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The new models of the human telomere d[AGGG(TTAGGG)$_3$] in K^+ solution

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Abstract—The human telomeric sequence d[AGGG (TTAGGG)$_3$] has been found to form different types of G-quadruplex structures. NMR revealed that in Na^+ solution this 22 nucleotide (nt) sequence exhibits an antiparallel structure, whereas crystallographic studies in the presence of K^+ showed a dramatically different parallel structure. The structure of this 22 nt sequence in the presence of K^+ has drawn intense interest as the intracellular K^+ concentration is greater than that of Na^+. However, the question of the type of structure for the 22 nt telomeric sequence in K^+ solution remains open. In this study, we substituted the Gs in the sequence with 8-bromoguanine and examined the resultant structures and thermal stabilities by circular dichroism (CD) spectroscopy. The results suggest that the 22 nt in K^+ solution exists as a mixture of mixed-parallel/antiparallel and chair-type G-quadruplex. To date, the exact structure of human telomeric G-quadruplex in K^+ solution is extremely controversial. The present study provides valuable information for understanding the discrepancies between the crystal and solution studies. We discuss the possible implications of the structure in understanding higher-order telomeric DNA structure and T-loop formation.

1. Introduction

The various conformations of DNA—the A, B, and Z forms, the protein-induced DNA kink, and the G-quartet form—are thought to play important biological roles in processes such as DNA replication, gene expression and regulation, and the repair of DNA damage.[1,2] In particular, G-quadruplex DNA formed from telomeric sequence repeats may be important for telomere maintenance,[3,4] and cell aging or death, which have become currently an attractive therapeutic target for the development of novel anticancer agents.[5–12]

G-quadruplexes are highly polymorphic, and a large number of different structures have been observed[13,14]. The different G-quadruplex topologies may be associated with some related aspects: the *syn*/*anti* conformation of guanine residues, the relative orientation of the G-quartet core, the types of linking loops, and the nature of associated metal cations.[15–22] Detailed structural studies have provided evidence for two distinct conformations of the human telomere G-quadruplex in the presence of Na^+ and K^+ ions.[23,24] The solution structure of the 22 nt sequence d[AGGG(TTAGGG)$_3$] in the presence of Na^+ ions has been elucidated by NMR analysis. This showed an antiparallel basket-type structure in which the opposing GGG columns are antiparallel, with one diagonal and two lateral TTA loops (Fig. 1a). On the other hand, the same 22 nt sequence adopts a completely different propeller-type structure in a crystal grown in the presence of K^+ ions. In this structure, four core GGGs are parallel, with the three linking external loops positioned on the exterior of the G-quartet core (Fig. 1b).

As the intracellular K^+ concentration is greater than that of Na^+, the structure of this sequence in K^+ solution has drawn intense interest. To investigate the solution structure of the 22 nt sequence, various studies have been performed using platinum cross-linking, FRET, ^{125}I-radioprobing, covalent ligation, sedimentation, and NMR.[25–30] Our laboratory used photochemical methods to detect the diagonal loops in the antiparallel structure, suggesting that in K^+ the sequence does not possess a diagonal loop.[31] Although these approaches gave some structural information, the definitive structural characteristics of the human telomere G-quadruplex in K^+ solution have not yet been

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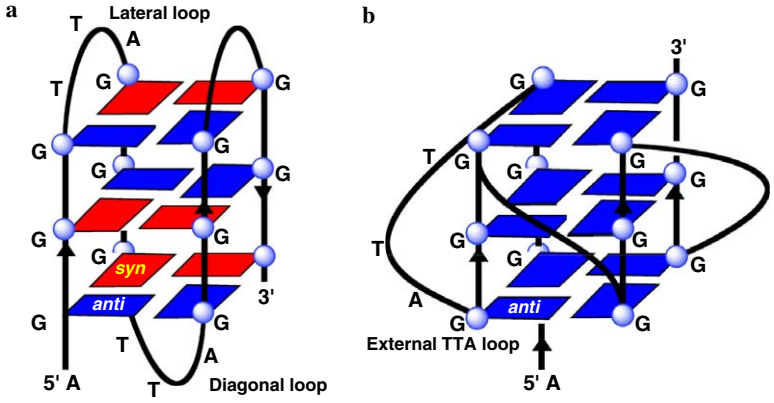

Figure 1. Schematic representations of the folded structures of d[$AG_3(T_2AG_3)_3$]. (a) The Na^+-stabilized solution structure, with one diagonal and two lateral TTAs. (b) The K^+-stabilized crystal structure, with external TTA loops abutting the sides of the G-quartet and parallel GGG columns. Blue and red boxes represent guanine bases in the *anti* and *syn* conformations, respectively.

determined. To define the structural features of 22 nt sequence in K^+ solution, we substituted the Gs in the 22 nt sequence with 8-bromoguanine (^{Br}G) and examined the resultant structures and their thermal stabilities in K^+ solution by circular dichroism (CD) spectroscopy. Based on these results, we propose a novel mixed parallel/antiparallel G-quadruplex structure containing two lateral loops and one external loop, together with an interconvertible chair-type G-quadruplex structure. These structures suggest the possible formation of models from the extended G-quadruplex: (i) individual quadruplexes form higher-order telomeric DNA structure, (ii) telomere sequences form two loop structures including a t-loop.[32] This may provide important information for understanding telomere structure and the development of telomere G-quadruplex-binding molecules as telomerase inhibitors.

2. Results and discussion

2.1. CD spectrum of 22 nt sequence in K^+ solution

The typical bands in the CD spectra show fundamental characteristics allowing G-quadruplex structures to be distinguished.[34–39] As shown in Figure 2a (blue), the CD spectrum of the human telomeric sequence d[$AGGG(TTAGGG)_3$] (ODN 1) in the presence of 100 mM Na^+ ions has a 295 nm positive band and a 265 nm negative band, which is characteristic of an antiparallel G-quartet structure consistent with the results of previous NMR studies[23]. In contrast, the CD spectrum of ODN 1 in 100 mM K^+ ion shows a stronger positive band at 290 nm, with weak negative peaks near 255 and 235 nm (Fig. 2a, purple). This spectrum is inconsistent with the reported specific features of the parallel structures, which exhibit a positive band around 260 nm and a negative band around 240 nm.[35,40] In fact, the 12 nt human telomeric sequence d(AGGGUTAGGGT) was demonstrated to form a homodimer parallel G-quadruplex by NMR studies.[30] The CD spectrum of the 12 nt sequence in K^+ shows a positive signal at 265 nm and a negative signal at 240 nm (Fig. 2b) consistent with the characteristic of the typical parallel G-quadruplex, which is clearly different from that of 22 nt sequence in K^+. These results clearly suggest that the structure of the 22 nt sequence in K^+ does not exist as the parallel structure observed in the crystal. Moreover, the Chaires group also demonstrated, based on sedimentation and fluorescence studies, that the crystal structure of the 22 nt sequence cannot be the predominant structure in K^+ solution.[29] The solution structure of the 22 nt sequence in K^+ has not been elucidated by NMR, presumably due to the presence of multiple conformational isomers.[30] To elucidate the major conformational constituents of the 22 nt sequence in K^+ solution, we carried out systematic chemical modification experiments.

2.2. The mixed parallel/antiparallel structure

Numerous studies suggested that the G residues are in a mixture of *syn*/*anti* conformation along the G_4 tracts in the antiparallel G-quadruplex, whereas the G resides are all in *anti* conformation in the parallel structure. The chemical and biochemical properties of the G-quartets could be better understood by controlling G-quartet-folding topologies in a specified manner.[41–44] The Williamson group determined the *anti*/*syn* arrangement of G-quadruplex of $d(TTTGG)_4$ using the modified nucleic acid ^{Br}G, which is known to adopt the *syn* conformation.[41] They showed that substitution of Gs in the *syn* conformation with ^{Br}G increased the thermal stability of the G-quadruplex, allowing determination of the arrangement of *anti*/*syn* conformations. Analogously, using the 8-methylguanine, we recently determined the arrangement of *anti*/*syn* conformations of the G-quadruplex structure at the 5′ end of the Rb gene.[45]

Recently, the Hurley laboratory showed that the binding of Se2SAP induces the parallel c-MYC G-quadruplex to form a mixed parallel/antiparallel G-quadruplex where all three anti-Gs in the first core GGG change to the *syn* conformation[46] (Fig. 2c). To examine the structural characteristics of the major components of the 22 nt sequence in K^+, we substituted three Gs in each of the four core GGGs of the 22 nt sequence with ^{Br}Gs to generate ODNs 2–5, and examined the resultant structures and

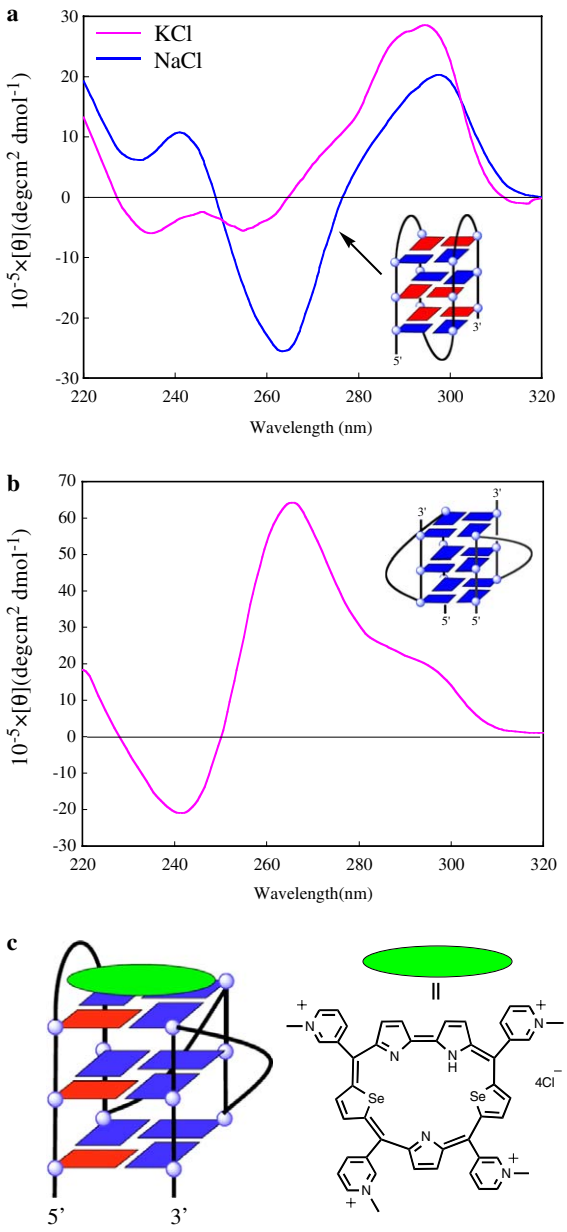

Figure 2. (a) CD spectra of the 22 nt sequence d(AGGGTT AGGGTTAGGGTTAGGG) ODN 1 in the presence of 100 mM NaCl or 100 mM KCl at 25 °C (0.3 mM base concentration). (b) CD spectrum of the 12 nt sequence d(TAGGGUTAGGGT) (1 mM base concentration). (c) Se2SAP stabilizes the mixed parallel/antiparallel G-quadruplex with *syn–syn–syn* orientation guanines in c-MYC 27 nt sequences,[46] red boxes represent guanine bases in *syn* conformations.

their thermal stabilities in K^+ solution by CD spectroscopy. ODNs 2–5 in 100 mM KCl showed two groups of different features in their CD spectra (Fig. 3a). The CD spectrum of ODN 2 showed a strong positive band at 290 nm and a strong negative band around 260 nm, whereas ODNs 3 and 5 showed a rather broad signal around 270 nm and a negative band at 240 nm indicating the features of a single strand. The CD spectrum of ODN 4 is similar to that of ODN 1, exhibiting the strong positive band at 290 nm and two weak negative peaks near 255 and 235 nm. The thermal stabilities of ODNs 1–5 were examined using CD melting experiments. ODNs 2 and 4 showed melting behavior with stability comparable with that of ODN 1. The melting profiles of ODNs 3 and 5 showed almost no transition, suggesting that ODNs 3 and 5 do not form a stable G-quadruplex structure (Fig. 3b).

Since ODN 4 exhibited slight increase of stability of G-quadruplex relative to ODN 1, we assumed that not all of the Gs in the third core GGG are in the *syn* conformation. The intriguing possibility is that two guanine residues form the *syn* conformation and one forms the *anti* conformation in the third core GGG. As one favorable incorporation of ^{Br}G in the *syn* conformation is estimated to stabilize the structure by 1–2 kcal/mol,[41] the energetic compensation might occur in ODN 4. To test this hypothesis, we prepared ODNs 6–8 in which two Gs are substituted with ^{Br}G in the third core GGG. As shown in Figure 3c, the CD spectra of ODNs 6–8 exhibited rather different profiles, and that of ODN 6 is similar to the CD spectrum of ODN 1. CD melting experiments indicate that ODN 6 possessing ^{Br}Gs at positions 1 and 2 of the third core $G_1G_2G_3$showed significant thermal stability compared to ODNs 7 and 8 containing ^{Br}Gs in other positions: the melting temperature of ODN 6 was about 75 °C (Fig. 3d). The results of the CD experiments suggest that a stabile G-quadruplex containing a *syn–syn–anti* orientation in third GGG exists in K^+ solution. Previous NMR studies revealed that the tetrahymena telomeric sequence $d(T_2G_4)_4$, which differs from the human sequence by one A to G mutation in each repeat, exhibits a mixed-type G-quadruplex structure with two lateral loops and an external loop[33]. Interestingly, the structure of $d(T_2G_4)_4$ contains a GGG in a *syn–syn–anti* orientation and a G-tetrad adopting *syn·syn·syn·anti* alignment. Recently, Zhang et al. found that a three-repeat human telomeric sequence can associate with a single-repeat human telomeric sequence in a (3 + 1) quadruplex structure with one GGG in the *syn–syn–anti* orientation with a *syn·syn·syn·anti* G-tetrad[47]. Taking into account these studies and our experimental results, we prepared ODN 9 which contained three ^{Br}Gs at the positions adopting *syn* conformations in the *syn · syn · syn · anti* G-tetrad. The CD spectrum of ODN 9 is very similar to that of ODN 6 (Fig. 3e). Significant stabilization of ODN 9 was observed compared to ODN 4 and 6: the melting temperature of ODN 9 was about 85 °C (Fig. 3f).

Furthermore, ODN 10 was prepared by incorporating three ^{Br}Gs at the positions in the *syn·syn·syn·anti* G-tetrad and two ^{Br}Gs in the *syn–syn–anti* orientation strand. The CD spectrum of ODN 10 was similar to those of ODNs 6 and 9. As expected, ODN 10 showed substantially higher stability than ODN 4, 6, and 9: the melting temperature of ODN 10 was over 100 °C (Fig. 4b). These data strongly suggest that a mixed parallel/antiparallel G-quadruplex exists in K^+ solution (Fig. 4c). Recently, the Yang laboratory also showed that a mixed parallel/antiparallel intramolecular G-quadruplex with five *syn* conformations Gs was formed the human BCL-2 promoter region in solution.[48] We found that the novel mixed structure has 5′–3′ directions different to previously reported mixed-type structures.[33,48] In our structure, the first loop is an external orientation

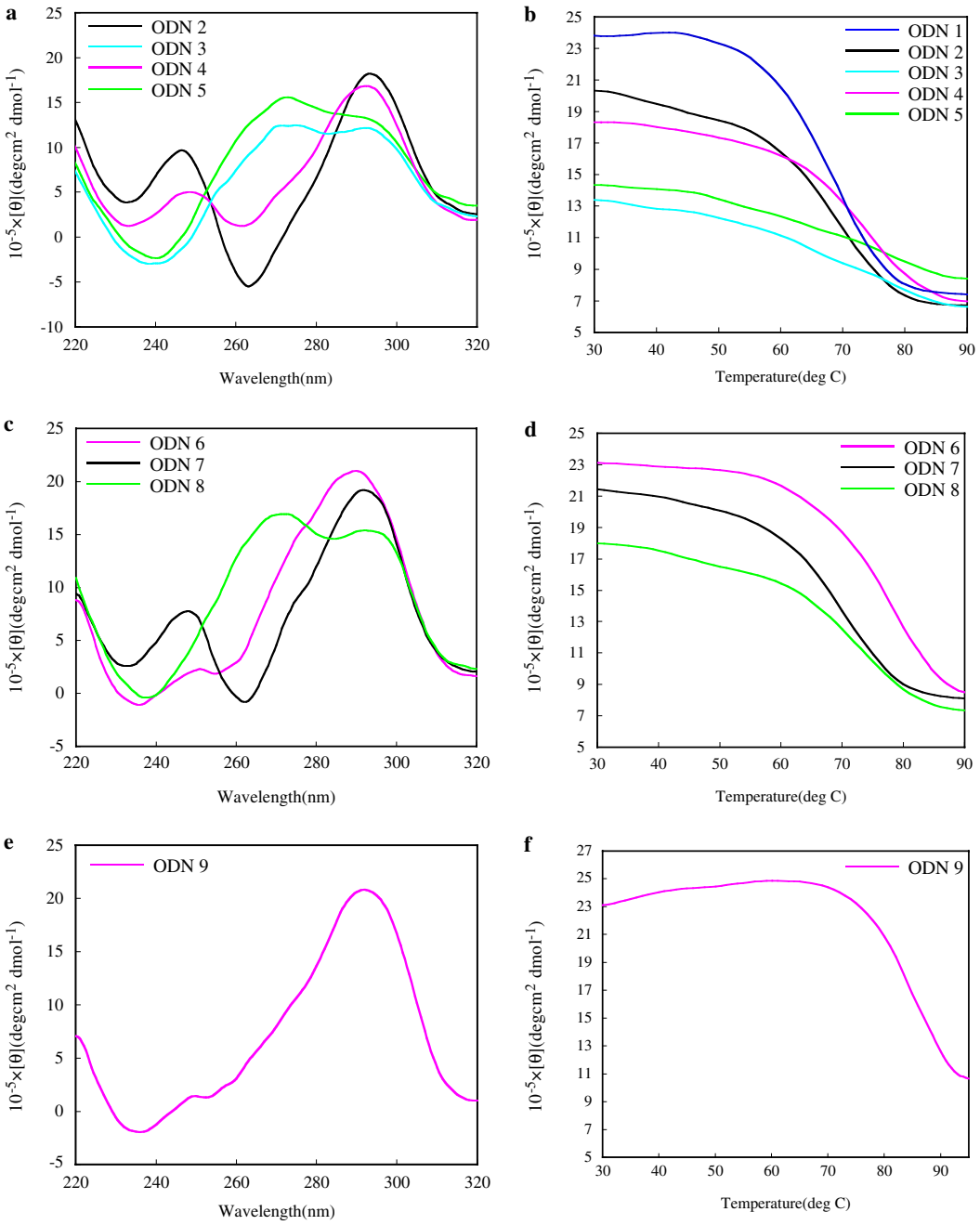

Figure 3. (a) CD spectra of the 22 nt sequences d(A^{Br}G^{Br}G^{Br}GTTAGGGTTAGGGTTAGGG), ODN 2; d(AGGGTTA^{Br}G^{Br}G^{Br}GTTAGGGT TAGGG), ODN 3; d(AGGGTTAGGGTTA^{Br}G^{Br}G^{Br}GTTAGGG), ODN 4; and d(AGGGTTAGGGTTAGGGTTA^{Br}G^{Br}G^{Br}G), ODN 5, in the presence of 100 mM KCl (0.3 mM base concentration) at 25 °C. (b) CD melting curves for ODN 1–5 monitored at 290 nm. (c) CD spectra of the 22 nt sequences d(AGGGTTAGGGTTA^{Br}G^{Br}GGTTAGGG), ODN 6; d(AGGGTTAGGGTTA^{Br}GG^{Br}GTTAGGG), ODN 7; and d(AGGGTTAGGGTTAG^{Br}G^{Br}GTTAGGG), ODN 8. (d) CD melting curves of ODN 6–8 monitored at 290 nm. (e) CD spectrum of d(A^{Br}GGGTTA^{Br}GGGTTAGGGTTA^{Br}GGG), ODN 9. (f) CD melting curve of ODN 9.

with the second and the third loops in a lateral orientation, whereas the previously reported mixed structure shows the first and second loops are in a lateral orientation and the third loop in the external orientation. An energy-minimized structure of the mixed G-quadruplex is shown in Fig. 4d, which is significantly more stable than the parallel structure in the AMBER force field.

In fluorescence studies using 2-aminopurine (Ap)-substituted 22 nt sequences, the Chaires laboratory reported that the order of fluorescence emission intensities was $Ap_{13} < Ap_{19} < Ap_7$ in the G-quadruplex[29]. These results are consistent with the proposed mixed structure and provide the explanation that Ap_{13} and Ap_{19} stack in the tetrad plane of the G-quartet, and that Ap_7 unstacks and tilts relative to the tetrad plane. The pattern of fluorescence emission intensities also agrees with the results of the Ap fluorescence quenching studies using acrylamide as a quencher to investigate the loop environments of the G-quadruplex.[29]

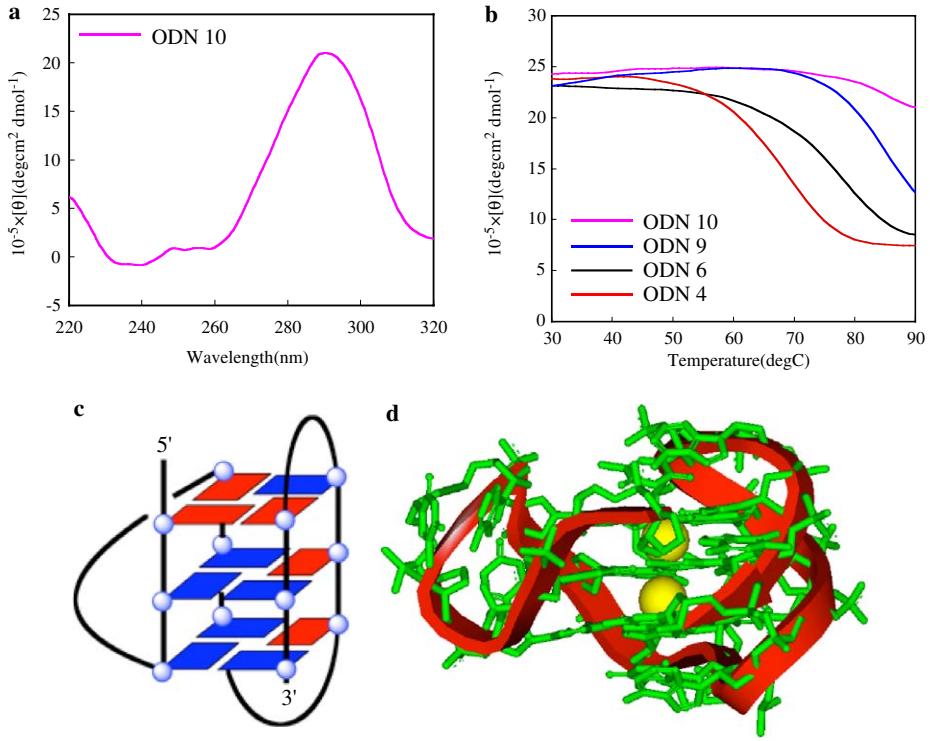

Figure 4. (a) CD spectrum of 22 nt d(A^{Br}GGGTTA^{Br}GGGTTA^{Br}G^{Br}GGTTA^{Br}GGG) ODN 10, in the presence of 100 mM KCl (0.3 mM base concentration) at 25 °C. (b) CD melting curves for ODN 10, ODN 4, ODN 6, and ODN 9 monitored at 290 nm. (c) Schematic of the mixed parallel/antiparallel G-quadruplex structure, red boxes represent guanine bases in the *syn* conformations. (d) An energy-minimized structure of the mixed parallel/antiparallel G-quadruplex.

2.3. The chair-type structure

The fact that ODN 2 shows comparable stability with ODN 1 suggests the existence of another conformational isomer in which two Gs of the first GGG core are in the *syn* conformation. Using ^{125}I-radioprobing, Panyutin et al. suggested that a chair-type G-quadruplex might coexist in K^+ solution.[27] It is assumed that a chair-type G-quadruplex may coexist in the K^+ solution with the mixed parallel/antiparallel G-quadruplex for ODN 1 (Fig. 5a). To test the hypothesis, we prepared ODN 11 by adding one ^{Br}G in the 2 position of the 5′ end of the $G_1G_2G_3$ core of ODN 10. ODN 11 showed high thermal stability, with a melting temperature of about 100 °C (Fig. 5b). Surprisingly, the CD spectrum of ODN 11 is similar to that of ODN 1 (Fig. 5c). These results are consistent with the fact that the CD spectra of ODNs 10 and 11 are similar to that of ODN 1. We note that the two structures could easily interconvert with each other only by changing the orientation of the 5′ end of the GGG core (Fig. 5a). To test this possibility, ODNs 12–14, each containing one ^{Br}G at 5′ end $G_1G_2G_3$ core, were prepared and examined their stability. CD melting of ODNs 12–14 showed that the order of stability of G-quadruplex was ODN 12 > ODN 13 > ODN14 (Fig. 5b), which is consistent with the predicted effect of ^{Br}G. ODN 12 with a $^{Br}G_1$ at *syn* position in the two structures, ODN 14 with a $^{Br}G_3$ *anti* position, and ODN 13 with a $^{Br}G_2$ at *anti* position in the mixed structure or *syn* position in the chair structure. The G_2 specificity might result in the moderate stability of ODN 13, which is consistent with the presumption that G_2 might change the *syn/anti* arrangement at equilibrium of the two structures. We note that only the G_2 should be changed in *syn/anti* conformation when the two structures interconvert each other. This G_2 specificity might result in ODN 2 not showing similar instability with ODNs 3 and 5 in the mixed structure (Fig. 3b) although they have the same pattern of Gs.

The features of the mixed and chair structures are fully consistent with the quantitative analysis of radioprobing experiments with ^{125}I [27]. We have previously demonstrated that the photoreactivity of ^{I}U-containing G-quadruplexes highly depends on differences in the loop structures[31]. Photochemical 2′-deoxyribonolactone formation efficiently and specifically occurs only in the diagonal loop of G-quadruplexes, whereas the reaction does not proceed in other loop structures. The mixed quadruplex and chair structures do not contain the diagonal loop, which is consistent with our previous observations. The structural features of human telomeric G-quadruplex in K^+ solution are extremely controversial, and this study provides valuable information to allow understanding of the discrepancies between crystalline and solution studies. The detailed structural features of the 22 nt human telomere sequence stabilized by ^{Br}G are currently under investigation by NMR analysis.

2.4. Possible biological implication

It is noteworthy that different structural forms may be important for participation in the different biological functions of telomeres. The unique topology of the

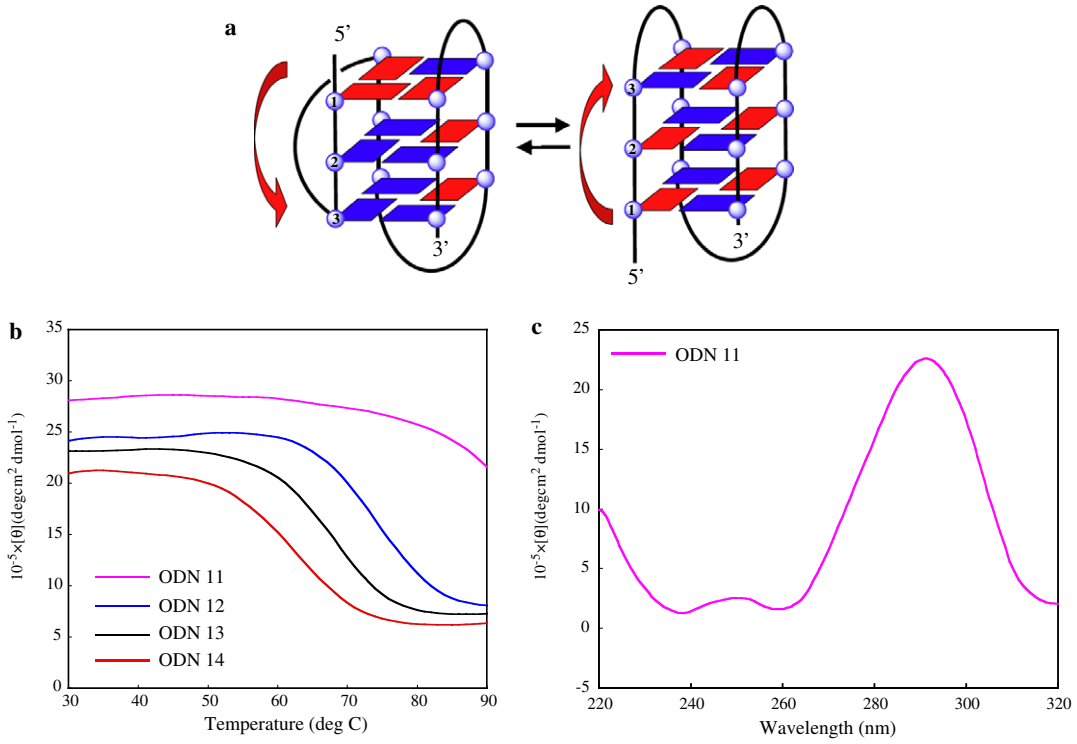

Figure 5. (a) Schematic presentation of interconversion of the mixed parallel/antiparallel and chair-type G-quadruplex structures by conversing the 5′ end GGG core, (b) CD melting curve of the 22 nt sequence ODNs 11, 12, 13, and 14, (c) CD spectrum of ODN 11.

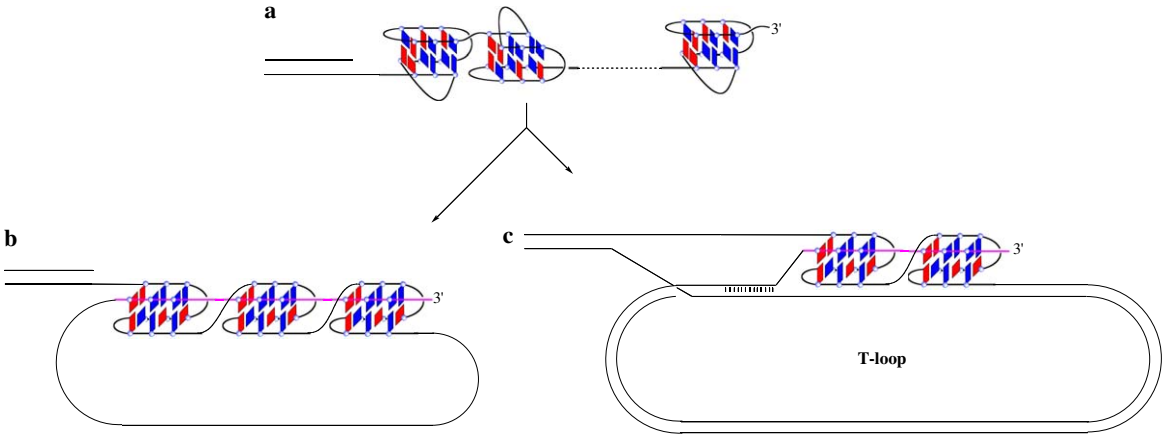

Figure 6. Models showing the higher-order telomeric DNA structure (a) and T-loop formation by the mixed parallel/antiparallel G-quadruplex (b,c).

mixed parallel/antiparallel structure could facilitate stacking of the 22 nt units into higher-order packing structures (Fig. 6a). The structures revealed here do not present any topological difficulties when extended to longer telomeric sequences: the chain ends are located on opposite faces of each of the quadruplexes. In fact, the oligomerization of individual quadruplexes can be simply performed by inserting an additional fourth TTA loop to each 22 nt structure. Furthermore, such a mixed structure could occur within 3′-end overhang and there is no barrier to the stacking and subsequent packaging to form a so-called t-loop at the end of the chromosome,[49] (Fig. 6b, c). Such oligomerization with higher-order structure and t-loop formation may provide flexibility for responding to environmental conditions as protein binding. These structures might also be important for effectively packing telomeric DNA into an active or inactive form upon chromosome condensation. The proposed structures might also have clinical implications. The structure-based design of telomerase inhibitors necessitates molecular understanding of the range of quadruplex topologies and these structures are valuable in providing such information.

3. Materials and methods

3.1. Synthesis of oligonucleotides

The phosphoamidite of 8-bromo-2′-deoxyguanosine was purchased from Glen Research. Oligonucleotides containing ^{Br}G were prepared by the phosphoamidite

Table 1. Oligonucleotides used in the studies

ODNs	
d(AGGGTTAGGGTTAGGGTTAGGG)	ODN 1
d($A^{Br}G^{Br}G^{Br}$GTTAGGGTTAGGGTTAGGG)	ODN 2
d(AGGGTTA$^{Br}G^{Br}G^{Br}$GTTAGGGTTAGGG)	ODN 3
d(AGGGTTAGGGTTA$^{Br}G^{Br}G^{Br}$GTTAGGG)	ODN 4
d(AGGGTTAGGGTTAGGGTTA$^{Br}G^{Br}G^{Br}$G)	ODN 5
d(AGGGTTAGGGTTA$^{Br}G^{Br}$GGTTAGGG)	ODN 6
d(AGGGTTAGGGTTA^{Br}GG^{Br}GTTAGGG)	ODN 7
d(AGGGTTAGGGTTAG$^{Br}G^{Br}$GTTAGGG)	ODN 8
d(A^{Br}GGGTTA^{Br}GGGTTAGGGTTA^{Br}GGG)	ODN 9
d(A^{Br}GGGTTA^{Br}GGGTTA$^{Br}G^{Br}$GGTTA^{Br}GGG)	ODN 10
d($A^{Br}G^{Br}$GGTTA^{Br}GGGTTA$^{Br}G^{Br}$GGTTA^{Br}GGG)	ODN 11
d(A^{Br}GGGTTAGGGTTAGGGTTAGGG)	ODN 12
d(AG^{Br}GGTTAGGGTTAGGGTTAGGG)	ODN 13
d(AGG^{Br}GTTAGGGTTAGGGTTAGGG)	ODN 14
d(TAGGGUTAGGGT)	

method on controlled pore glass supports (1 μmol) using an Applied Biosystems 3400 DNA synthesizer. After automated synthesis, the oligomers were detached from the support, deprotected, and purified by HPLC. The oligomers were identified by electrospray ionization mass spectrometry (ESI-MS) on a Perkin Elmer SCIEX API 165 mass spectrometer (negative mode). The purity and concentrations of these oligomers were determined by complete digestion of the oligomers with P1 nuclease and alkaline phosphatase to 2′-deoxymononucleosides. Oligonucleotides used in the studies are listed in Table 1.

3.2. CD measurements and analysis of CD melting profiles

CD spectra were measured using an AVIV MODEL 62 DS/202 CD spectrophotometer. CD spectra were recorded using a 1 and 0.1 cm path-length cell. Samples were prepared by heating the oligonucleotides at 90 °C for 5 min and gradually cooling to room temperature. In CD melting studies, diluted samples were equilibrated at room temperature for several hours to obtain equilibrium spectra. The melting curves were obtained by monitoring a 290 nm CD band. Solutions for CD spectra were prepared as 400 μL samples at 0.3 mM (base concentration) in the presence of 100 mM KCl at 25 °C.

3.3. Molecular modeling studies

Minimizations were performed with the Discover (MSI, San Diego, CA) program using cvff force-field parameters. The starting structure was built on the basis of the NMR structure of the tetrahymena telomeric sequence $d(T_2G_4)_4$[33] and the crystal structure of d[AGGG(TTAGGG)$_3$][24]. The connecting parts between them were built using standard bond lengths and angles. The initial structure was energy minimized using a distance-dependent dielectric constant of $\varepsilon = 4r$ (r stands for the distance between atoms i and j) and with convergence criteria having an RMS gradient of less than 0.001 kcal/mol Å. Eighteen Na cations were placed at the bifurcating position of the O–P–O angle at a distance of 2.51 Å from the phosphorus atom. The resulting complex was soaked in a 10-Å layer of water. The whole system was minimized without any constraint, to the stage where the RMS was less than 0.001 kcal/mol Å.

Acknowledgments

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