Fluorescence Resonance Energy Transfer as a Probe for G-Quartet Formation by a Telomeric Repeat

Jean-Louis Mergny*[a] and Jean-Claude Maurizot[b]

The secondary structure of guanine-rich oligodeoxynucleotides has been investigated with fluorescent probes. Intramolecular folding of a telomeric oligonucleotide into a quadruplex led to fluorescence resonance energy transfer (FRET) between a donor (fluorescein) and an acceptor (tetramethylrhodamine) covalently attached to the 5′ and 3′ ends of the DNA, respectively. Depending on oligonucleotide length, quenching efficiency varied between 0.45 and 0.72 at 20 °C. The conjugation of the dyes to the oligonucleotide had a limited, but significant, influence on the thermodynamics of G-quartet formation. Intramolecular folding was demonstrated from the concentration independence of fluorescence resonance energy transfer over a wide concentration range. Folding of the oligonucleotide was confirmed by UV absorption, UV melting, and circular dichroism experiments. The folding of the G-quartet could be followed at concentrations as low as 100 pM. Fluorescence resonance energy transfer can thus be used to reveal the formation of multistranded DNA structures.

KEYWORDS:

DNA structures · dyes · fluorescence spectroscopy · G-quartet · oligonucleotides

Introduction

Fluorescence resonance energy transfer (FRET) is a dipole – dipole resonance interaction between two "close" molecules, where one molecule, called the "donor", transfers its excitation energy to the other molecule, called the "acceptor". FRET has given valuable information on the structure of various nucleic acids,[1–7] because of its distance and orientation dependence.[8] Concerning multistranded structures, FRET has been succesfully applied to triple helices,[9–12] C-rich quadruplexes[13] (the so-called i motif), and a G-rich repeat of the protooncogene c-*myc*.[14] The present study was undertaken to determine the potential use of derivatized oligonucleotides as probes for a telomeric G-rich quadruplex nucleic acid structure by the technique of fluorescence energy transfer.

It is well known that guanine-rich oligodeoxynucleotides or polynucleotides may adopt a non-B-helical structure called the G-quartet.[15–17] In previous studies, we have determined the stability of the folded form(s) of different single-stranded oligodeoxynucleotides by using UV absorbance melting experiments.[18] We wanted to investigate whether fluorescence spectroscopy could provide useful information on the folding of a guanine-rich oligodeoxynucleotide. In the present study, we report that FRET can be used to probe the secondary structure of a guanosine-rich DNA fragment, provided a fluorescein molecule (donor) and a rhodamine derivative (acceptor) are attached to the 5′ and 3′ ends of the oligonucleotide, respectively. We chose to analyze short oligodeoxynucleotides mimicking repeats of the guanine-rich strand of vertebrate telomeres with four repeats of three guanines (Figure 1). A 22-base-long, closely related oligodeoxynucleotide was previously shown to form a G-quartet, as demonstrated by 2D NMR spectroscopy.[19] It is interesting to note that the terminal 3′ extremities of human chromosomes are prone to exhibiting unusual conformations.[20, 21]

Results

Fluorescence properties of the conjugated dyes

The spectroscopic properties of fluorescein (F) and tetramethylrhodamine (tamra, T) were affected by covalent linkage to the oligonucleotide. Maximum absorption and excitation wavelength for the 21-mer fluorescein conjugate was 496 nm, with an emission maximum at 519 nm, and a quantum yield (0.4, determined with fluorescent standards) that was lower than that of free fluorescein. The oligonucleotide linked to tamra had maximum excitation (561 nm) and emission (579 nm) wavelengths very similar to those of the protein-conjugated dye (555 and 580 nm, as given by the manufacturer). It should be noted

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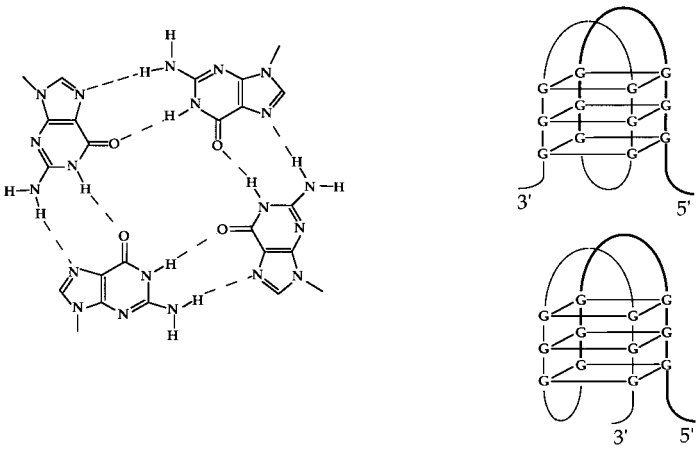

Oligonucleotide name	Sequence (5′ → 3′)
21	GGGTTAGGGTTAGGGTTAGGG
F21	fluo-GGGTTAGGGTTAGGGTTAGGG
21T	GGGTTAGGGTTAGGGTTAGGG-tamra
F21T	fluo-GGGTTAGGGTTAGGGTTAGGG-tamra
23	**TT**GGGTTAGGGTTAGGGTTAGGG
F23	fluo-**TT**GGGTTAGGGTTAGGGTTAGGG
23T	**TT**GGGTTAGGGTTAGGGTTAGGG-tamra
F23T	fluo-**TT**GGGTTAGGGTTAGGGTTAGGG-tamra
26	**TTTTA**GGGTTAGGGTTAGGGTTAGGG
F26	fluo-**TTTTA**GGGTTAGGGTTAGGGTTAGGG
26T	**TTTTA**GGGTTAGGGTTAGGGTTAGGG-tamra
F26T	fluo-**TTTTA**GGGTTAGGGTTAGGGTTAGGG-tamra
FconT	fluo-TTTTTTTTTTTTTTTTTTTTT-tamra

Figure 1. *Top: Schematic representation of a G-quartet (left) and two possible conformations of an intramolecular tetraplex (right). Bottom: List of the oligonucleotides used in the present study. Abbreviations used for the fluorescent oligonucleotides 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. fluo = fluorescein; tamra = tetramethylrhodamine.*

that the molar extinction coefficient of fluorescein at λ_{max} in the visible range was decreased by 20% as compared to that of free fluorescein ($\varepsilon = 4.1 \times 10^4\,\text{M}^{-1}\,\text{cm}^{-1}$) upon covalent linkage. Such a decrease has already been described for fluorescein conjugated to an oligonucleotide.[5] The properties of the fluorescein-conjugated oligonucleotides were also strongly dependent on pH[22] and a strong quenching was observed below pH 7 (data not shown). The spectral properties of both dyes conjugated to guanine-rich oligonucleotides were similar, but not identical, to the properties of the same molecules attached to cytosine-rich oligomers.[13]

UV melting studies with the 21- and 23-mers

We first confirmed that covalent attachment of the dyes did not prevent G-quartet formation of the 21-mer used in this study. A "parent" oligonucleotide (22-mer, 5′-d-AGGGTTAGGGTTAGGGTTAGGG) was previously shown to fold into a G-quartet structure by NMR spectroscopy, and UV melting experiments provided a good test to measure the stability of the structure. Therefore, T_m measurements were performed for the oligomers **21**, **F21**, **21T**, and **F21T** at pH 7.0 (see Table 1). As shown in Figure 2A, 260 nm is a poor wavelength to follow G-quartet formation, as reported previously.[18] It was possible to provide evidence for G-quartet dissociation by recording the absorbance at 295 nm (Figure 2B). In agreement with a previous study,[18] G-quartet formation induces an increase in absorbance at this wavelength. The induced hyperchromisms for **21**, **F21**, **21T**, and **F21T** were similar, but the melting temperatures were different. The T_m values of **21** and **21T** were close to each other (58.5 and 57.5 °C, respectively), whereas those of **F21** and **F21T** were shifted towards lower temperatures (50.5 and 47.0 °C, respectively), showing that the presence of a fluorescein group at the 5′ end had a destabilizing effect on G-quartet formation. From the shape of the melting curves one could determine a ΔH° value of $-201\,\text{kJ mol}^{-1}$ for **21**, as compared to -167 and $-192\,\text{kJ mol}^{-1}$ for **F21** and **21T**, respectively. The ΔH° value of the doubly substituted 21-mer (**F21T**) was even less favorable ($-150\,\text{kJ mol}^{-1}$, Table 1). It was also possible to determine the ΔG° value of quadruplex formation at 37 °C for all oligomers: These values, which were negative in all cases, were -12.5, -6.7, -11.3, and $-3.8\,\text{kJ mol}^{-1}$ for **21**, **F21**, **21T**, and **F21T**, respectively. Covalent addition of a rhodamine had hardly any effect (a difference of $1.3\,\text{kJ mol}^{-1}$ was not significant) whereas the fluorescein dye destabilized the quartet structure by $5.9\,\text{kJ mol}^{-1}$. This destabilization could be the result of the presence of a negative charge on the conjugated fluorescein dye: We have observed that the sole presence of a negatively charged terminal phosphate group at the 5′ end of a quadruplex also had a destabilizing effect. Nevertheless, G-quartet formation was favored at 37 °C for all oligonucleotides.

Figures 2C and D show the absorbance vs. temperature profiles at two other wavelengths in the visible region, close to

Table 1. *Thermodynamic parameters for the melting of the G-quadruplex structures.*

Oligonucleotide	Modification[a]	T_m [°C][b]	ΔH° [kJ mol^{-1}][b]	T_m [°C][c]
21	none	58.5	−201	–[d]
F21	5′-fluo	50.5	−167	–[d]
21T	3′-tamra	57.5	−192	–[d]
F21T	5′-fluo, 3′-tamra	47.0	−150	54.0 (0.72)
F23T	5′-fluo, 3′-tamra	45.0	−159	49.0 (0.6)
26	none	45.0	−163	–[d]
F26T	5′-fluo, 3′-tamra	39.0	−146	43.0 (0.45)

[a] 5′-fluo: A fluorescein moiety is covalently attached to the 5′ end of the oligonucleotide; 3′-tamra: a tetramethylrhodamine moiety is covalently attached to the 3′ end of the oligonucleotide. [b] T_m and ΔH° values were determined from UV melting experiments in a 10 mM sodium cacodylate buffer (pH 7.5) containing 0.1 M NaCl. [c] The T_m of doubly substituted fluorescent oligonucleotides was estimated from the analysis of the fluorescence emission at 515 nm as a function of temperature. The quenching of fluorescence emission at 515 nm (value at 20 °C compared to that at 80 °C) is indicated in parentheses. [d] Not applicable.

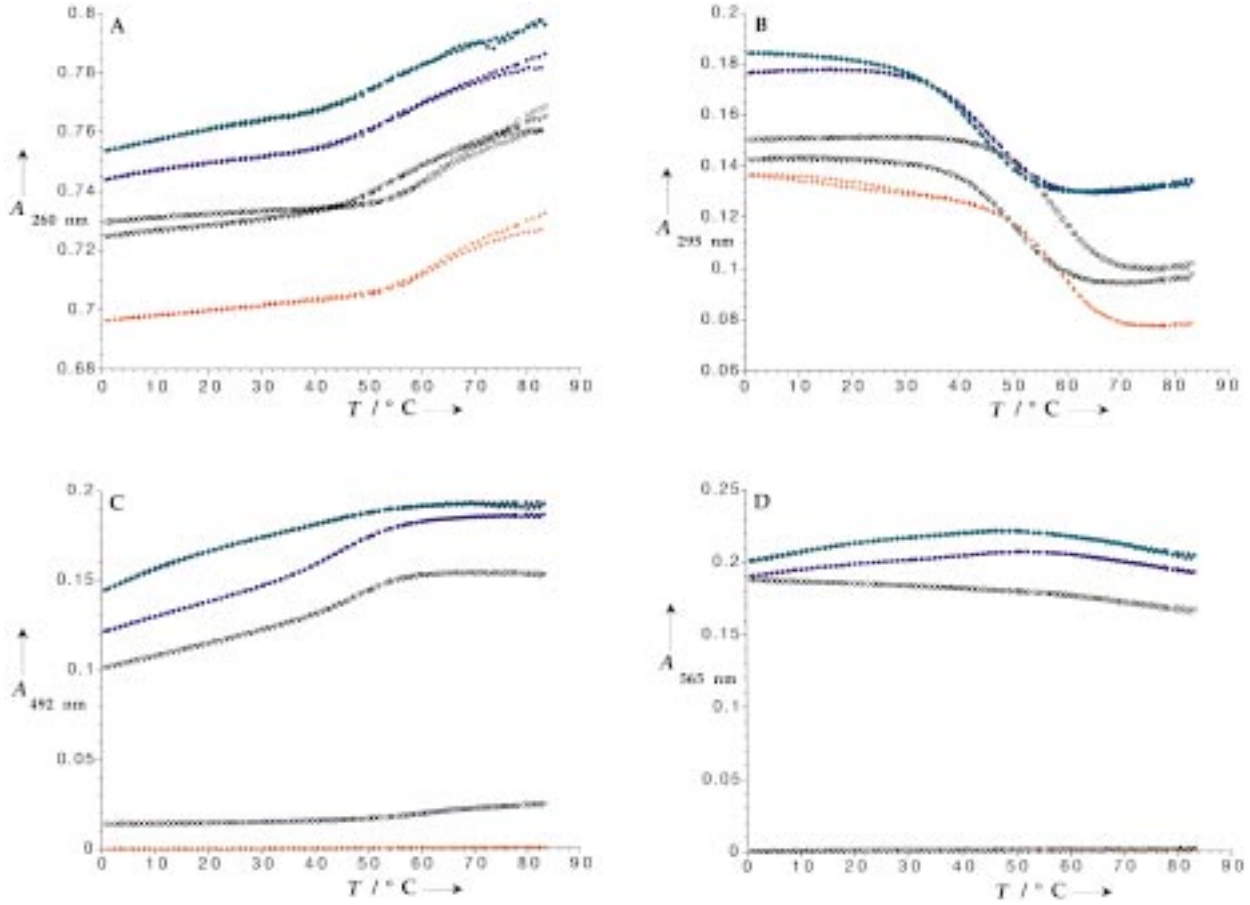

Figure 2. *UV denaturation profiles of **21** (red crosses), **F21** (black triangles), **21T** (black circles), **F21T** (blue squares), and **F23T** (green diamonds) in a 10 mM cacodylate buffer (pH 7.0) containing 0.1 M NaCl (4 μM strand concentration). A, B, C, D: Absorbance recorded at 265, 295, 492, and 565 nm, respectively.*

the absorbance maxima for fluorescein (492 nm, Figure 2C) and tamra (565 nm, Figure 2D). The unsubstituted 21-mer **21** does not absorb light in the visible region (crosses). The oligonucleotide **21T** shows a monotonous variation of absorbance at these two wavelengths (open circles). The absorbance of **F21** at 492 nm (Figure 2C, triangles) is somewhat affected by G-quartet formation. These absorbance measurements showed that, upon G-quartet formation, a significant hypochromism (15 – 20%) was observed for the fluorescein dye (in both **F21** and **F21T**) but not for the rhodamine dye.

CD spectroscopy

Circular dichroism (CD) spectroscopy can be used to distinguish between parallel and antiparallel G-quartet structures. The CD spectrum of the unsubstituted oligomer **21** (Figure 3, crosses) exhibited a positive band at 298 nm and a strong negative band at 266 nm, characteristic of an antiparallel G-quartet structure.[23–26] The intensity of the 298-nm band decreased when heating the sample over 50 °C (data not shown).

The presence of a single fluorescent group such as fluorescein at the 5′ end of **F21** and tamra at the 3′ end of **21T** had little effect on the spectrum of the oligomer in the UV region (Figure 3A, circles and triangles), showing that the presence of one dye did not significantly alter the overall structure of the quadruplex. This is in contrast to the spectrum of the doubly labelled oligomer **F21T**, which is characterized by the appearance of a strong positive band around 262 nm (solid line). The alteration of the CD spectrum indicated that either the quadruplex was disrupted and a new structure was formed, or that the dyes, which also absorb light in this region, interact with each other and/or with the quadruplex structure. The fact that the spectrum of **F21T** also exhibited a circular dichroism in both the fluorescein and the tamra region supports the latter hypothesis (Figure 3B). Altogether, these observations demonstrate that the simultaneous presence of both fluorophores allows new interactions to take place. If the CD changes observed both in the UV and visible regions were solely the result of the interactions between the dyes and the quadruplex, one would expect that the spectral modifications of **F21T** vs. **21** would be the sum of the modifications of **21T** and **F21**. This suggests that a significant contribution to the CD changes results from direct interaction between the two dyes, or that the G-quadruplex structure is strongly perturbed by the double labeling.

This phenomenon was much less pronounced in the case of **F23T** (Figures 3A and B, dashed lines), which was closer, but not completely identical, to the spectrum of a classical antiparallel

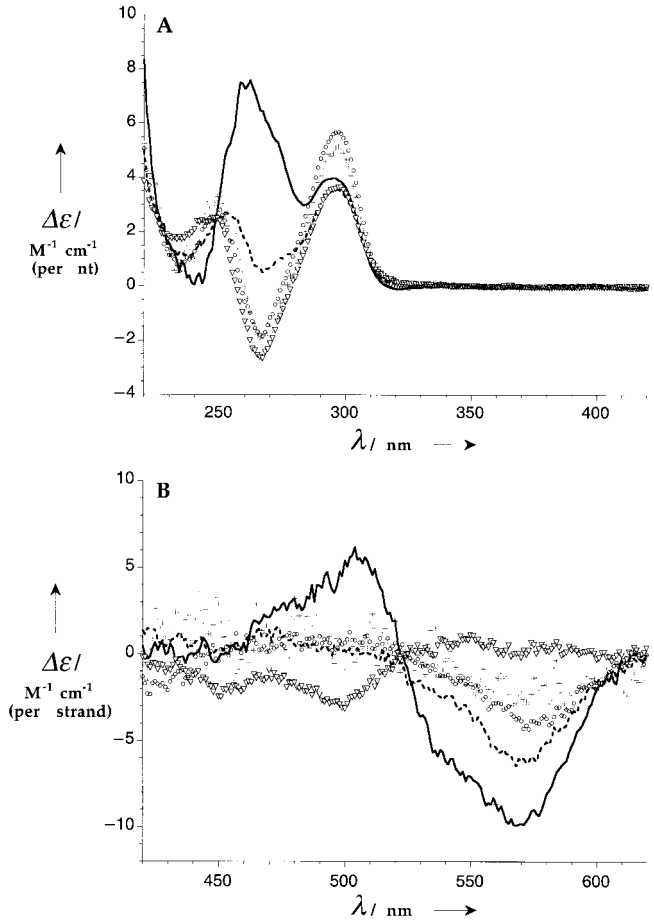

Figure 3. *CD spectra of **21** (crosses), **F21** (triangles), **21T** (circles), **F21T** (solid line), and **F23T** (dashed line) in a 10 mM cacodylate buffer (pH 7.0) containing 0.1 M NaCl (4 μM strand concentration) at 20 °C. A: Spectrum recorded between 220 and 420 nm (Δε is expressed per nucleotide). B: Spectrum recorded between 420 and 620 nm (Δε is expressed per strand).*

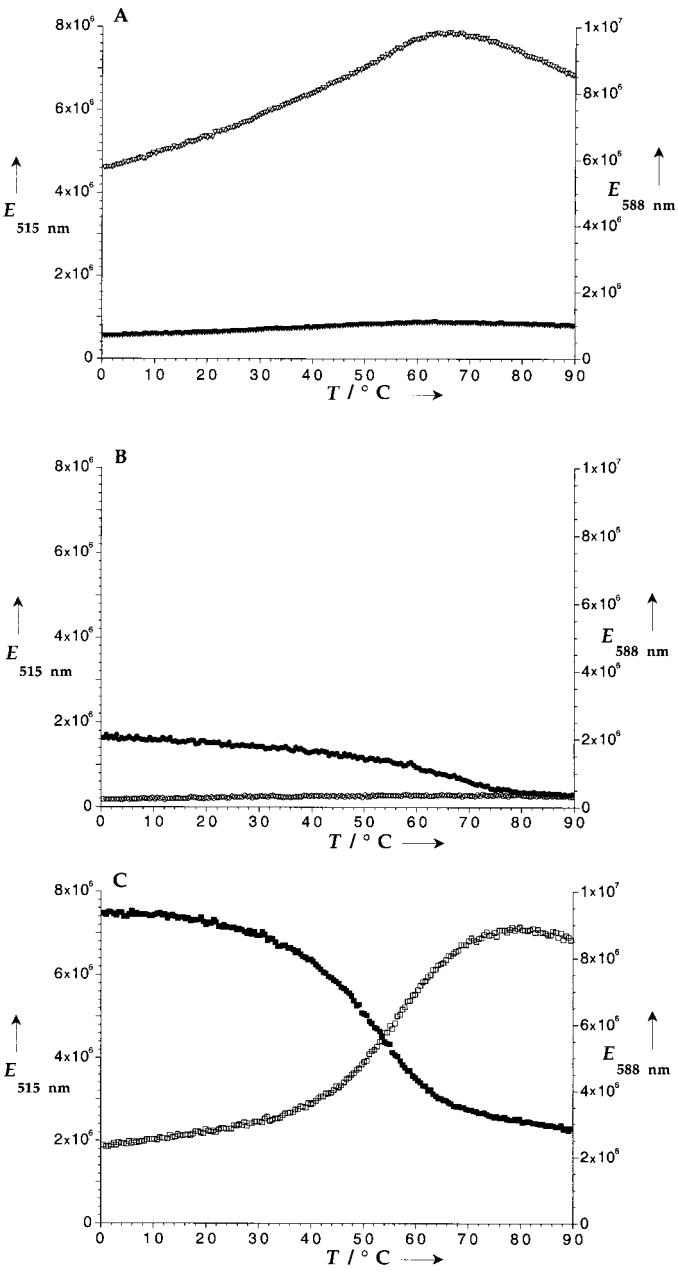

Figure 4. *Fluorescence vs. temperature measurements for different oligonucleotides in a 10 mM cacodylate buffer (pH 7.0) containing 0.1 M NaCl (0.2 μM strand concentration). Excitation was set at 480 nm, and emission was recorded at two different wavelengths, 515 and 588 nm. The temperature of the water bath was increased or decreased at a rate of 0.25 °C min⁻¹. A: **F21** (515 nm: open triangles; 588 nm: filled triangles). B: **21T** (515 nm: open circles; 588 nm: filled circles). C: **F21T** (515 nm: open squares; 588 nm: filled squares).*

G-quartet. Therefore, the presence of two extra bases at the 5′ end of the oligonucleotide largely prevented the occurence of the phenomenon observed with **F21T**.

FRET measurements with the 21-mers

In the unfolded form, little transfer is expected, as the average distance of the two chromophores should be larger than the critical Förster distance (calculated to be around 5.0 nm). Intramolecular folding should bring the two chromophores in close enough proximity for energy transfer to be observed. Therefore, FRET should be a convenient method to monitor the 3′-to-5′-end distance.

We performed fluorescence measurements as a function of temperature for the **F21** and **F21T** oligomers. As shown in Figure 4 A, when excitation was set at 492 nm, a decrease in fluorescence emission at 515 nm was observed at low temperature for **F21**. The shape of this fluorescence vs temperature profile was very similar to the absorbance at 488 nm vs temperature profile (Figure 2 C, triangles). G-quartet formation induces a 25% quenching of fluorescence. Upon excitation at 470 nm, no emission at 515 nm was recorded for **21T** (Figure 4 B, circles), whereas a reduced, slightly temperature-dependent emission at 588 nm was observed. This signal is the result of a direct excitation of the rhodamine dye. Although 470 nm was chosen as the excitation wavelength to minimize the excitation of the rhodamine, this phenomenon could not be totally avoided.

The temperature dependence of the **F21T** oligomer emission at 515 nm was even more pronounced, and a 72% quenching of

fluorescence was observed. This quenching was in part the result of a decrease in fluorescein absorbance (15 – 20%, Figure 2C): However, the decrease in fluorescein emission could not be solely explained by this hypochromism. Therefore, both fluorescein absorbance and fluorescence properties are altered in the presence of tamra. The emission profile at 588 nm followed an opposite trend as an increase in emission was observed at low temperature (Figure 4C, open squares). This result is in agreement with FRET from fluorescein to the rhodamine, although the CD spectra indicated that other phenomena, such as a direct interaction of the two dyes, play a role in the spectroscopic properties of these oligonucleotides. A parallel experiment, performed with a cytosine-rich oligomer led to a completely different result:[13] In that case a spacer of five bases was required to observe FRET.

Identical measurements were performed with a control oligonucleotide, **FconT** (see Figure 1), in which fluorescein and tamra are attached at the 5′ and 3′ ends, respectively. Monotonous variations of fluorescein and tamra emission were observed in the temperature range 0 – 80 °C, and no quenching of the emission of fluorescein was demonstrated, in agreement with an unfolded structure at all temperatures (data not shown).

Excitation energy transfer studies for the 23-mers

In the case of the 21-mers, fluorescein and tamra were directly attached to the 5′ and 3′ ends of the G-quartet structure. Folding of the oligonucleotide should lead to the juxtaposition of the two dyes, as suggested by CD spectroscopy. For this reason, we designed longer oligonucleotides having two extra bases at the 5′ end (**F23T**) to increase the distance between the two chromophores and prevent direct physical interaction.

Absorbance measurements at four different wavelengths (265, 295, 492, and 565 nm; only data for **F23T** are shown in Figure 2, diamonds) gave results similar to the melting curves presented in Figure 2 for the 21-mers: **F23T** has a slightly lower T_m value than **F21T** ($T_m = 45$ and 46.5 °C, respectively, deduced from the melting profiles at 295 nm shown in Figure 2C). Contrary to **F21T**, the absorbance at 492 nm and 565 nm of **F23T** was less affected by the formation of the G-quartet (Figures 2C, D, diamonds).

Fluorescence vs. temperature measurements were performed by simultaneously recording emission at 515 and 588 nm for **F23T** (Figure 5A). A 60% quenching of donor and an enhanced acceptor emission were observed at low temperature, and the T_m deduced from the fluorescence melting curves (49 °C) was in reasonable agreement with the T_m determined from the UV-absorbance melting curves (45 °C; Figure 2B, diamonds). The difference between these two values could be attributed to the differences in temperature measurement protocols. For technical reasons, the temperature indicated in UV-absorbance experiments, which is directly measured in the sample, is more accurate than the temperature of the fluorescence experiments (temperature of the cell holder). Therefore, fluorescence and absorbance melting curves reflect the same phenomenon; that is, the thermal denaturation of the G-quadruplex structure. The quenching of fluorescein emission was less pronounced in the case of the oligomer **F23T** (60%) than in the case of the oligonucleotide **F21T** (72%).

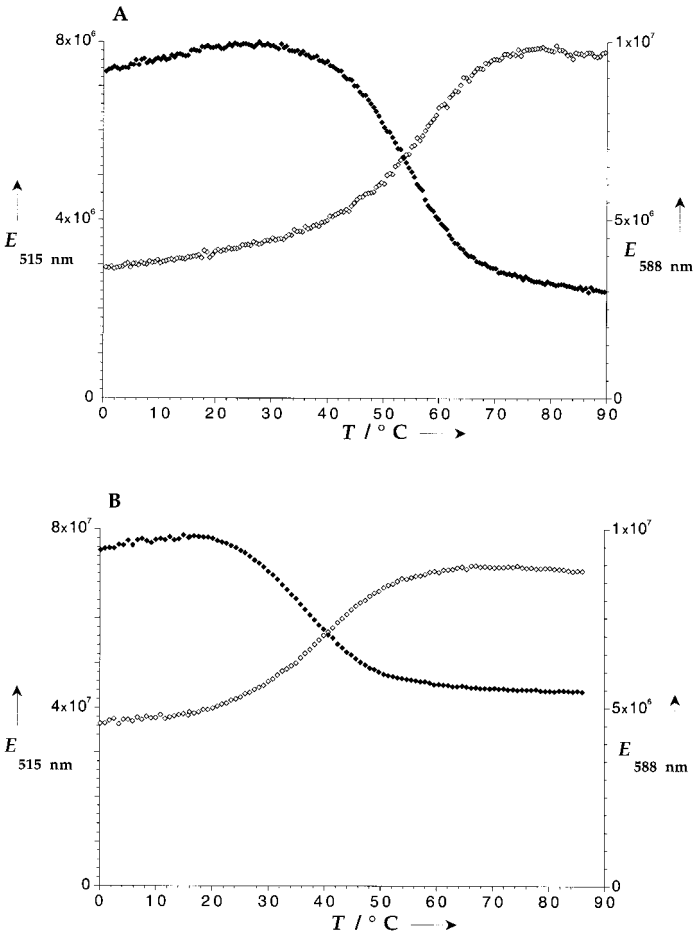

Figure 5. *A: Fluorescence vs. temperature measurements for **F23T** (515 nm: open diamonds; 588 nm: filled diamonds). B: Fluorescence vs. temperature measurements for **F26T** (515 nm: open diamonds; 588 nm: filled diamonds). All measurements were performed in a 10 mM cacodylate buffer (pH 7.0) containing 0.1 M NaCl (0.2 μM strand concentration). Excitation was set at 480 nm, and emission was recorded at two different wavelenghts, 515 and 588 nm. The temperature of the water bath was increased or decreased at a rate of 0.25 °C min^{-1}.*

Excitation energy transfer studies for the 26-mers

We then designed a longer oligonucleotide, with five extra bases at the 5′ end, to further increase the distance between the two chromophores and prevent direct physical interaction. Absorbance measurements at four different wavelengths (265, 295, 492, and 565 nm) were performed for the oligonucleotides **26**, **F26**, **26T**, and **F26T** (data not shown). The unsubstituted 26-mer had a lower stability than the parent 21-mer ($T_m = 45$ and 58.5 °C, respectively; see Table 1).[18] In other words, the addition of five extra bases at the 5′ end of the sequence had a deleterious impact on the stability of the G-quartet. However, the CD spectra of the unsubstituted 26-mer were similar to the spectra of the parent 21-mer. The addition of a fluorescein moiety at the 5′ end of the oligomer had a further destabilizing effect, in contrast to

rhodamine addition at the 3′ end, which did not seem to significantly affect the stability of the quadruplex. The CD spectrum of **F26T** was very similar to the spectrum of **F23T** (data not shown).

Fluorescence vs. temperature measurements were performed by simultaneously recording emission at 515 and 588 nm for the **F26T** oligomer (Figure 5B). As previously observed for **F21T** and **F23T**, the dissociation of the quadruplex was correlated with an increased emission at 515 nm and an opposite variation at 588 nm. A 45% quenching of donor and an enhanced acceptor emission were observed at low temperature. As for UV absorbance melting curves, these transitions were obtained at lower temperatures than for **F21T** (Figure 4C) and **F23T** (Figure 5A).

To demonstrate that FRET occurred effectively between the donor and the acceptor, we recorded the emission (Figure 6A) and excitation spectra (Figure 6B) of **F26** (triangles), **26T** (circles), and **F26T** (squares). The analysis of the emission spectra, after excitation at 480 nm, shows that the emission of **F26T** at 515 nm is strongly quenched, as compared to that of **F26**. In other words, the addition of a tamra group at the 3′ end of the 26-mer strongly quenches the emission of the fluorescein moiety. At longer wavelengths (around 588 nm), a relatively strong emission is observed for **F26T**. This peak may be explained only in part by a direct excitation at 480 nm of the tamra dye (see the emission of **26T** for comparison). The analysis of the excitation spectra (emission recorded at 588 nm) confirms that this enhanced emission is the result of the excitation of the fluorescein dye (compare the excitation spectra of **F26T** (squares) and **26T** (circles) in Figure 6B). Therefore, the emission peak at 588 nm is largely the result of FRET from fluorescein to tamra when excitation is in the fluorescence absorption band.

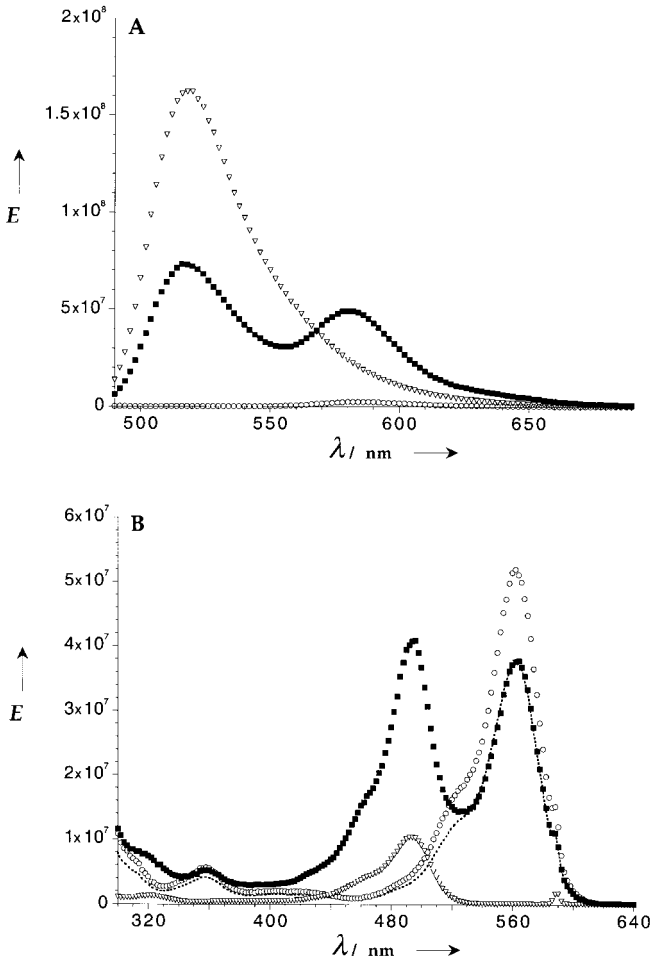

Figure 6. *Fluorescence spectra of* ***F26*** *(triangles),* ***26T*** *(circles), and* ***F26T*** *(squares) at 0.2 μM strand concentration in a 10 mM cacodylate buffer (pH 7.0) at 20 °C. The samples were equilibrated at 20 °C overnight before the measurements. A: Fluorescence emission spectra (excitation set at 480 nm). B: Fluorescence excitation spectra (emission set at 588 nm). The dotted line represents the contribution of tamra to the excitation spectrum of* ***F26T****.*

Effect of monovalent ions

It is known that the presence of various monovalent ions plays a role not only in the stability of the G-quadruplex structure, but also in the nature of the folded form (i.e., parallel or antiparallel).[27–32] We therefore wanted to test the influence of potassium to sodium replacement in our system. Absorption melting studies confirmed that the T_m of **F21T** is increased in the presence of potassium (data not shown). The T_m of the quadruplex was estimated to be over 70 °C for **F21T**, and the quenching of fluorescein emission was more or less identical in the presence of potassium or sodium ions at low temperature. The profiles obtained from fluorescence measurements at 588 nm were somewhat different: Little or no sensitized emission at 588 nm was associated with G-quartet formation in the presence of potassium ions. This could be the result of a difference of folding topology between the Na^+ and K^+ quadruplexes (see Figure 1, upper part, for an example of two conformers) which might bring the two chromophores in different relative orientations and distances. On the other hand, the replacement of sodium by lithium had the opposite effect: The T_m (determined by UV absorbance or fluorescence) of the oligonucleotide was dramatically lowered (see below).

Application of the test for G-quadruplex formation to ligands

Having designed a test for G-quadruplex formation, we wanted first to apply this method to the characterization of G-quadruplex ligands. These molecules are of great interest as they could lock a telomeric G-overhang into a folded conformation which cannot be extended by telomerase.[33–35] We have tested two known G-quartet ligands, 3,3′-diethyloxadicarbocyanine (DODC)[36] and a 2,6-diamido-functionalized anthraquinone (abbreviated as 2,6-AQ).[33] For comparison purposes, we chose the oligonucleotide **F21T**, the shortest intramolecular fluorescent G-quartet-forming oligonucleotide tested in the previous experiments. Similar results were obtained with **F23T** (data not shown), showing that other interactions than FRET between fluorescein and tamra were not a major threat to the method. We also chose a buffer somewhat unfavorable to quadruplex formation, containing 10 mM sodium cacodylate and 100 mM lithium cacodylate. If ligands are able to stabilize G-quartet DNA, they should increase its melting temperature: Starting from a relatively unstable quadruplex should magnify stabilization

effects and allow to avoid measurements at extremely high temperatures. We also selected a **F21T** oligonucleotide strand concentration of 0.2 μM and a 1 μM ligand concentration. Under these conditions, the dye is in molar excess compared to the oligonucleotide (0.2 μM), and to observe a significant stabilizing effect, dissociation constants in the micromolar range are required. The T_m of **F21T** was 42.3 °C in this lithium buffer, and DODC and 2,6-AQ gave stabilizations of 4.0 and 4.3 °C, respectively (Figure 7). In contrast, ethidium bromide gave little or no

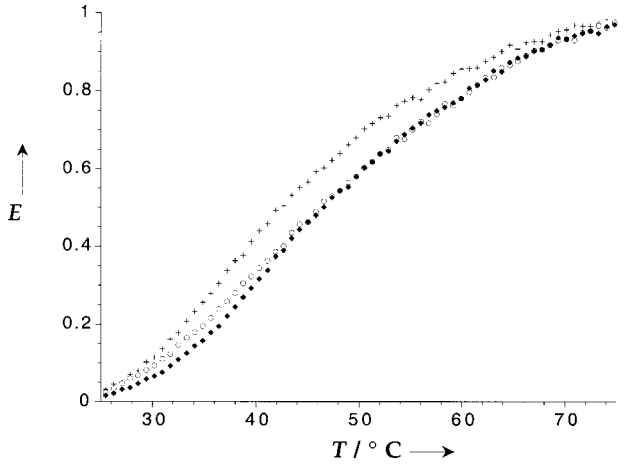

Figure 7. *Fluorescence vs. temperature measurements of* ***F21T*** *(0.2 μM strand concentration) in the absence (crosses) or the presence of G-quadruplex ligands (circles: 1 μM DODC, diamonds: 1 μM 2,6-AQ). Excitation was set at 480 nm and emission was recorded at 588 nm. The temperature of the water bath was increased at a rate of 0.25 °C min^{-1}. All these measurements were performed in a 10 mM cacodylate buffer (pH 7.4) containing 0.1 M LiCl.*

stabilization ($\Delta T_m < 1$ °C, dara not shown) under identical conditions: Although ethidium bromide has been reported to bind to G-quadruplexes,[37, 38] its affinity and specificity for this structure is low.[35] It should be noted that the same test may be performed in the presence of a large excess of a double-stranded nonfluorescent DNA competitor, allowing to identify ligands that show preference for quadruplex over triplex or duplex structures. Specifically, the stabilization provided by DODC was lost when 1 μM of a triplex structure was added to the mixture, in good agreement with a strong affinity of DODC for triplex rather than quadruplex structures[39] (data not shown).

Discussion

Choice of the linker and influence of the reporter groups

Donor – acceptor interactions other than dipole – dipole interactions should usually be avoided when FRET is to be used for the determination of distances. These interactions usually occur when the two molecules are brought in very close proximity (a few Å). Folding of the 21-mer, in which the fluorescein and tamra dyes are directly linked to the 5′- and 3′-terminal bases, led to a partial quenching of the donor fluorescence, which was correlated with a decrease in its absorption coefficient rather than in its quantum yield. It was possible to prevent such a close contact, for example by adding two or five extra bases between the two dyes. In the presence of sodium chloride, two bases were sufficient and a spacer of five bases near the 5′ end completely prevented undesired contacts between the two dyes. Upon folding of the resulting 26-mer, FRET was obtained between fluorescein and tamra, as shown by the quenching of fluorescein emission *and* the sensitized tamra emission. Identical results were obtained for the 23-mers. For the 21-mer, FRET was indeed present, but partial quenching of the dyes was also demonstrated in agreement with the peculiar CD spectrum of the **F21T** oligomer. Such possible interferences are avoided by adding two or five bases.

Quantitative measurement of fluorescence energy transfer

Measurement of the quenching of donor fluorescence should allow an estimation of the transfer efficiencies, provided that no other quenching phenomena are present. In the presence of sodium ions, quenching efficiencies were 72, 60, and 45% for **F21T**, **F23T**, and **F26T**, respectively. This leads to calculated maximum transfer efficiencies of 0.61, 0.58, and 0.44, taking into account the variation of the fluorescein absorption coefficient, and assuming that no other quenching phenomenon participates in the decrease in fluorescein emission.

As shown in this study, covalent attachement of the dyes has an impact on their fluorescence properties, especially in the case of fluorescein. It is also noteworthy that the extinction coefficient and relative quantum yield of covalently bound fluorescein is itself dependent on the state of the oligonucleotide (i.e. folded or unfolded), especially when it is directly attached to the G-quartet without any linker bases in between. Therefore, the critical Förster distance R_o between fluorescein and tamra is slightly different in the case of folded and unfolded oligomers. The quantum yield of fluorescein and to a lesser extent the absorbance properties of tamra are usually dependent on experimental conditions, which may explain the apparent discrepancies between the R_o values reported for this donor – acceptor pair (from 3.5 to 5.2 nm). In our case, a R_o distance of 4.6 nm was calculated: As the transfer efficiency was close to 0.5, one would deduce that the donor – acceptor distance is in the 4 – 5 nm range, provided that κ^2 does not differ too much from the assumed value of $^2/_3$.

Another important factor concerning FRET is its distance and orientation dependence. The orientation factor $\langle\kappa^2\rangle$ can be precisely determined in two extreme cases: Either the orientation of the two molecules has an isotropic distribution, or their position is fixed and known. For the experiments described in this study, all fluorophores experience a relative freedom of motion, due to a flexible linker with five carbon atoms. Nevertheless, the distribution of their orientation factors is probably not isotropic because the DNA strand sterically prevents some of the orientations. Furthermore, CD spectroscopy on **F21T** shows that the fluorophores are interacting closely with the quadruplex. This leads to an error in the orientation factor, which was assumed to be equal to $^2/_3$ (isotropic distribution) for all R_o determinations. For the shorter oligonucleotide, the presence of a CD signal in the visible region is a

clear indication that the dyes are not experiencing motional freedom compared to the quadruplex. The addition of a two- or five-base linker was sufficient to prevent this phenomenon. Therefore, for **F23T** and **F26T**, one can assume that fluorescein experiences a fair degree of motional freedom, whereas tamra, which is linked to the 3′ end of the quadruplex through a five-carbon linker, could be more restricted.

Why use FRET?

The advantages and drawbacks of the FRET approach were recently discussed for i-motif formation.[13] In the case of this peculiar DNA quadruplex, which is based on the formation of hemiprotonated $C \cdot C^+$ base pairs,[40] the labeling had a significant impact on the properties of the oligonucleotide. As nucleic acids are virtually nonfluorescent, attachement of a reporter dye(s) is mandatory to monitor the fluorescence of the oligomer. The structural effect of such modification is usually neglected. This was definitely not the case for i-motif formation,[13] and to a lesser extent for G-quartet formation (as shown in this study).

For both quadruplexes, the oligonucleotides are rather small, and therefore, the attachment of a dye has a significant impact on the molecular weight, charge, flexibility, and hydrophobicity of the molecule. However, a major difference between these tetraplexes lies in their relative stabilities at neutral pH: i-DNA is relatively unstable at pH 7.0, and the effect obtained in the presence of a terminal dye was easily demonstrated.

For i-motif analysis, five bases were necessary to allow independence of the two dyes and thus avoid static quenching. The situation was slightly different in the case of G-quartet formation. Direct attachment of both dyes at the 5′ and 3′ ends of the structure led to a significant alteration of the CD spectrum. Nevertheless, this interaction did not prevent FRET from occuring and no strong static quenching was observed. In the case of i-motif formation, a 96% quenching of fluorescein was observed, without coupling to a sensitized acceptor emission, showing that little, if any, FRET could be demonstrated with short oligomers.

There are justifications for the FRET approach: i) The technique is extremely sensitive, allowing detection of the phenomenon at 1×10^{-10} M strand concentration. ii) The dynamic response of most spectrofluorimeters is linear over a wide concentration range (typically five orders of magnitude). Association phenomena may thus be followed at different concentrations, allowing the determination of thermodynamic parameters. In our case, intramolecular folding led to a concentration-independent behavior demonstrated between 1×10^{-10} M and 2×10^{-7} M strand concentration (Figure 8). iii) A differential behavior was observed in buffers containing Na^+ or K^+: Enhanced acceptor emission was only demonstrated in the presence of sodium ions; folding of the G-quartet oligonucleotides in the presence of potassium ions only led to a quenching of fluorescein emission, without concomitant increase in rhodamine emission at longer wavelengths. This may suggest that the G-quartet structures in buffers containing sodium or potassium ions are significantly different, this may lead to different distances between the 3′ and 5′ extremities of the quadruplex (see Figure 1 A for an example).

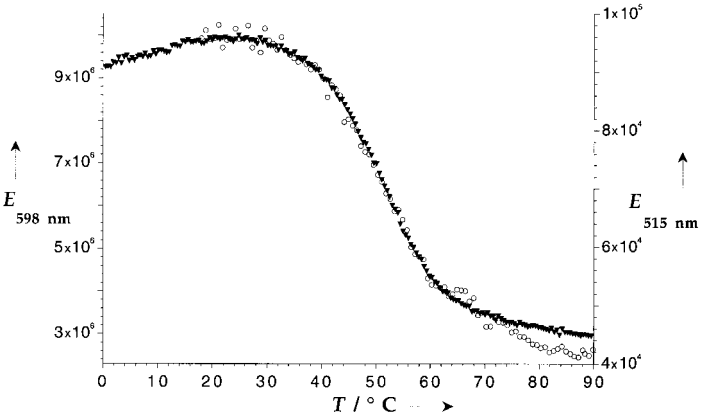

Figure 8. *Concentration dependence of the fluorescence vs. temperature measurements of* ***F23T*** *(circles: 0.2 μM, filled squares: 0.1 nM strand concentration). Excitation was set at 480 nm and emission was recorded at 598 nm. The temperature of the water bath was increased at a rate of 0.25 °C min⁻¹. Larger emission and excitation slits were used for the diluted sample (9.2 nm) as compared to the 0.2-μM sample (1.8 nm). All these measurements were performed in a 10 mM cacodylate buffer (pH 7.0) containing 0.1 M NaCl.*

A cation switch between different quadruplex conformers has already been reported,[30, 41, 42] and FRET might provide valuable information in these cases.

In conclusion, FRET is a valuable method for the investigation of the secondary structure of an oligonucleotide. We are currently exploring the possibility of analyzing the structure of a G-quartet oligodeoxynucleotide in the presence of various ligands and proteins, as G-quadruplex ligands increased the melting temperature of the quadruplex monitored by FRET. Many new G-quadruplex ligands have been described recently,[43–46] and such method could provide a semi-quantitative comparison of their stabilizing properties.

Experimental Section

Oligonucleotides and chemicals: All oligonucleotides and their fluorescein and tamra conjugates were synthesized and purified by Eurogentec (Belgium). The purity of doubly substituted oligonucleotides was checked by denaturing gel electrophoresis: Only one major fluorescent band was visible upon UV excitation, and shorter oligonucleotides or monosubstituted derivatives were hardly visible. The primary sequences and names of the fluorescent oligonucleotides are given in Figure 1 (bottom). Ethidium bromide and DODC were purchased from Molecular Probes and Sigma, respectively. 2,6-AQ was a kind gift of Dr. J. F. Riou (Aventis, Centre de Recherche, Vitry-Alfortville, France).

UV absorption studies: Unless otherwise specified, all experiments were performed in a 10 mM cacodylate buffer, 0.1 M NaCl or KCl, pH 7.0, at 4 μM oligonucleotide strand concentration. The ε values of derivatized fluorescein and rhodamine dyes was measured by recording the UV/Vis spectra of the purified oligomer in a 0.1 M KCl cacodylate buffer (pH 7.0, $T = 20\,^{\circ}C$). Thermal denaturation profiles were obtained with a Kontron Uvikon 940 spectrophotometer as previously described.[13] All T_m values are within $\pm 1\,^{\circ}C$. ΔG° values are within ± 1.3 kJ mol^{-1}.

Fluorescence studies: All measurements were performed on a Spex Fluorolog DM1B instrument, using a bandwidth of 1.8 nm (or 9.6 nm for the diluted samples) and 0.2×1 cm quartz cuvettes, containing 600 μL of solution. The temperature of the circulating water bath was recorded at regular time intervals. All measurements were made as previously described.[13]

Calculation of R_o: R_o is the critical Förster distance, at which the transfer efficiency E accounts for half of the deactivation processes of the donor. R_o was calculated as previously described.[5, 13] We used an average value of $^2/_3$ for the orientation factor κ^2 and 1.33 for the refractive index n. As the covalent linkage between the chromophore and the oligonucleotide has an effect on the absorption and fluorescence properties of the former, the absorption coefficient of the acceptor (ε) and the quantum yield of fluorescence of the donor (Φ_D) were calculated from the data obtained for the *conjugated* dyes. Due to the dependence on the sixth power of R, E very quickly drops to zero when $R > R_o$. The quenching of the donor, which is related to the transfer efficiency, was monitored at a wavelength where the emission of the acceptor was negligible (515 nm for the fluorescein/tamra couple).

CD measurements: All measurements were performed with a Jobin-Yvon Mark V instrument using 1-cm quartz cuvettes as previously described.[47] The CD intensity is expressed per nucleotide in the UV wavelength range and per oligonucleotide in the visible region.

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[1] A. I. H. Murchie, R. M. Clegg, E. Kitzing, D. R. Duckett, S. Diekmann, D. M. J. Lilley, *Nature* **1989**, *341*, 763 – 766.
[2] R. M. Clegg, A. I. H. Murchie, A. Zechel, C. Carlberg, S. Diekmann, D. M. J. Lilley, *Biochemistry* **1992**, *31*, 4846 – 4856.
[3] R. M. Clegg, A. I. H. Murchie, A. Zechel, D. M. J. Lilley, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 2994 – 2998.
[4] P. S. Eis, D. P. Millar, *Biochemistry* **1993**, *32*, 13 852 – 13 860.
[5] J. L. Mergny, A. S. Boutorine, T. Garestier, F. Belloc, M. Rougée, N. V. Bulychev, A. A. Koshkin, J. Bourson, A. V. Lebedev, B. Valeur, N. T. Thuong, C. Hélène, *Nucleic Acids Res.* **1994**, *22*, 920 – 928.
[6] E. A. Jares-Erijman, T. M. Jovin, *J. Mol. Biol.* **1996**, *257*, 597 – 617.
[7] G. S. Bassi, A. I. H. Murchie, F. Walter, R. M. Clegg, D. M. J. Lilley, *EMBO J.* **1997**, *16*, 7481 – 7489.
[8] T. Förster, *Ann. Phys.* **1948**, *2*, 55 – 75.
[9] M. S. Yang, S. S. Ghosh, D. P. Millar, *Biochemistry* **1994**, *33*, 15 329 – 15 337.
[10] J. L. Mergny, T. Garestier, M. Rougée, A. V. Lebedev, M. Chassignol, N. T. Thuong, C. Hélène, *Biochemistry* **1994**, *33*, 15 321 – 15 328.
[11] P. V. Scaria, S. Will, C. Levenson, R. H. Shafer, *J. Biol. Chem.* **1995**, *270*, 7295 – 7303.
[12] M. Yang, L. Q. Ren, M. Huang, R. Y. Kong, W. F. Fong, *Anal. Biochem.* **1998**, *259*, 272 – 274.
[13] J. L. Mergny, *Biochemistry* **1999**, *38*, 1573 – 1581.
[14] T. Simonsson, R. Sjoback, *J. Biol. Chem.* **1999**, *274*, 17 379 – 17 383.
[15] Y. Oka, C. A. Thomas, Jr., *Nucleic Acids Res.* **1987**, *15*, 8877 – 98.
[16] W. I. Sundquist, A. Klug, *Nature* **1989**, *342*, 825 – 829.
[17] J. R. Williamson, *Annu. Rev. Biophys. Biomol. Struct.* **1994**, *23*, 703 – 730.
[18] J. L. Mergny, A. T. Phan, L. Lacroix, *FEBS Lett.* **1998**, *435*, 74 – 78.
[19] Y. Wang, D. J. Patel, *Structure* **1993**, *1*, 263 – 282.
[20] J. D. Griffith, L. Comeau, S. Rosenfield, R. M. Stansel, A. Bianchi, H. Moss, T. de Lange, *Cell* **1999**, *97*, 503 – 514.
[21] C. W. Greider, *Cell* **1999**, *97*, 419 – 422.
[22] R. Sjoback, J. Nygren, M. Kubista, *Biopolymers* **1998**, *46*, 445 – 453.
[23] M. Lu, Q. Guo, N. R. Kallenbach, *Biochemistry* **1993**, *32*, 598 – 601.
[24] P. Balagurumoorthy, S. K. Brahmachari, *J. Biol. Chem.* **1994**, *269*, 21 858 – 21 869.
[25] P. Balagurumoorthy, S. K. Brahmachari, D. Mohanty, M. Bansal, V. Sasisekharan, *Nucleic Acids Res.* **1992**, *20*, 4061 – 4067.
[26] C. Gondeau, J. C. Maurizot, M. Durand, *J. Biomol. Struct. Dyn.* **1998**, *15*, 1133 – 45.
[27] J. R. Williamson, M. K. Raghuraman, T. R. Cech, *Cell* **1989**, *59*, 871 – 880.
[28] D. Sen, W. Gilbert, *Nature* **1990**, *344*, 410 – 414.
[29] V. M. Marathias, P. H. Bolton, *Biochemistry* **1999**, *38*, 4355 – 4364.
[30] S. Bouaziz, A. Kettani, D. J. Patel, *J. Mol. Biol.* **1998**, *282*, 637 – 652.
[31] N. V. Hud, F. W. Smith, F. A. L. Anet, J. Feigon, *Biochemistry* **1996**, *35*, 15 383 – 15 390.
[32] T. Miura, J. M. Benevides, G. J. Thomas, *J. Mol. Biol.* **1995**, *248*, 233 – 238.
[33] D. Sun, B. Thompson, B. E. Cathers, M. Salazar, S. M. Kerwin, J. O. Trent, T. C. Jenkins, S. Neidle, L. H. Hurley, *J. Med. Chem.* **1997**, *40*, 2113 – 2116.
[34] J. L. Mergny, C. Hélène, *Nat. Med.* **1998**, *4*, 1366 – 1367.
[35] P. J. Perry, T. C. Jenkins, *Expert Opin. Invest. Drugs* **1999**, *8*, 1981 – 2008.
[36] Q. Chen, I. D. Kuntz, R. H. Shafer, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 2635 – 2639.
[37] Q. Guo, M. Lu, L. A. Marky, N. R. Kallenbach, *Biochemistry* **1992**, *31*, 2451 – 2455.
[38] T. Shida, N. Ikeda, J. Sekiguchi, *Nucleosides Nucleotides* **1996**, *15*, 599 – 605.
[39] J. S. Ren, J. B. Chaires, *J. Am. Chem. Soc.* **2000**, *122*, 424 – 425.
[40] K. Gehring, J. L. Leroy, M. Guéron, *Nature* **1993**, *363*, 561 – 565.
[41] E. A. Venczel, D. Sen, *Biochemistry* **1993**, *32*, 6220 – 6228.
[42] T. Miura, G. J. Thomas, *Biochemistry* **1994**, *33*, 7848 – 7856.
[43] R. J. Harrison, S. M. Gowan, L. R. Kelland, S. Neidle, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2463 – 2468.
[44] P. J. Perry, A. P. Reszka, A. A. Wood, M. A. Read, S. M. Gowan, H. S. Dosanjh, J. O. Trent, T. C. Jenkins, L. R. Kelland, S. Neidle, *J. Med. Chem.* **1998**, *41*, 4873 – 4884.
[45] P. J. Perry, S. M. Gowan, M. A. Read, L. R. Kelland, S. Neidle, *Anti-Cancer Drug Des.* **1999**, *14*, 373 – 382.
[46] T. C. Jenkins, *Curr. Med. Chem.* **2000**, *7*, 99 – 115.
[47] P. B. Arimondo, F. Barcelo, J. S. Sun, J. C. Maurizot, T. Garestier, C. Hélène, *Biochemistry* **1998**, *37*, 16 627 – 16 635.

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