The Structure of Cyanine 5 Terminally Attached to Double-Stranded DNA: Implications for FRET Studies[†]

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Received April 30, 2008; Revised Manuscript Received May 30, 2008

ABSTRACT: Fluorescence resonance energy transfer, FRET, can be used to obtain long-range distance information for macromolecules and is particularly powerful when used in single-molecule studies. The determination of accurate distances requires knowledge of the fluorophore position with respect to the macromolecule. In this study we have used NMR to determine the structure of the commonly used fluorophore indocarbocyanine-5 (Cy5) covalently attached to the 5′-terminus of double-helical DNA. We find that Cy5 is predominantly stacked onto the end of the duplex, in a manner similar to an additional base pair. This is very similar to the behavior of Cy3 terminally attached to DNA and suggests that the efficiency of energy transfer between Cy3 and Cy5, that are attached to nucleic acids in this way, will exhibit significant dependence on fluorophore orientation.

Fluorescence resonance energy transfer (FRET) can provide distance information between fluorophores in the range of 10 to 80 Å (*1*). This size range is largely inaccessible to other solution methods, yet it is very much the scale of biological macromolecules, and FRET has been extensively applied to the analysis of global conformation, folding transitions and binding events in nucleic acids and proteins (*2–16*). Because of the sensitivity of fluorescence, FRET has become widely used in single-molecule studies (*17–21*).

In a typical FRET experiment, donor and acceptor fluorophores are attached to specific points on the macromolecule of interest. The efficiency of energy transfer between the fluorophores is then used to provide distance information from which aspects of the conformation of the macromolecule are deduced. However, both the extraction of distance information from FRET efficiency data and its interpretation are affected by the local conformation of the fluorophores at their sites of attachment. The relationship between FRET efficiency and fluorophore separation takes a particularly simple form if the fluorophores are flexibly tethered, but if this is not the case, then relative fluorophore orientation complicates the analysis as discussed below. Moreover, distance information between fluorophores affixed to a macromolecule can only be interpreted unambiguously if the positions of the fluorophores with respect to the molecule of interest are known. Both of these questions require better knowledge of the location of the fluorophores on the macromolecule.

The indocarbocyanine-3/indocarbocyanine-5 (Cy3/Cy5) donor−acceptor fluorophore pair has become a popular choice in single-molecule fluorescence studies, especially those involving nucleic acids (*15, 20, 22–25*). In a previous study we used nuclear magnetic resonance (NMR) to determined the structure of Cy3 covalently attached to the 5′ position of a DNA duplex via a 3 carbon and phosphodiester linkage (*26*). We concluded that the Cy3 is predominantly stacked onto the end of the helix, in a manner similar to that of an additional base pair. While defining the position of the Cy3 on the DNA, we were also able to couple this information with FRET-derived distances to determine an effective position for fluorescein attached at the opposite end of the duplex, and used as an energy transfer donor to Cy3 in many ensemble studies.

If Cy5, structurally very similar to Cy3, adopts a similar conformation on the end of a DNA helix, the combination of quite rigid Cy3 and Cy5 would (unlike the Cy3 fluorescein pair) be likely to generate an orientation dependence of energy transfer, leading to significant complication in the interpretation of interfluorophore distance. We have therefore used two-dimensional NMR to determine the solution structure of a self-complementary DNA decamer with Cy5 attached via a 3 carbon and phosphodiester linkage, to the 5′ position. This study shows that Cy5 is predominantly stacked on the end of the duplex DNA, leading to potential orientation dependence of energy transfer from Cy3.

MATERIALS AND METHODS

Construction of DNA Duplexes for NMR Studies. DNA oligonucleotides were synthesized using phosphoramidite chemistry (*27*) multiple times on a 1 μmol scale. Cy5 (Glen Research) was coupled to the 5′-terminus as a phosphoramidite. DNA was purified by reversed-phase HPLC in 100 mM ammonium acetate (pH 7.5) using a C18 column

[†] This work was supported by Cancer Research UK, and one of us, A.I., thanks the BBSRC, for funding.

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10.1021/bi800773f CCC: $40.75
Published on Web 07/03/2008

(μBondapak, Waters) and eluted with a linear gradient of acetonitrile. Samples were desalted and ion exchanged into sodium form before lyophilization.

Sample Preparation. Samples for NMR spectroscopy were dissolved in 0.6 mL of 0.1 M NaCl, 10 mM phosphate buffer, and 1 mM EDTA, in either 99.96% D_2O or 90% H_2O/10% D_2O (v/v). The solution pH was adjusted to 7.0. NMR spectra were acquired from 60 A_{260} units of Cy5-conjugated DNA.

NMR Experiments. All spectra were acquired using a Bruker Avance 500 NMR spectrometer equipped with CryoProbe. A very weak RF field at the HOD frequency was applied during the relaxation delay of 1.5 s. Spectral widths in F_2 and F_1 were 5482 Hz. The sign discrimination in F_1 was achieved by the States-TPPI method (*28*). The number of scans per increment was 32 for NOESY (*29*) and DQF-COSY (*30*) experiments. A series of NOESY spectra were acquired at 14 °C, with mixing times of 50, 75, 100, 150, and 250 ms. A NOESY experiment at 2 °C, with a mixing time of 250 ms was performed upon a sample dissolved in 90% H_2O/10% D_2O. The spectral width in F_1 was increased to 8500 Hz, and soft-pulse WATERGATE (*31*) was used for water suppression.

Data Processing and Analysis. NMR data were processed, assigned, and quantified using Felix (Accelrys). NOESY data were zero filled in F_1 to 1024 points and apodized using a 90°-shifted sine bell function. NOESY cross peaks from the D_2O spectra were quantified by volume integration at the five mixing times. COSY experiments were used for assignment purposes only. Spectra acquired in 90% H_2O were assigned, but no quantification of NOE cross peaks was attempted.

Structure Determination. Structures were calculated using restrained molecular mechanics and dynamics within the program X-PLOR-NIH (*32, 33*). B-form duplex DNA was constructed using Biopolymer within Insight II (Accelrys). Cy5 coordinate, parameter and topology files were generated within PRODRG (*34*). Hydrogen parameters and topologies were extrapolated from those previously used for Cy3 (*26*). Cy5 was initially placed on DNA in an extended position parallel to the DNA helix axis and at a distance of approximately 50 Å from the end of the DNA. The starting model was subjected to energy minimization followed by molecular dynamics, in the absence of experimental restraints, but with the oligonucleotide conformation fixed, to generate a family of 50 starting structures. Structural refinement was carried out using direct application of full relaxation matrix refinement utilizing the volume integral measurements from all available NOESY cross peaks and at all mixing times. The DNA structure (not including fluorophores) was restrained with a harmonic function against a standard parameter, B-form DNA helix. These restraints were relaxed on the terminal base pairs and removed from the C5′ position, connected to the Cy5 linker and phosphate. At the end of the process, a family of 41 unique structures remained after obvious outliers were removed, based on potential energies and restraint violations. An average structure was calculated and minimized using full relaxation restraints.

Calculation of Transition Dipole Moments. The direction and position of the transition dipole moments for the electronic transitions of the cyanine chromophores used in FRET studies were calculated using ab initio quantum mechanical methods. When used for FRET studies, Cy3 is the donor and Cy5 the acceptor, thus the relevant transition dipole moments are those associated with the first (vertical) electronic transitions of Cy3, when in the equilibrium geometry of its first electronic excited state, and of Cy5, when in the equilibrium geometry of its ground electronic state. The optimized excited-state geometry of Cy3 was obtained at the CIS/6-31G(d, p) level whereas the optimized geometry of Cy5 in its ground electronic state was obtained at the HF/6-31G(d, p) level. In both cases the molecules were confined to C_s symmetry during the optimization process but a subsequent frequency analysis revealed that true minima had been located, and removing the symmetry restriction did not lead to a lower energy structure. The direction of the transition dipole moments for the optimized structures were determined at the CIS/6-31++G(d, p) level. The Gaussian 03 suite of programs (*35*) running on a Linux workstation with dual Xeon processors (2.6 GHz) and 4 GB of memory was used for all calculations.

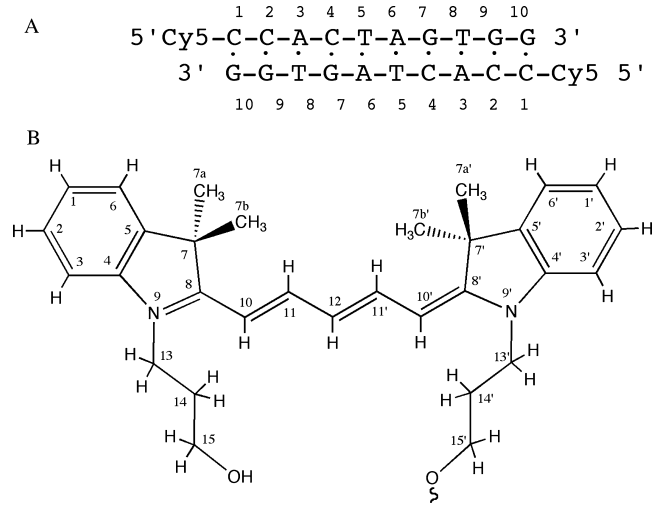

FIGURE 1: (A) Sequence and numbering scheme used for the DNA in this study. (B) Structure and numbering scheme for Cy5 used in this study.

RESULTS AND DISCUSSION

Cy5-Conjugated DNA Used for NMR Studies. The self-complementary oligonucleotide 5′-CCACTAGTGG-3′ with Cy5 attached via a three-carbon tether at the 5′-terminus was synthesized. The sequence was chosen to be directly comparable with that used in the previous determination of the structure of Cy-3 on DNA (*26*). The numbering system for the DNA nucleotides and Cy5 atoms is shown in Figure 1A and 1B respectively.

Assignment of DNA Proton Resonances. The nonexchangeable protons in the fluorophore-unconjugated DNA had been assigned previously (*26*), using standard methods (predominantly NOESY). Comparison between the unconjugated oligonucleotide and Cy5-labeled oligonucleotide spectrum showed only minor chemical shift differences in most cases, beyond the terminal base pairs. It was possible to assign the DNA proton resonances of the Cy5 derivitized oligonucleotide, without assumptions about the identity of the peaks derived from the Cy5 adduct. The chemical shifts of assigned protons from both the Cy5-conjugated oligonucleotide are presented in Supporting Information Table S1.

Assignment of Cy5 Proton Resonances. The Cy5 molecule comprises two indole ring systems connected by a planar pentamethyne linker. The Cy5 is covalently connected, via a three-carbon, hydrocarbon linker attached at N9′, to the 5′-phosphate terminus of the DNA. There is also an equivalent free chain attached at the corresponding N9 of the distal indole ring, which we term the pseudotether. Assignment was made in a similar manner to those for Cy3-DNA (*26*). The two indole rings of the Cy5 displayed a very similar pattern of NOESY and COSY peaks to those observed previously. The two sets of four aromatic resonances have chemical shifts between about 6.8 and 7.6 ppm. The proximal ring protons (1′, 2′, 3′, 6′) resonate between 7.2 and 7.6 ppm, and the distal ring protons (1, 2, 3, 6) resonate between 6.8 and 7.2 ppm. The absolute identity of the two indole rings was made by reference to the connectivity to the DNA protons of the DNA terminal base pairs. The clearest NOE interactions between the Cy5 and the DNA were those between the distal indole ring protons and the G10 H2′ and H2″ protons. In general the NOESY cross peaks arising from the Cy5 were not quite as well defined as those seen in the previous study on Cy3. However, full assignment of the Cy5 was possible and sufficient NOE interactions were measured to enable structural refinement. Volume integrals were measured from the assigned NOESY spectra at 50, 75, 100, 150, and 250 ms mixing times, and a restraints list constructed comprising 79 restraints involving only Cy5, 184 restraints between DNA protons only and 25 between DNA and Cy5.

Calculated Structures and Fluorophore Positions from NMR. Analysis of the NMR NOESY spectra of the Cy5-DNA construct (Figure 2) immediately identified similarities with the spectra previously obtained for the Cy3 construct (*26*). Full assignment and identification of cross peaks arising from the Cy5 moiety and almost complete assignment of the NOESY cross peaks arising from the associated DNA allowed us to determine the structure using a similar procedure to that previously employed for Cy3-DNA (*26*). The minimized average of the restrained dynamics calculations generated the Cy5-DNA structure labeled A and C in Figure 3, which may be directly compared with that previously determined for Cy3, labeled B and D in Figure 3. The structure of the Cy5 moiety relative to the DNA shows that the distal indole ring makes very similar stacking interactions with the terminal 3′ guanine base (structure C in Figure 3), compared with the equivalent portion of the Cy3 (structure D in Figure 3). The longer conjugated linker, between the two indole rings, displaces the proximal indole ring system further out from the DNA helical axis. NOE interactions between the proximal indole ring and the 5′ cytidine base were weak in the case of Cy3 and weaker still or missing in the case of Cy5.

The position of the planar heterocyclic body of Cy5 was well defined by its large number of NOE contacts to the terminal nucleotide pair of the DNA (Figures 3A and 3C). The structure of the Cy5 moiety does, overall, bear great resemblance to the structure determined previously for Cy3-DNA. Both fluorophores are positioned like an additional base pair on the end of the helix. The long axis of the Cy5 is rotated by approximately 32° relative to that of the terminal base pair.

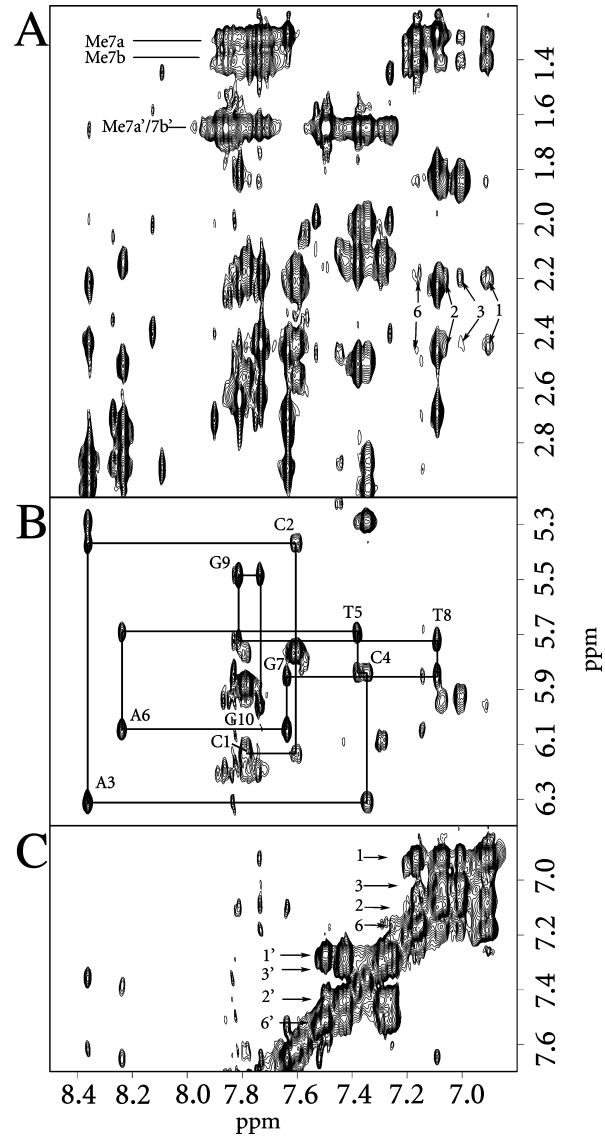

FIGURE 2: NMR NOESY spectra of the Cy5 labeled DNA duplex. Selected regions of the 250 ms NOESY between the distal aromatic ring of Cy5 and the H2′ and 2″ protons of G10. The positions of the NOE spectrum recorded in D_2O at 14 °C. (A) The base to H2′ and H2″ region, showing the positions of NOE interactions arising from the Cy5 methyl groups are also indicated. (B) The DNA base to H1′ region. The base to H1′ connectivities are represented by lines and annotation. (C) The aromatic region of the two-dimensional NOESY spectrum, showing diagonal resonance positions arising from the two Cy5 indole ring systems.

Our current work defines the structure of Cy5 in relation to the end of a DNA duplex to which it is covalently attached. Our previous study described the structure of its commonly used partner in single-molecule FRET studies, Cy3. The refinement of the Cy5 DNA structure was essentially identical to that performed for the Cy3 DNA structure, allowing direct comparison and analysis. Cartesian coordinates for the optimized structures of Cy3 and Cy5 are provided in the Supporting Information. Structural refinement from NMR data in a construct of this sort is complicated by the terminal location of the fluorophore, giving NOE interactions on one side only. Although the number of derived restraints is quite large, by nucleic acid standards, a composite view of the final structures would not necessarily be descriptive of the real distribution of structures in what

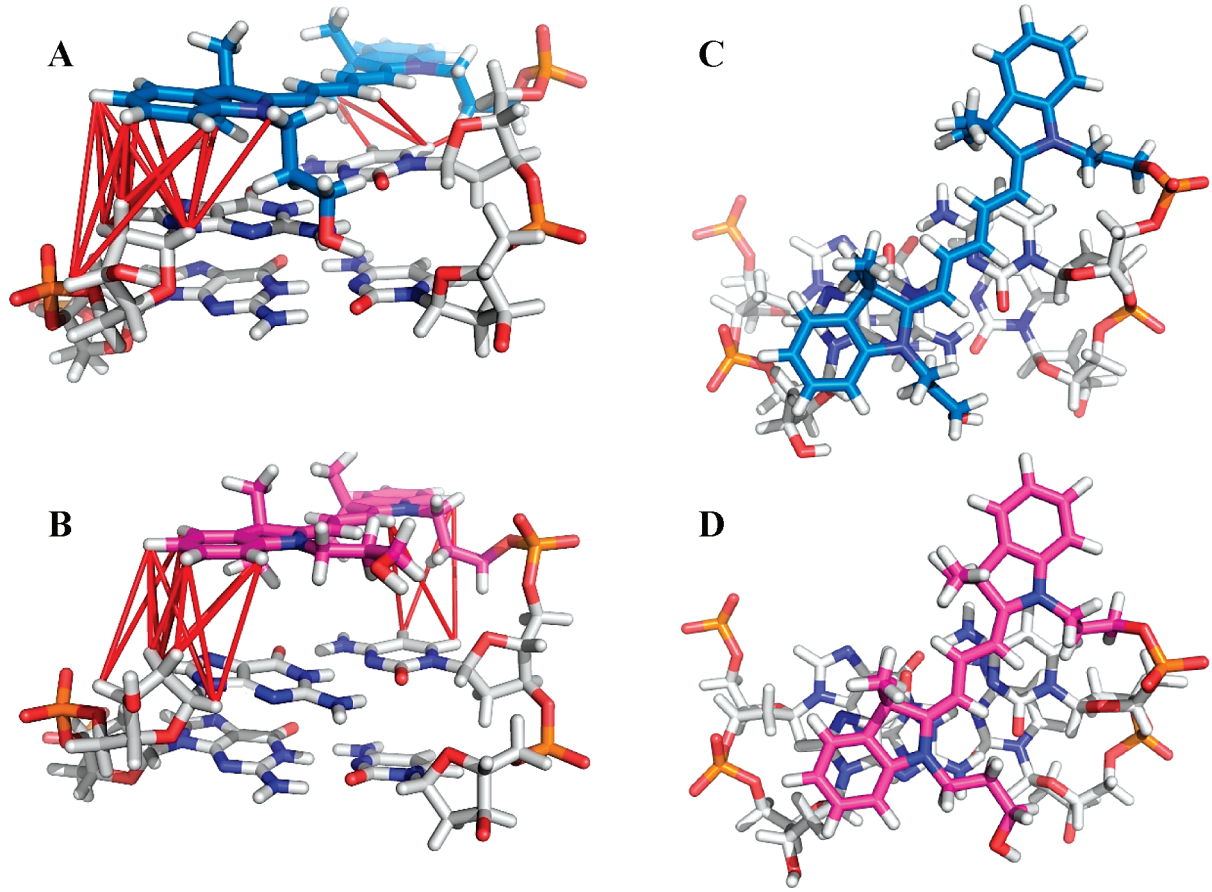

FIGURE 3: The average minimum structures for the terminal region of the (A) Cy5 adducted DNA and (B) Cy3 adducted DNA, showing NOE interactions as red sticks. Only a subset of all observed NOE interactions is shown to increase clarity. The position and stacking of fluorophores on the two terminal base pairs for (C) the Cy5 DNA structure and (D) the Cy3 DNA structure.

is almost certainly a dynamic system. The average structure probably represents quite accurately the mean position and orientation of the stacked fluorophore. However, any structure in which the fluorophore is lifted away from the DNA would fail to contribute to the observed DNA-Cy3 cross peaks. This, together with the absence of NOE interactions on the outer face of the fluorophore, would lead to a poor definition of partially stacked structures. As in the case of the Cy3 structure, the imino proton resonances of the terminal base pairs exhibited decreased intensity relative to the nonterminal imino protons, and no NOE cross peaks either to neighboring DNA imino protons or to the fluorophore. The implication of this observation is that the fluorophore position on top of the DNA helix must have some dynamic character, allowing proton exchange to occur between the terminal imino protons and water. Recent time-resolved fluorescence measurements of Cy3 attached to the 5′ end of duplex DNA show that approximately 17% of the Cy3 emission corresponds to a short lifetime that can be attributed to an unstacked form of the fluorophore (*36, 38*).

Calculation of the Transition Dipole Moments for Cy3 and Cy5. The direction of the transition dipole moments for the first electronic transition of Cy3, when in the equilibrium geometry of its first electronic excited state, and of Cy5, when in the equilibrium geometry of its ground electronic state, was found to lie along the polymethyne chain, as shown in Figure 4. Calculations performed using a C_3 linker joined to a phosphate group at one side showed the dipole vector pointing toward the phosphate linker side in both cases.

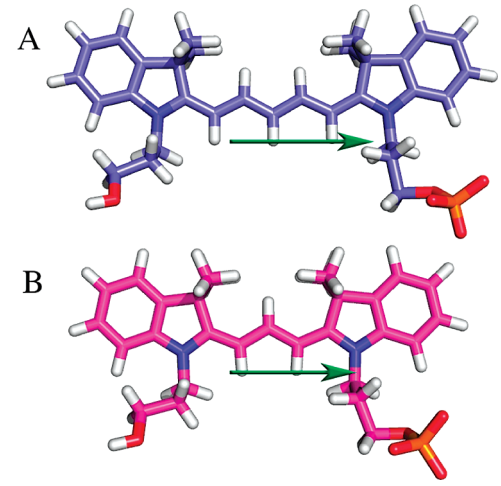

FIGURE 4: Directions of transition dipole moments (green arrow) for (A) Cy5 absorption and (B) Cy3 emission, calculated at the CIS/6-31++G(d,p) level. Cy3 is in the optimized geometry of its first electronic excited state. Cy5 is in the optimized geometry of its ground state.

Comparison between Cy5 and Cy3 DNA Structures. The structure determined for Cy5-DNA bears a close resemblance to that of Cy3-DNA. There are however some significant differences between the two structures. The distal part of the Cy5 fluorophore (specifically the indole ring numbered 1−9) occupies a position almost identical to that occupied by the distal portion of the previously studied Cy3. This indole ring is stacked on the base of the terminal G residue, with the three-carbon pseudolinker projecting away on the

minor groove side and the two methyl groups on the major groove side, making van der Waals contacts with the purine ring. Because the distal rings of Cy5 and Cy3 occupy similar positions, the positions of the proximal indole rings differ somewhat as a consequence of the extra length of the 5-carbon conjugated linker of Cy5, relative to the 3-carbon linker of Cy3. In Cy3 the proximal indole ring partially stacks on the terminal cytidine, though prevented from optimal stacking by the downward-projecting methyl group. In the case of Cy5, the proximal indole ring is displaced outward from the center of the DNA helix by approximately a further 4 Å compared to Cy3. The effect of moving the proximal indole ring out from stacking on the terminal cytidine residue can be seen in the observed chemical shifts both in the indole and in the terminal cytidine protons. A significant consequence of the Cy5-DNA structure is that the methyl groups of the proximal indole are displaced away from the terminal base pair, allowing the fluorophore to stack onto the DNA more closely than is possible for Cy3, with the conjugated linker making close van der Waals contacts across the terminal cytosine ring.

Implications for FRET Distance Measurement Using Cy3-Cy5. The NMR structure of Cy5 attached to DNA provides a point of reference when interpreting distance information obtained form FRET studies. Thus the local positions of both fluorophores are known when using the Cy3-Cy5 combination attached to duplex DNA in the manner described here.

However, the structure of these fluorophores introduces a potentially significant complication into the interpretation of distance information from FRET. The relationship between FRET efficiency (E_{FRET}) and the interfluorophore separation (R) is given by

$$E_{FRET} = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6} \quad (1)$$

where R_0 is given by $R_0^6 = 8.79 \times 10^{-5}[\kappa^2 \phi_D J(\lambda) n^{-4}]$, where κ^2 is the orientation factor, $J(\lambda)$ is the spectral overlap integral, ϕ_D is the quantum yield of the donor and n is the refractive index of the medium. Dipolar coupling depends on the relative orientation of the transition dipole moments of the FRET pairs, measured by κ^2 and so in principle R_0 will not be independent of orientation. If the fluorophores are flexible such that they reorient rapidly within the lifetime of the excited state of the donor, then κ^2 takes a constant, average value of 2/3 (*37*), and for fluorophores such as fluorescein this is a good approximation. However our NMR data, coupled with fluorescence lifetime information, suggest that a substantial fraction of the cyanine fluorophores are stacked onto the end of the helix. If this interaction fixes the relative orientation of the fluorophores rigidly, the simple relationship between distance and E_{FRET} no longer holds, and R_0 will be orientation dependent. If the planes of Cy3 and Cy5 are nearly parallel to each other and the fluorophores are coaxial, then $\kappa^2 = \cos^2 \theta$, where θ is the angle between the transition moments. The distance dependence of E_{FRET} will be modulated by the cosine function, and the efficiency will fall to zero when the dipoles are mutually perpendicular. In a separate study we have confirmed this modulation for FRET between Cy3 and Cy5 terminally attached to a series of DNA duplexes of different length, but shown that the effect is somewhat averaged by dynamic, probably lateral, motion of the fluorophores on the end of the helix (*36*).

CONCLUSION

Our NMR data have shown that Cy5 (like Cy3) that is attached at the 5′-terminus of duplex DNA becomes stacked onto the end of the helix, in the manner of an additional base pair. Knowledge of the position of the fluorophore will aid in the interpretation of FRET-derived distance measurements. However, the stacked location of the cyanine fluorophores suggests that alignment of the transition dipole moments could complicate the interpretation of FRET efficiency information. Although the orientation dependence may be lessened by flexibility both in the DNA helix and in the interaction between the cyanine dyes and the terminal base pairs, ignoring the effect could lead to significant errors in the estimation of either absolute or relative distance information. On the other hand the structural information detailed in this paper may allow the angular dependence to be used to provide valuable information regarding the underlying structure.

SUPPORTING INFORMATION AVAILABLE

Chemical shifts assignments (Table S1). Optimized coordinates for Cy3 and Cy5. Average minimized coordinates for hybrid Cy3 and Cy5 DNA. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Stryer, L., and Haugland, R. P. (1967) Energy transfer: a spectroscopic ruler. *Proc. Natl. Acad. Sci. U.S.A. 58*, 719–726.
2. Clegg, R. M. (1992) Fluorescence resonance energy transfer and nucleic acids. *Methods Enzymol. 211*, 353–388.
3. Clegg, R. M. (1996) Fluorescence resonance energy transfer, in *Fluorescence imaging spectroscopy and microscopy* (Wang, X. F., and Herman, B., Eds.) pp 179−252, John Wiley & Sons, New York.
4. Tuschl, T., Gohlke, C., Jovin, T. M., Westhof, E., and Eckstein, F. (1994) A three-dimensional model for the hammerhead ribozyme based on fluorescence measurements. *Science 266*, 785–789.
5. Clegg, R. M., Murchie, A. I., and Lilley, D. M. (1994) The solution structure of the four-way DNA junction at low-salt conditions: a fluorescence resonance energy transfer analysis. *Biophys. J. 66*, 99–109.
6. Bassi, G. S., Murchie, A. I., Walter, F., Clegg, R. M., and Lilley, D. M. (1997) Ion-induced folding of the hammerhead ribozyme: a fluorescence resonance energy transfer study. *EMBO J. 16*, 7481–7489.
7. Stuhmeier, F., Welch, J. B., Murchie, A. I., Lilley, D. M., and Clegg, R. M. (1997) Global structure of three-way DNA junctions with and without additional unpaired bases: a fluorescence resonance energy transfer analysis. *Biochemistry 36*, 13530–13538.
8. Murchie, A. I., Thomson, J. B., Walter, F., and Lilley, D. M. (1998) Folding of the hairpin ribozyme in its natural conformation achieves close physical proximity of the loops. *Mol. Cell 1*, 873–881.
9. Walter, F., Murchie, A. I., Duckett, D. R., and Lilley, D. M. (1998) Global structure of four-way RNA junctions studied using fluorescence resonance energy transfer. *RNA 4*, 719–728.
10. Murchie, A. I., Clegg, R. M., von Kitzing, E., Duckett, D. R., Diekmann, S., and Lilley, D. M. (1989) Fluorescence energy transfer shows that the four-way DNA junction is a right-handed cross of antiparallel molecules. *Nature 341*, 763–766.
11. Gohlke, C., Murchie, A. I., Lilley, D. M., and Clegg, R. M. (1994) Kinking of DNA and RNA helices by bulged nucleotides observed by fluorescence resonance energy transfer. *Proc. Natl. Acad. Sci. U.S.A. 91*, 11660–11664.
12. Jares-Erijman, E. A., and Jovin, T. M. (1996) Determination of DNA helical handedness by fluorescence resonance energy transfer. *J. Mol. Biol. 257*, 597–617.

13. Mergny, J. L. (1999) Fluorescence energy transfer as a probe for tetraplex formation: the i-motif. *Biochemistry 38*, 1573–1581.
14. Grainger, R. J., Norman, D. G., and Lilley, D. M. (1999) Binding of U1A protein to the 3′ untranslated region of its pre-mRNA. *J. Mol. Biol. 288*, 585–594.
15. Lilley, D. M. J., and Wilson, T. J. (2000) Fluorescence resonance energy transfer as a structural tool for nucleic acids. *Curr. Opin. Chem. Biol. 4*, 507–517.
16. Clegg, R. M. (2002) FRET tells us about proximities, distances, orientations and dynamic properties. *J. Biotechnol. 82*, 177–179.
17. Bokinsky, G., Rueda, D., Misra, V. K., Rhodes, M. M., Gordus, A., Babcock, H. P., Walter, N. G., and Zhuang, X. (2003) Single-molecule transition-state analysis of RNA folding. *Proc. Natl. Acad. Sci. U.S.A. 100*, 9302–9307.
18. Hodak, J. H., Fiore, J. L., Nesbitt, D. J., Downey, C. D., and Pardi, A. (2005) Docking kinetics and equilibrium of a GAAA tetraloop-receptor motif probed by single-molecule FRET. *Proc. Natl. Acad. Sci. U.S.A. 102*, 10505–10510.
19. Karymov, M., Daniel, D., Sankey, O. F., and Lyubchenko, Y. L. (2005) Holliday junction dynamics and branch migration: Single-molecule analysis. *Proc. Natl. Acad. Sci. U.S.A. 102*, 8186–8191.
20. McKinney, S. A., Declais, A.-C., Lilley, D. M. J., and Ha, T. (2003) Structural dynamics of individual Holliday junctions. *Nat. Struct. Biol. 10*, 93–97.
21. Nahas, M. K., Wilson, T. J., Hohng, S., Jarvie, K., Lilley, D. M. J., and Ha, T. (2004) Observation of internal cleavage and ligation reactions of a ribozyme. *Nat. Struct. Mol. Biol. 11*, 1107–1113.
22. Ha, T. (2001) Single-molecule fluorescence methods for the study of nucleic acids. *Curr. Opin. Struct. Biol. 11*, 287–292.
23. Mollova, E. T. (2002) Single-molecule fluorescence of nucleic acids. *Curr. Opin. Chem. Biol. 6*, 823–828.
24. McKinney, S. A., Tan, E., Wilson, T. J., Nahas, M. K., Declais, A. C., Clegg, R. M., Lilley, D. M. J., and Ha, T. (2004) Single-molecule studies of DNA and RNA four-way junctions. *Biochem. Soc. Trans. 32*, 41–45.
25. Rasnik, I., McKinney, S. A., and Ha, T. (2005) Surfaces and orientations: Much to FRET about? *Acc. Chem. Res. 38*, 542–548.
26. Norman, D. G., Grainger, R. J., Uhrin, D., and Lilley, D. M. (2000) Location of cyanine-3 on double-stranded DNA: importance for fluorescence resonance energy transfer studies. *Biochemistry 39*, 6317–6324.
27. Beaucage, S. L., and Caruthers, M. H. (1981) Deoxynucleoside phosphoramidites. A new class of key intermediates for deoxypolynucleotide synthesis. *Tetrahedron Lett. 22*, 1859–1862.
28. Marion, D., Ikura, M., Tschudin, R., and Bax, A. (1989) *J. Magn. Reson. 85*, 393–399.
29. Jeener, J., Meier, B. H., Bachmann, P., and Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546–4553.
30. Rance, M., Sorensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479–485.
31. Piotto, M., Saudek, V., and Sklenar, V. (1992) Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *J. Biomol. NMR 2*, 661–665.
32. Schwieters, C. D., Kuszewski, J. J., Tjandra, N., and Clore, G. M. (2003) The Xplor-NIH NMR molecular structure determination package. *J. Magn. Reson. 160*, 65–73.
33. Schwieters, C. D., Kuszewski, J. J., and Clore, G. M. (2006) Using Xplor-NIH for NMR molecular structure determination. *Prog. Nucl. Magn. Reson. Spectrosc. 48*, 47–62.
34. Schuttelkopf, A. W., and van Aalten, D. M. (2004) PRODRG: a tool for high-throughput crystallography of protein-ligand complexes. *Acta Crystallogr., Sect. D: Biol. Crystallogr. 60*, 1355–1363.
35. Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., Montgomery, J. A., Jr., Vreven, T., Kudin, K. N., Burant, J. C., Millam, J. M., Iyengar, S. S., Tomasi, J., J., Barone, V., Mennucci, B., Cossi, M., Scalmani, G., Rega, N., Petersson, G. A., Nakatsuji, H., Hada, M., Ehara, M., Toyota, K., Fukuda, R., Hasegawa, J., Ishida, M., Nakajima, T., Honda, Y., Kitao, O., Nakai, H., Klene, M., Li, X., Knox, J. E., Hratchian, H. P., Cross, J. B., Adamo, C., Jaramillo, J., Gomperts, R., Stratmann, R. E., Yazyev, O., Austin, A. J., Cammi, R., Pomelli, C., Ochterski, J. W., Ayala, P. Y., Morokuma, K., Voth, G. A., Salvador, P., Dannenberg, J. J., Zakrzewski, V. G., Dapprich, S., Daniels, A. D., Strain, M. C., Farkas, O., Malick, D. K., Rabuck, A. D., Raghavachari, K., Foresman, J. B., Ortiz, J. V., Cui, Q., Baboul, A. G., Clifford, S., Cioslowski, J., Stefanov, B. B., Liu, G., Liashenko, A., Piskorz, P., Komaromi, I., Martin, R. L., Fox, D. J., Keith, T., Al-Laham, M. A., Peng, C. Y., Nanayakkara, A., Challacombe, M., Gill, P. M. W., Johnson, B., Chen, W., Wong, M. W., Gonzalez, C. and Pople, J. A. (2003) *Gaussian 03*, Gaussian, Inc., Pittsburgh, PA.
36. Iqbal, A., Arslan, S., Okumus, B., Wilson, T. J., Giraud, G., Norman, D. G., Ha, T., and Lilley, D. M. J. (2008) Orientation dependence in fluorescent energy transfer between Cy3 and Cy5 terminally-attached to double-stranded nucleic acids. *Proc. Natl. Acad. Sci. U.S.A.*, in press.
37. Dale, R. E., Eisinger, J., and Blumberg, W. E. (1979) The orientational freedom of molecular probes. The orientation factor in intramolecular energy transfer. *Biophys. J. 26*, 161–193.
38. Sanborn, M. E., Connolly, B. K., Gurunathan, K., and Levitus, M. (2007) Fluorescence properties and photophysics of the sulfoindocyanine Cy3 linked covalently to DNA. *J. Phys. Chem. B, 111*, 11064–11074.

BI800773F