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Formation of (3+1) G-Quadruplexes with a Long Loop by Human Telomeric DNA Spanning Five or More Repeats

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Supporting Information

ABSTRACT: Structural studies of human telomeric repeats represent an active field of research with potential applications toward the development of specific telomeric quadruplex-targeting drugs for anticancer treatment. To date, high-definition structures were limited to DNA sequences containing up to four GGGTTA repeats. Here we investigate the formation of G-quadruplexes in sequences spanning five to seven human telomeric repeats using NMR, UV, and CD spectroscopy. A (3+1) G-quadruplex with a long propeller loop was isolated from a five-repeat sequence utilizing a guanine-to-inosine substitution. A simple approach of selective site-specific labeling of guanine residues was devised to rigorously determine the folding topology of the oligonucleotide. The same scaffold could be extrapolated to six- and seven-repeat sequences. Our results suggest that long human telomeric sequences consisting of five or more GGGTTA repeats could adopt (3+1) G-quadruplex structures harboring one or more repeat(s) within a single loop. We report on the formation of a Watson−Crick duplex within the long propeller loop upon addition of the complementary strand, demonstrating that the long loop could serve as a new recognition motif.

Telomeres serve as protective caps at the ends of linear eukaryotic chromosomes,[1] playing a crucial role in cell survival and proliferation.[2] Tandem repeats of the sequence $(GGGTTA)_n$ constitute the human telomeres,[3] with pendent G-rich single strands of 100−200 nt at the 3′-ends.[4] The propensity of these G-rich overhangs to form G-quadruplexes,[5] and the inhibitory effects of such structures on the catalytic activity of the enzyme telomerase,[6] have led to a growing interest in the development of specific telomeric quadruplex-targeting drugs for potential anticancer treatment.[7] A multitude of techniques have been applied to study the possible structures formed by these arrays in physiologically relevant conditions and concurred on their polymorphic nature.[8−10] At least five different intramolecular G-quadruplex folding topologies have been observed for sequences containing four GGGTTA repeats: the sequence d[A$(GGGTTA)_3$GGG] adopts in Na^+ solution[8a] and K^+-containing crystal[8b] an antiparallel-stranded basket-type and a parallel-stranded propeller-type G-quadruplex, respectively; the sequences d[TA$(GGGTTA)_3$GGG] and d[TA$(GGGTTA)_3$GGGTT] form in K^+ solution predominantly (3+1) G-quadruplexes Form 1[8d−f,h] and Form 2,[8g,h] respectively, differing in the successive order of loop arrangements; and the sequence d[$(GGGTTA)_3$GGGT] forms in K^+ solution predominantly a two-G-tetrad basket-type G-quadruplex[8i] (Form 3). These diverse structures might coexist in the telomere, and insights into their structures would contribute toward drug design efforts targeting telomeric G-quadruplexes. It remains a challenge to resolve the structures adopted by longer telomeric repeats.[9] Recent studies suggested the preferential formation of G-quadruplex at the 3′-end of sequences containing five or more repeats,[9d] as well as the stacking of two intramolecular G-quadruplexes within eight repeat sequences.[9e,f] Here we investigate the formation of G-quadruplexes in sequences containing five to seven human telomeric repeats using NMR, UV, and CD spectroscopy. Our results suggest that long human telomeric sequences consisting of five or more GGGTTA repeats could adopt (3+1) G-quadruplex structures harboring one or more repeat(s) within a single loop, which could represent a new recognition motif for the targeting of human telomeric DNA.

Imino proton spectra of the series of five-repeat natural human telomeric DNA sequences with different flanking ends (Table S1, Supporting Information (SI)) in K^+ solution indicated the presence of multiple G-quadruplex conformations (Figure 1a−e; Figure S1, SI). Here the 31-nt five-repeat sequence d[TA$(GGGTTA)_4$GGGTT] (denoted as *31htel*; Table 1) was chosen for subsequent analyses. This sequence bears the same flanking ends as the 25-nt four-repeat sequence d[TA$(GGGTTA)_3$GGGTT] (denoted as *25htel*; Table 1), which was previously studied by NMR.[8g,h] It has been shown that substitution of a guanine by the guanine derivative inosine in a DNA sequence could selectively disfavor particular G-quadruplex conformations.[11] Here, inosine replacement at the middle G of individual G-tract, i.e. giving rise to GIG, was separately applied to all five G-tracts of *31htel* (Table S2, SI) and showed successful moderation of the multiplicity of existing conformations, as evidenced by the presence of fewer and sharper imino proton peaks across the NMR spectra of all five modified sequences as compared to the parent sequence *31htel* (Figure 1f−j). Additionally, the absence of sharp imino proton peaks at 13−14 ppm[12] indicated that inosine bases did not take part in G-tetrad core formation. One might further suppose that the major conformations induced by each inosine substitution correspond to G-quadruplex structures in which the GIG tract was excluded from the G-tetrad core. Single inosine substitutions at the first, third, and fifth G-tracts did not favor a single major conformation (Figure 1f,h,j). In contrast, individual conversions of the second and fourth G-tracts to GIG resulted in a predominant species (estimated population >80%; Figure 1g,i). In particular, the latter oligonucleotide (denoted as *31htel[I22]*;

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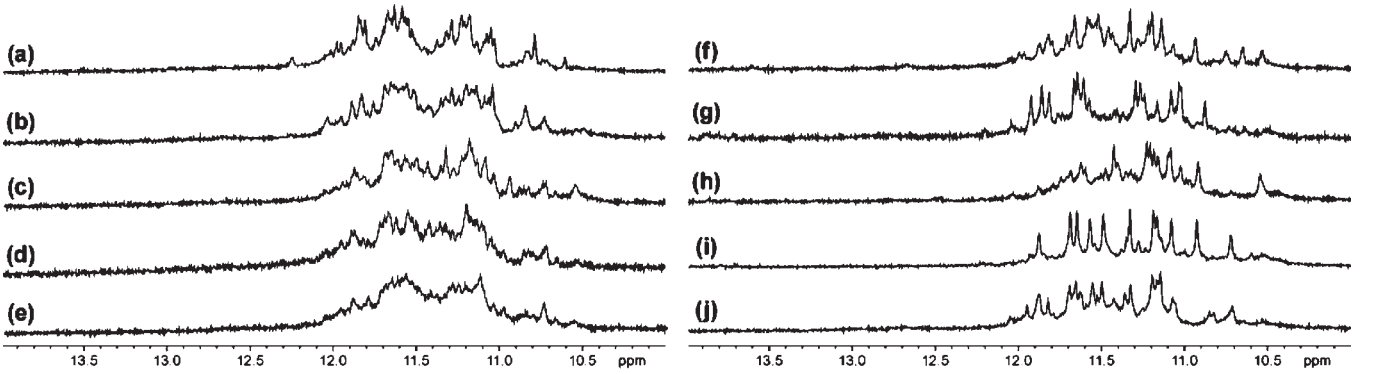

Figure 1. Imino proton spectra of (a−e) natural and (f−j) inosine-modified five-repeat human telomeric sequences in K^+ solution: (a) d[TA(GGGTTA)$_4$GGG], (b) d[TA(GGGTTA)$_4$GGGT], (c) d[TA(GGGTTA)$_4$GGGTT], (d) d[TA(GGGTTA)$_4$GGGTTA], (e) d[TTA(GGGTTA)$_4$GGGTTA], (f) d[TAGIGTTA(GGGTTA)$_3$GGGTT], (g) d[TAGGGTTAGIGTTA(GGGTTA)$_2$GGGTT], (h) d[TA(GGGTTA)$_2$GIGTTAGGGTTAGGGTT], (i) d[TA(GGGTTA)$_3$GIGTTAGGGTT], and (j) d[TA(GGGTTA)$_4$GIGTT].

Table 1. Representative Natural and Modified Human Telomeric DNA Sequences Used in This Study

name	sequence[a]			T_m[b] (°C)
25htel	TA GGG TTA GGG TTA GGG	TTA	GGG TT	62.0
31htel	TA GGG TTA GGG TTA GGG	TTA GGG TTA	GGG TT	–
31htel[I22]	TA GGG TTA GGG TTA GGG	TTA G**I**G TTA	GGG TT	49.8
37htel[I22/I28]	TA GGG TTA GGG TTA GGG	TTA G**I**G TTA G**I**G TTA	GGG TT	47.7
43htel[I22/I28/I34]	TA GGG TTA GGG TTA GGG	TTA G**I**G TTA G**I**G TTA G**I**G TTA	GGG TT	46.5
43htel[T22/T28/T34]	TA GGG TTA GGG TTA $^{\mathbf{Br}}$**G**GG	TTA G**T**G TTA G**T**G TTA G**T**G TTA	GGG TT	–

[a] Modified residues are in boldface. [b] Melting temperature in ~100 mM K^+ solution, as monitored by 295-nm UV absorbance.

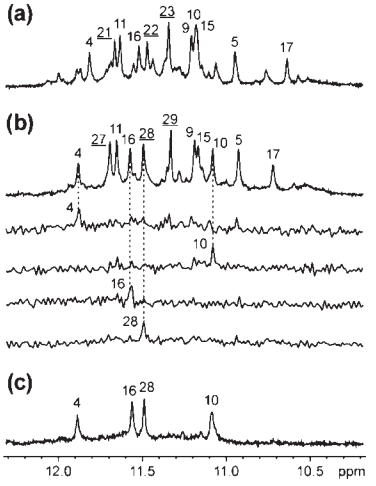

Figure 2. Imino proton spectra of (a) natural four-repeat human telomeric sequence d[TA(GGGTTA)$_3$GGGTT][8g,h] (*25htel*) and (b) modified five-repeat sequence d[TA(GGGTTA)$_3$GIGTTAGGGTT] (*31htel[I22]*) in K^+ solution, with examples of resonance assignments in ^{15}N-filtered spectra of samples, 2% ^{15}N-labeled at the indicated positions. Note the correspondence of the imino protons from the two sequences and the replacement of the fourth G-tract in *25htel* by the last G-tract in *31htel[I22]* (underlined) for G-tetrad core formation. (c) Imino proton spectrum of *31htel[I22]* after 1 h in ^2H_2O at 25 °C.

Table 1) exhibited remarkable resemblance to *25htel* (see below) and was the subject for further investigations.

The imino proton spectrum of *31htel[I22]* displayed 11 major peaks (Figure 1i), which were unambiguously assigned using site-specific 2% ^{15}N-labeled[13] samples (Figure 2b; Figure S2 and Table S3, SI). Up to the third G-tract, the guanine imino protons of *31htel[I22]* (Figure 2b) matched closely to those of *25htel* (Figure 2a). The remaining three imino protons originated from the last (fifth) G-tract (G27-G28-G29), consistent with the non-participation of the fourth tract (GIG) in G-tetrad core formation. Note that the imino protons of the fifth G-tract of *31htel[I22]* corresponded well with those from the fourth G-tract of *25htel* (underlined in Figure 2a,b). The similarity between *31htel[I22]* and *25htel* was also evident in the 2D NOESY spectra, both sequences show comparable spectral patterns, including the presence of five strong H8−H1′ cross-peaks for *syn* guanines (Figure S3a, SI). The imino protons of G4, G10, G16, and G28 were well-protected from exchange with solvent (Figure 2c), indicating their central placement in the G-tetrad core. We surmised that *31htel[I22]* might adopt the same (3+1) G-quadruplex topology as *25htel* and devised a strategy to unequivocally define its folding topology. Instead of the conventional thorough assignments of proton chemical shifts, which is costly and time-consuming, we performed a series of NOESY experiments on selective deuterated samples[14] (Table S3, SI) in order to determine the G-tetrad alignments. Specifically, the guanine residues making up the central G-tetrad (G4, G10, G16, and G28) were chosen for analysis, as their intra-tetrad imino-H8 cross-peaks were well-resolved (Figure 3b). Upon site-specific ^{2}H labeling at each H8 position, the respective imino-H8 cross-peak with the neighboring guanine vanished (Figure 3c−f), unambiguously establishing the alignment of the G4·G10·G16·G28 tetrad (Figure 3a). Moreover, the strong H8−H1′ intramolecular cross-peak corresponding to *syn* guanine G10 disappeared upon G10(^{2}H8)

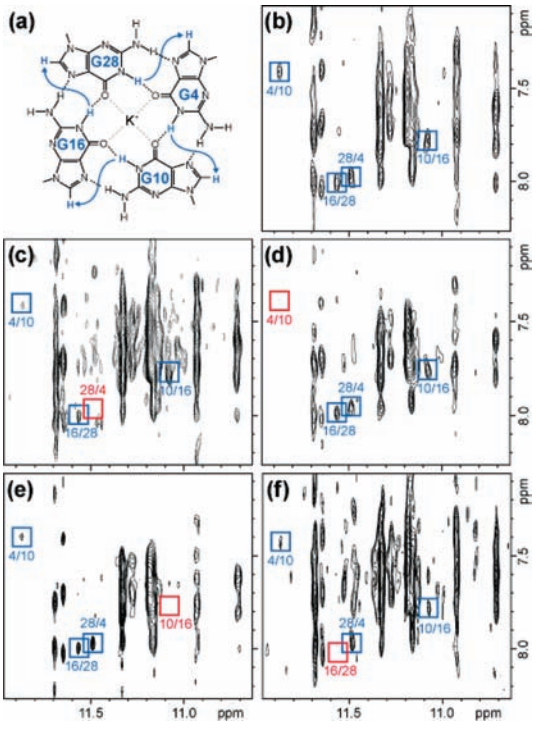

Figure 3. Alignment of the central G-tetrad of *31htel[I22]* as deduced by site-specific ^{2}H labeling.[14] (a) Characteristic guanine imino-H8 NOE connectivity patterns around the G4·G10·G16·G28 tetrad, as indicated with arrows. (b) NOESY spectrum (mixing time, 200 ms) showing the imino-H8 connectivity of *31htel[I22]* around the central G-tetrad. Cross-peaks that identify the arrangement of the G-tetrad are framed and labeled (in blue) with the residue number of imino protons in the first position and that of H8 protons in the second position. Site-specific ^{1}H8-to-^{2}H8 substitutions of (c) G4, (d) G10, (e) G16, and (f) G28 led to the disappearance of the respective imino-H8 cross-peaks (boxed in red), confirming the G-tetrad arrangement.

substitution (Figure S3, SI), further substantiating that *31htel[I22]* adopts the same (3+1) G-quadruplex Form 2 scaffold as *25htel*, albeit with a 9-nt long double-chain-reversal (or propeller) loop (Figure 4b). We propose this selective site-specific labeling approach as a strategy for fold determination with minimal resources.

Extension of the single inosine substitution approach to the 37-nt six-repeat sequence *37htel* having the same flanking ends as *25htel* and *31htel* (Tables S1 and S2, SI) considerably reduced the multiplicity of conformations in most cases (Figure S4, SI). However, all of these single-inosine-modified sequences still exhibited substantial polymorphism. Simultaneous inosine substitutions in the fourth and fifth G-tracts led to the emergence of a predominant species (~70%) in the 37-nt-modified sequence *37htel[I22/I28]* (Table 1), with the NMR spectrum somewhat resembling those of *25htel* and *31htel[I22]* (Figure S4h, SI). It was previously shown that 8-bromoguanine (^{Br}G) substitution at G15 of *25htel* favored the (3+1) G-quadruplex Form 2.[8h] Indeed, the application of ^{Br}G substitution at this position on *25htel*, *31htel[I22]*, and *37htel[I22/I28]* resulted in cleaner NMR spectra (Table S2 and Figure S5, SI). The remarkable match of major imino protons among these three sequences suggested that all of them form the (3+1) G-quadruplex Form 2 (Figure S6, SI).

We further probed the possibility of an even longer propeller loop in the 43-nt seven-repeat sequence *43htel[I22/I28/I34]*

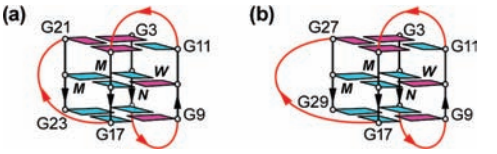

Figure 4. Schematic structures of (3+1) G-quadruplex Form 2 formed by (a) d[TA(GGGTTA)$_3$GGGTT][8g,h] (*25htel*) and (b) d[TA(GGGTTA)$_3$GIGTTAGGGTT] (*31htel[I22]*), with the latter adopting an extended 9-nt propeller loop. *Anti* and *syn* guanines are colored cyan and magenta, respectively. *W*, *M*, and *N* represent wide, medium, and narrow grooves, respectively. The backbones of the core and loops are colored black and red, respectively.

(Table 1), containing triple GIG substitutions. In this instance, the oligonucleotide exhibited multiple conformations (Figure S7b, SI), but heterogeneity was greatly suppressed as compared to the natural sequence *43htel* (Figure S7a and Table S1, SI). Appropriate ^{Br}G substitution further favored a major conformation as before (Table S2 and Figure S5, SI). The resemblance of the NMR spectrum of this sequence with those having fewer repeats described above suggested the adoption of the (3+1) G-quadruplex Form 2 scaffold with a 21-nt propeller loop (Figure S6d, SI).

UV-melting experiments were next carried out to investigate the stability of G-quadruplexes formed by human telomeric sequences of different lengths. Concentration-independent melting profiles of *31htel[I22]* (from 4 to 200 μM) confirmed the intramolecular nature of the structure. The melting temperature of *31htel[I22]* (T_m = 49.8 °C) was substantially lower than that of *25htel* (T_m = 62.0 °C), consistent with previous reports of decreasing G-quadruplex stability with increasing propeller loop length.[15] The modified six- and seven-repeat sequences *37htel[I22/I28]* and *43htel[I22/I28/I34]* displayed T_m of 47.7 and 46.5 °C, respectively (Table 1). Note that these could have consisted of contributions from the minor conformations.

CD characterization on this series of oligonucleotides was also performed (Figure S8, SI). In accordance with the CD signatures of (3+1) G-quadruplexes,[10e] there are two maxima at 260 and 295 nm coupled with a negative band at 240 nm. The ratio between the 260- and 295-nm peaks varied between sequences, which might reflect the difference in loop structures and/or the presence of other conformations.[11c]

The (3+1) G-quadruplex core represents a robust scaffold that could be adopted by the human telomeric repeats under various contexts,[8d−h] including the putative G-quadruplex formation within the telomeric t-loop structure.[8c,9g] For the Form 2 arrangement, the first and second TTA edgewise loops span wide and narrow grooves, respectively, while the third propeller loop connects across a medium groove.[8g,h] Previously, recurrence of structural elements was observed across different G-quadruplex structures, suggesting the transferability of structural elements between G-quadruplexes.[11b] It would be interesting to find out if the same long propeller loop could be transferred to the (3+1) G-quadruplex Form 1 scaffold.[8d−f] In retrospect, the *31htel[I10]* sequence with an inosine substitution in the second G-tract (Figure 1g; Table S2, SI) represents a promising candidate and warrants a separate investigation.

Our results suggest that the propeller loop in (3+1) G-quadruplexes could span up to three GGGTTA repeats (21 nt). There could be partial secondary structure formed within this loop, which could be stabilized under appropriate conditions, e.g. upon ligand binding. To test this hypothesis, we titrated a loop-modified seven-repeat

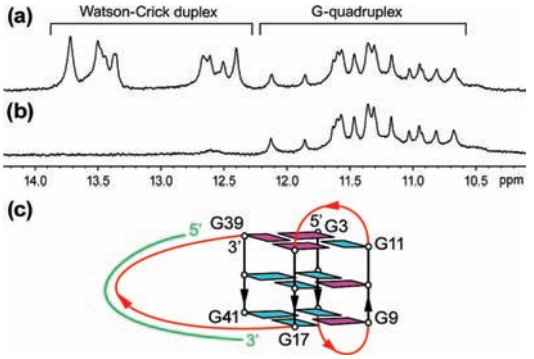

Figure 5. Targeting the long loop of a human telomeric G-quadruplex. (a,b) Imino proton spectra of (a) the 43-nt modified human telomeric sequence *43htel[T22/T28/T34]* bound with the 15-nt loop-complementary oligonucleotide d[CACTAACACTAACAC] and (b) the *43htel[T22/T28/T34]* sequence alone. (c) Schematic structure of a (3+1) G-quadruplex formed by a seven-repeat human telomeric sequence bound with a complementary oligonucleotide (colored in green).

sequence (denoted as *43htel[T22/T28/T34]*; Table 1) against a 15-nt oligonucleotide, which is complementary to the central segment of the long loop. Additional Watson−Crick imino proton peaks appeared at 12.3−13.8 ppm upon introduction of the loop-complementary oligonucleotide (Figure 5a,b), indicating its specific binding to the long propeller loop of the pre-formed G-quadruplex structure. This observation provides a proof-of-principle for the specific targeting of the loop with a ligand (Figure 5c). Such an interaction between the telomeric C-rich strand and a long loop in a G-quadruplex of the telomeric G-rich strand could also occur at natural telomeres, e.g. in the context of the t-loop.

In summary, we demonstrated the reduction of the multiplicity of structures in five- to seven-repeat human telomeric sequences using guanine-to-inosine substitutions. An efficient strategy was devised to rigorously determine the folding topology of a five-repeat sequence. We have shown that five- to seven-repeat sequences of this particular construct could adopt the (3+1) G-quadruplex Form 2 scaffold with a long propeller loop. A Watson−Crick duplex could be formed within this long loop upon addition of the complementary strand, suggesting that the long loop could serve as a new recognition motif.

■ ASSOCIATED CONTENT

Supporting Information. Tables S1−S3, Figures S1−S8, and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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