Pergamon

Tetrahedron 55 (1999) 9143–9150

TETRAHEDRON

Synthesis and Properties of Oligonucleotide Duplexes Containing Donor and Acceptor Fluorophores at 2'-Positions

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Received 15 April 1999; accepted 29 May 1999

Abstract : A method for introduction of dimethylamionapthamide and fluorescein labels as a fluorescence energy donor and acceptor pair into 2'-positions of DNA duplexes has been described. It has been shown that the attachment of these bulky fluorophores to the sugar 2'-position at the terminal fraying end of each oligonucleotide strand does not alter the normal thermal stability and global conformation of the DNA duplexes. A clear dependence of fluorescence energy transfer efficiency on the number of nucleotides in DNA has been observed, suggesting that the present donor-acceptor pair may be useful for FRET indicator of DNA.

Introduction

Fluorescence resonance energy transfer (FRET) is considered to occur through the dipole-coupling mechanism in which the efficiency of FRET depends on the distance between donor and acceptor fluorophores. It has therefore provided a useful mean for investigating structural and symmetry features concerning oligonucleotides that range over distances up to 100 Å in solution.[1] Several applications of FRET have been reported to resolve the problems of helical four-way junctions in DNA topology,[2] geometry of bent DNA molecules,[3] and relative orientation of the helical segments in RNA hammerhead ribozyme.[4] In these applications, the donor and acceptor fluorophores have been introduced covalently via a tether into the 5'- or 3'-end or the phosphate backbone of oligonucleotides. Although FRET has been successfully applied to analysis of complex nucleic acid structures, there remains the difficulty in generating a donor-acceptor pair at appropriate positions of nucleic acids.

We have shown that the sugar 2'-position of oligonucleotides is a suitable site for covalent attachment of several fluorophores.[5] Our approach to the synthesis of modified oligonucleotides involves the use of fluorescence labeled nucleoside phosphoramidites in the site-specific incorporation of fluorophores into oligonucleotides using automated phosphoramidite chemistry.[6] There is an other possible approach *so-called* a post-labeling technique in which oligonucleotides possessing an aliphatic amine are labeled in an aqueous buffered solution by appropriate fluorophore derivatives.[7] We felt that the combined use of these approaches would provide great flexibility for rapid construction of a donor-acceptor pair in oligonucleotides.

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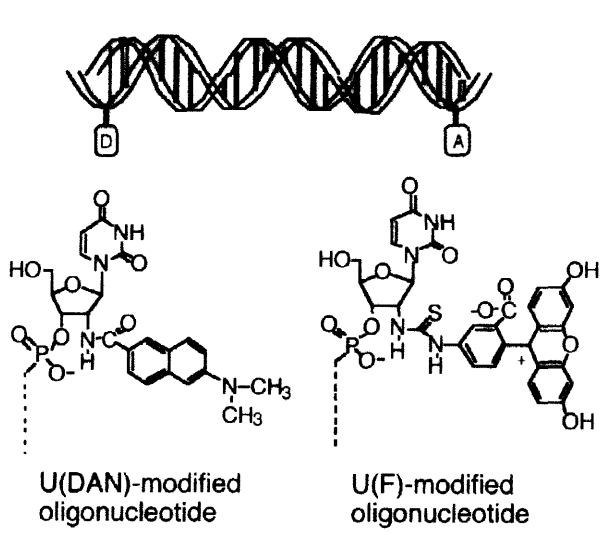
0040-4020/99/$ - see front matter
PII: S0040-4020(99)00484-6



DAN1: 5'-U(DAN)TTAGAGGTCAT
DAN2: 5'-U(DAN)ATAGAGGTCAT
DAN3: 5'-U(DAN)GTAGAGGTCAT
DAN4: 5'-U(DAN)CTAGAGGTCAT
DAN5: 5'-TCU(DAN)AGAGGTCAT
DAN6: 5'-TCTAGAGGU(DAN)CAT
DAN7: 5'-U(DAN)T_6A
DAN8: 5'-U(DAN)T_8A
DAN9: 5'-U(DAN)T_{10}A
DAN10: 5'-U(DAN)T_{12}A
DAN11: 5'-U(DAN)T_{14}A

F1: 5'-U(F)CTAGAGGTCAT
F2: 5'-TCU(F)AGAGGTCAT
F3: 5'-TCTAGAGGU(F)CAT
F4: 5'-U(F)A_6A
F5: 5'-U(F)A_8A
F6: 5'-U(F)A_{10}A
F7: 5'-U(F)A_{12}A
F8: 5'-U(F)A_{14}A

We have already synthesized a new fluorescent nucleoside, 2'-(6-dimethylamino-2-naphthamide)uridine [U(DAN)], which exhibits appropriate fluorescence that would be used as an energy donor to fluorescein (F) label as acceptor species in FRET experiments.[5d] The present report describes the procedures for incorporation of the DAN and F fluorophores into the 2'-positions of DNA. The binding and spectral properties of the labeled DNA were investigated to elucidate the potential utility of the donor and acceptor pair in FRET measure of DNA.

Experimental Procedures

General Methods. ^{31}P NMR spectra were obtained on a JEOL GX-400 spectrometer using 85 % H_3PO_4 as an external standard. ^{1}H NMR spectra were measured on a Bruker DRX-500 spectrometer. High-performance liquid chromatography (HPLC) was performed on a Waters 600E model apparatus with a Hitachi LC 4200 UV-VIS detector at 260 nm using a reversed phase Cosmosil 5C18 AR300 column (4.6 x 150 mm). Ultraviolet (UV) spectra were recorded with a Hitachi U-3000 spectrophotometer equipped with a thermoelectrically controlled cell holder (Hitachi SPR-10). Circular dichroism (CD) spectra were obtained on a JASCO CD J-720 spectrometer. Fluorescence spectra were measured on a JASCO FP-777 spectrofluorometer equipped with a temperature controller (ATTO superstat-mini).

Materials and Solvent. 5'-Dimethoxytrityl 2'-(trifluoroacetamide)uridine 3'-(2-cyanoethyl)-*N,N'*-diisopropylphosphoramidite [U(NH_2) phosphoramidite][8] and 2'-(6-dimethylamino-2-naphthamide)uridine [U(DAN)][5d] were synthesized according to the literature procedures. Snake venom phosphodiesterase (Sigma) and alkaline phosphatase (Funakoshi) were commercially obtained. Fluorescein isothiocyanate, isomer II (FITC) was obtained from Nacalai Tesque. 2-Cyanoethyl-*N,N,N',N'*-tetraisopropyl phosphordiamidite was obtained from Aldrich. Protected deoxynucleoside 3'-(2-cyanoethyl)-*N,N'*-diisopropylphosphoramidites and nucleoside-loaded controlled pore glass (CPG) supports were purchased from Cruachem. Oligonucleotides were prepared by a phosphoramidite chemistry on a Pharmacia LKB Gene Assembler Plus DNA/RNA synthesizer. For synthesis of the modified oligonucleotides, the X- or Y-bottle was used to supply the modified nucleoside amidite solutions. CH_3CN, pyridine, triethylamine (TEA), and diisopropylethylamine (DIEA) were refluxed

over CaH_2 for 5 h, distilled, and stored over CaH_2. THF was dried by refluxing over $LiAlH_4$, distilled, and stored over molecular sieves. DMF was distilled under the reduced pressure over ninhydrin and stored over molecular sieves.

5'-*O*-Dimethoxytrityl-2'-(6-dimethylamino-2-naphthamide)uridine [5'-DMT-U(DAN)]. U(DAN) (0.2 g, 0.45 mmol) was evaporated with dry pyridine. To a solution of U(DAN) in dry pyridine (4.0 mL) was added 4,4'-dimethoxytrityl chloride (0.19 g, 1.2 equiv.). The solution was continued to be stirred at room temperature overnight. The reaction mixture was concentrated in vacuo and the residual solution was extracted by EtOAc/H_2O. The organic phase was dried by Na_2SO_4 and evaporated in vacuo. The residue was applied to silica gel column chromatography (CH_2Cl_2 : MeOH = 50:1, v/v). The appropriate fractions were collected and evaporated in vacuo to give 5'-DMT-U(DAN) (0.17 g, 50 %). TLC (CH_2Cl_2:MeOH = 9:1, v/v) Rf 0.61; ^{1}H NMR (DMSO-d_6) : δ: 3.04 (s, 6, -N(CH_3)$_2$), 3.29 (m, 2, H5',5"), 3.74 (s, 6, -OCH_3), 4.11 (m, 1, H4'), 4.28 (t, 1, H3'), 4.89 (m, 1, H2'), 5.74 (d, 1, 3'-OH), 5.46 (d, 1, H5), 6.09 (d, 1, H1'), 6.89-6.95, 7.21-7.45, 7.66-8.21, 8.23,8.31 (m, total 21, aromatic plus C2'-amide), 11.34 (s, 1, uracil NH); FABMS (positive ion) m/z 742 [M+1].

5'-*O*-Dimethoxytrityl-2'-(6-dimethylamino-2-naphthamide)uridine-3'-phosphoramidite. To a solution of 5'-DMT-U(DAN) (150 mg, 0.20 mmol) and 1*H*-tetrazole (71 mg, 0.5 equiv.) and diisopropylamine (0.014 mL, 0.5 equiv.) in 5.0 mL of dry THF was added 2-cyanoethyl-*N,N,N',N'*-tetraisopropyl phosphordiamidite (0.13 mL, 2.0 equiv.). The solution was stirred at room temperature overnight. MeOH (0.1 mL) was added to terminate the reaction and then the solution was diluted with 5.0 mL of EtOAc containing a small amount of DIEA. The solution was washed with 10 % $NaHCO_3$ and the organic phase was dried by Na_2SO_4 and concentrated in vacuo. The residual solution was applied to silica gel column chromatography (EtOAc:CH_2Cl_2:TEA = 45:45:10, v/v). The desired fraction was collected and evaporated in vacuo to give the phosphoramidite (0.19 g, 92 %). TLC (EtOAc:CH_2Cl_2:TEA = 45:45:10, v/v) Rf 0.63 ; ^{31}P NMR (CH_3CN) 151.2, 152.6 ppm; FABMS (positive ion) m/z 942 [M+1].

Synthesis of Oligonucleotides Containing U(DAN). The synthesis of U(DAN) modified oligonucleotides was accomplished by a phosphoramidite chemistry beginning with 5'-DMT-nucleoside (0.2 μmol) bound to a CPG support. For the coupling of normal deoxyribonucleoside phosphoramidites, the standard protocol (50 μL of 0.1 M amidite and 50 μL of 0.1 M tetrazole in acetonitrile, 2 min) was used. For the coupling of U(DAN) phosphoramidite, 120 μL of the 0.12 M amidite and 120 μL of 0.1 M tetrazole in acetonitorile (10 min) were used. With these conditions, the coupling efficiency based on the DMT cation assay was 98 % for U(DAN) phosphoramidite. The CPG bound oligonucleotides were treated with concentrated ammonium hydroxide at 55 ℃ for 14 h. Purification of the modified oligonucleotides was performed with 20 % denaturing polyacrylamide gel electrophoresis as described.[6] The integrity of oligonucleotide structure was verified with ion spray mass analysis. The mass data for **DAN1-6** were 3872.6 (calcd. 3872.7), 3882.8 (calcd. 3881.7), 3897.5 (calcd. 3897.7), 3857.6 (calcd. 3857.7), 3857.7 (calcd. 3857.7), and 3857.9 (calcd. 3857.7).

Synthesis of Oligonucleotides Labeled by Fluorescein. The synthesis and purification of oligonucleotides containing 2'-amino-2'-deoxyuridine [U(NH_2)] were carried out by using U(NH_2) phosphoramidite with essentially same procedures as described for the U(DAN) modified oligonucleotides. The oligonucleotides containing U(NH_2) (3.0 A_{260} unit) were allowed to react with FITC (39 mg/mL) in 25 μL of DMF and 25 μL of 50 mM $NaHCO_3$ and 200 mM Na_2CO_3 (pH 9.0) at room temperature overnight. To the reaction mixture were added 100 μL of 0.1 N HCl and then 50 μL of H_2O. The solution was extracted by

EtOAc (4 x 200 μL). Saturated $NaHCO_3$ (10 μL) was added to the aqueous phase to neutralize the solution. The fluorecein labeled oligonucleotides were purified by reversed phase HPLC; elution was carried out with (i) 5 % CH_3CN (5 min), (ii) a linear gradient of CH_3CN (1 %/min, 5-35 min), and (iii) a linear gradient of CH_3CN (0.78 %/min, 35-80 min) in triethylammonium acetate (pH=7.0) each at a flow rate of 1 mL/min. The purified oligomers (1-2 A_{260} units) were obtained by lyophilization.

The integrity of oligonucleotides was verified by ion spray mass and enzymatic digestion analysis. Ion spray mass data for oligomer **F1-3** were 4050.6 (calcd. 4050.8), 4050.5 (calcd. 4050.8) and 4050.3 (calcd. 4050.8). The fluorecein oligonucleotides (0.2 A_{260} unit) were subjected to digestion with snake venom phosphodiesterase (10 units/mL) and alkaline phosphatase (100 units/mL) in 10 μL of 0.1 M Tris-acetate buffer (pH 8.2) containing 10 μL of 0.1 M $MgCl_2$ at 37 ℃ for 1 h. The reaction mixtures were analyzed by reversed phase HPLC; elution was carried out with (i) 4 % CH_3CN (5 min), (ii) a linear gradient of CH_3CN (1 %/min, 5-50 min) in 50 mM ammonium acetate (pH 6.0) each at a flow rate of 1.0 mL/min. The result for oligomer **F2** as an example is shown in Figure 1.

Preparation of Oligonucleotide Solution for UV Melting Measurements and CD Spectra. All solutions were prepared using a buffer containing 0.1 M or 1.0 M NaCl and 0.01 M sodium phosphate, adjusted to pH 7.0. Oligonucleotide concentrations were determined by absorbance at 260 nm and the calculated single-strand extinction coefficients based on a nearest neighbor model.[9] All duplex melting curves by UV spectra were measured at a common total single-strand concentration (4.0×10^{-5} M) containing a 1 : 1 molar ratio of oligonucleotides with an increase in temperature from 0 to 80 ℃ at a rate of 0.5 ℃/min. The solutions were heated to 80 ℃, kept there for 5 min, and then gradually cooled before melting experiments. CD spectra were measured for the same solutions used for the UV melting studies at room temperature.

Fluorescence spectra. All measurements were performed at a single strand oligonucleotide concentration of 2×10^{-5} M in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M or 1.0 M NaCl. An excitation wavelength was used at 331 nm for U(DAN)-modified oligonucleotides, and an excitation / emission band width of 10 nm / 3 nm were employed. The apparent efficiency of fluorescent resonance energy transfer (FRET) was determined from the quenching of the steady-state fluorescence spectra of the donor. For each set of experiments, fluorescence spectra were obtained for the duplex of donor-labeled DNA + unmodified complementary DNA (DU), the duplex of acceptor-labeled DNA + unmodified complementary DNA (AU), the duplex of donor-labeled DNA + acceptor-labeled DNA (DA). The apparent efficiency of FRET (Eapp) was calculated with the following equation, Eapp = 1 - [(DA - AU) / DU] where DA, AU and DU are the integration values for the fluorescence intensity of the donor- and acceptor-labeled DNA, acceptor-labeled DNA, and donor-labeled DNA, respectively. DA, AU, and DU were obtained from the emission values between 400 nm and 490 nm.

Results and Discussion

The synthetic pathway for 2'-(6-dimethylamino-2-naphthamide)uridine [U(DAN)], a new fluorescent nucleoside, has already been reported.[5d] U(DAN) was then converted by a usual way to 5'-*O*-dimethoxytrityl U(DAN) 3'-phosphoramidite which was used for the synthesis of U(DAN) containing oligonucleotides on an automated DNA synthesizer. The purification of oligomers were carried out by denaturing polyacrylamide gel electrophoresis. The purified DAN-modified oligonucleotides showed expected molecular masses.

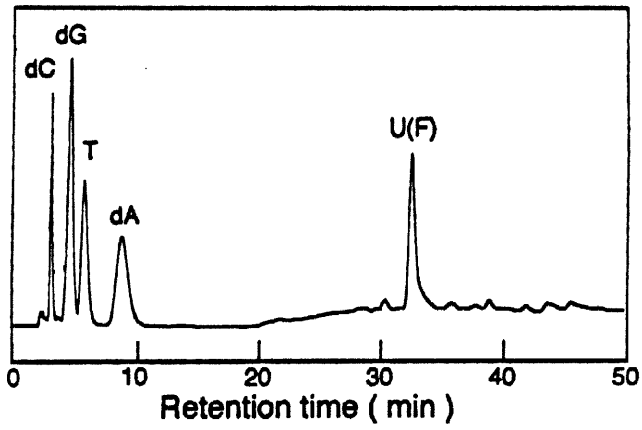


Figure 1. Reversed phase HPLC (Cosmosil 5C18 AR300, 4.6 x 150 mm) for the reaction mixture from the enzymatic digestion of fluorescein labeled oligonucleotide, **F2**. The elution was carried out by 4 % CH_3CN (5 min) and then a linear gradient of CH_3CN (1 %/min, 5-50 min) in ammonium acetate (50 mM, pH = 6.0) at a flow rate of 1 mL/min.

The fluorescein labeling of oligonucleotides at the 2'-position has been accomplished by the reaction of fluorescein isothiocyanate with the oligonucleotides containing 2'-amino-2'-deoxyuridine. The fluorescein labeled oligonucleotides were purified by means of reversed phase HPLC. The purified oligonucleotides were analyzed by usin ion spray mass spectroscopy and enzymatic digestion with snake venom and alkaline phosphatase. The experimentally determined molecular masses were found to be as expected. Figure 1 shows the HPLC of the enzymatic digestion products from the fluorescein labeled oligomer **F2** as an example. The digestion of the oligomer gave dC, dG, T, dA and the 2'-fluorescein modified uridine and no other modified nucleosides were detected. These results indicate that the fluorescein is covalently attached to the 2'-amino function of oligonucleotides. In this fluorescein labeling, the desired oligonucleotides were usually obtained in ca. 60 -70 % isolated yields (determined based on A_{260}) from the precursor oligonucleotides.

For the FRET analysis of DNA structural features in solution, the site-specific labeling of oligonucleotides is necessary without retarding the properties of the native oligonucleotide duplexes. It has been shown that incorporation of fluorescent derivatives of 2'-amino-2'-deoxypyrimidines[7b,c] into oligodeoxyribonucleotides resulted in lowered melting temperatures of the duplexes. We therefore first examined the binding and conformational properties of oligonucleotides containing U(DAN), **DAN1-6**, and fluorescein modified uridine [U(F)], **F1-3**, depending on the site of their incorporation. The binding of the modified oligonucleotides with a complementary DNA segment was investigated by UV melting measurements. All the duplex exhibited sigmoidal curves in their melting profiles. The tm values and hypochromicity of the duplex melting are summarized in Table 1. Analysis of these values revealed that the oligonucleotide containing U(DAN) or U(F) at the 5'-terminal end retains its normal affinity for DNA. The CD profiles of the oligonucleotide duplexes containing the labeled nucleosides at the 5'-end exhibited the most resemble profiles as those for the corresponding unmodified DNA duplexes. Therefore it can be concluded that the attachment of the bulky fluorophore to the sugar 2'-position at the terminal fraying end of one oligonucleotide strand does not alter the normal thermal stability and global conformation of the DNA duplexes. Similar observations have been reported for the oligonucleotides containing a bulky fluorophore at the sugar 2'[5c] and a metal ligand at the pyrimidine C-5 position.[10]

Table 1. Binding and fluorescence properties of U(DAN)- and U(F)-modified oligonucleotides.

oligonucleotide	Tm (℃)	hypochromicity (%)	Δλ (nm)	relative emission intensity at 455 nm
5'-dTTTAGAGGTCAT	37.0	21.3		
DAN1	39.0	20.0	1.0	2.35
5'-dTATAGAGGTCAT	37.0	20.7		
DAN2	39.0	20.1	3.0	0.63
5'-dTGTAGAGGTCAT	41.0	20.1		
DAN3	43.0	20.6	2.0	0.70
5'-dTCTAGAGGTCAT	40.0	21.3		
DAN4	43.0	20.1	1.5	0.76
DAN5	35.1	17.7		
DAN6	32.7	17.2		
F1	41.4	18.1		
F2	32.0	18.3		
F3	30.0	18.0		

UV melting measurements at 260 nm were carried out in a pH 7 phosphate buffer containing 0.1 M NaCl at a total strand concentration of 4.0×10^{-5} M. Fluorescence spectra (λex = 331 nm) were measured for the same solution used for the melting studies at 22 ℃. Solutions of U(DAN)-modified oligonucleotides (conc. = 2.0×10^{-5} M) in the same buffer were used as a reference to yield Δλ in blue shift and relative emission intensity. DNA fragments used in binding and fluorescence measurements are 5'-dATGACCTCTCTAAA for 5'-dTTTAGAGGTCAT and **DAN1**, 5'-dATGACCTCTCTATA for 5'-dTATAGAGGTCAT and **DAN2**, 5'-dATGACCTCTCTACA for 5'-dTGTAGAGGTCAT and **DAN3**, and 5'-dATGACCTCTAGA for 5'-dTCTAGAGGTCAT, **DAN4-6** and **F1-3**.

The fluorescence properties of the oligonucleotides containing U(DAN) at the terminal end, **DAN1-4**, and their duplexes with complementary DNA are also shown in Table 1. The single-stranded U(DAN)-modified oligonucleotides exhibited the emission maximum at around 455 nm which well overlapped with the absorption band (480 nm and 450 nm) of the fluorescein label. Upon hybridization of these oligonucleotides to complementary DNA, little shift in the emission maximum was observed with significant change in the emission intensity. Since both fluorescence maximum and intensity of DAN fluorophore should be highly sensitive to the environmental polarity,[5d] the observed fluorescence changes suggest that the DAN fluorescence does not

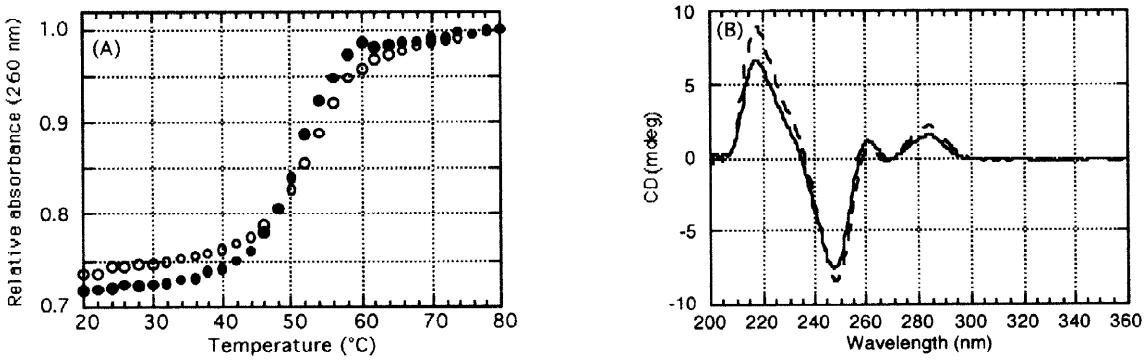


Figure 2. UV melting curves (A) and CD spectra (B) for doubly labeled DNA duplex at a total strand concentration of 4.0×10^{-5}M. The measurements were carried out in a buffer containing 1.0 M NaCl and 0.01 M sodium phosphate, adjusted to pH 7.0. (A) **DAN11** + **F8** (○), 5'-T_{15}A + 5'-TA_{15} (●). (B) **DAN11** + **F8** (solid line), 5'-T_{15}A + 5'-TA_{15} (broken line).

transfer to less polar base-pair pocket of the duplexes. It is most likely that the DAN fluorescence projects toward the minor groove of DNA duplex.

To test the validity of the present donor and acceptor pair in distance-dependent FRET measure, we next prepared the doubly labeled DNA duplexes possessing different numbers of base-pairs (A-T pairs) between these fluorophores (oligomers **DAN7-11** and **F4-8**). As shown in Figure 2, both the UV melting and CD profiles of the duplex containing U(DAN) and U(F) at each strand (oligomers **DAN11 + F8**) were almost similar to those for the unmodified duplex. Similar melting and CD profiles were observed for all the doubly labeled duplexes. These results clearly established that the duplexes doubly labeled by donor and acceptor fluorophores at the 2'-positions of the terminal ends also retain their normal thermal stability and global conformation as can be seen in the singly labeled duplexes.

Figure 3 shows the fluorescence spectra observed in the typical FRET experiment. The U(DAN) oligonucleotide (**DAN8**) exhibits the fluorescence whose maximum appeared at 458 nm by excitation at 331 nm. Upon hybridization to the complementary oligonucleotide labeled by fluorescein (**F5**), the DAN fluorescence was quenched. With this fluorescence quenching, enhancement of the emission derived from the fluorescein at 523 nm was observed. On contrary, no fluorescence changes were observed for **DAN8** in the presence of fluorescein labeled oligonucleotides with non-complementary sequence such as **F1-3**. All the labeled duplexes displayed similar fluorescence properties. These observations indicate that the intramolecular FRET occurs between DAN and fluorescein labels along the DNA duplex. The FRET efficiencies (Eapp) measured for each duplex are shown graphically in Figure 3. There is a clear dependence of Eapp on the number of nucleotides in the DNA, suggesting that the present donor and acceptor pair would be useful for FRET measure of DNA.

We have described an efficient method for introduction of the 2'-fluorescent labeled nucleosides as a donor and acceptor set at appropriate positions in DNA duplexes. The attachment of bulky fluorophores via a short

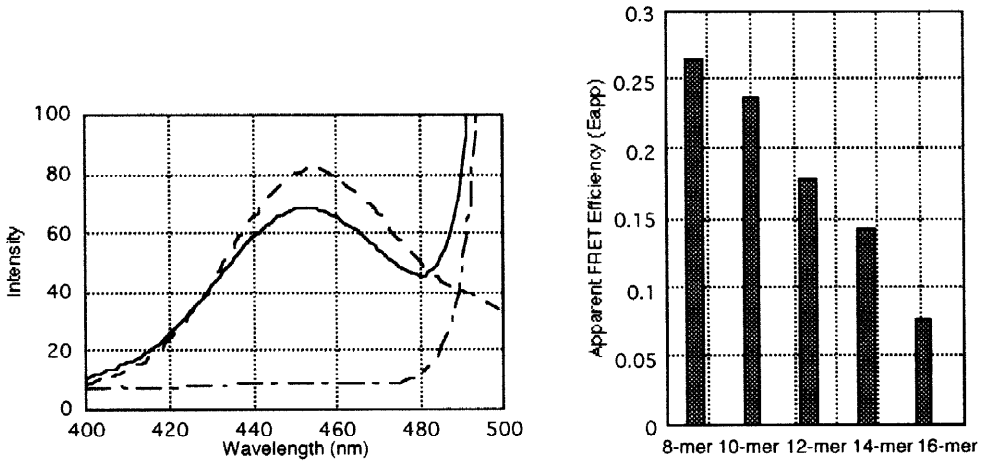


Figure 3. *Left Panel* : Fluorescent spectra for a typical FRET measurement of DNA duplex (10 mer) at a total strand concentration of 4.0×10^{-5} M. The measurements were carried out at 3 ℃ by excitation at 331 nm in a buffer containing 1.0 M NaCl and 0.01 M sodium phosphate, adjusted to pH 7.0. **DAN8** + 5'-TA_9 (- - - -), 5'-T_9A + **F5** (— · —), **DAN8** + **F5** (solid line). *Right Panel* : Apparent FRET efficiency (Eapp) in DNA duplexes containing various number of base pairs. **DAN7** + **F4** (8-mer), **DAN8** + **F5** (10-mer), **DAN9** + **F6** (12-mer), **DAN10** + **F7** (14-mer), **DAN11** + **F8** (16-mer).

tether to the sugar 2'-position at the fraying end of each oligonucleotide strand does not alter the normal thermal stability and global conformation of the DNA duplexes. The measurements of the apparent FRET efficiencies depending on the oligonucleotide length suggest that the FRET may be used as an indicator of DNA structures.

Acknowledgment

We are very grateful to Professor Hiroshi Sugiyama and Dr. Takeo Kaneko for measurements of ion spray mass and FAB mass spectra, respectively. We also thank Dr. Takashi Sugimura for use of his CD spectrometer. This research was in part supported by grant-in-aid for Scientific Research from Monbusho (072229241).

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