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A novel intramolecular G-quartet-containing fold of single-stranded $d(GT)_8$ and $d(GT)_{16}$ oligonucleotides☆

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ABSTRACT

Human genome is shown to be enriched with $(GT)_n$ stretches of lengths from 8 to 20 dinucleotides. Low temperature ($T \leq 10$ °C) conformations of $d(GT)_n$ oligonucleotides ($n = 7, 8, 12, 16, 20$) were studied by means of circular dichroism (CD), thermal melting, ethidium bromide (EtBr) probing and single nucleotide substitutions. Rotational relaxation times for EtBr:$d(GT)_n$ complexes confirmed a monomolecular state of the oligonucleotides. CD spectra indicated involvement of all guanines of $d(GT)_8$ and $d(GT)_{16}$ in G-quartets, while $dT(GT)_7$, $d(GT)_{12}$ and $d(GT)_{20}$ were shown to be only partially ordered. The schemes of the $d(GT)_8$ and $d(GT)_{16}$ folds are suggested.

1. Introduction

The four-stranded DNA G-quadruplexes are under extensive study due to their possible role in gene regulation. Appropriate guanine-rich sequences are known to be abundant in telomeres as well as in the promoter regions of many genes [1]. The structural features of the canonical G-quadruplexes containing uninterrupted runs of guanines G_n ($n \geq 2$) in each strand are studied rather well [1]. Formation of similar quadruplexes by $(GT)_n$ sequences is not so widely known. The polymorphic microsatellite $(GT)_n$ sequences are present in genomes of many eukaryotic organisms and are known to drastically affect regulation of gene expression [2–5]. Earlier, using a model oligonucleotide 3′-$d(GT)_5$-$(CH_2CH_2O)_3$-$d(GT)_5$-3′ we suggested the formation of a bimolecular parallel quadruplex with five G-quartets and the looped-out thymines ("GT-quadruplex") [6]. The substitution of G for T in position 10 of the model oligonucleotide resulted in the formation by 3′-$dG(TG)_4G$-$(CH_2CH_2O)_3$-$dG(GT)_4G$-3′ of a parallel double helix (hp-GT) stabilized by G·G and T·T pairs [7,8].

In this study, we address the question: whether $d(GT)_n$ sequences possess a wider conformational potential which may be exploited in cell regulatory processes.

2. Experimental

2.1. Sample preparations

Oligonucleotides $d(GT)_{8, 12, 16, 20}$, $dT(GT)_7$, $dAT(GT)_7$ and $d(GT)_7AT$ were synthesized and purified in gel by Syntol (Moscow). Samples contained 0.1 M NaCl, 10 mM Na-phosphate buffer, and pH 8.0 (PBS + Na^+). Molar extinction coefficient of $d(GT)_n$ at 90 °C in water was $\varepsilon_{260} = 10{,}550\ M^{-1}\ cm^{-1}$ (in nucleotides). Solutions containing EtBr (Serva) were prepared in the same buffer, $\varepsilon_{485} = 5600\ M^{-1}\ cm^{-1}$.

2.2. CD spectroscopy

CD spectra of $d(GT)_n$ were registered with Jasco 715 spectropolarimeter (JASCO). The optical path length was 1 cm. The CD values ($\Delta\varepsilon$) are given per moles of nucleotides, 1 cm of optical path ($M^{-1}\ cm^{-1}$). CD thermal melting was registered at 260 nm.

2.3. The fluorescence of EtBr:$d(GT)_n$ complexes

The fluorescence intensity (I_λ) and polarization (P) of EtBr were registered with a Photon Technology Instruments Spectrofluorimeter using a thermostatical cell holder. The fluorescence life time (τ) of EtBr was evaluated using "Easy Life V System". The fluorescence quantum yield (q) of EtBr:$d(GT)_n$ complexes was estimated as related to that of EtBr bound to *ds*DNA. The concentration of intercalated EtBr was determined fluorimetrically [8,9]. The intensity of EtBr fluorescence was measured at 610 nm under excitation at 540 nm.

Abbreviations: EtBr, Ethidium bromide; CD, circular dichroism.

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2.4. Rotational relaxation time of EtBr:d(GT)$_n$ complexes

Rotational relaxation time (ρ) of EtBr:d(GT)$_n$ complexes was estimated using Perrin equation:

$$\rho = 3\tau(1/P_o - 1/3)/(1/P - 1/P_o). \quad (1)$$

Here P is the measured fluorescence polarization of EtBr:d(GT)$_n$ complexes and $P_o = 41 \pm 1\%$ is its limiting value in the absence of rotational depolarization; τ is the fluorescence lifetime of the EtBr:d(GT)$_n$ complexes [6–8]. The polarization measurements were performed in PBS + Na^+ buffer at concentrations $3.5 \cdot 10^{-5}$ M(N) of d(GT)$_n$ and 10^{-6} M of EtBr. Under the experimental conditions used, the EtBr was essentially fully bound to the oligonucleotides, ensuring that the contribution of the free dye to the fluorescence signal was negligible.

3. Results

3.1. Length polymorphism of GT repeats in various genomes

The (GT)$_n$/(AC)$_n$ motif is known to be the most frequent tandem dinucleotide repeat in the mammalian genome [10]. Using GenBank data we have carried out the analysis of length dependent occurrence of (GT)$_n$/(AC)$_n$ repeats in various genomes. Fig. 1 shows the frequency of the repeat (GT)$_n$/(AC)$_n$ (average occurrence number per million of nucleotides) as dependent on the length (n) in genomes of *Homo sapiens*, mouse, invertebrates, plants and fungi. For all species, the repeats shorter than 8 dinucleotides are most frequent. The length distribution of GT repeats is close to random for invertebrates, plants and fungi (Fig. 1). Unlike this, in animal genomes the occurrence of (GT)$_n$ sequences with the lengths of $8<n<20$ is essentially non-random. Distinct local maxima are present at $n \approx 18$–20 for mouse and at $n \approx 15$–18 for *Homo sapiens* (Fig. 1). The non-random length distribution of GT repeats in higher organisms due to the accumulation of the certain (GT)$_n$ repeats may be of a functional importance. Regulatory microsatellite regions may contribute to such an enrichment of human and mouse genomes with the (GT)$_n$ repeats, $8<n<20$. Apart from specific structural features of the double-helical (GT)$_n$/(AC)$_n$ regions [11], conformational potential of single strands constituting the duplex may be functionally meaningful. In this study, we focus our attention on d(GT)$_n$ oligonucleotides ($7 \le n \le 20$).

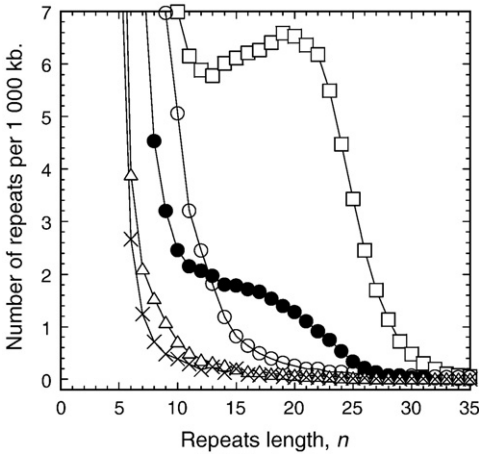

Fig. 1. Length distribution of (GT)$_n$ repeats in full genomes: open squares — mouse; filled circles — *Homo sapiens*; open circles — invertebrates; triangles — plants; crosses — fungi.

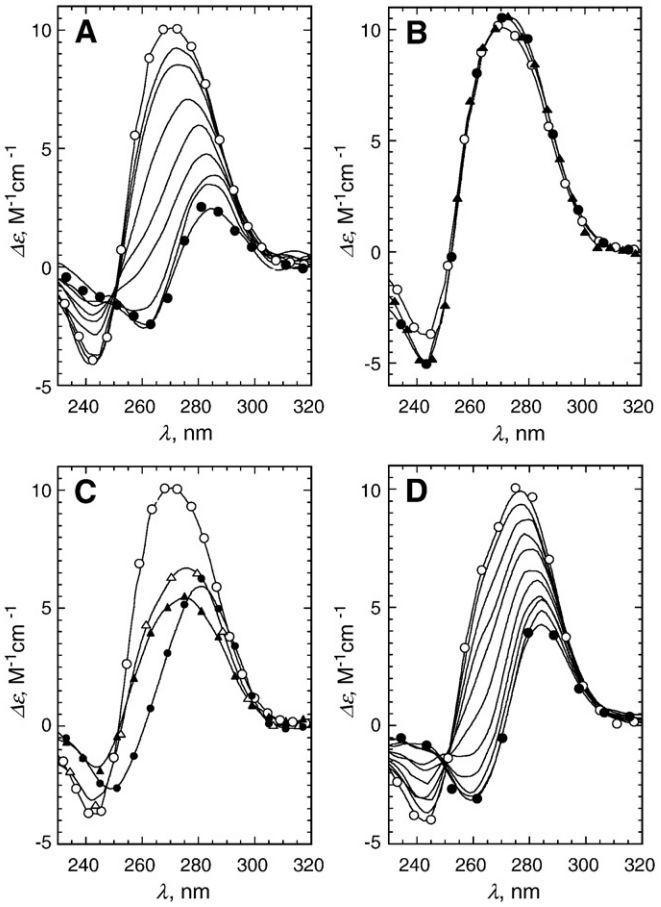

Fig. 2. (A) CD spectra of d(GT)$_8$ in PBS + 0.1 M NaCl at temperatures increasing from 1 °C (open circles) to 60 °C (filled circles). (B) CD of d(GT)$_8$ at $t = 1$ °C in varied solutions: (open circles) — PBS + 0.1 M NaCl; (filled triangles) — PBS + 0.1 M KCl; (filled circles) — 40% v./v. methanol + 0.1 M NaCl. (C) CD of d(GT)$_8$ and of its modifications in PBS + 0.1 M NaCl, $t = 1$ °C: (open circles) — d(GT)$_8$; (open triangles) — dT(GT)$_7$; (filled triangles) — dAT(GT)$_7$; (filled circles) — d(GT)$_7$AT. (D) CD spectra of d(GT)$_{16}$ in PBS + 0.1 M NaCl at temperatures increasing from 1 °C (open circles) to 60 °C (filled circles). Concentration of d(GT)$_n$ in all cases was $3.2 \cdot 10^{-5}$ M of nucleotides.

3.2. CD spectroscopy of d(GT)$_n$

Fig. 2(A) shows the CD spectra of d(GT)$_8$ in PBS + Na^+ at temperatures from 1 to 60 °C. At 1 °C the CD spectrum of d(GT)$_8$ (Fig. 2A, open circles) has a pronounced positive band with maximum around 268 nm and negative band with minimum around 240 nm. Upon increasing the temperature from 1 to 60 °C, the ordered oligonucleotide structure melts down to a disordered single-stranded conformation with its characteristic CD spectrum (Fig. 2A, filled circles) [6]. The isodichroic point at 250 nm is coherent with the presence of two d(GT)$_8$ conformations: the structured one and that of the denatured single strand. The low temperature CD spectra of d(GT)$_8$ at 1–3 °C (Fig. 2A) are very similar to the CD spectra of the parallel intermolecular [d(TTGGGGTT)]$_4$ G-quadruplex [12] and to the parallel intramolecular d(G$_3$T)$_3$G$_3$ G-quadruplex with the single-thymine double-chain-reversal loops [13]. These data suggest that d(GT)$_8$ fold comprises two G-quartets with all guanines in *anti*-conformation [1,12,13].

G-quadruplexes are known to be markedly stabilized by K^+ ions [12,14] as well as by ethanol [15] or methanol [16] in the solution. In contrast, the d(GT)$_8$ G-quartet-containing fold is not influenced by the presence of K^+ or methanol which is evidenced by the identity of its low temperature (1 °C) CD spectra in the presence of Na^+, K^+ and 40% methanol (Fig. 2B).

The involvement of definite guanines in $d(GT)_8$ folding can be tested by their replacement. The $dAT(GT)_7$ and $d(GT)_7AT$ oligonucleotides were taken to check the roles of 5′- and 3′-end guanines in the $d(GT)_8$ fold. Oligonucleotide $dT(GT)_7$ was also taken for comparison with $dAT(GT)_7$. The low temperature (1 °C) CD spectra of the four oligonucleotides are given in Fig. 2(C). In cases of deletion or replacement of the 5′-terminal guanine, rather moderate changes in the CD spectra (open and filled triangles) are accompanied by a reduction of the amplitude of characteristic positive band (260–268 nm) which evidently corresponds to a partial disordering of the G-quartet structure. Unlike this, the replacement of the 3′-terminal guanine entails spectral changes (filled circles) with characteristic CD signal at 260 nm dropping to zero. This implies the disruption of G-quartets with guanines in *anti*-conformation.

CD spectra of $d(GT)_{16}$ in PBS + Na^+ at temperatures from 1 to 60 °C are given in Fig. 2(D). At low temperatures (1–3 °C) they are almost indistinguishable from those for $d(GT)_8$. This indicates a close similarity of conformations for the two oligonucleotides and specifically, the involvement of all guanines of $d(GT)_{16}$ in four G-quartets.

CD spectra of $d(GT)_{12}$ and $d(GT)_{20}$ at 1 °C demonstrate markedly lower amplitude of the positive band around 260–268 nm in comparison with that of $d(GT)_8$ (Fig. 1, suppl. material). This allows to suggest that $d(GT)_{12}$ and $d(GT)_{20}$ oligonucleotides at 1 °C are less ordered and at least some of their guanines are not involved in G-quartets.

So, the analysis of CD spectra of oligonucleotides $d(GT)_n$ ($n = 8, 12, 16$ and 20) as well as of $dT(GT)_7$, $dAT(GT)_7$ and $d(GT)_7AT$ divulges that only $d(GT)_8$ and $d(GT)_{16}$ can fold in conformations with all guanines involved in two or four G-quartets, respectively. Oligonucleotides $dT(GT)_7$, $dAT(GT)_7$, $d(GT)_{12}$ and $d(GT)_{20}$ may form partially ordered conformations with only some of guanines involved in putative G-quartets. In contrast to this $d(GT)_7AT$ is totally deprived of G-quartet structure.

3.3. Thermostability of $d(GT)_8$ and $d(GT)_{16}$ folds

Thermal denaturation of $d(GT)_8$ and $d(GT)_{16}$ folds registered with CD magnitudes at 260 nm is depicted in Fig. 3. The thermodynamic parameters of the intramolecular folds formation were assessed using a two-state model. Thermodynamic parameters of $d(GT)_8$ fold formation in PBS + Na^+ (Fig. 3, filled triangles), in K^+ (filled circles) and in 40% methanol (open squares) are the same within experimental error: $T_m = (12 \pm 1)$ °C, $\Delta H = -(95 \pm 10)$ kJ/mol. Thermodynamic parameters of $d(GT)_{16}$ fold in PBS + Na^+ ($T_m = (9.5 \pm 0.5)$ °C; $\Delta H = -(98 \pm 4)$ kJ/mol) (Fig. 3, filled squares) are close to those of $d(GT)_8$.

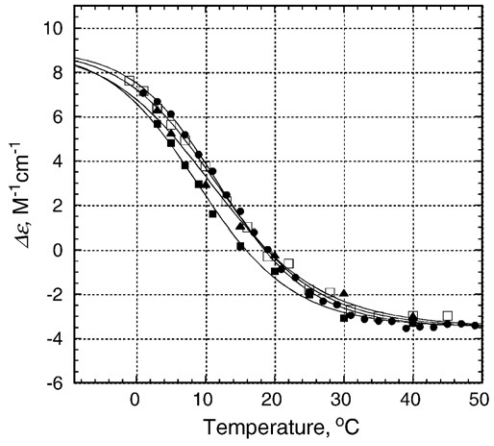

Fig. 3. $CD_{260\ nm}$ thermal denaturation curves for $d(GT)_8$ and $d(GT)_{16}$ oligonucleotides: (filled triangles) — $d(GT)_8$ in PBS + 0.1 M NaCl; (filled circles) — $d(GT)_8$ in PBS + 0.1 M NaCl + 0.1 M KCl; (open squares) — $d(GT)_8$ in 40% methanol + 0.1 M NaCl; (filled squares) — $d(GT)_{16}$ in PBS + 0.1 M NaCl. Concentration of $d(GT)_n$ in all cases was $3.2 \cdot 10^{-5}$ M of nucleotides.

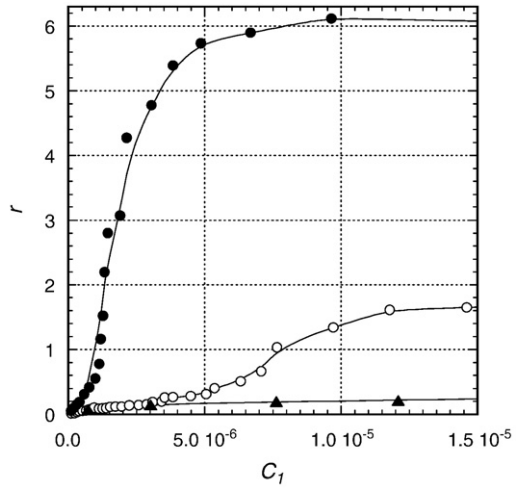

Fig. 4. EtBr adsorption on $d(GT)_8$ (open circles), $d(GT)_{16}$ (filled circles) and $dAT(GT)_7$ (triangles) registered by EtBr fluorescence. C_1 is the concentration of free dye in solution. The parameter r is the concentration of bound dye related to the concentration of the oligonucleotide strands. PBS + 0.1 M NaCl; $t = 3$ °C.

It is to be noted that $d(GT)_8$ and $d(GT)_{16}$ folds are inferior to canonical G-quadruplexes in thermostability [1,12–17].

3.4. Probing $d(GT)_n$ conformations with EtBr fluorescence

The secondary structure of $d(GT)_n$ ($n = 8, 12, 16, 20$) folds was assessed with EtBr probing. Fluorescence quantum yield q of EtBr:$d(GT)_n$ complexes as related to that of EtBr:dsDNA was determined to be $q / q_{DNA} = 0.9 \pm 0.02$. Fluorescence life time τ of EtBr:$d(GT)_n$ was measured to be $\tau = 24 \pm 0.5$ ns, which is close to $\tau = 25 \pm 0.3$ ns characteristic for EtBr:dsDNA. These data indicate the intercalation mode of EtBr binding to the studied oligonucleotide folds [6,8,9].

A predominance of intramolecular folds over intermolecular structures was verified by the determination of rotational relaxation times ρ based on EtBr:$d(GT)_n$ fluorescence polarization P measurements (see Eq. (1)). In PBS + Na^+ buffer, at 3 °C the fluorescence polarization was found to be $P = (8.7 \pm 0.3)\%$ for EtBr:$d(GT)_8$, $P = (13 \pm 0.2)\%$ for EtBr:$d(GT)_{12}$, and $P = (16 \pm 0.2)\%$ for EtBr:$d(GT)_{16}$. The estimated rotational relaxation times $\rho = 17 \pm 1$ ns for EtBr:$d(GT)_8$, $\rho = 29 \pm 2$ ns for EtBr:$d(GT)_{12}$ and $\rho = 39 \pm 2$ ns for $d(GT)_{16}$ correspond well to the monomolecular masses of the oligonucleotides (Supplementary material, Fig. 2) [6,8,18].

The study of EtBr binding to $d(GT)_n$ using a fluorimetric technique allows one to register the quantity of intercalation binding sites [8,9]. The adsorption isotherms were plotted in coordinates $r(C_1)$, i.e. as the number of bound dye molecules per an oligonucleotide against free dye concentration (Fig. 4). The shape of the adsorption isotherms for $d(GT)_8$ (open circles) and $d(GT)_{16}$ (filled circles) corresponds well to the cooperative type of binding. The maximal number of EtBr molecules intercalated into $d(GT)_8$ and $d(GT)_{16}$ equals to 2 and 6, respectively. The apparent dissociation constants were estimated to be $K_d = 7 \times 10^{-6}$ M for EtBr:$d(GT)_8$ and $K_d = 1.6 \times 10^{-6}$ M for EtBr:$d(GT)_{16}$.

The cooperative type of EtBr intercalation in $d(GT)_8$ and $d(GT)_{16}$ folds is a sign of a conformation quite different from DNA duplex or the parallel-stranded hairpin hp-GT, that bind EtBr anti-cooperatively [9]. EtBr binding curve to $dAT(GT)_7$ (Fig. 4, triangles) indicates the absence of intercalation binding sites in this oligonucleotide.

The observed stoichiometry of EtBr intercalation is in concordance with the maximal number of possible G-quartets in $d(GT)_8$ and

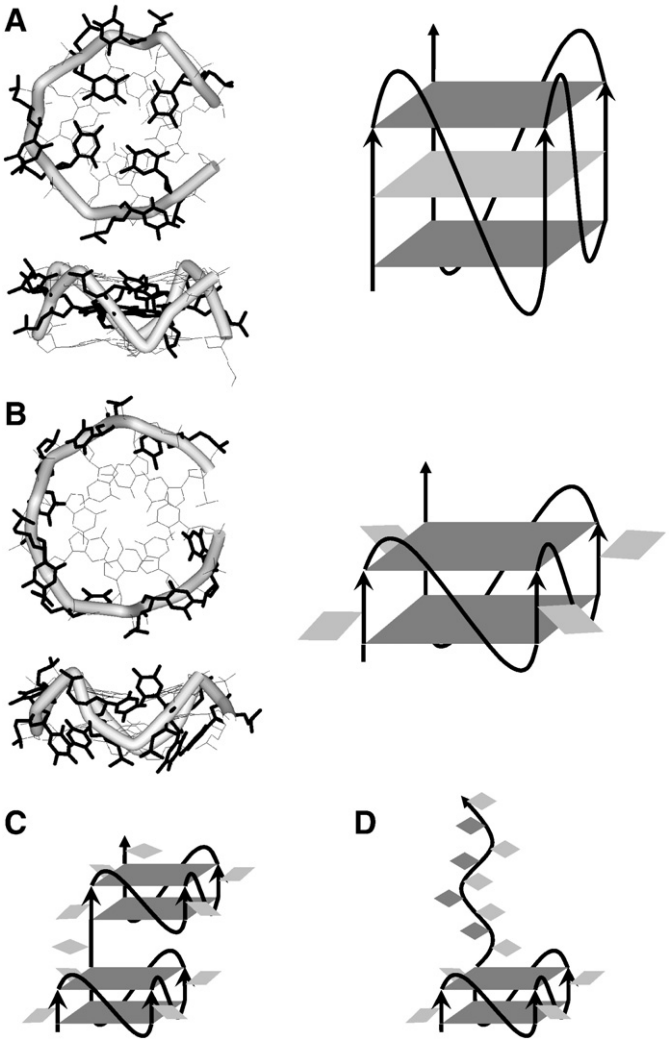

Fig. 5. Schemes of G-quartet-containing $d(GT)_n$ folds. All the guanines, forming G-quartets, are in *anti*-conformation. (A) $d(GT)_7G$ fold. Four thymines form a quartet between two G-quartets, while three others T form single-thymine double-chain-reversal loops. (B) $d(GT)_7G$ bi-quartet fold. All seven thymines are looped out. (C) $d(GT)_{16}$ fold comprising two bi-quartets. (D) $d(GT)_{12}$ fold comprising a bi-quartet and the unstructured $(GT)_4$ tail.

$d(GT)_{16}$ folds revealed with CD. Thus, two EtBr molecules can intercalate in the gap between two G-quartets of $d(GT)_8$, while three gaps between four G-quartets of $d(GT)_{16}$ can accommodate three pairs of EtBr molecules.

3.5. Scheme of G-quartet-containing $d(GT)_8$ fold

The structural basis of the $d(GT)_8$ folding is the propensity of eight guanines to form two G-quartets with all guanines in *anti*-conformation. The presence of this structural motif is confirmed by CD spectroscopy (Fig. 2A). We derive a scheme for $d(GT)_8$ folding as follows. The intramolecular parallel quadruplex structure formed by human telomeric sequence $dAG_3(T_2AG_3)_3$ (PDB code 1KF1) was chosen as a structure template [19]. Substitution of T loops for T_2A loops was performed as described earlier [20]. All guanines of the middle G-quartet were replaced by thymines, the 5′-adenine was removed. This resulted in a $(GT)_7G$ structure containing a T-quartet between two G-quartets (Fig. 5A). One of the two G-quartets is formed by G1, G5, G9 and G13 guanines while another is formed by G3, G7, G11 and G15. Geometry optimization done for all atoms with HyperChem using Amber force field with Amber99 parameter set (in the absence of solvent and counter ions) has also shown a possible alternative structure of a similar energy, which allows all thymines to be bulged out (Fig. 5B) and G-quartets to come into stacking contact. Earlier T-quartets formation by thymines had been demonstrated by X-ray analysis [21]; T-quartets had been detected in quadruplex $[d(G_5T_5)]_4$ using CD [22]. Single-thymine double-chain-reversal loops were identified in $d(G_3T)_3G_3$ quadruplex [20]. Similar to the case of $d(G_3T)_3G_3$ structure, the thymines in the single T loops in the $d(GT)_8$ fold can form H-bonds with N(2) of guanines involved in 3′-end G-quartet, thus contributing to a stabilization of the quartet.

4. Discussion

Analysis of CD spectra of oligonucleotides $d(GT)_n$ ($n = 8, 12, 16$ and 20) as well as of $dT(GT)_7$, $dAT(GT)_7$ and $d(GT)_7AT$ divulges that only $d(GT)_8$ and $d(GT)_{16}$ can fold in conformations with all guanines involved in two or four G-quartets, respectively. Oligonucleotides $dT(GT)_7$, $dAT(GT)_7$, $d(GT)_{12}$ and $d(GT)_{20}$ may form partially ordered conformations with only some of guanines involved in putative G-quartets. In contrast to this $d(GT)_7AT$ is totally deprived of G-quartet structure.

The suggested folding scheme for $d(GT)_8$ (Fig. 5A) differs from that of $d(G_3T)_3G_3$ quadruplex [13] by the replacement of the central G-quartet by T-quartet. Such a replacement reduces the thermostability of the $d(GT)_8$ fold dramatically (Fig. 3).

Our data on EtBr binding (Fig. 4) support the folding scheme given in Fig. 5B rather than that of Fig. 5A. In scheme (A), the fold with the three quartets connected by the single-thymine double-chain-reversal loops cannot stretch enough to accommodate intercalators. Therefore, in the $d(GT)_8$ fold the T-quartets either do not form or become destroyed upon EtBr intercalation.

The $d(GT)_{16}$ fold may be conceived of as composed of two rather independent $(GT)_7G$ structural units connected by the single T16 linker (Fig. 5C). According to such a folding scheme all guanines in $d(GT)_{16}$ participate in four G-quartet formation, in compliance with the CD data (Fig. 2D). Three gaps between four G-quartets allow three pairs of EtBr molecules to intercalate (Fig. 4). The smaller estimated K_{diss} for EtBr binding to $d(GT)_{16}$ in comparison with that to $d(GT)_8$ may be due to a greater accessibility of the gap formed by two central G-quartets connected by a single T linker (Fig. 5C). The observed close similarity of the thermodynamic parameters of $d(GT)_{16}$ and $d(GT)_8$ folds testifies in favor of the $d(GT)_{16}$ folding scheme as of doubling the $d(GT)_8$ structural units. More generally, one should expect a beads-on-a-string architecture [22,23] in cases of $(GT)_{8n}$ repeats. Meanwhile for $d(GT)_{12}$, $d(GT)_{20}$ oligonucleotides there is no possibility to incorporate all guanines in G-quartets (Fig. 5D).

Canonical G-quadruplexes formed by G_n ($n>2$) blocks are known to be so stiff, that they can accommodate intercalators only via stacking with the terminal G-quartets (capping) [1,24]. Unlike this, monomolecular G-quartet-containing $d(GT)_8$ and $d(GT)_{16}$ folds exhibit remarkable flexibility. Their structure is able to compress by bulging out thymines and drawing G-layers together, and to extend by intercalation of either thymines, or ligands like EtBr between the G-quartets.

The conformational flexibility of the $d(GT)_n$ sequences may be connected with their regulatory functions in the cell and may be exploited by a number of biologically active ligands.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2009.05.006.

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