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G-Quadruplex Motifs Arranged in Tandem Occurring in Telomeric Repeats and the Insulin-Linked Polymorphic Region

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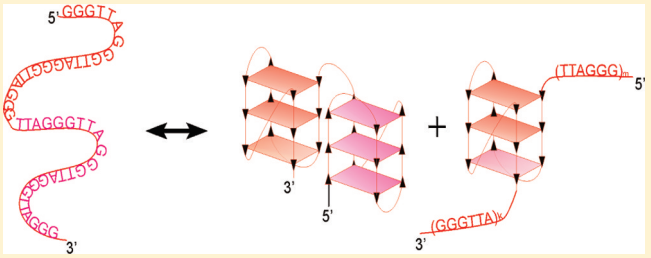
ABSTRACT: To date, various G-quadruplex structures have been reported in the human genome. There are numerous studies focusing on quadruplex-forming sequences in general, but few studies have focused on two or more quadruplexes in the same molecule, which are most commonly found in telomeric DNA and other tandem repeats, e.g., insulin-linked polymorphic region (ILPR). Although the human telomere consists of a number of repeats, higher-order G-quadruplex structures are discussed less often because of the complexity of the structures. In this study, sequences consisting of 4−12 repeats of d(G_4TGT), d(G_3T_2A), and/or d(G_4T_2A) have been studied by circular dichroism, ultraviolet spectroscopy, and temperature-gradient gel electrophoresis. These sequences serve as a model for the arrangement of quadruplexes in the telomere and ILPR in solution. Our major findings are as follows. (i) The number of G-rich repeats has a great influence on G-quadruplex stability. (ii) The evidence of quadruplex−quadruplex interaction is confirmed. (iii) For the first time, we directly observed the melting behavior of different conformers in a single experiment. Our results agree with other calorimetric and spectroscopic data and data obtained by single-molecule studies, atomic force microscopy, and mechanical unfolding by optical tweezers. We propose that the end of telomeres can be formed by only a few tandem quadruplexes (fewer than three). Our findings improve our understanding of the mechanism of G-quadruplex formation in long repeats in G-rich-regulating parts of genes and telomere ends.



Guanine-rich DNA sequences tend to form four-stranded G-quadruplex structures that are involved in a variety of biological functions; it is suggested that they may also be important causal factors in cell aging and human diseases such as cancer.[1,2] The presence of several tracts containing guanines can favor the formation of topologically various scales of G-quadruplex structures. The conformational plasticity of DNA depends on the environment (buffer, pH, temperature, etc.) and the sequence of the particular DNA molecule.[3] The formation of G-quartets and their subsequent stacking are fundamental to quadruplex formation and stability. Intramolecular quadruplexes form stable structures, especially in the presence of potassium, although small changes in the sequence can have large effects on structure and stability.[4]

Telomeres are structures at the end of chromosomes that protect chromosomal DNA from degradation and recombination.[1,2] Eukaryotic telomeres consist of tandem repeats of G-rich sequences, e.g., $(T_2AG_3)_n$ repeats in humans. Several kilobases of that sequence are paired with a complementary strand to form duplex DNA, but more than 100 nucleotides of the sequence remain unpaired and form a single-stranded overhang. The 3′-unpaired telomeric region has the ability to form quadruplex structures.[5,6] Structures of oligonucleotides containing four repeats of the 5′-TTAGGG sequence have been well established under a range of different solution conditions.[6−13] Several recent studies have shown that sequences closely related to the human telomeric repeat adopt a (3+1) topology with one double-chain reversal and two edgewise loops.[14−16]

A variable number of tandem repeats (VNTR) or minisatellite regions associated with many human diseases[17,18] often contain G-rich segments, many of which have the potential to form noncanonical DNA structures. A VNTR region of particular interest is the insulin-linked polymorphic region (ILPR) that contains G-quadruplex-forming $ACAG_4TGTG_4$ sequences located −363 bp upstream of the insulin coding sequence.[18−21]

However, a fuller understanding of VNTR biology is hampered by the lack of knowledge of the structure of the full-length DNA that is more relevant biologically. No high-resolution crystallographic and nuclear magnetic resonance (NMR) structural data are available for longer lengths of telomeric repeats and ILPR with longer lengths [$(G3T2A)_{n\geq5}$ or $ILPR_{n\geq4}$]. Longer sequences may indeed be difficult to study via standard methods, e.g., NMR or crystallography. Because of the lack of structural evidence for sequences that have the potential to form multiple quadruplexes, our current knowledge of quadruplex structures in these sequences is based mostly on simulation studies and on the predictions from the observation of single G-quadruplex-forming units.[22,23] However, the validity of these predictions has yet to be experimentally confirmed. Experimental studies conducted with multiple intramolecular quadruplex-forming sequences have been focused primarily on the telomere regions where different models have been

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postulated. No QQI in long human telomere sequences was recorded in the first models; it was suggested that telomeres form a "bead on a string" structure in which each G-quadruplex exists as a distinct unit.[24] Initial results from a novel strategy that integrates molecular dynamics simulations with experimental validation to obtain realistic structural models for higher-order telomeric DNA for $(T_2AG_3)_8$ repeats have been reported.[25] High-resolution quadruplex structures obtained for the four-repeat sequence were used to construct multimeric structures containing several quadruplex units. However, this model also points toward a different type of higher-order interaction involving loop−loop stacking between the two adjacent quadruplexes.

In this study, we analyze different quadruplex-forming sequences using circular dichroism (CD), ultraviolet (UV) absorption spectroscopy, native polyacrylamide gel electrophoresis (PAGE), and temperature-gradient gel electrophoresis (TGGE) in the presence of potassium (Table 1). The Q3 sequence represents a human repeat able to form a single intramolecular quadruplex unit; an artificial Q4 sequence contains one more guanine in a G-run, and sequences Q3-Q3, Q4-Q4, and hybrid Q3-Q4 represent sequences consisting of those mentioned above that have the potential to form two quadruplexes in tandem (Figure 1). We suggest that quadruplexes containing four guanines in a G-run melt at higher temperatures than quadruplexes containing only three guanines in a G-run; this can help to discriminate between the melting processes of quadruplexes containing three and four G-tetrads ordered in tandem. Our results show that the conformation adopted by multiple intramolecular quadruplexes cannot be automatically predicted from structures of individual quadruplex units. Furthermore, our results show the existence of a QQI in the folded structure of Q3-Q3, Q3-Q3-Q3, Q3-Q4, and ILPR4. While higher-order structures have been predicted in the ILPR,[23] this study provides experimental evidence that directly supports the predictions and observations of Schonhoft et al.[26]

MATERIALS AND METHODS

All chemical and reagents were obtained from commercial sources. The acrylamide/bisacrylamide (19:1) solution and ammonium persulfate were purchased from Bio-Rad; polyethylene glycol (PEG-200) and *N,N,N′,N′*-tetramethylethylenediamine were purchased from Fisher Slovakia. DNA oligomers were obtained from Metabion (Table 1). PAGE-purified DNA was dissolved in double-distilled water before use. Single-strand concentrations were precisely determined by measuring the absorbance (260 nm) at 95 °C using molar extinction coefficients.[27]

Circular Dichroism Spectroscopy. CD spectra were recorded on a Jasco (Easton, MD) J-810 spectropolarimeter equipped with a PTC-423L temperature controller using a quartz cell with a 1 mm optical path length in a reaction volume of 300 μL and an instrument scanning speed of 100 nm/min, with a 1 nm pitch, a 1 nm bandwidth, and a response time of 2 s, over a wavelength range of 220−320 nm. All the CD spectra are baseline-corrected for signal contributions caused by the buffer. CD spectra were recorded in units of molar circular dichroism versus wavelength. Before the CD measurement, each DNA sample was dissolved in an appropriate buffer, heated to 95 °C, and slowly cooled to the initial temperature of the CD−UV measurement. The amount of DNA oligomers

Table 1. Oligodeoxynucleotides Used in This Study

abbreviation	no. of nucleotides	ε^a (mM^{-1} cm^{-1})	5′ → 3′ sequence[b]
Q3	21	215.0	GGGTTAGGGTTAGGGTTAGGG
Q3-Q3	45	459.4	GGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGG
Q3-Q3-Q3	69	703.8	GGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGG
Q4	25	255.4	GGGGTTTAGGGGTTAGGGGTTAGGGG
Q3-Q4	49	499.8	GGGTTTAGGGTTTAGGGTTTAGGGTTTAGGGGTTTAGGGGTTTAGGGG
Q4-Q4	53	540.2	GGGGTTTAGGGGTTTAGGGGTTTAGGGGTTTAGGGGTTTAGGGGTTTAGGGG
ILPR2	28	283.9	ACAGGGGTGTGGGGACAGGGGTGTGGGG
ILPR4	55	552.3	CAGGGGTGTGGGGACAGGGGTGTGGGGACAGGGGTGTGGGGACAGGGGTGTGGGG

[a]Millimolar extinction coefficient. [b]G-Runs are underlined.

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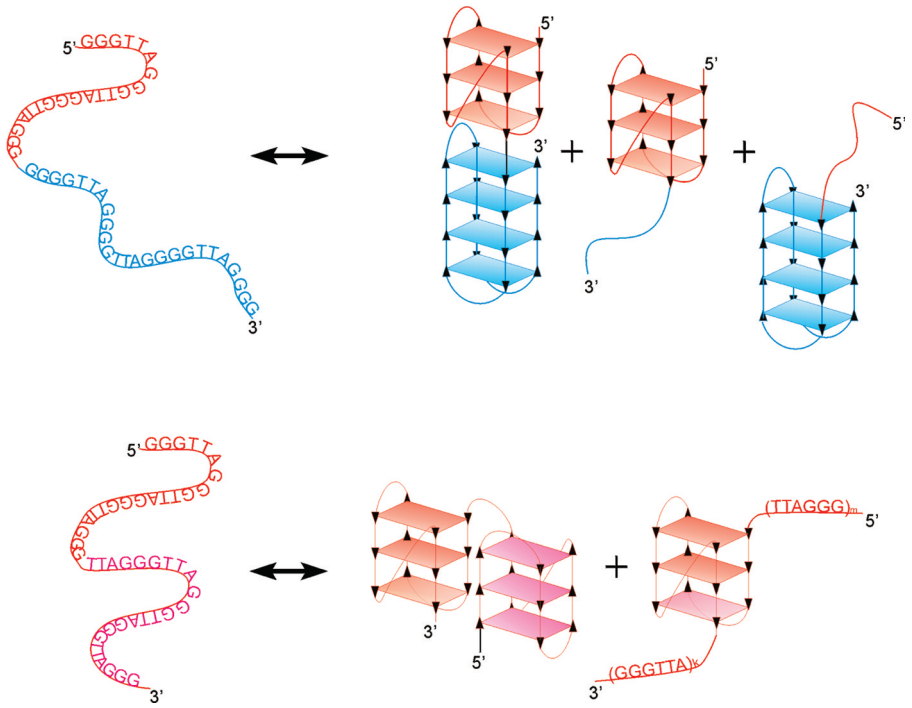

Figure 1. Schematic representation of Q3-Q4 and Q3-Q3 quadruplex folding. Q3-Q4 can fold in different manners: dual quadruplexes consisting of Q3 and Q4 in one DNA molecule, one Q3, or Q4 quadruplexes with protruding sequences. In analogy, the Q3-Q3 molecule can be folded into dual quadruplexes arranged in tandem or form only one quadruplex with different lengths of protruding sequences not associating at the G-quadruplex structure, $(G_3T_2A)_{k,m}$, where $k + m + 4 = n$.

used in the experiment was kept close to values where the absorbances reach ~0.4−0.8 at the absorption maximum of ~260 nm. Circular dichroism was expressed as the difference in the molar absorption of the right-handed and left-handed circularly polarized light ($\Delta\varepsilon$) in units of M^{-1} cm^{-1}. The molarity was related to DNA oligomers; the final spectra express the same DNA strand concentration of the oligomer that is essential for accurate CD amplitude information. CD data represent three averaged scans taken over a temperature range of 20−95 °C in the presence of potassium. The modified Britton-Robinson buffer was used in all experiments where Tris was used instead of KOH (NaOH): 25 mM H_3PO_4, 25 mM boric acid, and 25 mM acetic acid supplemented with 2.5 mM KCl or 50 mM NaCl; the pH was adjusted with Tris to a final value of 7.0.

TGGE and UV−CD Melting Curves. The CD melting profiles were recorded at 293 nm. The thermal stability of different quadruplexes was also measured by recording the UV absorbance and CD ellipticity at 293 nm as a function of temperature, by a method similar to that previously described.[27] The temperature ranged from 10 to 95 °C, and the heating rate was 0.25 °C/min. The melting temperature (T_m) was defined as the temperature of the midtransition point. T_m was estimated from the peak value of the first derivative of the fitted curve. The van't Hoff enthalpy can be used only for a two-state equilibrium in which only two species are present: fully folded and fully unfolded states. The two-state process of spectral measurements was justified by a dual-wavelength parametric test.[28] However, the declination from two-state melting behavior was 2.5−18%; therefore, the van't Hoff enthalpy does not reflect the intrinsic value.

Electrophoresis. PAGE was conducted in a temperature-controlled vertical electrophoretic apparatus (Z375039-1EA, Sigma-Aldrich, San Francisco, CA). The gel concentration was 14% (19:1 monomer:bis ratio, Applichem, Darmstadt, Germany). Approximately 2 μg was loaded onto 14 cm × 16 cm × 0.1 cm gels. Electrophoreses were run at 10 °C for 4 h at 126 V (~8 V/cm). A description of the TGGE equipment used has been published previously.[29] However, for this kind of experiment, ~10−15 μg of DNA was loaded into the electrophoretic well. In some TGGE experiments, two different oligomers were loaded separately in the same electrophoretic gel after electrophoresis had been conducted for 15 min without applying the temperature gradient. DNA oligomers were visualized with Stains-all after the electrophoresis, and the electrophoretic record was photographed with an Olympus Camedia 3000 camera.

RESULTS AND DISCUSSION

We recorded the CD spectra, PAGE data, CD−UV thermal melting profiles and TGGE melting bands of human telomeric sequences $G_3(T_2AG_3)_n$, Q3-Q4, Q4, Q4-Q4, ILPR2, and ILPR4 in Britton-Robinson buffer containing 2.5 mM K^+, where n equaled 3, 7, or 11. The presence of potassium has a significant effect on G-quadruplex stability; therefore, its concentration had to be adjusted to a range in which the quadruplexes consisting of Q4 sequences display whole pre- and postmelting regimes of the quadruplex transition obtained with TGGE and spectral methods allowing the application of curve fitting analysis.[30−32] To capture the whole quadruplex transition, only 2.5−10 mM KCl could be used, because the temperature range of the TGGE experiment is limited to a maximum of 80 °C. Nevertheless, the results obtained can be extrapolated to a higher potassium concentration in solution.[27] A potassium concentration of 2.5 mM was sufficient for the formation of quadruplexes and the capture of the entire melting

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transition for all oligomers that were used. Previously, we have performed CD−UV measurements in which the Q4 oligomer was studied at different potassium concentrations.[27] Measurements have shown that this oligomer forms an extremely stable G-quadruplex; its stability can yield a melting transition above 90 °C, depending on the potassium concentration.

CD Spectra of G3- and G4-like Oligomers. The CD spectra of quadruplexes may indicate whether they fold into a parallel conformation or an antiparallel conformation, because quartet stacking and the polarity of DNA strands are the determining factors of the intensity and shape of the CD spectrum, in particular the angle of rotation between the stacks.[33] However, the interpretation of optical properties such as hypochromicity or the shape and the sign of CD bands can be controversial.[33−35] Usually, antiparallel quadruplexes exhibit positive CD signals at ∼295 nm, with a negative signal at 260 nm, and in addition, the (3+1) conformer exhibits a shoulder at 265−270 nm.[36] In contrast, parallel G-quadruplex structures give a positive band at ∼265 nm and a negative peak at 240 nm. Unfolded oligomers do not display these spectral signatures. These spectral features are mainly attributed to specific guanine stacking in various G-quadruplex structures.[35] Figure 2a shows

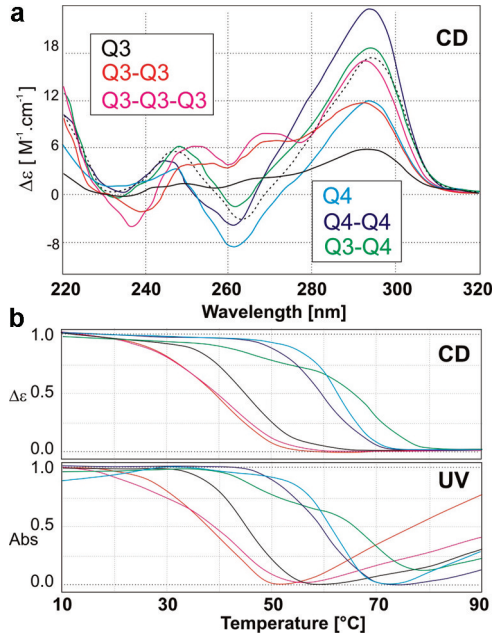

Figure 2. (a) CD spectra of Q3 (black), Q3-Q3 (red), Q3-Q3-Q3 (magenta), Q4 (blue), Q3-Q4 (green), and Q4-Q4 (navy blue) in modified 25 mM Britton-Robinson buffer (pH 7.0) in the presence of 2.5 mM KCl. The sum of the spectra for Q3 and G4 is shown as a dotted line. (b) Corresponding CD and UV melting curves obtained at 293 nm. Each melting curve represents original data that are shown in the interval between 0 and 1. With the exception of the biphasic melting curve of Q3-Q4, all curves are S-shaped. However, a two-wavelength parametric test excludes clear two-state transitions. The melting transition temperature (T_m) of the individual G-quadruplex was determined from the melting curves generated by monitoring the 293 nm feature.

the CD spectra of oligonucleotides in modified Britton-Robinson buffer containing 2.5 mM KCl. Q3, Q3-Q3, and Q3-Q3-Q3 oligomers show spectra corresponding to (3+1) arrangements of strand orientations. However, oligomers consisting of the Q4 segment show spectra that correspond to antiparallel strand arrangements of DNA. An increase in the potassium concentration to 50 mM does not significantly change the spectral profiles. The CD spectrum and first derivative of Q3-Q4 in the presence of 2.5 mM KCl also show signatures that correspond to the (3+1) conformer (not shown);[37] therefore, it is very likely that this spectrum represents a convolution of antiparallel and (3+1) spectra.

If the first quadruplex in Q3-Q4 does not have any influence on the formation of the second quadruplex in tandem, then the spectral sum of Q3 and Q4 provides the same CD spectrum and thermodynamic parameters as a hybrid Q3-Q4 sequence (dashed line in Figure 2a). One of the goals of this study is to explore this suggestion further, because the topological aspects of longer sequences that are able to form highly ordered tandem quadruplexes with more than one individual G-quadruplex structure have been studied very rarely.[22,25,26,38−41] The Q3-Q4 sequence serves as a model of the arrangement of quadruplexes in telomeres in solution.

Interestingly, we had expected that the same molar concentration of Q3-Q3 and Q3-Q3-Q3 would give a molar dichroic signal 2- and 3-fold higher than that of Q3 because Q3-Q3 and Q3-Q3-Q3 have the potential to form two and three equivalent quadruplexes per oligomer, respectively. The maximum molar circular dichroism values at 293 nm for Q3, Q3-Q3, and Q3-Q3-Q3 are ∼5, 12, and 18, respectively. These experimental values are close to values at which the formation of quadruplexes could achieve a saturated state. This is, however, a surprising result, because the Q3-Q3 oligomer, for example, can also form a structure containing only one G-quadruplex motif; the result is a weaker signal of total molar circular dichroism (see also TGGE results depicted in Figures 4 and 5). This fact can be explained as follows. (i) The connective TTA link between two G-quadruplexes contributes to a dichroic signal in this spectral area. (ii) Each Q3 oligomer is not necessarily folded into a quadruplex, but an increase of the number of G-tracts somehow facilitates quadruplex formation, e.g., Q3-Q3, Q3-Q3-Q3, etc. (iii) QQI contributes to heighten the magnitude of the CD signal perhaps because of G-quartet stacking stabilization.[26] Therefore, the CD spectral profile of Q3-Q3 and Q3-Q3-Q3 cannot differ from that of Q3 within the whole 250−300 nm region only by a multiplying factor. It means that the number of repeats influences the spectral profiles.

A simple experiment was performed to clarify the issue of QQI. The CD spectra of Q3 and Q4 were obtained individually, and immediately after this, the spectrum of their mix was obtained. The arithmetic value of Q3 and Q4 spectra perfectly fit the spectrum of the Q3/Q4 mix, but after incubation of this mix for a few hours at room temperature, the CD signal at 293 nm was ∼10% higher. This effect can be easily explained; there is a slow kinetic of G-quartet translocation in solution contributing to circular dichroism. However, this process is facilitated and the kinetic of reaction faster when dual quadruplexes can be formed in a single DNA molecule, because their relative distance and mobility in solution are significantly restricted. The same effect was observed for the ILPR4 quadruplex; here again non-zero QQI cannot be excluded. However, the QQI quantification has to be performed by other experimental routes, e.g., single-molecule methods.[26]

Another suggestion was also refuted during the study. Quadruplexes formed within Q3 and Q4 differ by approximately one G-tetrad; therefore, it would be expected that

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molar dichroism would be approximately one-third higher for Q4 than for Q3. However, experimental results do not support this suggestion, because Q3 and Q4 form topologically different G-quadruplexes; *syn*/*anti* arrangement and stacking of the G-quartet are different in antiparallel and (3+1) conformations. Molar circular dichroism is different for these G-quadrupex structures; therefore, it is incorrect to suggest that it is an arithmetic sum of quartet contributions as has been published previously.[42] Nonetheless, it seems on the basis of the CD spectral profile and their first-derivative function (not shown) that Q3 and ILPR2 form topologically equivalent quadruplexes where this calculation is allowed; the intensity of the dichroic signal differs by approximately one-third (Table 2).

Table 2. Apparent Melting Temperatures (T_m) of G-Quadruplexes in Britton-Robinson Buffer with 2.5 mM KCl[a]

		CD			
	conf.	T_m (°C)	$\Delta\varepsilon_{max}$ (M^{-1} cm^{-1})	UV T_m (°C)	TGGE T_m (°C)
Q3	H	46.1	5.2 ± 0.6	46.3	45.9
Q3-Q3	H	41.7	11.6 ± 1.6	42.4	44.4
Q3-Q3-Q3	H	41.4	17.6 ± 1.9	43.1	42.0
Q4	A	69.7	9.8 ± 1.3	69.6	69.5
Q3-Q4	H	46.4	5.3 ± 0.4	43.3	39.4
	A	68.2	11.8 ± 0.7	68.2	62.4
Q4-Q4	A	60.5	20.3 ± 1.3	61.5	61.7
ILPR2	A	66.5	5.9 ± 1.3	67.1	67.3
ILPR4	A/P	64.2	14.3 ± 1.9	66.3	64.9
ILPR2[b]	A	44.3	4.7 ± 0.4	44.0	47.3
ILPR4[b]	A	46.5	11.1 ± 0.7	46.8	46.3

[a]Thermodynamic parameters were obtained by CD−UV and by TGGE melting curves. The standard deviation of T_m is ±0.5 °C, and the ΔH_{vH} error is approximately ±5% for CD and UV and 10% for TGGE. CD and UV melting curves were obtained at 293 nm. In TGGE, only the most abundant conformers (the most intensive band) were evaluated. [b]Instead of potassium ion, 50 mM NaCl was used.

CD and UV Melting Curves. Thermodynamic parameters are obtained by van't Hoff analysis through the evaluation of CD and UV melting curves. Although the determination of ΔH_{vh} is not sufficiently precise for the correct evaluation of noncalorimetric measurements, the information obtained about the stability of the quadruplex motif in model oligonucleotides can offer important information about the formation of two tandem quadruplexes in one oligomer.

Several spectroscopic techniques can be used to monitor the formation and stability of these structures, for example, UV molecular absorption, circular dichroism (CD), or nuclear magnetic resonance. Among these, CD in the UV region can be considered the most appropriate technique because the measured instrumental response is extremely sensitive to the distance between the interacting strands, the inclination, and the distance between the bases and the axis of the structure, while the measurements can be taken at an acceptable cost. Another frequently used technique is electrophoresis. During electrophoretic separation under a nondenaturing condition, a sample of negatively charged DNA folds into various structures that can move with different mobilities.[38]

With the exception of the Q4-Q3 oligomer where a biphasic melting curve is observed, oligomers exhibit clear sigmoidal curves (Figure 2b). These melting curves were obtained at 293 nm with UV absorption and CD spectroscopy, and all results obtained by both types of measurement are summarized in Table 2. Slopes may arise from intrinsic physical phenomenon, such as the intrinsic temperature dependence of absorbance changes resulting from solvent expansion.[43]

A T_m of 64.7 °C was obtained in Britton-Robinson buffer containing 50 mM KCl with CD, as compared to a value of 46.1 °C in a buffer with 2.5 mM KCl.[27] The melting transitions of G3-G3 and G3-G3-G3 in the same buffer were 41.7 and 41.4 °C, respectively. The destabilizing effect of protruding sequences on melting temperature has been described previously.[44,45] The T_m values agree with the data previously published by our group and others.[26,27,29,38]

Similarities between the CD spectral profiles of Q3, Q3-Q3, Q3-Q3-Q3, ILPR2, and ILPR4 and the fact that melting curves are sigmoidal support the suggestion that quadruplexes formed in Q3-Q3, Q3-Q3-Q3, and ILPR4 are arranged in tandem and that they are topologically very close, and because of QQI, they melt cooperatively (Figures 2, 4, and 5). However, the Q4 oligomer forms a topologically different G-quadruplex structure, so Q4, Q3-Q4, and Q4-Q4 sequences were analyzed. Q4 quadruplexes should fold into a structure that contains one G-tetrad more than Q3, and its stability is significantly higher.[26] Because of this property, the Q3-Q4 sequence consisting of Q4 and Q3 forms two different tandem G-quadruplexes; biphasic melting transitions were recorded.

Electrophoresis. Electrophoretic separation can provide information about the molecularity of G-quadruplexes and the presence of multimeric conformers (Figure 3). Electrophoresis

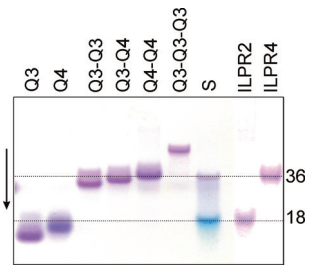

Figure 3. G-Quadruplexes resolved by gel electrophoresis and visualized by Stains-all staining. The oligomers were loaded as follows: Q3, Q3-Q3, Q3-Q3-Q3, Q4, Q3-Q4, Q4-Q4, standard (mix of $d(A)_{18}$ and $d(AC)_{18}$), ILPR2, and ILPR4. Electrophoretic separation was performed in a 14% polyacrylamide gel at 10 °C in 25 mM Britton-Robinson buffer (pH 7.0) and 2.5 mM KCl. Prior to being used, the DNA sample was heated in the same buffer for 5 min at ~98 °C and slowly cooled to room temperature within 50 min.

was performed in the presence of 2.5 mM KCl. Oligomers $d(A)_{18}$ and $d(AC)_{18}$ were used as standards. The decrease in mobilities corresponds to molecular mass as follows: Q3, Q4, Q3-Q3, Q3-Q4, Q4-Q4, and Q3-Q3-Q3. Q3 moves even faster in the presence of 2.5 mM KCl than the shorter oligomer $d(A)_{18}$, which agrees with previous studies suggesting that this oligomer folds into a G-quadruplex. Although we can clearly observe the most intense band, a slower moving smear band is also present in telomeric sequences. It could indicate the presence of alternative and/or unfolded conformers. This effect is strongly dependent on the potassium concentration, but it is not absolutely eliminated at the physiological concentration.[27,37] The electrophoretic experiment, with $G_3(T_2AG_3)_n$ (n = 1−16), was performed in 100 mM KCl.[38] The results

obtained under these conditions were generally similar to those of the previous experiment. This previous experiment showed that when the number of G-runs is not multiplied by 4 then the multimeric forms are likely to occur, mainly for 7 and 10 G-runs. However, when there are 4, 8, and 12 G-runs, no multimers are observed. In spite of these findings, we are not focusing here on oligomers containing different numbers of repeats, although these experiments were also performed under the same conditions. Interestingly, if the number of repeats reaches 5, 6, or 7, the DNA oligomer folds into a G3 quadruplex with a protruding sequence at the 3′- and/or 5′-termini (TGGE records are not shown). Once again, the average melting temperatures, obtained by TGGE, UV, and CD, for oligomers containing 5, 6, and 7 repeats are lower than for G3 oligomers: 43.9, 41.8, and 42.2 °C, respectively. These oligomers are analogous to oligomers containing a protruding sequence that are not associated with quadruplex formation. The effect of G-quadruplex destabilization with protruding sequences has been described in detail previously.[45] Digestion of $(G_3T_2A)_nG_3$, Q3-X, and X-G3 with S1 nuclease, where n equals 5−12 and X is T_7, T_{11}, $(AC)_7$, or $(AT)_7$, confirms that S1 preferentially digests a quadruplex with protruding sequence, but when n = 3, 7, or 11 (Q3, Q3-G3, and Q3-Q3-Q3), these structures were nuclease resistant (not shown). However, the nuclease reaction mix contains a higher concentration of monovalent ions stabilizing the G-quadruplex. It means the conditions for digestion and electrophoretic analyses were different.

TGGE of G-Quadruplexes. TGGE is powerful method that helps solve the problem of quadruplex polymorphism, because electrophoresis allows us to distinguish between different conformers and evaluate the most abundant conformers. TGGE offers an objective melting profile over a temperature gradient.[27] The results depicted in Figures 4 and 5 clearly demonstrate that the number of G-runs in quadruplexes is one of the determining factors in the thermal stability of intramolecular G-quadruplexes. The TGGE results elucidate and confirm many of the crucial suggestions described above. The thermal stability of intramolecular monomers in the presence of 2.5 mM potassium is as follows: Q3-Q3-Q3 ≤ Q3-Q3 < Q3 < Q3*-Q4 < Q4-Q4 < Q3-Q4* ≤ Q4 (asterisks mark which of those quadruplexes in Q3-Q4 unfold) (Figure 4 and Table 2). These results are in agreement with the spectral data. TGGE directly demonstrates that, for example, the Q3-Q4 oligomer forms structures consisting of (i) two Q3 and Q4 quadruplexes arranged in tandem, (ii) one Q3 quadruplex with a protruding sequence of Q4, and (iii) one Q4 quadruplex with a protruding sequence of Q3 (Figure 4A). Similarly, Q4-Q4 can form two Q4 quadruplexes in tandem and one with protruding sequences (Figure 4B). Finally, the Q3-Q3 oligomer forms two tandemic Q3 quadruplexes and structures containing one Q3 with various lengths of protruding sequences (their melting temperature is highlighted by double arrows in Figure 4D). However, their melting temperatures are different. In analogy, the longest Q3-Q3-Q3 oligomer can form various conformers containing one to three Q3 quadruplexes, but TGGE does not allow us to ascertain whether these oligomers form three or only two quadruplexes in tandem (Figure 4). Similar problems have been studied recently by AFM, where only two quadruplexes in tandem were detected instead of three in Q3-Q3-Q3.[46] Tang et al. showed that the most likely occurrence of G-quadruplex structure in telomere is close to the 3′-end using DMS footprinting and T4 DNA polymerase.[45]

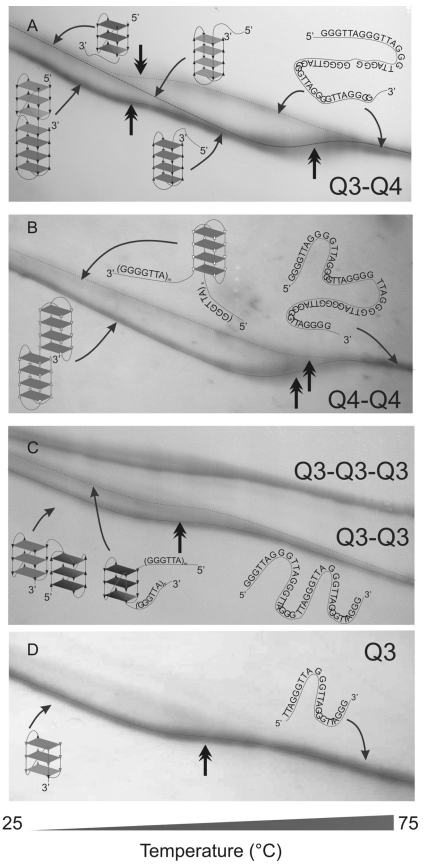

Figure 4. Representative TGGE records of DNA oligomers in Britton-Robinsson buffer in the presence of 2.5 mM KCl: (A) Q3-Q4, (B) Q4-Q4, (C) Q3-Q3 and Q3-Q3-Q3, and (D) Q3. A representative graphic interpretation is included for each electrophoretic melting curve. The vertical double arrows represent the positions of melting points.

Quadruplexes forming from oligomers at the 3′-end are more stable than quadruplexes at the 5′-end. On the basis of these results, we may suggest that the propensity to form G-quadruplexes arranged in tandem decreases in the 3′- to 5′-end direction with the number of repeats. Therefore, we propose that the end of the telomere can form only a few tandem quadruplexes, fewer than three, which some other authors call "beads on the string". G-Quadruplexes at the end of the telomere can play an important biological role; it was recently shown how these terminal structures affect reactions at the telomere end.[47]

An objective melting curve obtained from electrophoretic records by ridge tracking analysis as applied for proteins was used for this purpose.[29] T_m values are summarized in Table 2. Here again, temperatures and enthalpies of Q4, Q4-Q4, Q3-Q4, ILPR2, and ILPR4 are significantly higher than those for Q3-like sequences, which supports the suggestion that these quadruplexes are stabilized by four stacked G-quartets.[26] It is important to note that spectral measurements of any type, including microcalorimetry, do not always allow us to distinguish between conformers occurring in solution. This means that for a mixed population of conformers in any sequence, TGGE can offer more relevant results. The TGGE results clearly demonstrate that CD spectroscopy results are a convolution of several conformers of G-quadruplexes. Interest-

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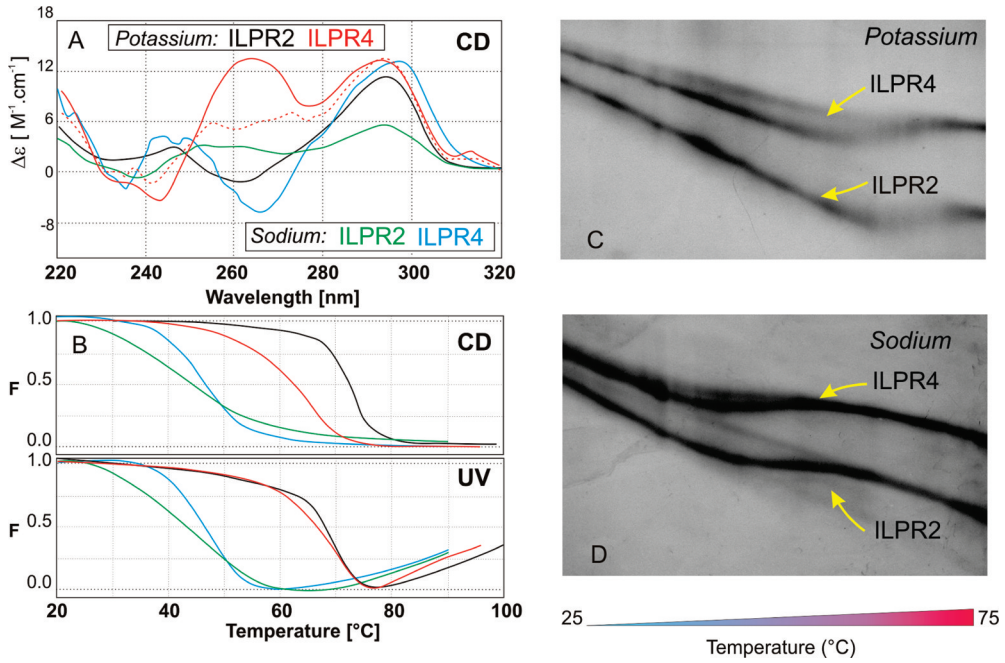

Figure 5. (A) CD spectra of ILPR2 (black) and ILPR4 (red) in modified 25 mM Britton-Robinson buffer (pH 7.0) in the presence of 2.5 mM KCl. For this panel, instead of potassium, 50 mM NaCl has been used: ILPR2 (green) and ILPR4 (blue). The DNA sample prior to use was heated in the same buffer for 5 min at ~98 °C and slowly cooled to room temperature within 50 min. The ILPR4 maximum at ~265 nm in the presence of potassium is strongly dependent on the postannealing incubation time. The spectrum obtained after incubation for 24 h at 15 °C is represented as a dotted line. (B) Corresponding CD and UV melting curves that were obtained at 293 nm. Each melting curve is shown in the interval between 0 and 1. (C and D) TGGE records of ILPR2 and ILPR4 obtained in the presence of 2.5 mM KCl (C) and 50 mM NaCl (D).

ingly, intramolecular quadruplexes after denaturation move more slowly because of strand unfolding (Figures 4 and 5).

Study of ILPR2 and ILPR4. The same analytical methods were applied for the study of ILPR2 and ILPR4 oligomers (Figure 5). CD spectra in the presence of 2.5 mM KCl are shown in panel A, CD and UV melting curves in panel B, and TGGE results in panels C and D. However, the TGGE results also show where potassium was used in place of 50 mM NaCl. The melting transitions in potassium are smeared because of the slow kinetics of transition, but in the presence of sodium, this kinetics is faster and the melting transition is less blurred. The slow kinetics during electrophoretic separation results in a number of intermediate states of DNA, which are in either imperfectly folded or unfolded states.[29] ILPR-derived oligomers confirm our conclusions about telomeric and artificial model repeats. (i) Dual quadruplexes in ILPR4 melt cooperatively at temperatures lower than those in ILPR2. (ii) The maximum circular dichroism at 295 nm for ILPR4 is a little more than double that of ILPR2; therefore, QQI is not excluded. (iii) PAGE confirms that only intramolecular G-quadruplexes are formed for both variants of ILPR. (iv) Conformers consisting of only one G-quadruplex can also occur in ILPR4.

Generally, our results agree with results published recently by other research groups. In addition, our results support the existence of QQI among quadruplexes arranged in tandem in one DNA molecule. Although the validity of this preliminary conclusion has yet to be adequately tested by other experimental routes, all experimental measurements were performed at a low ionic strength and not in "crowded" conditions, which is different from the case in cellular environments. Nevertheless, our experimental method reveals some new facts about highly ordered quadruplex structures.

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ABBREVIATIONS

PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; ILPR, insulin-linked polymorphic region; QQI, quadruplex−quadruplex interaction; TGGE, temperature-gradient gel electrophoresis; UV, ultraviolet absorption spectroscopy.

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