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The orientation of the ends of G-quadruplex structures investigated using end-extended oligonucleotides

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ABSTRACT

Human telomere DNA is of intense interest because of its role in the biology of both cancer and aging. The single-stranded telomere terminus can adopt the structure of a G-quadruplex, which is of particular important for anticancer drug discovery many researchers have reported various G-quadruplex structures in the human telomere. Although the human telomere consists of a number of tandem repeats, higher-order G-quadruplex structures are less discussed due to the complexity of the structures. Here we examined the orientation of the ends of the G-quadruplex structures with consideration given to higher-order structures. We prepared end-extended and ^{Br}G-substituted oligonucleotides. Native PAGE analysis, CD measurements and NMR spectroscopy showed that the ends of stable G-quadruplex structures point in opposite directions. Our results indicate that the human telomere DNA is likely to form rod-like higher-order structures. This may provide important information for understanding telomere structure and the development of telomere G-quadruplex-binding molecules as telomerase inhibitors.

1. Introduction

Telomeres are essential structures at the ends of chromosomes. They play an important role in various biological events, such as aging and apoptosis.[1] The human telomere consists of tandem repeats of d(GGGTTA)[2] and is typically 5–8 kb long with a 3′ single-stranded overhang of 100–200 nt.[3–5] In this G-rich region, a tetraplex structure called a G-quadruplex can be formed in vitro. In 80–85% of cancer cells, telomerase is activated to maintain the length of telomere.[6] Because the G-quadruplex can inhibit the activity of telomerase[7], the elucidation of G-quadruplex structure is of great interest in the development of anticancer agents.[8–10]

To date, various G-quadruplex structures have been reported in the human telomere.[11] First, Patel et al. reported an antiparallel structure in Na^+ solution, based on NMR analysis (Fig. 1a).[12] Next, Neidle et al. showed a parallel structure, based on X-ray crystallography (Fig. 1b).[13] However, the conditions in these experiments were far from physiological one. In 2006, three groups, including our group showed the (3+1) hybrid G-quadruplex structure in physiological K^+ solutions (Form b in Fig. 1c).[14–16] Shortly after these reports, another (3+1) hybrid G-quadruplex structure was reported (Form a in Fig. 1).[17,18] Each G-tetrad have the same glycosidic conformations in both Forms a and b. The difference between Forms a and b is the loop orientation. With this in mind, Patel suggested four possible G-quadruplex structures, including the two above conformations (Fig. 1c).[19]

As mentioned above, various structures of unimolecular G-quadruplexes have been reported.[20] Considering the length of the telomeric DNA single-stranded overhang, higher-order G-quadruplex structures such as dimeric or multiplex G-quadruplexes, are at least feasible in vivo. A beads-on-a-string model was proposed for the long human telomeric sequences[21], with the assumption that adjacent G-quadruplexes are not likely to stack on each other. On the other hand, the stability of human telomeric G-quadruplex multimers has already calculated by means of molecular dynamics simulation.[22,23] In addition to these reports, some reports have discussed long sequences with more than four human telomeric repeats.[24–26] Among them, Nakatani et al. proposed that a small molecule could induce the stacking of G-quadruplexes.[26]

In this study, we took a different approach to this problem by investigating the orientation of the ends of the G-quadruplex structures. The orientation is very important in considering the packing state of G-quadruplex structures. When the ends of G-quadruplex structures point in the same direction, double strands can be formed easily (Forms c and d in Fig. 1c). As can be seen in Figure 1, the end-extended oligonucleotides in Forms c and d are expected to be more compact than those in Forms a and b. To examine the

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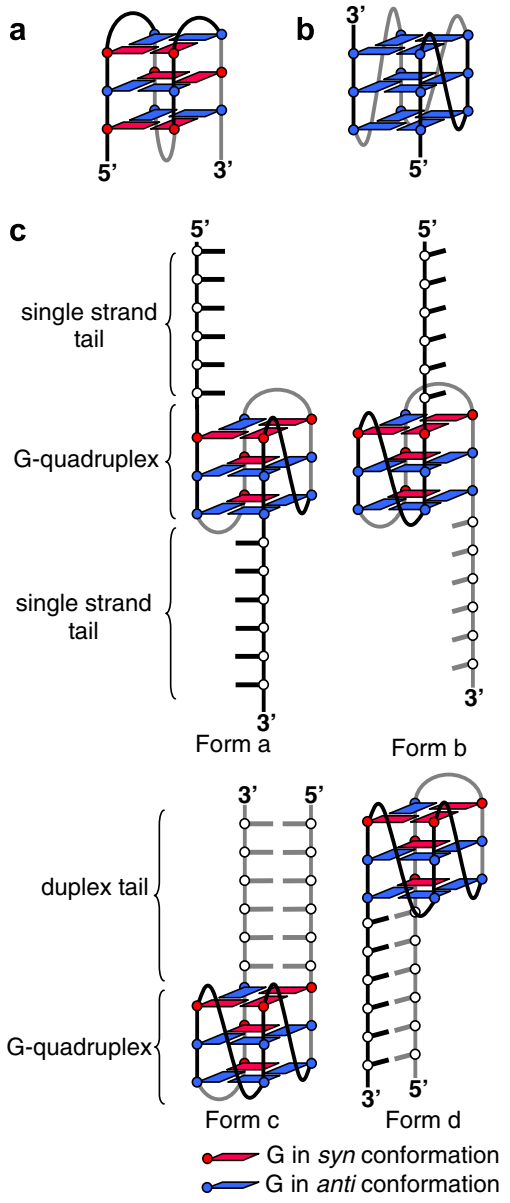

Figure 1. Schematic structure of (a) antiparallel and (b) parallel G-quadruplex structures. (c) Schematic four possible G-quadruplex structures of end-extended oligonucleotides. Forms c and d are more compact than Forms a and b. The red and blue circles represent guanine bases in *syn* and *anti* conformations, respectively.

differences in apparent molecular mass, we carried out native polyacrylamide gel electrophoresis (PAGE). Furthermore, we carried out CD measurements and NMR analysis to determine the structure of these oligonucleotides.

2. Results

2.1. Native PAGE analysis of end-extended oligonucleotides

To examine the orientation of the ends of the G-quadruplexes, we prepared ODNs 1–5, containing the same human telomeric sequence, $d(GGGTTA)_3GGG$ (Table 1). In each oligonucleotide, the ends of the human telomeric sequences were extended with various sequences, called 'tails' (Fig. 1c). The tail sequences of ODN 1 consisted of polyadenine, whereas the tail sequences in ODNs 2–5 were complementary. The native PAGE experiments showed

Table 1
The sequence of oligonucleotides used in this study (^{Br}G = 8-bromoguanine)

Name	Sequence
ODN1	5′-AAAAAAGGGTTAGGGTTAGGGTTAGGGAAAAAA-3′
ODN2	5′-TATATAGGGTTAGGGTTAGGGTTAGGGTATATA-3′
ODN3	5′-TAGATAGGGTTAGGGTTAGGGTTAGGGTATCTA-3′
ODN4	5′-TAGCTAGGGTTAGGGTTAGGGTTAGGGTAGCTA-3′
ODN5	5′-TCGCTAGGGTTAGGGTTAGGGTTAGGGTAGCGA-3′
ODN6	5′-A^{Br}GGGTTA^{Br}G^{Br}GGTTA^{Br}GGGTTA^{Br}GGG-3′
ODN7	5′-A^{Br}GGGTTA^{Br}GGGTTA^{Br}G^{Br}GGTTA^{Br}GGG-3′
ODN8	5′-A^{Br}GGGTTA^{Br}GGGTTA^{Br}GGGTTA^{Br}G^{Br}GG-3′
ODN9	5′-A^{Br}G^{Br}GGTTA^{Br}GGGTTA^{Br}GGGTTA^{Br}GGG-3′
ODN10	5′-TATATA^{Br}GGGTTA^{Br}GGGTTA^{Br}GGGTTA^{Br}GGGTATATA-3′
ODN11	5′-TAGATA^{Br}GGGTTA^{Br}GGGTTA^{Br}GGGTTA^{Br}GGGTATCTA-3′
ODN12	5′-TCGCTA^{Br}GGGTTA^{Br}GGGTTA^{Br}GGGTTA^{Br}GGGTAGCGA-3′
ODN13	5′-TAGATA^{Br}GGGTTA^{Br}G^{Br}GGTTA^{Br}GGGTTA^{Br}GGGTATCTA-3′
ODN14	5′-TAGATA^{Br}GGGTTA^{Br}GGGTTA^{Br}G^{Br}GGTTA^{Br}GGGTATCTA-3′
ODN15	5′-TAGATA^{Br}GGGTTA^{Br}GGGTTA^{Br}GGGTTA^{Br}G^{Br}GGTATCTA-3′
ODN16	5′-TAGATA^{Br}G^{Br}GGTTA^{Br}GGGTTA^{Br}GGGTTA^{Br}GGGTATCTA-3′

broad bands for all oligonucleotides except ODN 1 (Fig. 2). These broad bands indicated the existence of various conformations. This result clearly suggests that the shift of mobility is attributed on the sequence of tails.

2.2. CD measurements of various oligonucleotides

To evaluate the structures of G-quadruplex, the CD spectra of ODNs 1–5 were measured (Fig. 3a). All the spectra of ODNs 1–5 were very similar though the mobility of oligonucleotides were different in gel experiment. These spectra have a positive band at ~290 nm and negative band at ~245–255 nm, which are characteristic spectra of (3+1) hybrid G-quadruplex structures.[14,27] Next, we prepared the 8-bromoguanine ($d^{Br}G$) substituted oligonucleotides, ODNs 6–9, which are designed to take on Forms a–d, respectively. All oligonucleotides showed the similar CD spectra (Fig. 3b). The polarity of stacked G-quadruplex is believed to be the determining factor for G-quadruplex CD spectra.[28] Thus, the spectra of ODNs 6–9 are consistent with the structure of Forms a–d having the same glycosidic conformations and different loop orientations. Based on these results, we concluded that end-extended oligonucleotides, ODNs 1–5, can take on Forms a–d, not parallel and antiparallel G-quadruplex structures.

2.3. Analysis of ^{Br}G-substituted oligonucleotides using native PAGE and NMR spectrometry

To avoid the structural diversity, we substituted the dG with 8-bromoguanine ($d^{Br}G$). As can be seen in the four proposed G-quadruplex structures in Fig. 1c, all of the initial Gs in the G-tract take a *syn* conformation. Therefore we introduced the $d^{Br}G$ in place of the

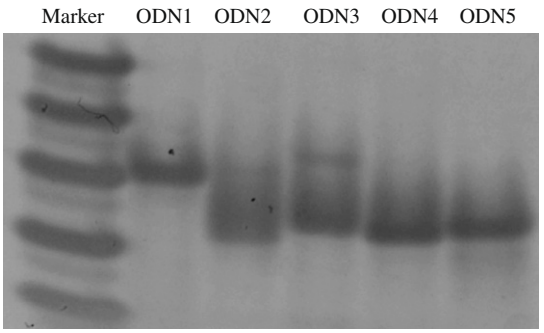

Figure 2. Native gel electrophoresis of ODNs 1–5.

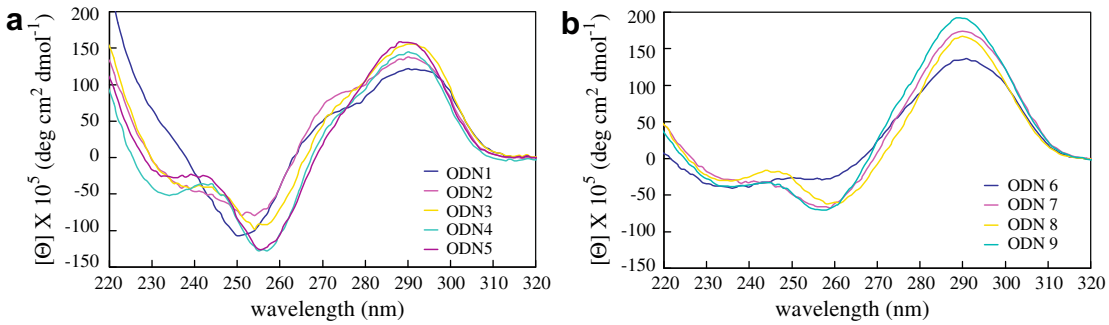

Figure 3. CD spectra of (a) end-extended oligonucleotides, ODNs 1–5 and (b) 8-bromoguanine substituted oligonucleotides, ODNs 6–9.

first dG in the G-tracts, to give ODNs 10–12 (Table 1). The tails of ODNs 10–12 are supposed to form 0,1,3 G–C base pair(s), respectively. The result of the electrophoresis is shown in Fig. 4. Two bands can be clearly seen in each lane. The slower migrating bands are predominant in ODNs 10 and 11, whereas the faster migrating band is predominant in ODN 12. Considering the sequence of ODN 12, the tails of ODN 12 are likely to form a duplex tightly. NMR analysis also supported the formation of the duplex in ODN 12 (Fig. 5). Imino protons in Watson-Click base pairs were previously shown in the lower field to 12.7 ppm.[29] In ODN 12, the proton peaks were observed around 13 ppm, which confirms that there are Watson–Crick base pairs in ODN 12. Other peaks are thought to be derived from G-tetraplex bases. Based on these results, we anticipate that the compact structure in ODN 12 is as described in Fig. 1c. In ODN 12, the ends of the G-quadruplex structure appear to point in the same direction due to the formation of the duplex. In contrast, the slower migrating bands were predominant for ODNs 10 and 11. Because of the lack of the duplex formation, the ends of the G-quadruplex structures in ODNs 10 and 11 appear to point in opposite directions.

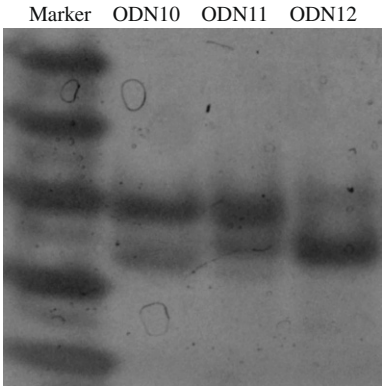

Figure 4. Native gel electrophoresis of ODNs 10–12.

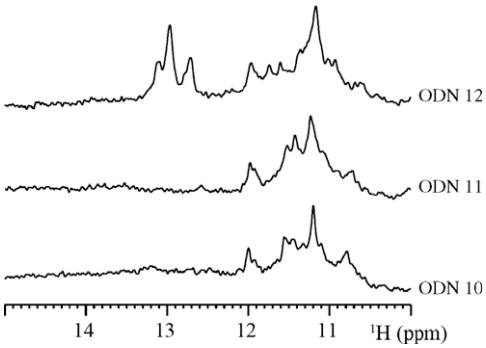

Figure 5. Imino proton spectra of ODNs 10–12.

2.4. Investigation of each conformation, Forms a–d, using native PAGE and NMR spectrometry

To evaluate the G-quadruplex structures, Forms a–d, we substituted all dGs in the syn conformation with $d^{Br}G$. These oligonucleotides, ODNs 13–16 (Table 1), are designed to take on Forms a–d, respectively. Figure 6 shows the results of the electrophoresis of ODN 11 and ODNs 13–16. All oligonucleotides show two bands and the slower migrating band is predominant in ODN 11, which are in good agreement with the results shown in Figure 4. Thus, in the slower migrating bands, the ends of the G-quadruplex structures are predicted to point in the opposite direction and the oligonucleotides do not take on a double-strand formation. The slower migrating bands are predominant in ODNs 13 and 14, whereas the faster migrating bands are predominant in ODNs 15 and 16 (Table 2). Because ODNs 15 and 16 are designed to take on a compact formation, these results in Figure 6 are consistent with what we expected. In the slower migrating bands, the oligonucleotides

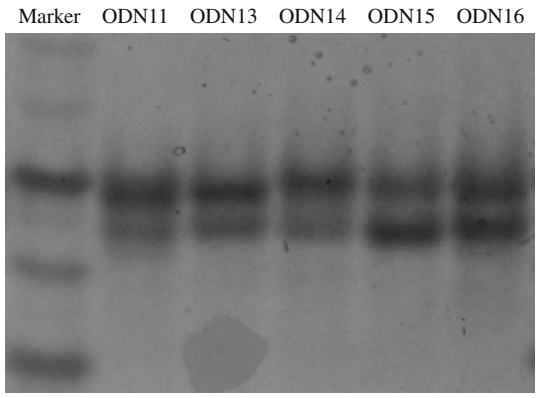

Figure 6. Native gel electrophoresis of ODNs 11, 13–16.

Table 2
Relative trace quantity of each bands in Fig. 6

	Slower migrating band (%)	Faster migrating band (%)
ODN 11	62	38
ODN 13	61	39
ODN 14	64	36
ODN 15	43	57
ODN 16	48	52

take on Forms a and b, while in the faster migrating bands, the oligonucleotides take on Forms c and d.

Next we investigated the intact G-quadruplex structures using NMR analysis. To avoid the complexity of the structures, the tail sequences were removed from ODNs 13–16. These oligonucleotides, ODNs 6–9, were designed to take on Forms a–d, respectively, and we have already measured CD spectra of these oligonucleotides (Fig. 3b). The imino proton spectra of ODNs 6–9 are shown in Figure 7. Observation of the 12 distinct imino protons in ODNs 6 and 7 indicate that each oligonucleotide takes on only one conformation. However, the more than 12 imino proton resonances in ODNs 8 and 9 explain the existence of exchange between multiple G-quadruplex conformations.

3. Discussion

To investigate the orientation of the ends of G-quadruplex structures, various end-extended oligonucleotides were prepared (Table 1). Because the sequences of the tail strands are complementary, these oligonucleotides were expected to show the different apparent molecular masses depending on their conformations. Indeed, the sequence of tail strands did affect the mobility of the oligonucleotides in the native PAGE experiment (Fig. 2).

We then measured the CD spectra of these oligonucleotides (Fig. 3). Interestingly, all oligonucleotides showed similar and characteristic spectra of (3+1) hybrid G-quadruplex structures. It is believed that a fundamental interpretation of the origin of the CD bands is in the stacking interactions of neighboring G-quartets.[28] With this in mind, we anticipate that G-quadruplex structures of these end-extended oligonucleotides are Forms a–d. To stabilize the G-quadruplex formations, we substituted oligonucleotides with $d^{Br}G$. Because $d^{Br}G$ favors a syn conformation[30], substitution with $d^{Br}G$ in the proper positions can stabilize the G-quadruplex structures and limit the structural diversity.[14,31] ODNs 6–9 can take on Forms a–d, respectively. The CD spectra of ODNs 6–9 were similar to those of ODNs 1–5. This results indicate that these oligonucleotides are likely to take on (3+1) hybrid G-quadruplex structures.

Next we prepared ODNs 10–12 which can take on all conformations, Forms a–d. In native PAGE experiments, only two bands were observed in each lane (Fig. 4). For ODN 12, the faster mobility band was predominant. Considering the stability of duplex formation in ODN 12, the ends of the G-quadruplexes structures appear to point in the same direction in the faster migrating bands. This prediction is also supported by the NMR analysis. ODN 12 showed imino proton peaks around 13 ppm, which confirm the existence of Watson–Crick base pairs (Fig. 5).

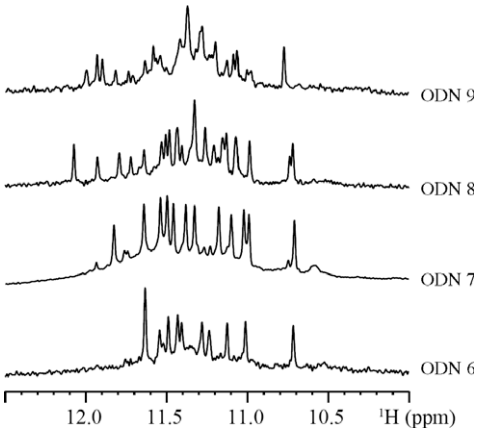

Figure 7. Imino proton spectra of ODN 6–9.

When the stability of the double strand in tails was decreased, the slower migrating band became predominant. We speculate that G-quadruplex structure is formed before the formation of duplex because Tm of G-quadruplex structure is higher than that of duplex. For instance, in the case of ODN 12 in Figure 4, the formation of duplex in tails seems to be strong enough to affect the formation of G-quadruplex structure. When compared to ODNs 2 and 3, ODNs 10 and 11 have the same respective sequences in their tails. When the G-quadruplex structure was stabilized by $d^{Br}G$-substitution, the slower migrating bands were predominant. In these cases, the G-quadruplex structures are less affected by formation of duplex because G-quadruplex structures are stabilized by $d^{Br}G$-substitution. These results indicate that the ends of the stable G-quadruplex structures point in opposite direction.

ODNs 13–16 are $d^{Br}G$-substituted oligonucleotides which are designed to take on Forms a–d, respectively. The electrophoretic mobility of each oligonucleotide was consistent with the proposal structures, Forms a–d (Fig. 6). Thus, the slower migrating bands were predominant in ODNs 13 and 14, whereas faster bands were predominant in ODNs 15 and 16. A difference of only one $d^{Br}G$-substitution could affect the proportion of the bands, which suggests a lower free energy barrier between each conformation. To examine the G-quadruplex structures by NMR spectroscopy, the tails of ODNs 13–16 were removed in order to reduce the complexity. These oligonucleotides, ODNs 6–9, were designed to take on Forms a–d, respectively. The number of imino protons in ODNs 8 and 9 was more than 12, which indicates the existence of multiple conformations (Fig. 7). In contrast, we observed 12 distinct imino proton resonances in ODNs 6 and 7. Although we could not strongly confirm the presence of Forms c and d, these results can provide the evidence that in stable G-quadruplex structures the ends of the G-quadruplex structures point in opposite direction.

Considering the length of the human telomeric single-stranded overhang, it is an interesting question as to whether adjacent human telomeric G-quadruplexes stack on each other. According to a beads-on-a-string model proposed by Sugimoto and co-workers,[21] individual G-quadruplexes do not interact with each other. However, groups using electrospray mass spectrometry have reported the existence of dimeric G-quadruplexes with nontelomeric oligonucleotides.[32,33] Recently, the stability of human telomeric G-quadruplex multimers was calculated.[22,23] Therefore experimental evidence for higher-order human telomeric G-quadruplex is required. To the best of our knowledge, there are three reports using long oligonucleotides with more than four human telomeric repeats.[24–26] Among them, Nakatani et al. found by means of polymerase stop assays that G-quadruplexes could stack on each other in the presence of small molecules.[26] We then studied further details of the G-quadruplex multimers. The direction of the ends of the G-quadruplex is quite important to consider in the rod-like higher-order G-quadruplex formations. To form the rod-like higher-order G-quadruplexe structures, the ends of the G-quadruplex structure should point in opposite directions (Fig. 8). Here we found that the ends of the stable G-quadruplex structures do point in opposite directions, which indicates that most G-quadruplexes in the human telomere are likely to take on end-to-end stacking structures. However our results are not sufficient to prove the existence of high-order G-quadruplex structures. Further experimental evidence is needed.

4. Conclusions

The orientation of the ends of G-quadruplex structures was investigated using native PAGE, CD measurements and NMR analyses. End-extended ^{Br}G-substituted oligonucleotides showed different mobilities based on the G-quadruplex structures. We

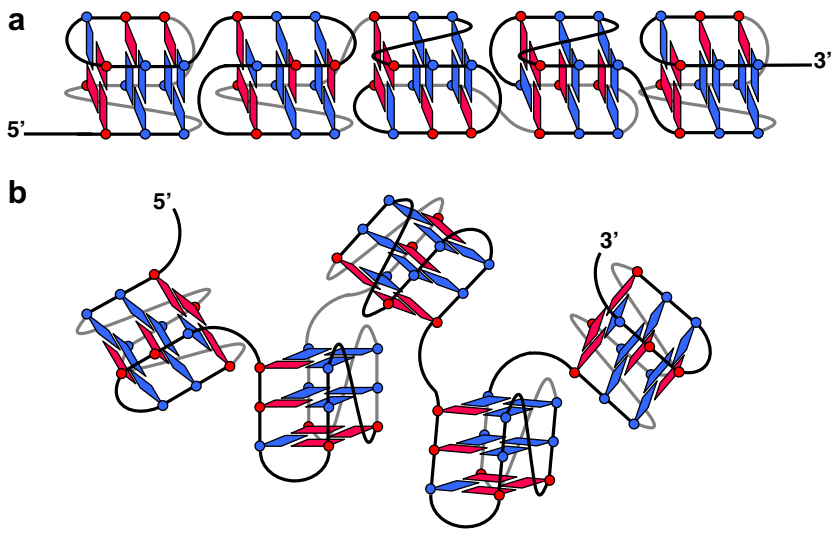

Figure 8. Schematic representation of the G-quadruplexes in long telomeric sequences. When the ends of the G-quadruplex point in the opposite direction, telomeric DNA can form rod-like higher-order G-quadruplex structures. Thus, adjacent G-quadruplexes can stack on each other (a). When the ends of the G-quadruplex structure point in the same directions, adjacent G-quadruplexes cannot stack on each other (b).

found that the ends of the stable G-quadruplex structure point in opposite direction. This can provide important information for understanding telomere structure and the development of telomere G-quadruplex-binding molecules as telomerase inhibitors.

5. Experiments

5.1. Preparation of oligonucleotides

All oligonucleotides used in this study are listed in Table 1. ODNs 1 and 4 were purchased from Sigma Aldrich Japan, while the other oligonucleotides were purchased from Japan Bio Service Co. Ltd. Those oligonucleotides were used without further purification.

5.2. Native PAGE experiments

Oligonucleotide samples were prepared in running buffer (50 mM Tris–HCl; pH 7.0, 25 mM $K_2B_4O_7$) with loading dye. The concentrations of oligonucleotides were 0.25 OD at 260 nm. Samples were heated at 95 °C for 10 min, followed by gradual cooling to 4 °C. Native 20% polyacrylamide gels with 4% stacking gels were run at 11.7 V/cm for 6 h at 4 °C. The gels were stained with SYBR Gold and observed with FUJIFILM LAS-3000UV mini. The observed data was analyzed with Multi Gauge Ver 3.1. Trace quantity is the amount of intensity of a band on user-defined region of interest. Relative trace quantity is a percentage of total intensity. Self-complementary oligonucleotides of 12, 14, 16, 18, 20 nt were used as a marker.

5.3. CD measurements

The CD spectra were measured using an AVIV MODEL 62 DS/202 CD spectrometer. A quartz cell of 1 cm optical path length and an instrument scanning speed of 1 nm/s was used for the measurements. The buffer used for CD was 50 mM Tris–HCl (pH 7.0) and 25 mM $K_2B_4O_7$. Oligonucleotides samples (5 μM) were heated at 95 °C for 10 min and gradually cooled to room temperature.

5.4. NMR spectroscopy

NMR spectra were recorded with Bruker DRX600 spectrometers. Each oligonucleotide was dissolved in a solution comprising 100 mM KCl, 20 mM K phosphate (pH 6.2), 0.01 mM DSS and 5% D_2O. Samples were heated at 95 °C for 10 min, followed by gradual cooling to 4 °C.

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