may have two different structures, depending on whether the breakpoint in bcr is located downstream of exon b2 or of exon b3. These two possibilities are abbreviated as b2a2 or b3a2, respectively. The two modified oligodeoxynucleotides linked to either fluorescein '18a2−fluorescein' or rhodamine 'rhodamine−18b' are also presented. A synthetic DNA fragment, 38 base-long, whose sequence corresponds to the most frequent translocation (including exon b3 of bcr, thus leading to a 'b3a2' translocation) was used as a template for fluorescence energy transfer experiment. Lower Panel: Emission spectra of 18a2-fluorescein in the absence (full line) and in the presence (broken line) of rhodamine−18b3 upon excitation at 444 nm. Result of direct excitation of acceptor in the absence of donor is shown by the crosses. All strand concentrations were 1μM, in a 100 mM NaCl, 10 mM cacodylate buffer at pH 6.8. Temperature was fixed at 35°C, to avoid formation of an unstable secondary structure of the 38-mer.

complementary sequence (41) (figure 5). No binding or transfer was detected with an [α]-oligonucleotide whose sequence was $^{5'}$TTTCCTCCTCT$^{3'}$, i.e., with an antiparallel orientation with respect to the target sequence. This experiment confirms that [α]-oligodeoxynucleotides bind in a parallel orientation with respect to their complementary β target sequence.

Detection of gene translocation

Many diseases are associated with gene translocation between different chromosomes. A chimerical gene is thus obtained, as in chronic myeloid leukemia, where an oncogene from chromosome 9, abl, encoding a tyrosine kinase, is fused with another gene, bcr, from chromosome 22, which codes for a GTPase activating protein for $p21^{rac}$ (42). After transcription and splicing, a messenger RNA, containing exons from both bcr and abl is obtained. Detection of the presence of such a chimerical RNA could be achieved by fluorescence energy transfer between two oligonucleotides, one bound to the bcr part and the other one to the abl part. The normal mRNA, transcribed from a non-fused abl gene does not contain the 5′ half of the chimerical RNA.

To test such a possibility, a 38 nucleotides single-stranded DNA fragment containing the sequence at the junction between exon b3 of bcr and exon a2 of abl was synthesized (figure 5). As shown in figure 6, transfer occurred from 18a2-fluorescein to rhodamine−18b3 in presence of the target, whereas no transfer occurred when rhodamine was linked to another non-complementary 18 base-long oligonucleotide (5′ TTCCTTATTG-ATGGTCAG). An increase in temperature from 35°C to 60°C abolished transfer, as rhodamine−18b3 was no longer bound to its target at this temperature. Direct excitation of rhodamine at 460 nm could not account for the emission intensity at 590 nm (see figure 5). Fluorescence excitation spectra with the emission set at 610 nm clearly demonstrated that this sensitized emission corresponded to excitation of fluorescein, and not rhodamine, thus indicating that transfer occurred from fluorescein to rhodamine (not shown). The fluorescein−rhodamine couple was suitable because of the relatively high fluorescence quantum yield of both molecules, and the important spectral overlap between the emission of fluorescein and the absorption of rhodamine, leading to a critical Förster distance of 3.0 nm. This calculated value is lower than the one usually measured or proposed (47), and this can be explained by the lower fluorescent quantum yield Φ_D of the donor (0.2 for fluorescein) measured after coupling to the oligonucleotide, which plays a direct role in the calculation of Ro (see Eq. 1). The small but significant (−15%) variation of the extinction coefficient of rhodamine also decreased Ro by 2−3 %.

DISCUSSION

Demonstration of fluorescence energy transfer

Evidence for fluorescence energy transfer can be provided by several methods: (i) quenching of the donor fluorescence, (ii) sensitized (enhanced) emission of the acceptor, (iii) analysis of excitation spectra and (iv) decrease of the fluorescence lifetime of the donor. To quantify the transfer phenomenon, the simplest method relies on the quenching of the donor fluorescence (transfer efficiency is directly proportional to the quenching, in the absence of any other deactivation process). All chosen D−A couples allowed us to find a wavelength where only the donor had a measurable emission (480 nm for the coumarin−ethidium and for the acridine−ethidium couples; 525 nm for the fluorescein−rhodamine couple), and thus where the quenching can be directly monitored. The sensitized emission of the acceptor should also be proportional to the quenching of the emission of the donor. Such behavior was observed in figure 2. Unfortunately, the decrease of the fluorescent lifetime of the donor could only be qualitatively fitted with fluorescence quenching. The major reason for the lack of quantitative correlation is due to the complex fluorescence decay of all fluorescent oligonucleotides analyzed so far, especially when they are bound to their target. The existence of several folded conformations of the conjugated dye

has an impact on the fluorescence decays (37). Thus, quantitative analysis of the changes in fluorescent decay behavior of the conjugated oligonucleotides is difficult.

Donor–Acceptor interactions other than dipole–dipole should be avoided as much as possible. These interactions usually occur when the two molecules are brought in very close proximity (a few Å). It is usually possible to prevent such a close contact, for example by inserting a few bases between the two target sequences. The spacer was 5 bases for the experiments described in figure 2, 3 and 5. But even with a spacer of only 2 bases, no other interaction was observed between fluorescein and rhodamine on the abl/bcr target. No donor/acceptor interaction was observed in the absence of the template at the concentrations ($\sim 1\ \mu M$) used in the energy transfer experiments. The presence of the negatively charged phosphate backbones strongly inhibits close encounters between the dyes. Aggregation and stacking of chromophores tend to change their spectral characteristics. We found no evidence for such interactions: no change in the absorption spectra of the donor and acceptor was observed upon simultaneous binding to the target, and the emission of the acceptor was not affected by the presence of the donor, at an excitation wavelength where the donor absorption is negligible. In all cases, in the absence of the target, no transfer, and no fluorescence modification were observed, demonstrating that the donor and acceptor oligonucleotides did not interact in solution: the presence of neighboring targets for both oligonucleotides was a necessary condition to observe FET.

Choice of donor–acceptor pair

Many donor–acceptor pairs are potentially available (43). The first criteria for D–A choice is the presence of a spectral overlap between the donor emission and the acceptor excitation. This should lead to an important overlap integral, and thus a large Förster distance (a few nm). However, the fact that the spectroscopic properties of the donor and the acceptor can be affected by covalent linkage to the oligonucleotide should be kept in mind.

The absorption maximum of the donor is not necessarely the optimal excitation wavelength. To minimize the relative contribution due to direct excitation of the acceptor, the excitation wavelength has to be chosen such that the ratio between the extinction coefficients of the donor and the acceptor is maximum. This condition may not be always fulfilled (see figure 6: the contribution from a direct excitation of the acceptor is important, and has to be taken into account to quantify energy transfer).

Five different fluorophores have been tested here for fluorescence energy transfer. 2-methoxy-6-chloro-9-aminoacridine is not a good donor as its fluorescence is quenched by stacking with guanines, thus limiting its use to sequences where no G is present. Ethidium oligonucleotides are interesting because of the fluorescence enhancement observed upon binding to their complementary target. However, chemical synthesis of ethidium oligonucleotide conjugates first required derivatization of the dye, and is not as straightforward as that of other conjugates. The coumarin-ethidium couple gave unequivocal results (see figure 2). The fluorescein/rhodamine pair is a well documented donor/acceptor pair. Chemical linkage to the oligonucleotides is easy, and these modified oligonucleotides can be purshased from several companies. The fluorescence quantum yield of fluorescein linked to an oligonucleotide is relatively high (0.2) and energy transfer from fluorescein to rhodamine is easily detected. This pair of dyes has previously been used to analyse the structure of nucleic acids (8, 11, 47). However, the spectroscopic properties of these dyes are modified upon conjugation to oligonucleotides (11), and are dependent upon experimental conditions, such as pH, ionic concentration (11) and temperature. Therefore, the critical Förster distances (Ro) have to be calculated for each experiment.

Quantitative measurement of fluorescence energy transfer

Another important factor concerning FET is its distance and orientation dependence. The orientation factor $<\kappa^2>$ can be precisely determined in two extreme cases: the orientations of the two molecules have an isotropic distribution, or if their position is fixed and known. For the experiments described in this study, all fluorophores had a limited freedom, and the distribution of their orientation factors was certainly not isotropic, mainly because the target strand sterically prevents some of the orientations, and some of the fluorophores (acridine, ethidium) are in close interaction with the target sequence. This leads to an error on the orientation factor, which was assumed to be equal to 2/3 for all Ro determination, but could well take any value between 0 and 4. Thus, one should be careful when two structures are compared, based on the transfer efficiency. No transfer at all was observed when 45 C was used as a template in figure 4; on the other hand, limited but significant transfer occurred between acridine and ethidium in the experiment presented in figure 3 'B'. The 'linear' distance between donor and acceptor are similar (16 bases): the difference of transfer efficiencies could be the result of an unfavorable orientation in the 45C case. The geometry and the eventual folding of the nucleic acid linker between the donor and the acceptor has also an influence on the average distance between the two molecules. Thus, both distance and orientation of the two molecules might be affected. Pertinent conclusions can only be drawn if the fluorophores experience complete motional freedom (11, 51). Other methods should be used to confirm the model suggested by FET (10).

Some authors have observed that FET in nucleic acids does not seem to fit the equation **4** (9). In the experiment presented in figure 3, E was 0.5 for a Donor–Acceptor distance of 5 bases, and 0.1 for a distance of 16 bases. This result can be explained by the geometry of double-helical DNA (47).

Choice of donor and acceptor concentrations

In this study, oligonucleotide concentrations have been kept in the micromolar range, to allow comparison of FET with UV melting experiments. The sensitivity of this method is better that obtained by absorption measurements (30). 10–100 fold lower concentrations still led to a fluorescence emission above detection threshold of most fluorometers. However, two problems might arise, as the binding of an oligonucleotide is a bimolecular process: (i) The melting temperature of the complex is temperature dependent; one should check that the oligonucleotide is still bound at low concentrations. The ΔH values for binding of the conjugated oligonucleotides that we have used in our studies (11 to 18 nt) were estimated to be in the -56 to -90 kcal/mol range. A ten-fold lower concentration for all oligonucleotides would then lead to a 5 to 8°C decrease of the melting temperature. Dye conjugation to the oligonucleotide may also have a significant effect on its thermodynamic parameters of binding (13, 44). (ii) Kinetics of association are slower. This should be a problem only if very low concentrations are used (30).

Practical use of fluorescence energy transfer requires a correct choice of the molar ratio between the donor, the acceptor and

the template. (i) The donor concentration should be equal or lower than the target concentration; otherwise, the excess of donor molecules, not bound to the target, would not contribute to FET, and therefore mask the FET phenomenon. (ii) On the other hand, the acceptor concentration should be equal or higher than the target concentration. A lower concentration would lead to a decrease in transferred energy, as some donor molecules would be far from an acceptor on their targets (see the titration in figure 2).

CONCLUSION

FET, which does not always provide sufficient information to calculate exact distances, is a valuable method to investigate the binding of fluorescent oligonucleotides to their complementary targets. Several potential applications of FET between two adjacent oligonucleotides hybridized to single-stranded sequences have been presented here. The two target sequences can be adjacent on the same nucleic acid fragment or separated by a few bases only. They may also be separated by longer distances in the primary sequence provided they are brought close to each other in space by folding of the nucleic acid chain (e.g. hairpin formation). Provided proper controls are performed, this method gives rapid and clear-cut results and avoids requirement for radioactively-labelled oligonucleotides. The possibility to bind an oligonucleotide to a *double-stranded* DNA target via triple-helix formation is now well documented (see reference (27) for a review). FET between two triplex-forming oligonucleotides is presently under investigation.

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