Direct experimental evidence for quadruplex–quadruplex interaction within the human ILPR

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Received January 22, 2009; Revised March 4, 2009; Accepted March 5, 2009

ABSTRACT

Here we report the analysis of dual G-quadruplexes formed in the four repeats of the consensus sequence from the insulin-linked polymorphic region (ACAGGGGTGTGGGG; ILPR$_{n=4}$). Mobilities of ILPR$_{n=4}$ in nondenaturing gel and circular dichroism (CD) studies confirmed the formation of two intramolecular G-quadruplexes in the sequence. Both CD and single molecule studies using optical tweezers showed that the two quadruplexes in the ILPR$_{n=4}$ most likely adopt a hybrid G-quadruplex structure that was entirely different from the mixture of parallel and antiparallel conformers previously observed in the single G-quadruplex forming sequence (ILPR$_{n=2}$). These results indicate that the structural knowledge of a single G-quadruplex cannot be automatically extrapolated to predict the conformation of multiple quadruplexes in tandem. Furthermore, mechanical pulling of the ILPR$_{n=4}$ at the single molecule level suggests that the two quadruplexes are unfolded cooperatively, perhaps due to a quadruplex–quadruplex interaction (QQI) between them. Additional evidence for the QQI was provided by DMS footprinting on the ILPR$_{n=4}$ that identified specific guanines only protected in the presence of a neighboring G-quadruplex. There have been very few experimental reports on multiple G-quadruplex-forming sequences and this report provides direct experimental evidence for the existence of a QQI between two contiguous G-quadruplexes in the ILPR.

INTRODUCTION

Guanine rich DNA can form quadruplex, tetraplex or G4 DNA by associating stacks of G-tetrads that are formed by Hoogsteen hydrogen bonding of four guanine nucleotides (Figure 1A). The most extensively characterized examples of quadruplex DNA are found in telomeres at the ends of eukaryotic chromosomes (1,2). Recent genome-wide analysis has identified a large number of potential quadruplex-forming sequences (3). Interestingly, these sequences have been found to occur with a higher abundance in the promoter regions (4), which suggests their potential involvement in regulatory processes. Several of these G-rich sequences have been correlated with gene function, particularly in proto-oncogenes where they are found in a higher abundance (5). Furthermore, other structural and functional studies have suggested a link between the presence of a G-quadruplex and the control of regulation of key genes (2,6–9) such as c-MYC (10,11), VEGF (12), bcl-2 (13), c-kit (14,15) and KRAS (16).

Variable number of tandem repeats (VNTR) or minisatellite regions associated with many human diseases (17,18) often contain purine-rich segments, many of which have the potential to form noncanonical DNA structures. A VNTR region of particular interest is the insulin-linked polymorphic region (ILPR) that contains G-quadruplex and i-motif-forming sequences located at –363 bp upstream of the Insulin coding sequence (18–20) (Figure 1B). It contains tandem repeats of the most prevalent sequence ACAGGGGTGTGGGG (variant ‘a’) as well as other variants (‘b’–’k’) (20,21). The number of these variant repeats has been correlated with the probability of developing insulin-dependent diabetes.

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sequence containing two ILPR repeats (ILPR repeat sequences such as the ILPR (24–30). While a common finding in telomeric DNA and other tandem sequences in general, few studies have focused on two or more quadruplexes in the same molecule, which are most likely to form an intramolecular G-quadruplex (19,20), no structural studies have focused on the ILPR with longer lengths (ILPR<sub>n-2</sub>) that are biologically more relevant. Based on simulation studies, Gupta and co-workers have predicted that longer ILPR sequences could potentially form a higher order quadruplex structure (31). Due to the lack of structural evidence on sequences that have the potential to form multiple quadruplexes, the current knowledge of quadruplex structures in these sequences is based mostly on simulation studies, and on the predictions from observations on single G-quadruplex forming units (27,31). However, the validity of these predictions has yet to be adequately tested.

Experimental studies conducted on multiple intramolecular quadruplex-forming sequences have been focused primarily on the telomere regions where different models have been postulated. Recently, Yu et al. (25) proposed that long human telomere sequences form a ‘bead on a string’ structure where each G-quadruplex exists as distinct noninteracting units. In contrast, based on crystal structure and simulation studies, a model of higher-order interactions between stacked telomeric G-quadruplexes has been proposed (27,32) and confirmed in a GGA triplet-repeat sequence (29,33). However, Petraccone et al. (34) in a recent biophysical and simulation study has suggested a different type of higher-order interaction involving loop–loop stacking between the two adjacent quadruplexes. In general, other higher-order quadruplex–quadruplex interactions (QQI) could also be envisaged, such as loop-mediated interactions via small molecules or intermolecular intercalation of quartet planes (28,35). However, there is still a paucity of experimental evidence for interactions between contiguous intramolecular quadruplexes (QQI).

Current understanding of multiple intramolecular quadruplex structure is often based upon knowledge gained from studies on single quadruplexes. However, formation of multiple intramolecular quadruplex structures, stacking or nonstacking, may be influenced by a neighboring quadruplex, although the extent of such effect is not well understood and is difficult to predict. Thus, it can be proposed that findings on single quadruplexes may not necessarily be extended to predict structures of multiple quadruplexes.

In this study, we have used native PAGE, dimethyl sulfate (DMS) footprinting, circular dichroism (CD) spectroscopy and mechanical unfolding at the single molecular level by optical tweezers to study a dual quadruplex forming ILPR sequence (ILPR<sub>n-4</sub>). Our results show that the conformation adopted by multiple intramolecular quadruplexes cannot be automatically predicted from structures of individual quadruplex units. Furthermore, our results suggest the existence of a QQI in the folded structure of the ILPR<sub>n-4</sub>. While higher-order structures have been predicted in the ILPR (31), this study provides experimental evidence in direct support of this prediction.

MATERIALS AND METHODS

Materials

DNA samples were purchased from Integrated DNA Technologies™ (www.idtdna.com) and purified by denaturing PAGE. Concentrations of the DNA were calculated using the UV absorbance value at 260 nm and the extinction coefficients for each single-stranded oligonucleotide were calculated by the nearest-neighbor approximation (36). The sequences of the oligonucleotides used in this study are of the following composition:

ILPR<sub>n</sub>: 5′-(ACAGGGGTGTGGGG)ₙ₋₄ (n = 1,2,3)
ILPR<sub>n-4</sub>: 5′-(ACAGGGGTGTGGGG)ₙ₋₄ACAGGGGTGTTGGGACAGGGGTGTTGGG
IQ56: 5′-(ACAGGGGTGTGGGG)₂ATAATAATAAAATATATAATAATAAAAT

CD spectroscopy

For the current study, 5 µM of the DNA samples were prepared in 10 mM Tris–HCl buffer pH 7.8 with 100 mM potassium chloride or 100 mM lithium chloride. Before measurement, the DNA samples were heated to 97 °C for 10 min and cooled at a rate of 0.2 °C/min to 25 °C. CD spectra were recorded on a Jasco-810 spectropolarimeter (Easton, MD) using a quartz cuvette with a 1-mm optical path length at a temperature of 25°C. The spectra obtained were the average of three scans over a range of 200–320 nm at a 0.5-nm interval with a 1-mm optical path length at a temperature of 25°C.
scan rate of 50 nm/min. The resulting spectra were then subtracted from a buffer only baseline and smoothed using a Savitzky–Golay function.

Thermal denaturation studies
For all melting experiments 5 μM of ILPR DNA in 10 mM Tris–HCl pH 7.8, 100 mM KCl was heated to 97°C for 10 min and annealed by cooling to 25°C at a rate of 0.2°C/min. The sample was then melted at an identical rate and CD values were recorded at 0.5°C intervals. Before melting, ~100 μl of mineral oil was placed on top of the solution to eliminate evaporation. The CD melting experiments were performed on the previously mentioned spectropolarimeter with a 1-mm optical path length quartz cuvette and the temperature was controlled by a Jasco (model PFD-425S) peltier temperature controller (Easton, MD, USA).

Preparation of 5’ end radiolabeled DNA
DNA samples were radiolabeled at the 5’ end by incubating the DNA with T4 polynucleotide kinase (Promega™) and [γ-32P]ATP (Perkin Elmer™). The labeled products were then purified using denaturing PAGE.

Native PAGE
Radiolabeled oligonucleotides were prepared in 10 mM Tris–HCl pH 7.8 with 100 mM KCl. Before electrophoresis each sample was heated at 97°C for 10 min and cooled to 25°C at a rate of 0.2°C/min. The samples were analyzed on a 10% native acrylamide (19:1 acrylamide:bisacrylamide) gel containing 100 mM KCl and run in 1 X Tris/Borate/EDTA buffer at room temperature. The running buffer was periodically recirculated to maintain the salt concentration at the same level throughout the duration of the experiment.

DMS footprinting
Oligonucleotide samples radiolabeled at the 5’-end were prepared in 10 mM Tris–HCl pH 7.8, 100 mM KCl with 1 μM of cold ILPR DNA. Mixed samples measuring 100 μl were then heated at 97°C for 10 min and cooled to 25°C at 0.2°C/min. The samples were analyzed on a 10% native acrylamide (19:1 acrylamide:bisacrylamide) gel containing 100 mM KCl and run in 1 x Tris/Borate/EDTA buffer with 100 mM KCl at room temperature. The running buffer was periodically recirculated to maintain the salt concentration at the same level throughout the duration of the experiment.

Laser tweezers instrument
For the single molecule experiment, a diode pumped solid-state (DPSS) laser (1064 nm, 4 W, CW mode, BL-106C, Spectra-physics) was used as a laser source. Details on the laser tweezers instrument have been described previously (37). Briefly, a single laser source was used to create two laser traps consisting of P and S polarized light, respectively. The S polarized light was controlled by a steerable mirror (Nano-MTA, Mad City Labs) at a conjugate plane of the back focal plane of a focusing objective (Nikon CFI-Plan-Apochromat 60x, NA 1.2, water immersion, working distance ~320 μm). The exiting P and S polarized beams were collected by an identical objective and detected by two position sensitive photodetectors (PSD, DL100, Pacific Silicon Sensor).

DNA construct for the single molecule studies
The DNA construct comprises of ILPR-59 (the underlined part in 5’–CTA GAC GGT GTG AAA TAC CGC ACA GAT GCG (ACA GGG GTG TGG GG)4ACA GCC AGC AAG ACG TAG CCC AGC GCG TC) sandwiched between two dsDNA handles with unequal lengths. The longer and shorter dsDNA handles have digoxigenin and biotin attached to their free ends, respectively. The synthesis was performed according to previous report (37). Briefly, the ILPR-59 was annealed with XbaI oligo (5’-CGCATCTGCTGCGGTATTTCACACCGT-3’), Eagl oligo (5’GGCGGACGCGCTGGGCTACGTCTTCTGGC-3’) at the two ends in order to hybridize with the dsDNA handles. XbaI oligo was hybridized with the shorter dsDNA handle (615 bp) whereas the Eagl oligo was hybridized with the longer dsDNA (2690 bp) (Scheme 1).

Single molecular experiments
For the single molecular experiment, the appropriate amount of DNA construct obtained above was incubated with streptavidin-coated polystyrene beads (diameter: 0.97 μm, Bangs Laboratory) for 1 h at room temperature and diluted to 700 μl working buffer (100 mM KCl, 2 mM EDTA, 10 mM Tris buffer, pH 8.0). The streptavidin coated beads linked with the DNA construct and the anti-digoxigenine coated beads (diameter: 2.17 μm, Spherotech) were injected separately into a reaction chamber via different channels.

These two types of beads were captured separately by the two laser traps. The two beads were rubbed against each other until the free end of a DNA molecule on

Scheme 1. Illustration of DNA construct used for the mechanical unfolding experiments.
the streptavidin-coated bead was tethered to the anti-
digoxigenine coated bead via the digoxigenine and anti-
digoxigenine complex. The two traps were then moved away from each other via the steerable mirror. A sudden drop in the tension was observed whenever there was an unfolding of a secondary structure. The force-extension curve for each tether was recorded in a Labview® program and the detail data treatment was performed using the programs in Matlab® and Igor® softwares. The single tether was confirmed by observing the single breakage of the tether during extension. Only these single tethered force-extension curves were considered for data analysis. To find the change in contour length during the rupture of a folded structure, individual force-extension curves were fitted with an extensive WLC formula (38),

\[
x = \frac{L}{\ell} = 1 - \left(\frac{k_BT}{FP} \right)^\frac{1}{2} + \frac{F}{S}
\]

where \(F\) is the force, \(T\) is absolute temperature, \(S\) is the stretch modulus, \(P\) is the persistence length, and \(x\) and \(L\) are end-to-end distance and contour length of the folded structure, respectively. The values of \(P\) and \(S\) at room temperature were taken from literature (38).

**Prediction of probability of simultaneous unfolding**

If the probability of unfolding a single domain at a particular force \(F_i\) is \(P(F_i)\), then the probability of unfolding both domains simultaneously \(P_{\text{simultaneous}}\) between the observed minimum \((F_1)\) and maximum \((F_2)\) unfolding force is given by

\[
P_{\text{simultaneous}} = \sum_{i=1}^{\text{No. of bins}} \left[P(F_i)\right]^2
\]

For continuous force range between \(F_1\) and \(F_2\),

\[
P_{\text{simultaneous}} = \int_{F_1}^{F_2} \left[P(F)\right]^2 \, dF
\]

\(P(F)\) can be estimated from a Gaussian distribution of unfolding forces of a single domain between \(F_1\) and \(F_2\). To obtain \(P_{\text{simultaneous}}\), numerical integration of the above function was performed with 1000 intervals from 5 to 55 pN.

**RESULTS AND DISCUSSION**

**ILPR sequences indicate length-dependent structural features**

To elucidate the effect of a G-quadruplex on the conformation adopted by its neighboring quadruplex, we decided to focus in depth on a multiple G-quadruplex-forming sequence containing four repeats of the predominant ILPR variant ‘a’ (ILPR\(_n=4\), see ‘Materials and Methods’ section for sequence details). Based upon the number of G-rich stretches, the ILPR\(_n=4\) can form two contiguous quadruplexes. We chose this sequence because the occurrence of homomeric repeats of any variants greater than four are very rare in the ILPR (data not shown).

First, we used native gel electrophoresis to show that the ILPR\(_n=4\) form compact structures. Figure 2A shows the ILPR oligonucleotides of various lengths separated on a native gel with 100 mM KCl both in the gel and the running buffer. The ILPR\(_n=4\) (see ‘Materials and Methods’ section for sequences) showed an increased mobility compared to nonstructure-forming oligonucleotides of identical sizes (Figure 2A, lane M), indicating the formation of intramolecular structures. Upon quantitation of the bands in the gel, ILPR\(_n=4\) formed \(~99\%\) intramolecular structures, with the exception of \(n=3\), which formed \(~5\%\) intermolecular structures. However, ILPR\(_n=1\) containing only two guanine stretches, incapable of forming an intramolecular G-quadruplex, showed mobilities consistent with either an intermolecular structure (see ** in Figure 2A) or the single stranded DNA form (see * in Figure 2A). Then, we used CD spectroscopy to confirm that the intramolecular structures observed in ILPR\(_n=4\) are G-quadruplexes. G-quadruplexes with a parallel folded topology have been shown in most cases to have a positive peak at \(~265\) nm and a negative peak at \(~240\) nm; while antiparallel folded topologies are generally associated with a positive \(~295\) nm peak and a negative peak at \(~260\) nm (39). In case of ILPR\(_n=4\) that is capable of forming one G-quadruplex, the CD spectrum in buffer containing 100 mM KCl is consistent with an antiparallel quadruplex structure (Figure 2B). However, with the addition of a second G-quadruplex forming unit to the ILPR\(_n=2\), which forms ILPR\(_n=4\), under identical conditions the CD spectrum implies the presence of parallel and antiparallel quadruplex forms (Figure 2B, see discussions below). This result demonstrates that the quadruplex conformation of ILPR\(_n=4\) may not be a simple addition of two ILPR\(_n=2\) quadruplex units. In addition, CD spectra of ILPR\(_n=2\) in 100 mM LiCl were absent of any quadruplex type features, demonstrating the dependence of quadruplex formation in the ILPR sequences on the monovalent cation (Supplementary Figure S1).

The ILPR\(_n=4\) can potentially form two G-quadruplexes or a single G-quadruplex utilizing appropriate four G-stretches. To eliminate the latter possibility, we designed a 56-nt mutant of ILPR\(_n=4\) containing a quadruplex forming sequence 5’-(ACAGGGGTGTGGGG)\(_{2-3}\) linked to a 28-mer nonstructure forming oligonucleotides at the 3’ end (1Q56, see ‘Materials and Methods’ for full sequence). The 1Q56 has the same length as the ILPR\(_n=4\), but can only form one G-quadruplex linked to an unstructured 3’ DNA tail. CD spectrum confirmed such structural conformation in 1Q56, which showed a \(~295\) nm peak characteristic of an antiparallel quadruplex structure, and the spectral features at shorter wavelengths (40,41) implying the unstructured DNA tail (Supplementary Figure S2). This conformation of 1Q56 was further supported by DMS footprinting which revealed the protection of the four G-tracts at the 5’ end, most likely due to the formation of a G-quadruplex (10). Additionally, when equal concentrations of the 1Q56 and ILPR\(_n=3\) were analyzed, the magnitudes of the 295 nm peaks in both CD spectra
were almost identical, suggesting the formation of equal amount of quadruplex structures in each of the sequences (Supplementary Figure S2). These data led us to conclude that the 1Q56 can form a single quadruplex and that the unstructured DNA tail in the 1Q56 has no effect on the adjacent quadruplex. As 1Q56 and ILPR\(_n=4\) are equal in length, but 1Q56 can only form a single quadruplex, the mobility of the 1Q56 in native gel can be used to indicate whether two G-quadruplexes are formed in ILPR\(_n=4\). The native gel in Figure 2C showed that both ILPR\(_n=4\) and 1Q56 had higher mobilities than the fully unstructured 56-nt marker; and that the mobility of the ILPR\(_n=4\) was greater than 1Q56. These results strongly suggested the formation of two quadruplexes in the ILPR\(_n=4\). Further evidence of the dual quadruplex formation came from DMS footprinting, which revealed that all eight G-tracts in ILPR\(_n=4\) were protected in the presence of potassium cations (Supplementary Figure S3), very likely due to the formation of two G-quadruplexes (10). Thus, results from DMS footprinting, CD, and native gel collectively established that the ILPR\(_n=4\) forms an intramolecular structure with two G-quadruplexes.

As mentioned before, the CD spectra showed a dramatic change between ILPR\(_n=2\) and ILPR\(_n=4\). This change is entirely different from that observed in the spectra between one and two G-quadruplex containing units in human and Oxytricha telomeres under nearly identical conditions (25,34) (Figure 3). In contrast to the ILPR sequences, as the length of Oxytricha (25) and Human telomere (25,34) sequences increased, the relative distribution of the CD peaks at 265 nm and 295 nm that suggested parallel and antiparallel strands respectively, remained almost identical (Figure 3). The length-dependent CD features in ILPR provides strong evidence to our hypothesis that conformation of two quadruplexes in ILPR\(_n=4\) cannot be extracted directly from individual quadruplex structures as suggested by CD signals in telomeres (25,34).

So far our results indicate that the ILPR\(_n=4\) forms an intramolecular structure with two contiguous quadruplexes. In addition, CD results from ILPR\(_n=2\) sequences
show quadruplex structural features that suggest a quadruplex in *cis* may influence the conformation of its adjacent unit.

**CD and single-molecule force measurements suggest the presence of hybrid parallel/antiparallel structures**

To investigate the effect of the neighboring quadruplexes on the conformation of the dual quadruplexes in the ILPR<sub>n=4</sub>, we performed CD melting of the sequence. At temperatures up to 80°C, the 295 nm and 265 nm peaks remained essentially unchanged. However, at temperatures above 80°C, there was a simultaneous change in both peaks with a concurrent increase in the 260 nm peak (Figure 4). The 295 nm and 265 nm melting showed clear transitions with identical inflection points (T<sub>1/2</sub> = 87°C, inset Figure 4) calculated from the first derivative. It should be noted that, the deconvolution of the 265 nm melting is more complicated as several factors (change in parallel/antiparallel) quadruplex features (positive and negative peaks at ~265 nm, respectively) and ssDNA formation (positive peak at ~260 nm) with increasing temperature contribute to its magnitude.

The appearance of the 260 nm peak with concurrent loss of the 295 nm and 265 nm peaks has been observed in the formation of unstructured ssDNA during simultaneous melting of quadruplex strands (41,42). Thus, it appears that the antiparallel and parallel strands in ILPR<sub>n=4</sub> quadruplexes melt simultaneously. Similar simultaneous melting observed previously on a human telomeric sequence has been attributed to a hybrid-type intramolecular G-quadruplex (42), therefore, quadruplexes in the ILPR<sub>n=4</sub> may adopt a hybrid conformation.

Previously, similar CD melting of a single ILPR<sub>n=2</sub> quadruplex by our group revealed two different transitions, which were assigned to the melting of distinct parallel and antiparallel G-quadruplexes, respectively (37). It should be noted that the ILPR<sub>n=2</sub> CD spectrum is dependent on the rate of annealing with slower rates favoring the antiparallel structure and faster rates favoring the parallel structure (data not shown). In the current study, quadruplexes formed in the ILPR<sub>n=4</sub> were annealed slowly to match the conditions used for the multiple quadruplex forming telomere sequences (25). From our results so far, it is possible that both quadruplexes in the ILPR<sub>n=4</sub> adopt a hybrid conformation, which will then be entirely different from the coexisting mixture of parallel and antiparallel G-quadruplex structures observed in ILPR<sub>n=2</sub> (37).

The formation of hybrid G-quadruplexes in ILPR<sub>n=4</sub> is fully supported by the single molecular studies using laser tweezers. Previously, we have applied laser tweezers to mechanically unfold G-quadruplex structures in the ILPR<sub>n=2</sub> (37). The presence and property of specific quadruplex species can be revealed from the histograms of unfolding forces of these species. Using the same approach (see ‘Materials and Methods’ section), we have observed a total of three types of events that corresponded to the unfolding of one or two G-quadruplexes in a DNA construct containing the ILPR<sub>n=4</sub> sequence (see ‘Materials and Methods’ for the details of the DNA construct). The first type showed a single rupture event with a change in contour length (<i>DL</i>) <15 nm in the force-extension curve (Figure 5A). The value of <i>DL</i> is consistent with that of a 25-mer ILPR G-quadruplex (37), suggesting only one G-quadruplex is present in the DNA construct.

**Figure 4.** Temperature-dependent change in CD spectra of ILPR<sub>n=4</sub>. Inset: CD melting curves at 265 nm and 295 nm. Dotted lines represent sloping baselines of 265 nm and 295 nm melting curves.

**Figure 5.** (A) Force-extension curves of the 59-mer ILPR sequence (see ‘Materials and Methods’ section for detailed sequence) showing a single rupture event. The rupture event is indicated inside a dotted circle. The red and green lines represent WLC model (see text) fittings for extension and relaxing curves, respectively. (B) Histogram of the rupture forces from (A). The data are fitted with a Gaussian curve shown in blue.
The histogram of the rupture force for this event showed a single peak centered at 28.4 ± 0.4 pN (Figure 5B). Previously, we observed that the ILPR\textsubscript{n}=2 containing a single G-quadruplex unit could be mechanically unfolded either at 22.6 pN or 36.9 pN (37), two forces corresponded to unfolding of parallel and antiparallel G-quadruplexes, respectively. The single unfolding force observed in ILPR\textsubscript{n}=4 was located between these two values, strongly suggesting that the G-quadruplex formed in the ILPR\textsubscript{n}=4 was neither purely parallel nor purely antiparallel. Instead, it most likely assumed a single hybrid G-quadruplex conformation with both parallel and antiparallel strand orientations.

The second type of mechanical unfolding revealed two sequential unfolding events (Figure 6A). Each event had the $\Delta L < 15 \text{ nm}$, indicating two G-quadruplexes were unfolded successively. Force distribution histogram for either of the quadruplexes during this sequential unfolding was similar to that of the first unfolding type, suggesting the hybrid conformation for quadruplexes. Therefore, our mechanical unfolding experiments provided compelling evidence to support the finding from CD studies that G-quadruplexes with hybrid conformation may exist in ILPR\textsubscript{n}=4. This reinforces our proposal that the G-quadruplex conformation in ILPR\textsubscript{n}=4 cannot be simply derived from the structure of quadruplex in ILPR\textsubscript{n}=2.

The force-extension curve shown in Figure 6B represented the third type of rupture events. It had only a single rupture whose $\Delta L$ was between 15 and 30 nm. Since this value was twice the value obtained from the rupture of a single G-quadruplex, it could be inferred that the rupture represents simultaneous unfolding of two G-quadruplexes. These two G-quadruplexes may exist independently or via QQI.

In contrast to the biochemical finding that majority of the ILPR\textsubscript{n}=4 forms two G-quadruplexes, mechanical unfolding experiments revealed that one G-quadruplex was more favored in the same ILPR sequence, perhaps due to the constraints imposed on the ILPR\textsubscript{n}=4 by the flanking dsDNA handles (see Scheme 1 and ‘Materials and Methods’ section). Compared to almost all of the DNA constructs used in current systems to study quadruplexes (43), the constraints presented in our system provided a unique resemblance to the in vivo situation where G-rich regions are almost always flanked by dsDNA regions.

Analysis of the simultaneous unfolding events suggests the existence of QQI in ILPR\textsubscript{n}=4

To clarify whether QQI exists between the two G-quadruplexes during the simultaneous unfolding of quadruplexes in ILPR\textsubscript{n}=4, we plotted the histogram of change in contour length ($\Delta L$) for both sequential and simultaneous ruptures. The histogram clearly showed two populations that can be well fitted by a two-peak Gaussian equation (Figure 6C, notice the two
ΔL peaks at 8.6 ± 0.3 nm and 18.1 ± 0.5 nm). Within experimental error, the ΔL of the simultaneous unfolding (18.1 ± 0.5 nm) was twice that of individual ruptures in the sequential unfolding events (8.6 ± 0.3 nm). This result confirmed that the simultaneous rupture of two G-quadruplexes occurred in ILPR<sub>n=4</sub>. Our calculations (see ‘Materials and Methods’ section) further showed that the predicted probability for the two independent G-quadruplexes to be ruptured simultaneously was 10.96% (see dotted line in Figure 6C for the expected population). However, the probability of the simultaneous unfolding observed here was 33.77% (notice the population fitted with the blue line Figure 6C), a value significantly higher than that predicted from the concurrent unfolding of the two independent G-quadruplexes (10.96%). This discrepancy can be well explained if QQI exists between the two G-quadruplexes in the ILPR<sub>n=4</sub>. The increased tension during the stretching of the ILPR<sub>n=4</sub> might destroy this interaction, leading to cooperative unfolding of both G-quadruplexes. Similar interpretation has been used to explain the hairpin–hairpin interaction between two RNA hairpins in a mechanical unfolding investigation (44).

DMS footprinting reveals nucleotides possibly involved in the QQI

To further investigate the potential QQI predicted by the single molecule studies, we used DMS footprinting to probe the N7 of guanines (45) involved in the G-tetrads (Figure 1A).

In order to explore directly the possible structural effect due to the presence of an adjacent quadruplex, we compared the DMS footprints of the ILPR<sub>n=4</sub> to the IQ56 that forms only one G-quadruplex at the 5′ end. Compared to DMS treatment in the absence of K⁺ in which G-quadruplex formation is minimal, when 100 mM K⁺ was added, both sequences showed significant protection of nearly all Gs except the ones in the TGT loops and in the 5′ terminal G-tract [G₄(1), Supplementary Figure S3]. It is not unusual to see enhanced cleavage at the 5′ and 3′ ends of G-quadruplexes as the terminal nucleotides are more accessible to DMS (11) possibly due to ‘breathing’ of the structure. The more pronounced cleavage of the two terminal G-tracts [G₄(1) and G₄(4) in Figure 7A] in IQ56 conforms to this pattern, suggesting that the nonstructured segment at the 3′ tail does not contribute any protection to the terminal Gs of the G-quadruplex. However, ILPR<sub>n=4</sub> only shows enhanced cleavage at the 5′ end [G₄(1)], but not the 3′ end [G₄(4)] of the 5′ terminal G-quadruplex, most likely due to the presence of the downstream G-quadruplex. The effect of the neighboring G-quadruplex is more obvious after normalizing the bands of ILPR<sub>n=4</sub> against those of IQ56, which clearly demonstrates a maximal protection of the last G in the G₄(4) of the ILPR<sub>n=4</sub>, but not IQ56 (Figure 7B, see ‘Materials and Methods’ section for details on quantitation; see ‘→’ denoting the last G in Figure 7A). These observed protections in the 5′ end G-quadruplex can be ascribed either to the presence of the neighboring quadruplex itself, or to the QQI between the two tandem quadruplexes.

To determine the probable cause of the observed protection, we intended to compare ILPR<sub>n=4</sub> to the most widely studied example of the human telomere (TTAGGG₃₅₅)₅, or hTel. Under DMS footprinting conditions identical to the ILPR<sub>n=4</sub> treatment, the first four G-stretches from the 5′ end of the hTel [G₃(1)-G₄(4)] showed footprinting patterns very similar to the IQ56 (Figure 7A). Remarkably, the last guanine of the fourth G-stretch [see ‘→’ in the G₄(4)] showed strikingly enhanced methylation (or less protection) akin to the IQ56, which is markedly different from that observed in the ILPR<sub>n=4</sub>. In the most recent model of the dual quadruplex in hTel (34), the QQI is through the loop nucleotides without the involvement of the terminal guanine of the 5′ G-quadruplex. This may explain the enhanced methylation of the terminal guanine possibly due to ‘breathing’ akin to that of a DNA duplex (46,47). Such ‘breathing’ could also be contributed by the bending or flexibility in the linker region (ACA) between the two quadruplexes in ILPR<sub>n=4</sub>. Compared to the hTel, the better protection of the terminal guanines in the ILPR<sub>n=4</sub> strongly suggests that the two quadruplexes in ILPR<sub>n=4</sub> are more closely associated to effectuate a better suppression of the structural ‘breathing’, the lack of which leads to the easier methylation of the guanines in current hTel models (25,32,34). The presence of different linkers in hTel and ILPR<sub>n=4</sub> suggests the important contribution of linker composition toward the suppression of ‘breathing’. Interestingly, there is a similar increase in methylation at the 5′ end of the first G-quadruplexes of both hTel and ILPR<sub>n=4</sub>, which not only supports the ‘breathing’ at that end, but also implies a relatively constrained fourth G-stretch [G₄(4)] in the ILPR<sub>n=4</sub>; a possible result from the QQI in the ILPR. Therefore, combining with the IQ56 DMS footprinting results and the single molecular data, we suggest that there is a QQI, which may be different from current models in hTel (25,32,34).

Structural features of multiple ILPR G-quadruplexes

In RNAs and proteins, higher-order structures are commonplace, however, in the context of DNA, there is significantly less structural diversity. Several higher-order intermolecular G-quadruplex structures, such as interlocking or ‘slipped’ G-quadruplexes (48), G-octaplex (35), G-wire formation (49) or Frayed Wires (50), have been reported. Although, many of these structures are very stable, the higher-order intermolecular interaction seems improbable in vivo where intramolecular interactions are likely to prevail. However, only very few studies, including those on the ILPR (24), have focused on ‘intra-molecular’ multi G-quadruplex forming sequences. Although, lacking in specific details of the interactions, simulation studies on multimeric ILPR sequence have predicted intramolecular higher-order structures (QQI) (31).

The exact nature of the proposed QQI is unclear at this point. It is possible that the protection is due to a direct contact involving the fourth G-stretch. Alternatively, the protection may be attributed to a
distal interaction that increases the rigidity of the 5’ quadruplex structure to suppress the ‘breathing’ of its terminal G-stretch (see discussion above).

Based upon our data, we propose that the ILPR\textsubscript{n=4} sequence has the following characteristics: (i) quadruplexes in the ILPR\textsubscript{n=4} adopt a hybrid structure containing both parallel and antiparallel strands based on CD and single molecular studies, which elucidates the fact that the conformation of quadruplexes in ILPR\textsubscript{n=4} is not a simple addition of two ILPR\textsubscript{n=2} units, and (ii) presence of a QQI that may lead to a more rigid G-stretch between the two quadruplexes in the ILPR\textsubscript{n=4}.

**Potential biological significance**

Higher-order quadruplex structures in the ILPR could be postulated to have diverse biological consequences. The ILPR has been shown to have extreme polymorphism
with a ~90% heterozygosity rate (18). To explain this phenomenon it has been hypothesized that replication errors in the ILPR and other tandem repeat regions are  

resulted from the inhibition of replication by G-quadruplex or other noncanonical DNA structures (17,18). Experimental evidence for this hypothesis has been provided by Gupta and co-workers who demonstrated in vitro that inhibition of replication can be ascribed to the structure formation in the ILPR in a manner that is dependent on the number of variant ‘a’ repeats (23,31). Since, they also proposed that higher-order quadruplex structure may form in the ILPR with larger number of repeats, it is tantalizing to speculate that the higher-order structures may contribute to the observed increase in replication errors. Additionally, the transcription factor Pur-1 and the Insulin protein have been shown to bind specifically to ILPR G-quadruplexes (24,51). The formation of higher-order quadruplex structures via QQI may therefore, affect the binding of various proteins, which potentially can affect gene regulatory processes.

Conclusion

We have shown that the ILPR<sub>2-4</sub> capable of forming dual G-quadruplexes adopts a folded structure through a possible QQI. DMS footprinting suggests that the QQI in the ILPR is different from that observed in the telomere sequence. In addition, it is clear from our study that knowledge gained from single quadruplex structures may not always be automatically extended to describe the formation in multiple quadruplexes, whose structures must merit individual attention. This work will begin to elucidate the structural basis of the polymorphism in the ILPR as well as other mini-satellite regions, and will also provide insights into the basic mechanisms of the contribution of these regions to diseases such as IDDM. However, the contribution of sequence and length polymorphism to the quadruplex structure and more importantly, to the biological function of the ILPR, is still not well understood, and will become important subjects for future investigations.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Dr Scott Strobel, Dr Fred Walz, and Mark Morris for their critical reading of this manuscript.

FUNDING

The Kent State University (KSU) and an Ohio Board of Regent grant (to S.B. and H.M.); a New Faculty Award Program at Camille and Henry Dreyfus Foundation (to H.M.); and NIH 810008116 (to H.M. and S.B.).

Conflict of interest statement. None declared.

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