DNA Tetraplex Formation Studied with Fluorescence Resonance Energy Transfer*

(Received for publication, February 5, 1999, and in revised form, April 7, 1999)

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It is emerging that DNA tetraplexes are pivotal for many major cellular processes, and techniques that assess their structure and nature to the point are under development. Here we show how the structural conversion of largely unstructured single-stranded DNA molecules into compact intrantrad DNA tetraplexes can be monitored by fluorescence resonance energy transfer.

We recently reported that intrantrad tetraplex formation takes place in a nuclease hypersensitive element upstream of the human c-myc proto-oncogene. Despite the highly repetitive guanine-rich sequence of the hypersensitive element, fluorescence resonance energy transfer measurements indicate that only one well-defined tetraplex structure forms therein. The proposed structure, which is specifically stabilized by potassium ions in vitro, has a core of three stacked guanine tetradsthat is capped by two intrantrad A-T base pairs.

It was early discovered that guanine-rich sequences were capable to self-associate into higher order structures termed DNA tetraplexes (1). For a long time these entities were considered laboratory curiosities and nearly three decades passed before they were suggested to possess biologically significant functions (2, 3). A growing body of evidence now indicates that formation of DNA tetraplexes are important to many major cellular processes, and they form in the telomeres (4), the immunoglobulin switch regions (2), the control regions of the retinoblastoma susceptibility genes (5) and the chicken β-globin gene (6), in L1 retrotransposons (7), and upstream of the insulin gene (8).

In general, the term tetraplex describes any four-stranded DNA structure containing guanine tetrads without reference to strand connectivity. Tetraplexes exist in a number of structural variations and strand stoichiometries; they form by association of one (9–11), two (3, 12), or four strands (2, 13). In principle, three strand arrangements are conceivable but have yet to be substantiated. Moreover, a sequence that has the ability to form a unimolecular intrantrad structure can also form bimolecular or quadrimolecular interstrand structures (14–17). What determines a priori which of these structures form depends on strand concentration.

DNA tetraplexes are exceedingly stable structures with a core of stacked guanine tetrads, which arise from the association of four guanines into a cyclic hydrogen-bonding arrangement that involves N1, N7, O6, and N2 of each guanine base. Furthermore, they are specifically stabilized by cations, which are coordinated by the eight carbonyl oxygens that line the cages formed by stacking of guanine tetrads (18).

Several excellent DNA tetraplex studies have been performed by x-ray crystallization (13, 19), NMR (20–22), and CD (23, 24). Notably, the majority of these structural studies have focused on telomeric DNA and been confined to interstrand DNA tetraplexes.

We have previously reported that formation of an intrantradly folded tetraplex takes place in the control region of the c-myc gene (25). The region in which tetraplex formation occurs is hypersensitive to nuclease and is referred to as the c-myc nuclease hypersensitive element (NHE)†(26–28). The NHE is central to activation of c-myc (29, 30), and we have suggested that tetraplex formation therein not only accounts for its hypersensitivity but also provides a link between the formation of hypersensitive sites and gene activation. The c-myc allele is normally present in two copies and four copies during mitosis, which in principle permits formation of bimolecular and quadrimolecular structures that might be of biological relevance. However, in the proposed mechanism for initiation of c-myc expression only the intramolecular tetraplex is likely to be pertinent. To characterize this structure in more detail, we have engaged in a number of studies based on x-ray crystallography, NMR, and CD. Alas, these techniques require strand concentrations that favor formation of intermolecular species, and we constantly observe mixtures containing unimolecular intrantrad, bimolecular, and quadrimolecular interstrand tetraplexes, as well as higher order aggregates. Irrespective of which type of tetraplex one is to scrutinize, it is necessary to circumvent formation of heterogeneous mixtures that limit the resolution. To study activation of c-myc expression from the NHE it is important to find tools that are sensitive to precise intrantrad tetraplex formation.

Fluorescence resonance energy transfer (FRET) is a process by which the excited state energy of a fluorescent donor chromophore is transferred to an unexcited acceptor chromophore. This yields quenched donor and increased acceptor fluorescence. The efficiency of FRET depends largely on the donor-acceptor distance, and FRET-based techniques are widely used to study interchromophoric distances between ~10 and ~80 Å. More specifically, they have been employed to study conformational changes of nucleic acids and have elucidated the overall geometry of four-way DNA junctions (31) and the hammerhead ribozyme (32). In addition FRET-based methods have been used as a tool to study DNA bending (33), to investigate DNA hybridization (34), DNA triple helix formation (35), and the

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1 The abbreviations used are: NHE, nuclease hypersensitive element; FRET, fluorescence resonance energy transfer; TEMED, N,N,N’,N’-tetramethylethlenediamine.
kinetics of both DNA unwinding (36), and cleavage (37).

Also the conversion of a flexible single-stranded DNA molecule into a compact intrastrand DNA tetraplex is a major structural transition that might be suitable to monitor by FRET. To investigate this we have conjugated fluorescein as donor chromophore to the 5'-ends, and tetramethyl rhodamine as acceptor chromophore to the 3'-ends of tetraplex-forming oligonucleotides. The oligonucleotides comprise the guanine-rich strand of the c-myc NHE, which is known to adopt an intrastrand tetraplex in the presence of potassium ions (25).

**EXPERIMENTAL PROCEDURES**

**Oligonucleotide Preparation**—The 33-base-long oligonucleotide fluorescein-5'-d(TGG GGA GGG TGG GGA GGG TGG GGA AGG)-3'-amine, whose sequence corresponds to bases 2180–2212 of the human c-myc locus (38), was from Eurogentec, as were the 27-base-long oligonucleotides fluorescein-5'-d(TGG GGA GGG TGG GGA GGG TGG GGA AGG)-3'-amine and 5'-d(TGG GGA GGG TGG GGA GGG TGG GGA AGG)-3'-amine corresponding to bases 2186–2212, the 22-base-long oligonucleotides fluorescein-5'-d(GGG GAG GGT GGG GAG GGT GGG G) G)-3'-amine and 5'-d(GGG GAG GGT GGG GAG GGT GGG G)-3'-amine corresponding to bases 2190–2211, and the 16-base-long oligonucleotide fluorescein-5'-d(TGG GGA GGG TGG GGA GGG TGG GGA)-3'-amine, corresponding to bases 2195–2210 of the human c-myc locus. All oligonucleotides were purified by high pressure liquid chromatography and 3'-amines were conjugated to tetramethyl rhodamine-5'-isothiocyanate from Molecular Probes according to a previously described procedure (39), except here the reactions were carried out in 0.2 mM carbonate buffer, pH 10. All labeled oligonucleotides were purified on 20% polyacrylamide gels by standard denaturing electrophoresis, recovered by electroelution, ethanol precipitated, and redissolved in TE buffer (pH 7.6). Acrylamide solutions and ammonium persulfate were from Bio-Rad, TEMED was from Fluka, TRIS was from Amresco, boric acid was from J. T. Baker, and EDTA was from Merck.

**Preparation of the NHS Tetraplexes**—The purified doubly labeled 22-base-long oligonucleotide, fluorescein-5'-d(GGG GAG GGT GGG GAG GGT GGG G)-3'-tetramethyl rhodamine, was incubated in TE buffer (pH 7.6) containing 100 mM KCl for 48 h at 37 °C. It was then subjected to preparative native polyacrylamide gel electrophoresis as described previously (25), except now the running buffer contained 25 mM KCl. The intramolecularly folded tetraplex form of the oligonucleotide was recovered from the gel by electroelution in presence of 25 mM KCl.

**Ion Specificity of Tetraplex Formation**—The purified doubly labeled 22-base-long oligonucleotide, fluorescein-5'-d(TGG GGA GGG TGG GGA GGG TGG GGA)-3'-amine fluorescein-5'-d(TGG GGA GGG TGG GGA GGG TGG GGA AGG)-3'-tetramethyl rhodamine, was incubated in TE buffer (pH 7.6) containing either lithium, sodium, potassium, rubidium, or cesium chloride for 48 h at 37 °C before absorption and fluorescence measurements. All salts were from Sigma.

**Absorption and Fluorescence Measurements**—Oligonucleotide concentrations were determined by absorption spectroscopy in absence of monovalent alkali cations. The molar absorbivities at 260 nm were assumed to equal the sum of the individual base contributions (40), and the absorption of attached chromophores served as internal reference. At pH 7.6 the donor chromophore fluorescein has an absorption maximum at 494 nm with a molar absorptivity of 52,000 M⁻¹ cm⁻¹, and a fluorescence emission maximum at 514 nm (41). The acceptor chromophore tetramethyl rhodamine has an absorption maximum at 556 nm with a molar absorptivity of 90,000 M⁻¹ cm⁻¹, and a fluorescence emission maximum at 580 nm (41). Absorption spectra were recorded on a Cary 4 spectrophotometer using a path-length of 1 cm and a spectral bandwidth of 1 nm. Fluorescence measurements were made on a Spex FL 1222 spectrophotometer. The excitation wavelength was 460 nm. The oligonucleotide strand concentration was 0.1 μM in all fluorescence measurements to minimize the inner filter effect (42) and to favor the formation of unimolecular intrastrand fold-back tetraplex structures. Both absorption and fluorescence measurements were performed at 37 °C.

**RESULTS**

First, we incubated doubly labeled oligonucleotides at 37 °C in the presence of 100 mM potassium chloride and recorded their fluorescence emission spectra. The 22-base-long oligonucleotide demonstrated high energy transfer efficiency (Fig. 1, dotted line), albeit lower, also the 27-base-long oligonucleotide demonstrated FRET, whereas neither the 33-base-long nor the 16-base-long oligonucleotide exhibited detectable FRET (data not shown). After an identical incubation, we subjected the 22-base-long fluorescein-5'-d(GGG GAG GGT GGG GAG GGT GGG G)-3'-tetramethyl rhodamine oligonucleotide to preparative electrophoresis in the presence of potassium chloride as described under “Experimental Procedures.” This produced two bands (data not shown); one band had the expected electrophoretic mobility of an unstructured single-stranded DNA molecule, whereas an additional band migrated through the gel with higher electrophoretic mobility. The latter, which corresponds to an intramolecularly folded tetraplex (25), was recovered from the gel. The fluorescence emission spectrum of the purified tetraplex was recorded and demonstrated very efficient energy transfer (Fig. 1, dashed line). If instead the doubly labeled 22-base-long oligonucleotide was hybridized to its complementary strand in the absence of potassium ions to adopt an extended double-stranded conformation, the recorded fluorescence emission spectrum revealed no FRET (Fig. 1, solid line). Second, we monitored how tetraplex formation in the purified doubly labeled 22-base-long oligonucleotide depends on the concentration of potassium ions. Potassium chloride was titrated to the oligonucleotide, and fluorescence emission spectra were recorded after each addition. The individual spectra of 22-base-long oligonucleotides, singly labeled with either fluorescein in the 5'-end or tetramethyl rhodamine in the 3'-end, were fitted to the recorded spectra. The value of the fitting parameter that reflects the fluorescence efficiency of fluorescein decreases with increasing concentration of potassium ions (Fig. 2). The quenching is maximal already at 1 mM and further increasing the concentration of potassium ions does not appreciably alter it. No quenching is observed at potassium chloride concentrations up to 1 μM (Fig. 2). When the same experiment was repeated with the oligonucleotide singly labeled with only fluorescein, some quenching could be observed. However, this was negligible compared with that of the doubly labeled oligonucleotide in which a corresponding increase in tetramethyl rhodamine fluorescence confirmed that energy transfer was the dominating phenomenon observed.

Third, after incubation in the presence of different alkali ions at individual concentrations of 5 mM, we recorded fluorescence...
emission spectra of the doubly labeled synthetic 27-base-long oligonucleotide. At this concentration potassium ions induce maximal FRET (Fig. 3, dashed line). Although far less efficiently than the potassium ion, the rubidium ion induces detectable FRET (Fig. 3, dotted line), whereas lithium, sodium, and cesium ions completely lack the ability to induce FRET in the 27-base-long oligonucleotide (Fig. 3, all solid superimposed).

**DISCUSSION**

We have previously shown that the guanine-rich strand of the c-myc NHE undergoes intranstrand tetraplex formation in the presence of potassium ions (25). Upon sequence inspection, the NHE appears capable of forming many different tetraplex structures. Here we have constructed four doubly labeled oligonucleotides comprising sequences from the guanine-rich strand of the NHE. All four oligonucleotides, which range from 16 to 33 bases in length, exhibit motifs for the formation of intramolecularly folded tetraplexes.

The 16-base-long oligonucleotide, fluorescein-5′-d(GGG GAG GGT GGG GAG GGT GGG G)-3′-tetramethyl rhodamine, has the minimal motif to adopt an intranstrand tetraplex with three planes of guanine tetrads, two one-base loops (the two thymines) and a central two-base-long loop (the GA dinucleotide). However, the 16-base-long oligonucleotide does not fold intramolecularly as judged by the absence of FRET. Most likely the two shorter loops impose excessive strain on the intramolecular fold and prevent the structure from forming.

The 22-base-long oligonucleotide, fluorescein-5′-d(GGG GAG GGT GGG GAG GGT GGG G)-3′-tetramethyl rhodamine, can adopt a tetraplex with three planes of guanine tetrads, two two-base loops (the foremost GA and endmost TG dinucleotides) and a central six-base-long loop (the GA dinucleotide). However, the 16-base-long oligonucleotide does not fold intramolecularly as judged by the absence of FRET. Most likely the two shorter loops impose excessive strain on the intramolecular fold and prevent the structure from forming.

The 27-base-long oligonucleotide, fluorescein-5′-d(TGG GGA GGG TGG GGA GGG TGG GGA AGG)-3′-tetramethyl rhodamine, demonstrates moderate FRET efficiency (Fig. 3, dashed line). The presence of energy transfer, although lower than in the 22-base-long oligonucleotide, suggests that identical core structures form. The flanking sequences in the 27-base-long oligonucleotide (the 5′ thymine and the 3′ AAGG tetranucleotide) are not involved in structure formation but increase the interchromophoric distance. This explains why the 27-base-long oligonucleotide displays less efficient FRET.

The 33-base-long oligonucleotide, fluorescein-5′-d(TGG GGA GGG TGG GGA AGG TGG GGA)-3′-tetra-
methyl rhodamine, has the possibility to adopt an intramolecularly folded tetraplex with four planes of guanine tetrads and three five-base-loops (the two AGGGT and the AAGGT pentanucleotides). The lack of FRET in this oligonucleotide precludes formation of such a structure. Instead we believe the same core structure that produces FRET in both the 22- and 27-base-long oligonucleotides forms also in the 33-base-long oligonucleotide. If so, the flanking sequences in the 33-base-long oligonucleotide (the 5’ thymine and the 3’ decanucleotide AAGGTGGGGGA) separate the chromophores beyond the detection limit for energy transfer, which explains the absence of FRET.

When the 22-base-long doubly labeled oligonucleotide has been incubated in the presence of potassium ions and is subjected to preparative electrophoresis as described under “Experimental Procedures,” two distinct bands are observed. High electrophoretic mobility is characteristic of compact structures like DNA tetraplexes (9). Accordingly, and as previously shown (25), the band of higher electrophoretic mobility corresponds to the intramolecularly folded tetraplex, whereas the band of normal electrophoretic mobility corresponds to a largely unstructured single-stranded DNA molecule. Indeed, the doubly labeled oligonucleotide recovered from the band of higher electrophoretic mobility exhibits very efficient FRET (Fig. 1, dashed line). This confirms that formation of the intramolecularly folded tetraplex yields high FRET and validates FRET as a suitable tool to study tetraplex formation. The absence of FRET, as demonstrated by the doubly labeled oligonucleotide recovered from the band of normal electrophoretic mobility, verifies that this contains unstructured single-stranded DNA molecules (data not shown). The fluorescence emission spectrum of the oligonucleotide incubated in presence of 100 mM potassium chloride shows less efficient energy transfer than that of the electrophoretically purified intramolecular tetraplex. We attribute this discrepancy to the extensive structural polymorphism exhibited by DNA tetraplexes.

To determine how tetraplex formation depends on the concentration of potassium ions, we titrated potassium chloride to the doubly labeled 22-base-long oligonucleotide. The fluorescence intensity of fluorescein decreases with increasing amounts of potassium ions in the range 1 mM to 1 mM (Fig. 2). The major cause of the quenching is likely to be formation of the intramolecularly folded tetraplex, but other mechanisms may contribute to the decreased fluorescence of fluorescein. For instance, if somehow parallel four-stranded tetraplexes form, the excited state energy can be transferred to nearby fluorescein chromophores resulting in self-quenching. However, such an arrangement would not account for the increased tetramethyl rhodamine fluorescence we observe. The spectroscopic properties of fluorescein is also known to change upon conjugation to synthetic oligonucleotides (41), but the effect of guanine tetrads remains to be clarified. Notwithstanding, we posit that the fluorescein quenching reflects tetraplex formation.

In the proposed structure (Fig. 4), the two chromophores are in close proximity to each other. If the interchromophoric distance becomes too small (<10 Å) close contact energy transfer can occur via the Dexter mechanism (43). To verify that FRET is the dominating mechanism observed, we used the 27-base-long oligonucleotide in the final set of experiments. Its five additional bases exclude the possibility that the chromophores are close enough for Dexter energy transfer to occur.

The third and final set of experiments addresses another characteristic of DNA tetraplexes, namely their selective interactions with cations that fit well in the cavities formed by the stacking of guanine tetrads. The cavity between two planes of guanine tetrads is lined by eight carbonyl oxygens, which can all participate in the precise coordination of cations. It is well established that coordination of cations, especially from the alkali series, adds both thermodynamically and kinetically to the stability of tetraplexes (44). In general, ionic radius is a parameter that reflects how well DNA tetraplexes are stabilized by coordination of different cations. More specifically, the potassium ion is believed to fit exceptionally well in the cavities between guanine tetrads. The ionic radii for the alkali series are as follows: Li⁺ (0.68 Å), Na⁺ (0.97 Å), K⁺ (1.33 Å), Rb⁺ (1.47 Å), and Cs⁺ (1.67 Å) (45). Our measurements demonstrate that the potassium ion induces FRET most efficiently (Fig. 3), which manifests its superior capacity to promote formation of intramolecularly folded tetraplexes. We believe the reason why the rubidium ion to some extent appears to promote tetraplex formation is that it has an ionic radius similar to that of the potassium ion. However, in human live cell nuclei the free cations are dominated by four species whose respective concentrations typically are as follows: K⁺ (150 mM), Na⁺ (5 mM), Mg²⁺ (0.5 mM), and Ca²⁺ (0.1 mM). Any other cation, including the rubidium ion, has normal physiological concentrations below 1 mM. Except for the potassium ion, only the sodium ion is abundant in the cell nucleus. At its physiological concentration of 5 mM, the sodium ion has negligible tetraplex promoting capacity (Fig. 3).

In summary, we draw the following conclusions from our FRET measurements. First, in addition to the aforementioned conformational changes of nucleic acids, fluorescence resonance energy transfer can also be used to study DNA tetraplexes and to monitor their real-time formation. Second, the c-myc NHE is a highly repetitive guanine-rich sequence that upon sequence inspection appears capable of forming many different tetraplex structures. Despite this, only one well-defined tetraplex structure involving bases 2190–2211 of the human c-myc locus forms (Fig. 4). A plausible explanation for this observation may be the two intrastrand A-T base pairs that cap the guanine tetrad core and stabilize the structure. Third, the intrastrand tetraplex structure is specifically stabilized by potassium ions in vitro. It has been shown that the potassium ion promotes formation of intrastrand tetraplexes, whereas the sodium ion favors the formation of four-stranded parallel structures (46). Because the potassium ion is far more abundant than any other cation in human cell nuclei we propose that the guanine-rich strand of the c-myc NHE folds into an intramolecular tetraplex in vivo.

Acknowledgments—We thank Professor M. Kubista for discussion and advice.

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