Measurement of Local DNA Reorganization on the Picosecond and Nanosecond Time Scales

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Abstract: Picosecond and nanosecond dynamics in the interior of DNA are observed for the first time as a dynamic Stokes shift in the fluorescence of a specially designed base-pair analogue. A recently synthesized coumarin C-riboside has been incorporated into an oligonucleotide in place of a normal purine·pyrimidine base pair. The dynamic Stokes shift of this probe occurs with components near 300 ps and 13 ns. These times are too long for solvent relaxation or simple vibrational motion of the DNA framework. They imply that the interior of DNA is a unique dynamic environment unlike either a fluid or a molecular crystal.

Introduction

The detail and precision of DNA crystal structures can divert attention away from the importance of thermal fluctuations around these structures. Recent computer simulations have raised the possibility that there are relatively large fluctuations away from the average structure with lifetimes in the picosecond range.[1−5] The existence of such fluctuations has profound implications for a variety of biologically important processes. The sequence dependence of static deviations in DNA structure has been implicated in gene recognition and DNA/protein interactions,[6−8] and a similar role has been hypothesized for sequence-dependent variations in flexibility and relaxation rates.[9,10] The possibility of rapid electron transfer through DNA is being intensely debated.[11−14] Strong fluctuations in the DNA structure would dramatically affect electron-transfer rates, both by modulating the interbase coupling and by providing a reorganization coordinate capable of localizing charge. The dynamics of structural fluctuations are also critical to the rates of chemical reactions occurring within the DNA, because they determine how quickly the DNA can adapt to the product's new geometry.[15−18] We have directly time-resolved the dynamic fluctuations within the interior of DNA using ultrafast spectroscopy in concert with a specifically designed photophysical probe. The results show that DNA provides a unique dynamical environment that is distinctly different from either a simple-fluid or a rigid-crystalline environment.

Although there have been many observations of DNA motion over longer lengths or longer times,[19,20] experiments measuring motions at the level of individual bases and in the picosecond time range have been difficult. The inherent time scales of nuclear and electron magnetic resonance limit their ability to quantify motion on the picosecond time scale.[20−24] Nonetheless, modeling of indirect effects often indicates the presence of significant localized, internal motion within DNA in the picosecond time range.[22,24−30] Fluorescence anisotropy decays

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of intercalated fluorophores have picosecond time resolution, but are dominated by overall tumbling or collective bending and twisting motions.[31,32] Nonetheless, small components in these decays have been attributed to local motion.[33] Anisotropy decay of intrinsic DNA-base fluorescence has been attributed to localized motions on the picosecond time scale,[34] but the interpretation is obscured by the possibility of excited-state mixing and the complex photophysics of the bases.[35] Vibrational spectroscopy provides evidence for changing or multiple conformations within DNA, but gives no direct information on the associated time scales.[36−40]

We introduce time-resolved Stokes shifts (TRSS) as a method of directly observing the local dynamics of DNA. The TRSS technique has been used extensively to measure the dynamics of simple liquids by using fluorophores free in solution.[41−43] When a fluorophore is excited, its properties, e.g., polarity or polarizability, change significantly, causing the local solvent structure to reorganize. This reorganization reduces the energy of the system and causes the fluorescence to shift to longer wavelengths, i.e., a Stokes shift develops. The rate of shifting of the fluorescence spectrum directly reflects the rate of reorganization of the fluorophore's environment. By the fluctuation−dissipation theorem,[44] this rate also gives the lifetime of thermal fluctuations in the unperturbed system.

This method has not been applied to DNA primarily because of the poor photophysical properties of either native DNA or currently available forms of modified DNA. To address this problem, we synthetically replaced a normal DNA purine·pyrimidine base pair with a fluorophore specifically designed to optimize TRSS measurements. The DNA becomes the fluorophore's "solvent", and the TRSS measures the dynamic motion of the DNA. Because the TRSS is caused by intermolecular interactions between the fluorophore and nearby groups in its environment, the TRSS is insensitive to either global motion of the oligomer or long-range twisting and bending motions. Instead, it results almost entirely from movement of groups proximal to the fluorophore.

In earlier work, we used a high viscosity, cryogenic solvent to slow the DNA dynamics, so they could be measured by steady-state techniques.[45] We showed that DNA does reorganize through a slow, diffusive process. However, under those conditions, the DNA's reorganization rate matched the solvent's reorganization rate. We hypothesized that the solvent was exerting a rate-limiting effect, and that under physiological conditions, the intrinsic properties of the DNA would determine its reorganization time.

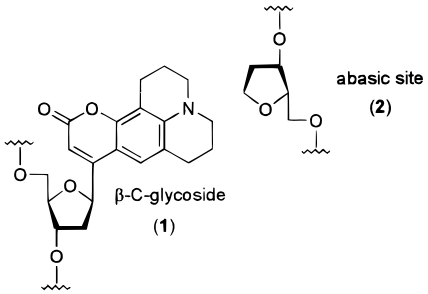

Figure 1. Structures of the coumarin C riboside **1** and abasic-site analogue **2** that form a base-pair analogue with good photophysical properties.

By introducing the TRSS experiment, we have tested that hypothesis. In a room temperature, aqueous solution, the DNA's dynamics are several orders-of-magnitude slower than the solvent's. The reorganization is multiexponential, showing that at least two reorganization processes are involved. Moreover, a large fraction of the dynamics is still unresolved with the current 100 ps time resolution. However, straightforward extensions of the current techniques can explore those faster processes as well.

Results

Incorporating a Photophysical Probe into DNA. Two of us have recently reported the synthesis of the coumarin C-riboside **1** (Figure 1), a fluorescent molecule designed specifically for the present studies.[46] Several considerations went into the design of this molecule. It incorporates the well-characterized Coumarin 102 molecule as its fluorophore. Work on solution-phase TRSS has demonstrated that Coumarin 102 and related dyes are nearly ideal probes.[41−43,47,48] Coumarins have simple photophysics uncomplicated by competing processes: they have no low-lying electronic states that interact with their first excited states[48] (although see ref 49) and nonradiative relaxation and intersystem crossing are weak, resulting in near unity quantum yields.[50]

The amine group of **1** is rigidified, preventing intramolecular twisting or pyramidalization in the excited state, as occurs in other coumarins.[50] Extensive measurements by Seidel, Schulz, and Sauer show that Coumarin 102 is unlikely to either donate or accept electrons from DNA bases,[51] processes which would quench fluorescence. The absorption maximum of **1** is near 400 nm, a wavelength long enough to prevent energy transfer to or electronic mixing with the transitions of the normal DNA bases.

Molecular modeling of compound **1** in duplex B-DNA (Figure 2, details in the Experimental Section) shows that when it is positioned opposite the abasic-site analogue **2** in helical B-DNA, the coumarin portion effectively replaces a normal purine·pyrimidine base pair. There is no significant distortion of the

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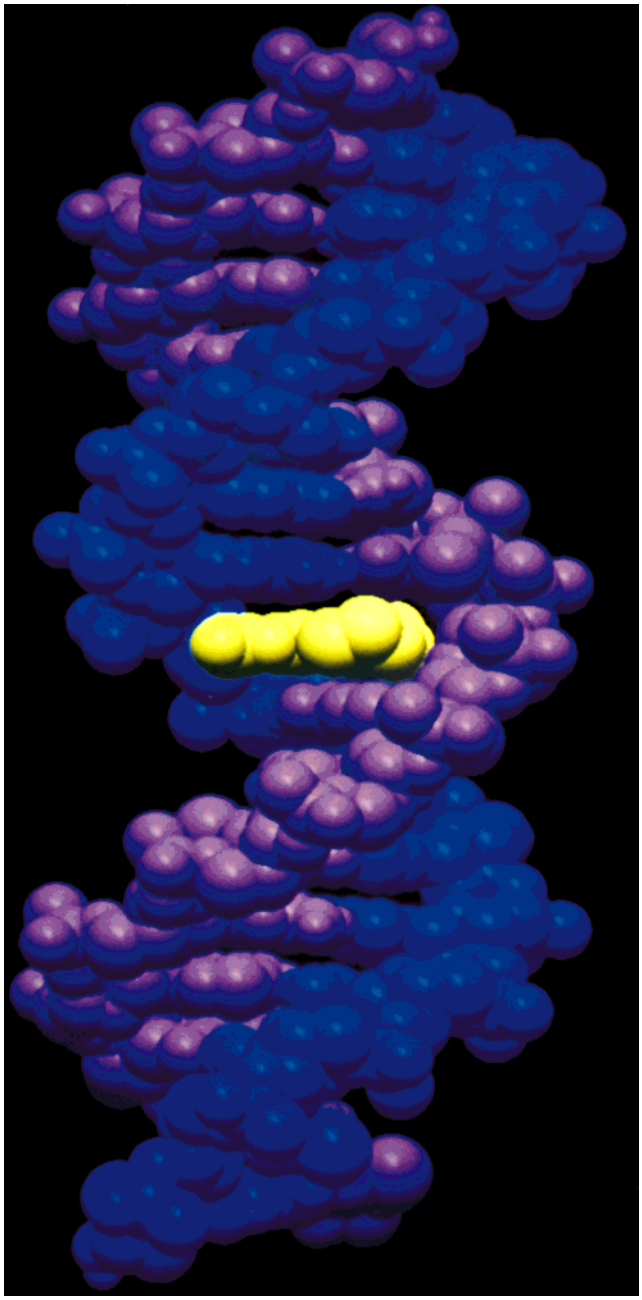


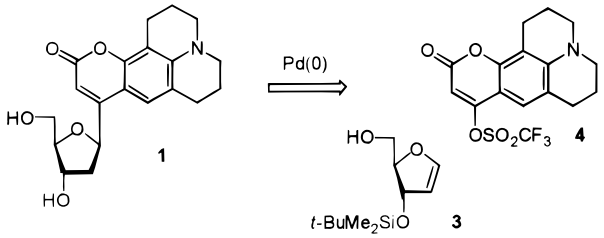
Figure 2. Molecular modeling structure of the modified 17-mer duplex showing the synthetic chromophore occupying the site of a base pair. The yellow fluorophore **1** is covalently attached to the blue DNA strand. The purple DNA strand has the abasic site **2** complementary to the fluorophore.

double helix, as measured by C1′ to C1′ distances.[52] Coumarin 102 itself has a low solubility in water relative to organic solvents, so hydrophobic forces will also favor the coumarin unit occupying the DNA interior as opposed to extending into the aqueous medium.

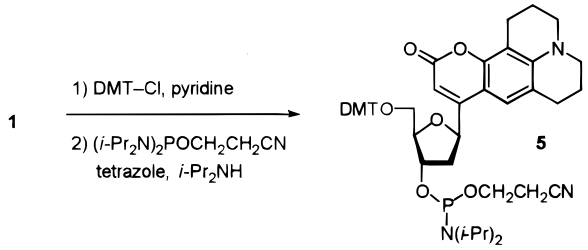
Coumarins have a large increase in dipole moment in their excited states, and as a result, the energy of their excited states is primarily sensitive to the local electric field. Thus as a TRSS probe, compound **1** will respond specifically to movement of charged groups in the dye's local environment.

In the synthesis of fluorophore **1**, a Coumarin-102 unit is covalently attached to a 2-deoxyribose via a β-C-glycosidic bond

Scheme 1



Scheme 2



formed using a palladium-promoted Heck coupling between glycal **3** and coumarin triflate **4** (retrosynthetic Scheme 1). This arylation reaction sequence occurred with complete control of stereochemistry.[53]

Incorporation of this fluorophore into an oligonucleotide proceeded by protection of the 5′-hydroxyl group as the dimethoxytrityl (DMT) ether followed by phosphitylation of the 3′-hydroxyl group to afford phosphoramidite **5** (Scheme 2). This reagent was incorporated directly into a 17 base-pair DNA oligomer using standard automated oligonucleotide synthesis protocols. For the photophysical experiments described below, we prepared the modified T7-RNA-polymerase-promoter sequence 5′-d(TAATACGA**1**CCACTATA)-3′ as a "typical" B-DNA sequence. The complementary sequence 5′-d(TATAGTGG**2**TCGTATTA)-3′ was prepared with abasic model **2** in the position complementary to coumarin **1**.[52]

The two sequences anneal to form a normal DNA duplex. The circular dichroism spectrum of the oligomer indicates a B-form double helix. Melting studies show a decrease in stability compared to the native duplex containing a G·C base pair. In pH 7.2 phosphate buffer (100 mM) containing 100 mM NaCl, the native duplex (**1**→C, **2**→G) has a T_m of 58.5 °C, whereas the modified duplex containing **1·2** melts at $T_m = 45.5$ °C. This destabilization is typical for a non-native base-pair substitution.[54]

The coumarin remains strongly fluorescent in the double helix. In fact, the fluorescence lifetime is increased relative to aqueous solution (7.4 vs 4.2 ns). Because the coumarin in this sequence is surrounded by one of each of the normal DNA bases, we infer that electron transfer or other quenching processes will not be a problem for this fluorophore in other sequences as well.

Spectroscopic measurements (Figure 3) support the molecular modeling prediction that **1** adopts a position in the interior of the DNA. The excitation spectrum of **1** (Figure 3) is red-shifted in the duplex (c) relative to the spectrum free in aqueous buffer (a), and the spectrum in the melted oligonucleotide is intermediate between the two (b). These results are consistent with the probe being shielded from the solvent in the duplex and partially exposed in the single-stranded oligomer. Melting experiments performed with detection in the coumarin absorbance band at

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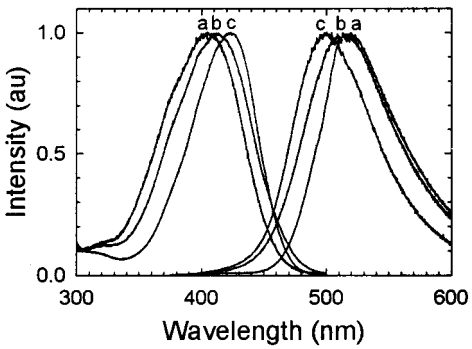

Figure 3. Steady-state fluorescence excitation (left) and emission (right) spectra of **1** (a) free in solution, (b) in the melted, single-stranded oligonucleotide (90 °C), and (c) in the double-stranded oligonucleotide (25 °C).

380 nm show that the coumarin spectral changes occur in concert with the melting of the DNA, as monitored by the absorption at 260 nm. Additionally, the Stokes shift of **1** in the duplex is smaller than that in solution (Figure 3), indicating that its environment cannot reorganize as effectively as in water. Again, the melted oligomer has an intermediate Stokes shift, consistent with partial exposure to the solvent. With an appropriate fluorophore positioned in the interior of the DNA duplex, TRSS measurements of the DNA dynamics become possible.

Time-Resolved Measurements of DNA Dynamics. In earlier studies, we measured steady-state Stokes shifts of the intercalated fluorophore acridine orange.[45] In the current work, we have not only replaced the structure-perturbing intercalator acridine orange with the structure-preserving fluorophore **1**, we have also turned to fluorescence measurements with picosecond time resolution.

Fluorescence decay measurements of **1** in the oligomer were made at 10 wavelengths, but the qualitative features are evident by examining just two decays. Figure 4 shows decays on the blue (460 nm) and red (550 nm) sides of the fluorescence spectrum. The long exponential decay common to both wavelengths corresponds to the fluorescence lifetime (7.4 ns). At short times, the long wavelength measurement shows a rise in intensity, whereas the short wavelength measurement shows an additional fast decay. These effects are caused by the fluorescence spectrum shifting from short to long wavelengths.

The difference between the blue and red decays is shown in Figure 4. The TRSS contribution to the signal is much larger than the noise and has components much longer than the instrument response function. The presence of at least two relaxation times is noticeable.

A more quantitative analysis comes from using the full set of wavelength-dependent decays to reconstruct the time-dependent fluorescence spectra (Figure 5).[55] The spectra show a continuous shift with time, as expected for a continuous evolution of structure. In contrast, a transfer between two discrete conformers or substates would have given an isosbestic point in the spectra.

The time evolution of the spectral peak gives the standard solvation response function for the DNA (Figure 6).[41−43] The function is well fit by a biexponential with time constants of 300 ps and 13 ns. (The fit function for the peak fluorescence frequency is 19856 cm^{-1} + 312 cm^{-1}[0.47 exp(−t/300 ps) + 0.53 exp(−t/13.4 ns)].) The dynamics clearly occur on two distinct time scales and at least two distinct mechanisms must be involved.

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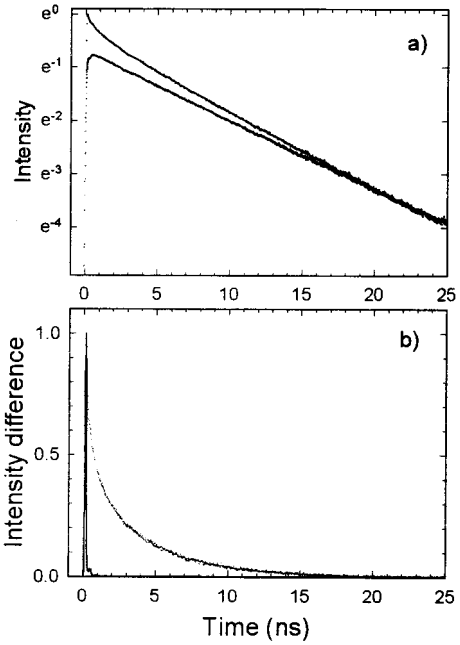

Figure 4. Wavelength resolved fluorescence decays of the modified oligomer: (a) full decays collected at 460 nm (upper points) and 550 nm (lower points) (the decays have been normalized to match at long time) and (b) subtraction of these two decays (data (points) and instrument response function (solid line)). The difference is caused by a dynamic Stokes shift induced by DNA motion.

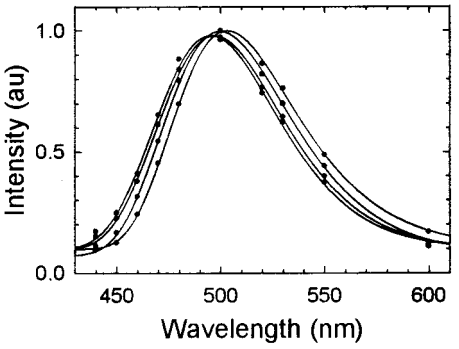

Figure 5. Representative time-resolved fluorescence spectra of the modified oligomer. Left to right on the short wavelength side: 100 ps, 300 ps, 3.0 ns, and 30 ns. Reconstructed data (points) and log-normal fits (curves). Spectra are normalized to constant area.

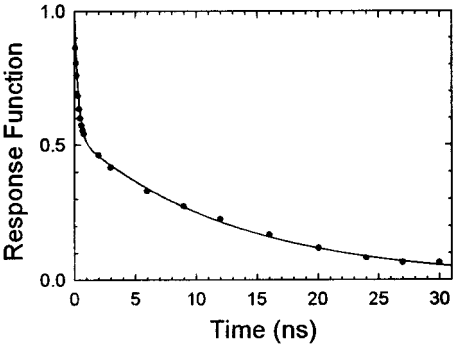

Figure 6. Solvation response function for the interior of DNA derived from the relaxation of the peak of the time-dependent fluorescence spectrum (Figure 5). The curve is a biexponential fit with time constants of 300 ps and 13 ns.

We note that there are likely to be other fast relaxation processes in addition to those observed here. We only observe a Stokes shift of 312 cm^{-1} within our time range, whereas the

total Stokes shift is expected to be roughly 1500 cm^{-1}. (The Stokes shift in cyclohexane is a bound on the $t = 0$ Stokes shift. In cyclohexane, the emission and excitation spectra cross at 65% of their maxima.[56] Our estimate assumes the same crossing point occurs in our samples at $t = 0$.) Presumably, the missing Stokes shift occurs faster than we can resolve with our current instrumentation. This hypothesis is certainly consistent with molecular dynamics simulations, which see the majority of the relaxation occurring before 10 ps.[2,57,58]

Discussion

The relaxation times seen in the TRSS experiments are too slow for many of the most obvious processes. They are too slow to represent even low-frequency vibrational motion of the DNA or quasiharmonic oscillation of the fluorophore within a static DNA structure. The relaxation of water itself is subpicosecond,[59] so we are not directly detecting bulk solvent motion. Times this slow must result from complex, cooperative reorganization of the DNA structure and its immediate surroundings. These motions are more similar to the diffusive reorganization of a liquid than to the vibrational motions of a crystal.

A similar situation occurs with proteins. Proteins have crystallographically well-defined average structures, but suffer dynamic fluctuations about this average.[60−62] These fluctuations are often described in terms of diffusion on a rugged potential energy landscape,[63] and related ideas may be useful in describing DNA dynamics.

In previous work, we found that in a high-viscosity, cryogenic medium, the average DNA relaxation rate exactly equals the solvent relaxation rate.[45] At that time, we hypothesized that movement of portions of the DNA also involves displacement of solvent. In a high-viscosity solvent, the solvent reorganization becomes rate limiting. In the current work, the aqueous solvent relaxes very quickly, in under 1 ps.[59] Under these conditions, the DNA relaxation rates are much slower than the solvent relaxation rates. These results support the conclusion that the DNA relaxation requires significant motion of the external solvent, but also show that there are significant internal constraints on the motion as well.

A more specific assignment of the DNA motions being observed is still somewhat speculative. The coumarin probe we used is specifically sensitive to motions of charges. A variety of charged groups exist in the DNA proper. The hydrogen bonds in the base pairs have strong dipole and quadrupole moments. These moments produce strong, but short ranged electric fields. Thus distortions of the base pairs are likely to contribute to the observed dynamics, but the contribution from the pairs neighboring the fluorophore will dominate over more distant base pairs. The phosphate groups on the DNA backbone have full charges, which produce long-range monopole fields. Motion of the phosphates is also likely to contribute to the TRSS.

Molecular dynamics (MD) simulations indicate that there are a variety of relatively large fluctuations in DNA structure with lifetimes extending into the 100's of picoseconds range.[1−4,64] Thus, it is very plausible that our 300 ps time scale is connected with relaxation of the DNA structure. Because of limitations on the length of computer trajectories, it is not known if these fluctuations also extend out to the 13 ns time seen in our experiment.

On the other hand, the TRSS may be sensing motion in the perturbed environment outside the DNA proper. For example, the counterion atmosphere of the DNA may affect the electric field in the interior of the DNA. The δ-relaxation seen in dielectric measurements of DNA solutions is attributed to diffusion of the counterions along the DNA chain.[65] It occurs on the tens of nanoseconds time scale and could be related to the long time seen in our TRSS measurements.

The water next to and in the grooves in the DNA is strongly perturbed, up to the point of forming a crystallographically structured "spine of hydration".[66] The constrained motion of this water[67] or exchange of this water with ions[68] may also contribute to the TRSS.

These initial results establish that the interior of DNA experiences an unusual dynamic environment. They also raise numerous questions about the origin of the dynamics, their dependence on sequence and external conditions, and the possibility of even faster processes. The techniques introduced here are easily extended to systematically modified DNA's and to shorter times.[59] These future studies will provide an extensive and detailed exploration of the unique dynamics of DNA.

Experimental Section

Coumarin Dimethoxytrityl Ether. A solution of **1** (100 mg, 0.28 mmol), coevaporated with pyridine (2 × 3 mL), dissolved in dry pyridine (4 mL) under N_2 at 25 °C was treated with bis(4-methoxyphenyl)phenylmethyl chloride (114 mg, 0.34 mmol, 1.2 equiv). After 3 h at 25 °C, the reaction mixture was diluted with saturated aqueous $NaHCO_3$ (10 mL) and extracted with CH_2Cl_2 (3 × 10 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated, and the residue was purified by flash chromatography (2.5 × 15 cm Et_3N deactivated silica, Et_2O) to afford the corresponding DMT ether (164 mg, 89%) as a yellow foam: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.25 (m, 9H, ArH), 6.91 (s, 1H, ArH), 6.83 (m, 4H, ArH), 6.26 (s, 1H, ArH), 5.34 (dd, J = 6.4, 9.5 Hz, 1H, C1′-H), 4.33 (m, 1H, C3′-H), 4.11 (m, 1H, C4′-H), 3.71 (s, 6H, 2 OCH_3), 3.35 (m, 1H, C5′-H), 3.15 (m, 5H, 2 CH_2 + C5′-H), 2.85 (m, 2H, CH_2), 2.71 (m, 2H, CH_2), 2.40 (m, 1H, C2′-H), 1.85 (m, 5H, 2 CH_2 + C2′-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.8, 158.5, 156.3, 151.2, 145.6, 144.7, 135.7, 130.0, 129.9, 128.1, 127.8, 126.8, 120.9, 117.9, 113.1, 106.9, 106.2, 104.0, 103.4, 86.3, 85.9, 75.2, 64.3, 58.5, 55.2, 49.8, 49.4, 21.5, 20.6, 20.4; FABMS, m/z 659 (M^+), 303, 118.

Coumarin Phosphoramidite 5. A solution of the above ether (100 mg, 0.15 mmol) in anhydrous CH_2Cl_2 (3 mL) under N_2 at 25 °C was treated sequentially with tetrazole (6 mg, 75 μmol, 0.5 equiv), dry diisopropylamine (11 μL, 75 μmol, 0.5 equiv), and (2-cyanoethyl)-*N*,*N*,*N*′,*N*′-tetraisopropyl phosphorodiamidite (55 mg, 0.18 mmol, 1.2 equiv). After 3 h at 25 °C, the reaction mixture was diluted with CH_2Cl_2 (10 mL) and dried (Na_2SO_4). The solvent was removed in vacuo and the residue was purified by flash chromatography (2 × 12 cm Et_3N deactivated silica, 5% CH_3OH/CH_2Cl_2) to afford **5** (85 mg, 71%) as a yellow foam: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.30 (m, 9H, ArH), 6.9

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(s, 1H, ArH), 6.8 (m, 4H, ArH), 6.32 (s, 1H, ArH), 5.3 (dd, $J = 6.2$, 9.5 Hz, 1H, C1′-H), 4.45 (m, 1H, C3′-H), 4.10 (m, 1H, C4′-H), 3.80 (s, 6H, 2 OCH_3), 3.65 (m, 6H, CH_2CH_2CN, and 2 $CH(CH_3)_2$), 3.4 (dd, $J = 5.2$, 9.8 Hz, 1H, C5′-H), 3.2 (m, 5H, CH_2 + C5′-H), 2.8 (m, 2H, CH_2), 2.7 (m, 2H, CH_2), 2.40 (ddd, $J = 2.4$, 6.2, 13.1 Hz, 1H, C2′-H), 1.9 (m, 5H, 2 CH_2 + C2′-H), 1.19 (d, $J = 3.1$ Hz, 6H, $CH(CH_3)_2$, 1.17 (d, $J = 3.1$ Hz, 6H, $CH(CH_3)_2$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.7, 158.5, 156.1, 151.2, 145.6, 144.7, 135.7, 130.0, 129.9, 128.1, 127.8, 126.8, 120.9, 117.9, 117.6, 113.2, 106.9, 106.2, 103.5, 86.3, 85.8, 75.1, 74.1, 64.3, 59.2, 58.2, 51.2, 49.8, 49.4, 42.9, 42.1, 24.6, 24.5, 21.5, 20.6, 20.4, 20.3, 20.2, 16.9, 16.8.

DNA Synthesis. Oligonucleotides were synthesized (0.2 μmol scale) using an Applied Biosystems 392 DNA/RNA synthesizer with commercially available reagents and protocols. Phosphoramidite **5** was incorporated using standard coupling times for oligomers containing **1**. Cleavage from the support and base deprotection was accomplished by treatment with concentrated NH_4OH (16 h, 25 °C). Following HPLC purification of the 5′-DMT-protected DNA (95 to 50% A over 40 min), the product fraction was lyophilized and detritylated with 80% acetic acid (400 μL, 0.5 h, 25 °C). The mixture was concentrated to dryness and dissolved in 0.2 mL of H_2O, and the final oligonucleotide product was purified by HPLC (92 to 75% A over 30 min). ESIMS for DNA containing coumarin **1**: found 5254.0; calcd 5252.5. ESIMS for DNA containing tetrahydrofuran **2**: found 5108.5; calcd 5105.6.

HPLC purification of the oligonucleotides was performed using a Perkin-Elmer Model 250 Biocompatible LC pump, LC-290 detector, and PE Nelson 1022 Plus computing integrator with a 4.1 × 250 mm Hamilton PRP-1 reverse-phase column (mobile phases: A = 0.1 M triethylammonium acetate, pH 6.5; B = CH_3CN).

Molecular Modeling. Energy minimization was performed to convergence (RMS = 0.01 Å/mol) with the AMBER* force field and long-range electrostatic corrections as implemented in MacroModel v 5.5, using explicit sodium counterions and GB/SA water treatment.

Sample Preparation and Spectroscopy. All experiments were performed in 100 mM phosphate buffer, pH 7.2, at 25 °C. The concentration of the DNA was ~1.75 mM (nucleotide). Melting studies show that the oligomer is double helical under these conditions.

Both time-resolved and steady-state spectroscopy were performed with magic-angle polarization. Steady-state emission spectra were collected with excitation at 390 nm and excitation and emission band-passes of 8 and 4 nm, respectively. Excitation spectra were recorded with detection at 520 nm and emission and excitation band-passes of 4 and 8 nm, respectively. Melting studies were done on a Beckman DU 640 UV/VIS spectrophotometer with Peltier temperature control. A_{260} values were recorded every 0.5 min from 15 to 80 °C (0.5 deg C/min).

Fluorescence decays were collected using standard time correlated single-photon counting techniques.[69] Subpicosecond excitation pulses at 390 nm were generated from the output of a home-built, mode-locked, Ti:Sapphire laser by using an external acousto-optic pulse selector, followed by second harmonic generation in a 1 mm BBO crystal. Fluorescence was collected by a subtractive double monochromator with a band-pass of 3 nm. The instrument response function typically had a fwhm of 100 ps.

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Supporting Information Available: A circular dichroism spectrum of the modified DNA oligomer (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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