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Structural polymorphism of the four-repeat *Oxytricha nova* telomeric DNA sequences

Rashid M. Abu-Ghazalah, Robert B. Macgregor Jr. *

Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, Canada

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ABSTRACT

G-quadruplexes are four-stranded nucleic acid complexes that exhibit a great deal of polymorphism. Recently a group described the polymorphism exhibited by the four-repeat of the *Oxytricha nova* telomeric sequences (Lee, J.Y., Yoon, J., Kihm, H.W., Kim, D.S., Biochemistry 2008, 47, 3389–3396). In this study we evaluated the effects of G-tract and loop lengths on this behaviour using circular dichroism (CD) and gel electrophoresis. The largest changes were detected for oligonucleotides with different numbers of consecutive G residues. Furthermore, decreasing the number of residues between the G runs, the loops, from four to three only results in minor alteration in the polymorphism. However, the shortening of the G-tract from four to three guanine residues led to characteristically anti-parallel G-quadruplex CD spectra. Finally, we show that adenine bases in the loop sequences are less likely to form G-quadruplexes in the presence of Na^+ cations than those comprised of thymine residues. The results presented here are an addition to the modest information available for predicting the type of G-quadruplex to be formed from G-rich sequences in aqueous solutions containing sodium or potassium ions.

1. Introduction

The conformational plasticity of DNA depends on the environment (buffer, pH, temperature, etc.) and on the sequence of the particular DNA molecule [1,2]. Among the naturally occurring DNA bases, guanine residues are unique in the way they self-interact to form G-quartets, a cyclical, planar arrangement of four guanine bases [3].

The formation of G-quartets underlies the stability of a group of structures called G-quadruplexes. These are four-stranded DNA structures that are composed of a single, folded oligodeoxynucleotide (ODN) strand, a bimolecular quadruplex, arising from two ODNs, or four separate strands combined to create a tetramolecular quadruplex. In general, ODNs with sequences composed of four or more clusters of guanine residues have the potential to fold into a monomolecular quadruplex. A sequence with two clusters of guanine residues can dimerize into a bimolecular motif; while a single run of guanines could lead to the formation of a tetramolecular quadruplex. In all three cases the formation of G-quartets and their subsequent stacking is fundamental to quadruplex formation and stability. Cations also play a critical role in facilitating the creation of G-quartets. As a result of G-quartet formation, eight carbonyl oxygens (between two stacked G-quartets) are brought into close proximity and the presence of a cation becomes necessary to stabilize the structure. A number of mono- and di-valent cations are effective; however, potassium ions lead to the greatest stability [4].

Another important aspect of folded G-quadruplexes is the orientation of the strands in relation to each other. The direction of the strands in a quadruplex comprised of one or two ODNs can either be anti-parallel, if at least one of the strands is of opposite orientation in relation to the other three; or parallel if all strands have the same directionality [5]. Antiparallel G-quadruplexes are composed of mixed *syn* and *anti* glycosidic angles [5]. In the case of tetramolecular quadruplexes, all four strands tend to be parallel to each other and the glycosidic angles are all in the *anti* configuration [5–8]; however, one may also observe a topology in which three strands are parallel and the fourth strand is in the anti-parallel orientation [8].

Previous studies have shown that there is an interesting interplay between the length and composition of the G-tract and loops and the structures that form [9–20]. In general, the stability of the folded quadruplex increases with the length of the guanine runs. An exception to this rule appears to be $d(G_3T)_4$; Rachwal et al. found that it is more stable than $d(G_7T)_4$ [11]. Other work was shown that the number of guanine residues in a G-cluster does not necessarily reflect the number of G-quartets present in a folded G-quadruplex [16]. Substituting rG for dG in the thrombin binding aptamer (TBA), d($G_2T_2G_2TGTG_2T_2G_2$), was found to transform the TBA quadruplex from an anti-parallel monomolecular conformation into a parallel bimolecular fold [19].

Guo et al. studied the stability of the tetramolecular structures formed by $d(T_nG_4)$ where $n=1$–8 and found that the most stable structure was formed by the $n=1$ sequence [14]. In a study undertaken by Hazel et al. it was determined that the length of the loop region of a

* Corresponding author. Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, 144 College St., Toronto, Ontario, Canada M5S 3M2. Tel.: +1 416 978 7332; fax: +1 416 978 8511.
E-mail address: rob.macgregor@utoronto.ca (R.B. Macgregor).

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sequence can have an impact on the folding of G-quadruplexes [17]. They found that parallel-stranded monomolecular G-quadruplexes are the only permitted conformation when the three loops are composed of a single nucleotide. A previous study with d($G_4T_nG_4$), $1 \le n \le 4$, established similar results [21]. When $n = 1$, CD spectra characteristic of a parallel-stranded G-quadruplex are observed [21]. With $n = 2$ the CD signal appears to reflect the presence of a mixture parallel- and anti-parallel G-quadruplex formations [21]. Rachwal et al. showed that single-nucleotide loops, dT, form a more stable monomolecular G-quadruplex than those containing four thymine residues in the loop region [13]. Furthermore, it was found that a monomolecular quadruplex with single-base linkers comprised of dA form the least stable structures when compared to those containing dT, dC or 1′,2′-dideoxyribose with a T_m that is ~25 °C lower [20], in support of other studies [22,23]. The crystal structure of the bimolecular G-quadruplex formed by d($G_4T_3G_4$) shows that the loops are of a lateral type [24] while a similar sequence with an extra thymine in the linker region d($G_4T_4G_4$) has both loops arranged diagonally [25]. Generally, it can be stated that longer loops lead to lower stability of the G-quadruplex [23,26,27].

Other than sequence-dependant effects on the final folded G-quadruplex, the type of cation present plays an important role. Sen and Gilbert reported a sodium-potassium switch in G-rich oligonucleotides; potassium ions led to the formation of a monomolecular G-quadruplex and sodium ions caused the same oligonucleotide to form a tetramolecular G-quadruplex [28]. One of the most studied G-quadruplex-forming sequences are those of the human telomeric repeats $(TTAGGG)_n$. The NMR structure of a four-repeat human telomeric sequence d$(TTAGGG)_4$ was shown to be a folded antiparallel monomolecular G-quadruplex in the presence of Na^+ [29]. In contrast, the resolved crystal structure of the same sequence in the presence of K^+ showed that the G-quadruplex was indeed monomolecular, but is an all-parallel-stranded structure with the loops all being of the propeller type [30]. However, the solution structure(s) of this molecule failed to show that the all-parallel conformation was the predominant structure in the presence of K^+. Instead, the structure adopted in the presence of K^+ is a mixture of two hybrid structures comprised of three parallel strands and a fourth pointing in the opposing direction [31].

Another cation-induced structural transition was demonstrated by Miura et al. who showed that at low concentrations of Na^+ and K^+, d$(T_4G_4)_4$, forms monomolecular G-quadruplexes and at higher cation concentrations tetramolecular G-quadruplexes arise, with K^+ being more effective at initiating this transition [32]. However, another group found that in the presence of K^+, a mixture of parallel and antiparallel monomolecular G-quadruplexes exists while in the presence of Na^+ only the antiparallel species are present [33]. In the work we present here CD and polyacrylamide gel electrophoresis (PAGE) were used to evaluate the effects of systematically varying the G-tract and loop lengths of the four-repeat *Oxytricha nova* telomeric repeat, d$(T_4G_4)_4$, on the observed cation-induced structural transition. We show that the loop and G-tract lengths play important roles on the folded G-quadruplex structure. Furthermore, we show that loops comprised solely of dA residues behave differently than those containing dT bases.

2. Materials and methods

2.1. Oligonucleotides

The cartridge-purified oligonucleotides were purchased from Cortec DNA Service Laboratories Inc. (Kingston, Ontario, Canada). Their concentrations were estimated spectrophotometrically, using extinction coefficients at 260 nm calculated from the nearest-neighbour model [34]. The samples were aliquoted into 50 μM stock solutions, lyophilized, and stored at −20 °C. The DNA samples were subsequently re-dissolved in Tris–HCl 10 mM (pH 7.5) for experimentation. A 10 b.p. DNA ladder was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Sample preparation

Prior to electrophoresis or measuring CD spectra, all were prepared with a final concentration of 5 μM of DNA strands. Samples were heated to 95 °C for 5 min and, left to cool slowly to room temperature, and then stored at 4 °C overnight.

2.3. Circular dichroism spectroscopy

CD spectra were collected at 1.0 nm increments at 4 °C from 220–300 nm on an Aviv model 62A DS circular dichroism spectrometer (Lakewood, NJ, USA) using a cuvette with a 0.1-cm pathlength. The CD spectra presented are the average of three consecutive scans. Samples were degassed prior to loading into the cuvette.

2.4. Electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) was run on 10% polyacrylamide gels containing 50 mM Tris-borate buffer (pH 7.5) and 20 mM KCl or NaCl. Electrophoresis was carried out at 10 V/cm, at 4 °C. Oligonucleotides were radiolabelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Fermentas). Band patterns of the resolved gels were visualized on a STORM 840 (GE Healthcare).

3. Results

Table 1 lists the ODNs that were investigated in this study. The CD spectra of the four-repeat of the *O. nova* telomeric sequences, d$(T_4G_4)_4$, is presented in Fig. 1. In the presence of 100 mM Na^+, the CD spectrum appears to be that of an antiparallel G-quadruplex: a positive peak at about 295 nm and a trough at about 260 nm. In contrast, in the presence of 100 mM K^+ the CD spectrum maintains two positive peaks at ~265 nm and a shoulder at ~295 nm. This could indicate the presence of a mixture of two or more species of G-quadruplexes that are parallel- and anti-parallel stranded. These findings are consistent with a recent study by Lee et al. who showed that a similar sequence, d$(G_4T_4)_4$, forms only antiparallel G-quadruplexes in the presence of Na^+ while in K^+-containing buffers a mixture of parallel and antiparallel monomolecular G-quadruplexes arise [33].

We varied the lengths of the G-tracts and loop regions to gain a better understanding of sequence factors that contribute to the propensity of *T4* to form G-quadruplexes in the presence of K^+ and Na^+. The effect of loop length on the CD spectra of ODNs is presented in Fig. 2A. *T7* appears to assume a parallel G-quadruplex in the presence of K^+ and a mixture of parallel- and anti-parallel G-quadruplexes in the presence of Na^+. However, decreasing the lengths of the loops, as in the case of *T3*, results in CD spectra similar to those observed for *T4*. There are two positive peaks around 265 and 295 nm in K^+, and a single positive peak at 295 in the presence of Na^+. The difference between the *T4* and *T3* CD spectra lies in the relative intensity of the 295 nm peak to that of the 265 nm peak in K^+. In the potassium *T4* CD spectrum, the intensity of the 265 nm is relatively higher than that of the shoulder at 295 nm. Under the same conditions, the 295 nm band of the *T3* CD spectrum is similar in intensity to the peak at 265 nm.

Table 1
Oligodeoxyribonucletides used in these experiments.

Oligo		Sequence	Length (bases)
T4	d$(T_4G_4)_4$	TTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG	32
T7	d$(T_7G_4)_4$	TTTTTTTGGGGTTTTTTTGGGGTTTTTTTGGGGTTTTTTTGGGG	44
T3	d$(T_3G_4)_4$	TTTGGGGTTTGGGGTTTGGGGTTTGGGG	28
G5	d$(T_4G_5)_4$	TTTTGGGGGTTTTGGGGGTTTTGGGGGGTTTTGGGGG	36
G3	d$(T_4G_3)_4$	TTTTGGGTTTTGGGTTTTGGGTTTTGGG	28
A4	d$(A_4G_4)_4$	AAAAGGGGAAAAGGGGAAAAGGGGAAAAGGGG	32

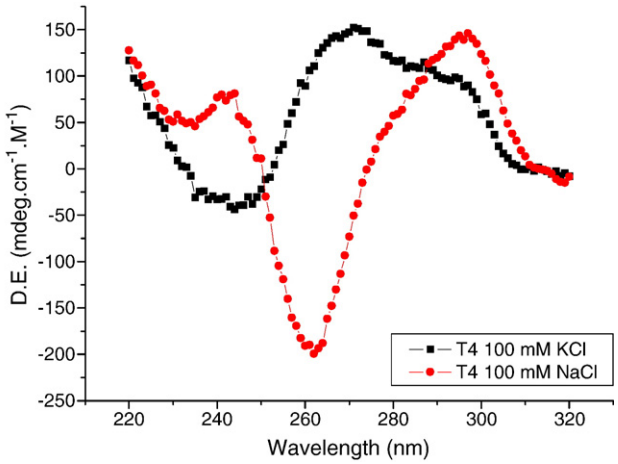

Fig. 1. CD spectra of *T4* in Tris–HCl, pH 7.5 and 100 mM KCl (black squares) or 100 mM NaCl (red diamonds). The DNA strand concentration equals 5 μM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Next, we investigated the effects of G-tract length alterations for their ability to form G-quadruplexes, Fig. 2B. The addition of one guanine residue to the four-guanine G-tracts of the *O. nova* telomeric sequences while maintaining the loop lengths, *G5*, results in CD spectra that are characteristic of parallel G-quadruplexes (positive peak at 265 nm) in K^+ and antiparallel conformations (positive peak at 295 nm) in Na^+ (Fig. 2B). In contrast, under both cation solutions, the CD spectra of *G3* are consistent with that of antiparallel G-quadruplexes. However, the intensity of the CD band at 295 nm was higher in K^+ solutions.

Fig. 3 displays autoradiagrams of native (non-denaturing) 10% polyacrylamide gels illustrating the relative mobilities of the ODNs under study in the presence of K^+ (Fig. 3A) and Na^+ (Fig. 3B). *T4* appears to form two species in K^+ with differing mobilities, while a single band is found in the presence of Na^+. The results are in accord with the information provided from the CD spectra of *T4* in K^+ that more than one species are present in solution. Moreover, the fast electrophoretic mobility exhibited by *T4* in both gels is consistent with the mobility of monomolecularly folded G-quadruplexes. *T7* appears to be composed of a single species in K^+-containing buffers as evidenced by the presence of a single resolved band on the gel (Fig. 3A), whereas two species with differing electrophoretic mobilities appear on the Na^+ gel (Fig. 3B). This observed variance of the gel mobility of the two electrophoretic bands in Na^+-containing solutions sheds light on the stoichometric difference between the two apparent species. The slower moving band indicates that the G-quadruplex formed is composed of more than one strand (most likely dimeric). On the other hand, the faster moving band could signify that the resultant structure is monomeric in nature and hence is of lower molecular weight. It appears that a reduction in the number of

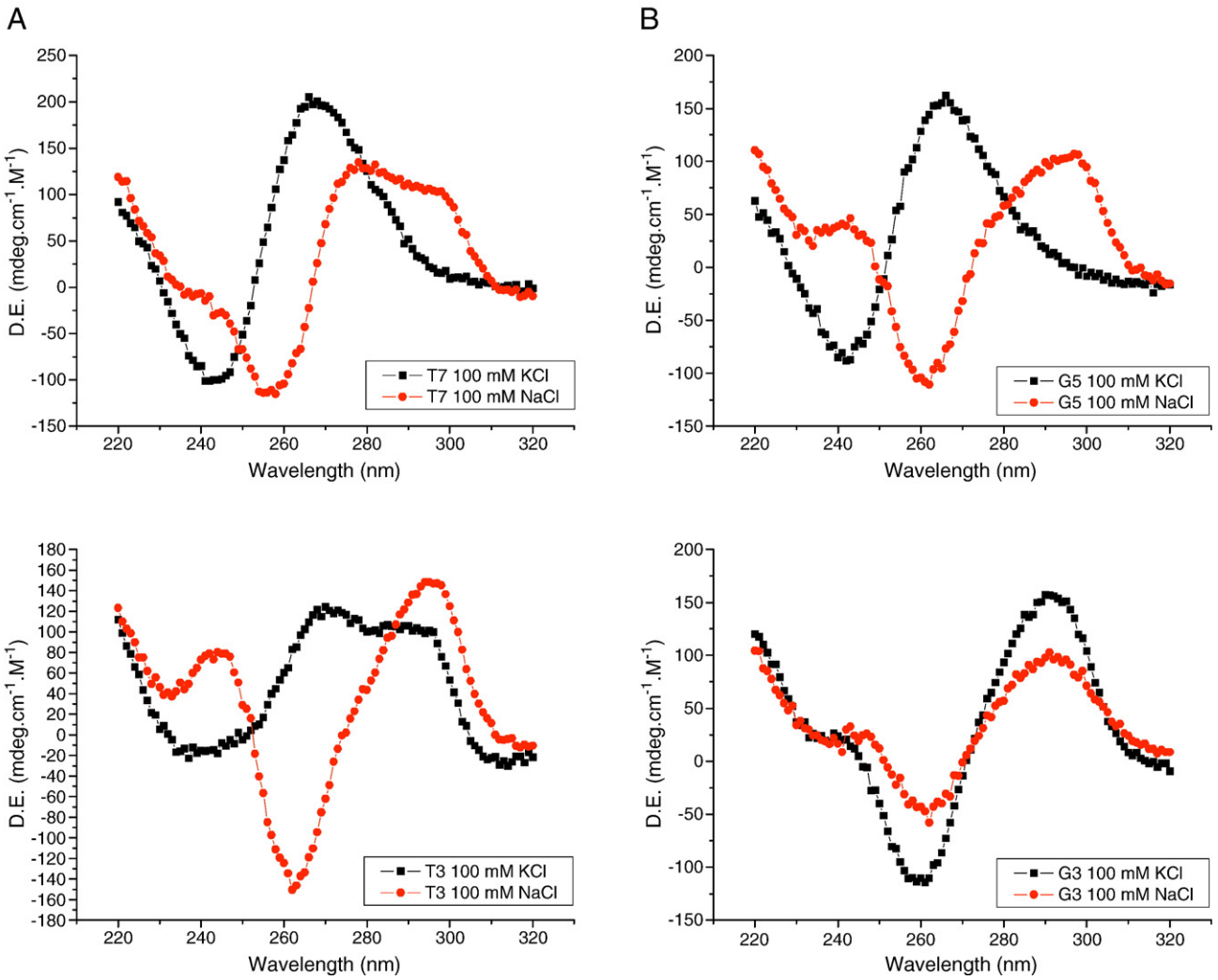

Fig. 2. CD spectra of *T7* and *T3* (A) and of *G5* and *G3* (B). The DNA was dissolved in Tris–HCl, pH 7.5, containing either 100 mM KCl (black squares) or 100 mM NaCl (red diamonds). The DNA strand concentration equals 5 μM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

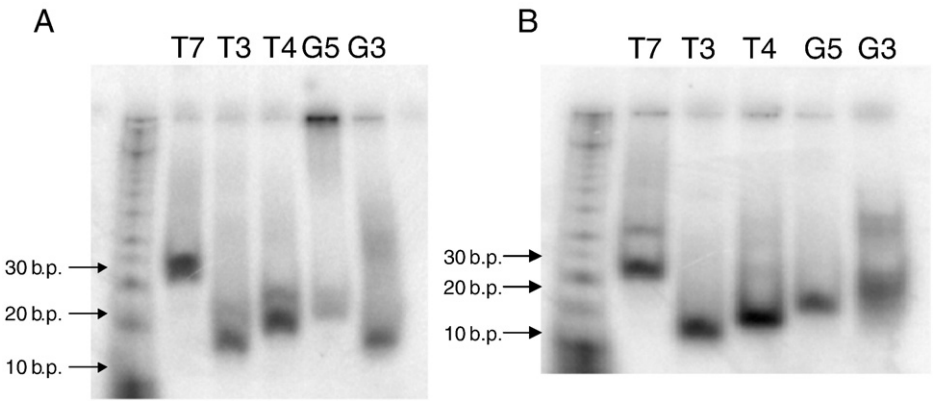

Fig. 3. Autoradiograms of ^{32}P-labelled oligonucleotides following electrophoresis on native 10% polyacrylamide gels containing 50 mM Tris–borate (pH 7.5) + 20 mM (KCl or NaCl). DNA samples were prepared in Tris 10 mM (pH 7.5) and contained either 100 mM KCl (A) or 100 mM NaCl (B).

residues that connect the G-tracts from four to three thymidines, *T3*, results in a behaviour that is comparable to *T4*. The gel containing K^+ shows two high mobility bands with the faster moving one being more intense. In contrast, only one fast moving band could be visualized in the presence of Na^+.

The ODNs with modified G-tract lengths display highly contrasting behaviours in the two cation solutions. In the presence of K^+, *G5* consistently formed highly aggregated species that exhibit little or no electrophoretic mobility with a fraction of the of the sequence forming what appears to be monomolecularly-folded G-quadruplexes (Fig. 3A). In sharp contrast, a single high-mobility band appears in gels that contain Na^+ (Fig. 3B). Shortening the G-tract from four consecutive guanine residues to three, i.e. *G3*, results in distinctive electrophoretic behaviours depending on the species of cations present. A fast moving band appears in the presence of K^+ while two bands resolve on the Na^+ gel. Both bands in the latter gel have reduced mobility in relation to the observed band in the K^+ gel. Of the two resolved bands of *G3* in the Na^+ gel, the one exhibiting slower electrophoretic migration indicates that it is a multimer G-quadruplex (possibly dimeric). The other band displaying faster electrophoretic mobility may arise from a monomolecular G-quadruplex that is less compact than the corresponding G-quadruplex in K^+ and hence it exhibits lower mobility. It is also possible that this band corresponds to the unfolded single stranded *G3*.

Lastly, we investigated whether changing the base composition of the sequences intervening between the G-tracts of *T4* would lead to the same observed polymorphic behaviour. The substitution of the thymine residues of *T4* for adenines, (*A4*), resulted in distinctive behaviour from that seen in *T4*. *A4* resolved as a single band exhibiting little to no mobility on a gel in the presence of K^+ with no evidence of the two fast-moving bands as evidenced with *T4* (Fig. 4A). However, in the presence of Na^+, a band appears that corresponds to the single-stranded species appears along with another band that exhibited little to no mobility (Fig. 4B). In the presence of K^+, the CD spectrum of *A4* provided a positive band at 265 nm without a major shoulder in the 295 nm region (Fig. 5). The CD spectrum of A4 in Na^+-containing solutions provide a low intensity positive peak around 260 nm (Fig. 5).

4. Discussion

Circular dichroism spectroscopy is a reliable method to monitor the secondary structures adopted by DNA; it is particularly useful for differentiating between anti-parallel and parallel-stranded topologies associated with G-quadruplexes [35]. A spectrum displaying a maximum at ~260 nm and a minimum at ~240 nm is indicative of a parallel-stranded conformation while that of an anti-parallel conformation has a maximum at ~295 nm and a minimum at ~260 nm [7,21,36,37]. However, one cannot assess the molecularity of the species solely on the basis of CD spectroscopy. Therefore, we employed gel electrophoresis, in tandem with CD, to obtain a more thorough understanding of structures adopted by DNA sequences under investigation.

Lee et al. demonstrated that an oligonucleotide containing four repeats of the *O. nova* telomeric repeats, $d(G_4T_4)_4$, exhibits structural polymorphism that is dependent on the type of cation present [33]. In Na^+-containing buffers, an antiparallel monomolecular G-quadruplex is present and in the presence of K^+, there appears to be a mixture of parallel and antiparallel G-quadruplexes [33]. Furthermore, they show that these monomolecular G-quadruplexes exhibit extreme thermostability, in agreement with another study [38]. We have systematically varied the length of the G-tracts and the loops to investigate the polymorphic nature of a similar four-repeat *O. nova* telomeric sequence, $d(T_4G_4)_4$ (*T4*). A sequence with three consecutive guanines, $d(T_4G_3)_4$ (*G3*), was chosen because three is reported to be the minimum number of guanine residues necessary for the formation of a stable G-quadruplex [27]. On the other hand, five or more consecutive guanine residues could possible lead to the formation of higher-order structures [39,40], hence we chose *G5* as our sequence for an increased G-tract ODN.

We have also systematically varied the length of the linker, or loop, regions. G-rich sequences with three single-nucleotide loops have been reported to be conformationally restricted to form a parallel-stranded G-quadruplex with double-chain-reversal loops [17,27]. In addition to forming monomolecular parallel G-quadruplexes, it was

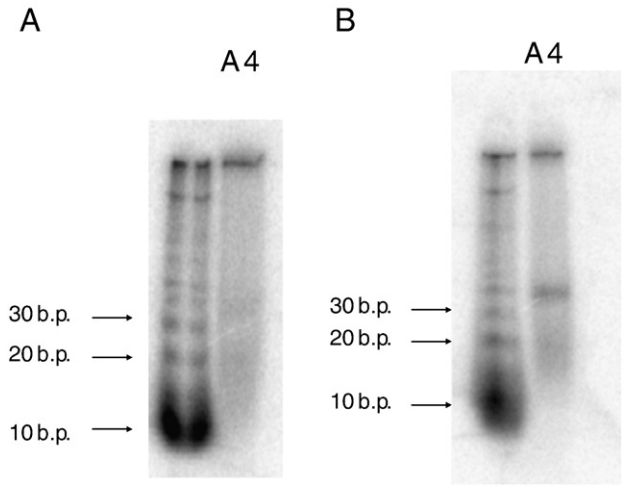

Fig. 4. Autoradiogram of ^{32}P-labelled *A4*, $d(A_4G_4)_4$, following electrophoresis on native 10% polyacrylamide gels containing 50 mM Tris-borate (pH 7.5) + 20 mM (KCl or NaCl). DNA samples were prepared in Tris 10 mM (pH 7.5) and contained either 100 mM KCl (A) or 100 mM NaCl (B).

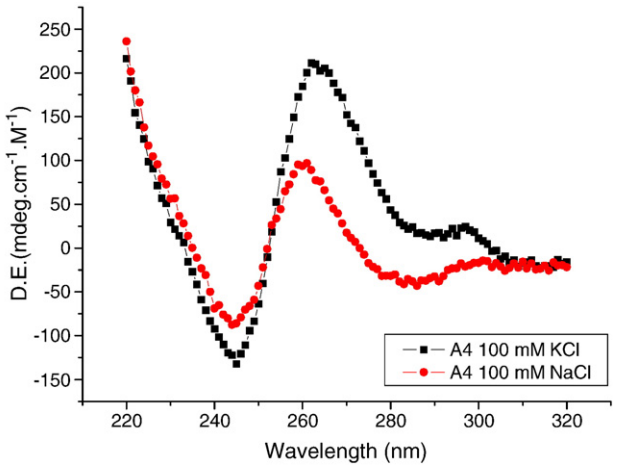

Fig. 5. CD spectra of *A4*. The DNA was dissolved in Tris–HCl, pH 7.5, containing either 100 mM KCl (black squares) or 100 mM NaCl (red diamonds). The DNA strand concentration equals 5 μM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

recently revealed that sequences comprised of short loops are prone to form higher-order structures [41]. Therefore we chose oligonucleotides with loop sequences consisting of three bases, $d(T_3G_4)_4$, and seven bases, $d(T_7G_4)_4$ [17].

We observed the behaviour of the oligonucleotides to be strongly dependent on the lengths of the G-tract and loops. We had anticipated that a reduction of the number of residues in the loops would result in the depletion of the antiparallel conformation due to the preference of parallel-stranded conformations imposed by short loops. However, *T4* and *T3* exhibited the most similar behaviour of the ODNs under study; it was evident that both ODNs adopt at least two different structures in the presence of K^+, which are presumably monomolecular G-quadruplexes (Fig. 3A). The sole difference lies in the relative intensities of the 265 and the 295-nm bands of the CD spectra. It appears that decreasing the loop length by one residue results in the increase of the antiparallel species in solution. Both bands of the CD spectrum of *T3* have equal intensities (Fig. 2A); under the same conditions, the band at 295 nm in the spectrum of *T4* is present as a low-intensity shoulder (Fig. 1). We were unable to distinguish between the species present in solutions containing K^+ by gel electrophoresis. In Na^+-containing solutions, *T3* behaved similarly to *T4* and formed what appears to be an anti-parallel monomolecular G-quadruplex. Hazel et al. showed that longer loop lengths in the general sequence $d(TG_3T_nG_3T_nG_3T_nG_3T)$, where $1 \leq n \leq 7$ favour anti-parallel quadruplexes in the presence of 100 mM KCl [17]. However, in our case, *T7* provided a CD spectrum consistent with parallel G-quadruplex in K^+ and what appears to be a mixture of parallel and anti-parallel conformation in the presence of Na^+.

Next we assessed the effect of having loops comprised of adenine bases instead of thymidines. *A4* formed species of low electrophoretic mobility which are most likely hyper-aggregated structures similar to those of G-wires [42] in K^+ (Fig. 4A). Additionally, there was no evidence for polymorphism under these conditions. In solutions containing Na^+, *A4* appeared to remain mostly as single-stranded species with some evidence of what is believed to be species similar to G-wires (Fig. 4B). Finally, the CD spectra of *A4* (Fig. 5) in the presence of K^+ appear to indicate the presence of parallel-stranded G-quadruplexes. The spectra together with the electrophoretic mobility of *A4* in K^+-containing buffers suggest the possible formation higher-order G-quadruplexes such as G-wires by *A4* in the presence of potassium ions. On the other hand, in the presence of Na^+ the intensity of the 265 nm peak is lower than that of *A4* in K^+ conditions (Fig. 5). These results are in agreement with previous studies that show that pyrimidines are favoured over purines in loop sequences [16,23].

Single nucleotide G-tract length alterations resulted in the most dramatic changes in the behaviour of the ODNs in relation to that of *T4*. An increase of one guanine in the G-tracts led to the formation of what appears to be parallel-stranded high-order aggregates in the presence of K^+ and an anti-parallel monomolecular G-quadruplex in the presence of Na^+. In contrast, a decrease in the G-tract lengths resulted in CD spectra that correspond to anti-parallel G-quadruplexes regardless of the type of cation present.

In this study, our starting point was the behaviour of *T4*, and ODN with four repeats of the telomeric sequence of *O. nova*, $d(T_4G_4)_4$; we varied the number of consecutive guanine residues as well as the number and nature of the linking residues. From our results it is evident that modifying the number of consecutive guanine residues has the most profound effect on the polymorphic behaviour exhibited by *T4*. The ODNs with three bases between the guanine residues behaved most similarly to *T4* in both Na^+- and K^+-containing solutions. On the other hand, increasing the number of bases linking the guanine regions leads to polymorphism in the presence of sodium ions but no in the presence of potassium ions. In agreement with previous studies [23,41], it appears as though the interaction with K^+ results in the formation of parallel G-quadruplexes while Na^+ displays preference for anti-parallel conformations. An exception to this rule was found with *G3*. In this case, the shortening of the G-tract resulted in consistently anti-parallel G-quadruplexes in the presence of either K^+ or Na^+. It has been reported that increasing the length of the loops in ODNs with three consecutive guanines leads to the formation of antiparallel structures even in the presence of K^+ [17]. However, the length of the G-tracts appears to override this observed effect. Therefore, based on our results, G-tract length modifications appear to have a more profound effect on the folding topology of a given sequence.

The polymorphic nature of the four-repeat of the *O. nova* telomeric sequence has been described previously by Miura et al. [32]. Using Raman spectroscopy, they showed that $d(T_4G_4)_4$ forms a monomolecular antiparallel G-quadruplex at low concentrations of NaCl and KCl. Upon increasing the concentrations of either salt, this ODN appears to form a tetra-molecular G-quadruplex. This conformational switch appears to occur at lower concentrations of KCl than NaCl indicating that KCl is more efficient at inducing this structural transformation. However, it should be noted that the DNA concentrations used in this particular study were 2 mM of strands compared to 5 μM in ours.

In summary, the data presented here will aid in the prediction of the conformational states of G-rich ODNs in solutions of K^+ and Na^+. This is important for our understanding of the interactions that stabilize these species and for predicting the structure that G-rich ODNs will adopt. In addition, it will be of use to those striving to design therapeutic agents that target different DNA conformations.

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