Stability of intramolecular quadruplexes: sequence effects in the central loop

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ABSTRACT

Hundreds of thousands of putative quadruplex sequences have been found in the human genome. It is important to understand the rules that govern the stability of these intramolecular structures. In this report, we analysed sequence effects in a 3-base-long central loop, keeping the rest of the quadruplex unchanged. A first series of 36 different sequences were compared; they correspond to the general formula GGGTTTGGGHNHGGGTTTGGG. One clear rule emerged from the comparison of all sequence motifs: the presence of an adenine at the first position of the loop was significantly detrimental to stability. In contrast, adenines have no detrimental effect when present at the second or third position of the loop. Cytosines may either have a stabilizing or destabilizing effect depending on their position. In general, the correlation between the $T_m$ or $\Delta G^*$ in sodium and potassium was weak. To determine if these sequence effects could be generalized to different quadruplexes, specific loops were tested in different sequence contexts. Analysis of 26 extra sequences confirmed the general destabilizing effect of adenine as the first base of the loop(s). Finally, analysis of some of the sequences by microcalorimetry (DSC) confirmed the differences found between the sequence motifs.

INTRODUCTION

G-quadruplexes are unusual nucleic acids structures which result from the hydrophobic stacking of several quartets; each quartet being a planar association of four guanines held together by eight hydrogen bonds (1–5). Four (or more) tracts of two or more guanines are required to form an intramolecular structure. A cation (typically Na$^+$ or K$^+$) is located between two quartets and forms cation–dipole interactions with eight guanines. Quadruplex-prone regions abound in the human genome (6–8) and the sequence repertoire of sequences compatible with quadruplex formation is far more diverse than initially imagined (9). Intramolecular G-quadruplexes may form at telomeres, oncogene promoter sequences and other biologically relevant regions (10,11). Therefore, it is important to understand the rules that govern the formation of these intramolecular structures and to determine their stabilities. Previous works support the important role played by the nature and length of the loops in quadruplex stability (12–22) and reports suggest a strong influence of loop length on quadruplex stability. However, the stability cannot simply be deduced from total loop length, as elegantly demonstrated by Kumar and Maiti (21).

To understand the contribution of loop sequence, we chose to study model sequences involving three medium loops composed of three nucleotides. This leads to a general sequence of the type GGGTTTGGHGGGGTTTGGG and gives a total of 36 (3 × 3 × 3) different sequences, which are presented in Table 1. All sequences were able to form a quadruplex. A detailed quantitative and exhaustive analysis of all sequences allowed to obtain conclusions that we also validated in different sequence contexts.

MATERIALS AND METHODS

Nomenclature, synthesis and purification of oligonucleotide sequences

Oligonucleotides were synthesized by Eurogentec (Seraing, Belgium) at the 40-, 200- or 1000-nmol scale. Several sequences were re-synthesized; inter-lot reproducibility was excellent. Concentrations were estimated using
extinction coefficients provided by the manufacturer and calculated with a nearest-neighbour model (23) as described before. Sequences are given in the 5′ to 3′ direction. H corresponds to A, C, or T while N = A, C, T or G. We chose to exclude loop sequences that create an ambiguity in G tract definition, such as GNN or NNG, as there would be two different possibilities to select three consecutive guanines in a block of 4 G.

Absorbance measurements. Melting experiments were conducted as previously described (19, 24, 25) by recording the absorbance at 240 and 295 nm (26, 27). Most sequences were tested at least twice at 5 μM strand concentration by two independent experimentators. As the \( T_m \) variations studied here are relatively modest, all samples were tested using strictly identical preparation protocols and buffers to maximize reproducibility. Despite all these precautions, the mean difference between two independent \( T_m \) determinations was 0.5°C. Hence, differences between two \( T_m \)s below 0.5°C were not considered significant. All transitions were reversible, indicating that the denaturation curves correspond to a true equilibrium process (28, 29). The intramolecular formation of the G-quadruplexes was evaluated by varying concentration in the 5–241 μM range for subset of sequences.

Thermodynamic and statistical analysis. One can extract from the raw absorbance data the fraction of folded oligonucleotide as a function of temperature, assuming linear baselines. One may then perform a van’t Hoff analysis of the melting curves using previously described procedures (30, 31). However, most \( \Delta H' \) values found were very similar and this analysis generally assumes that \( \Delta C_p = 0 \). This conventional assumption of a near-zero value for \( \Delta C_p \) has been invalidated in a number of cases (32), including for quadruplexes (also see the ‘DSC analysis’ section).

Rather than extrapolating \( \Delta G' \) values to the physiologically relevant temperature (37°C), we chose reference temperatures that are close to the average \( T_m \) values (50°C in sodium, 62°C in potassium). Hence, \( \Delta G' \) values may be directly determined from the folded fraction \( \theta \) at this temperature, \( \Delta G' = -RT \ln (K) \) without any extrapolation or hypothesis on the temperature independency of the \( \Delta H' \). As the transition is predominantly intramolecular, \( K = \theta / (1 - \theta) \), in which \( \theta \) (between 0 and 1) is deduced from the absorbance versus temperature profiles, with linear baselines assumption. These \( \Delta G'_{50°C(\text{Na}^+)} \) or \( \Delta G'_{62°C(\text{K}^+)} \) are of course less biologically relevant than an extrapolated \( \Delta G'_{37°C} \) but they are determined with a much higher confidence and fewer hypotheses. Comparison of \( T_m \) values (linear fits, Student’s t-tests) were performed with Kaleidagraph 3.6 software. Error bars correspond to standard deviation values between independent experiments or sequences.

**TDS and CD spectra.** Thermal difference spectra (TDS) were obtained by difference between the absorbance spectra from unfolded and folded oligonucleotides that were recorded much above and below their \( T_m \) (33).

Circular dichroism (CD) spectra were recorded on a JASCO-810 spectropolarimeter as previously described (19).

**Gel electrophoresis.** For some experiments, formation of G4-DNA was confirmed by non-denaturing PAGE. In that case, oligonucleotides were either \( S' \) labelled with T4 polynucleotide kinase or directly visualized by UV-shadow (see Supplementary Data). Prior to the incubation, the DNA samples were heated at 90°C for 5 min and slowly cooled (2 h) to room temperature. Samples were incubated at 50 nM or 4 μM strand concentration in Tris/HCl 10 mM pH 7.5 buffer with 100 mM NaCl or KCl. Ten percent sucrose was added just before loading. Oligothymidylate markers (dT15, dT21 or dT30) or double-stranded markers (DX10: 5’d-GCGTGACTTCGG + 5’d-CCGA TACGC; DX12: 5’d-GCGTGACTTCGG + 5’d-CCGAA GTCACGC) were also loaded on the gel. One should note that the migration of the dTn oligonucleotides does not necessarily correspond to single strands (34): these oligonucleotides were chosen here to provide an internal migration standard, not to identify intramolecular or higher-order structures.

**Differential scanning calorimetry.** Microcalorimetry experiments were carried out using a Nano DSC-II micro-calorimeter as previously described (35, 36). Buffer and oligo solutions were carefully degassed prior to their utilization and their thermal profiles were analysed in the 0 to 100°C temperature range at a scan rate of 1°C/min. A minimum of 12 scans (six heating/six cooling) was collected for each experiment (see Supplementary Data for examples of raw data). For all the scans, the oligo versus buffer scan was subtracted by the previously performed buffer scan, which allowed us to obtain the best scan shapes. Subtraction of the constructed baseline and calculation of the thermodynamic parameters were carried out using the Cp-calc software (Applied Thermodynamics). The oligonucleotides were dissolved at concentrations ranging from 200 to 241 μM in 10 mM lithium cacodylate buffer at pH 7.2 containing 100 mM KCl or NaCl.

**RESULTS**

**Evidence for quadruplex formation.**

Our objective is to compare the stability of close sequences under strictly identical conditions. All oligonucleotides (except one, see below) studied here form quadruplexes under both reference conditions (10 mM Lithium cacodylate pH 7.2 supplemented with 100 mM NaCl or KCl, abbreviated hereafter K+ or Na+ conditions). One can follow the typical evolution on temperature of the folded fraction determined at 295 nm in the presence of 100 mM KCl or NaCl (Figure 1A and B). One notices the presence of a cation-dependent conformational change associated with the temperature increase. As expected, stability of the quadruplex was always lower in NaCl and higher in KCl (Table 1). The TDS in K+ and Na+ buffers are shown in Figure 1C and D. These TDS spectra were obtained by difference between the absorbance spectra recorded above
and below the observed transition. They exhibit the typical pattern of a G-quadruplex structure with two positive maxima at 240 and 275 nm and a negative minimum around 295 nm (26,33,37). Furthermore, the CD spectra of these structures were in agreement with the formation of quadruplexes (Figure 1E and F) (38,39). Representative examples are shown in this figure. Spectra in sodium (Figure 1E) and in potassium (Figure 1F) are different, suggesting that the folding schemes for all these sequences are different in the two salts; nevertheless, CD spectra do not provide direct evidence for the folding topology of quadruplexes and this conclusion should be treated with caution. Finally, formation of a quadruplex was confirmed by the stability dependence of the structure on the nature of the monocation (Table 1).

For some sequences, concentration-independent melting temperatures confirmed that the folding process was intramolecular (Figure 1A and B). Non-denaturing gel electrophoresis allowed us to compare the behaviour in sodium and potassium. In the example provided in Figure 2B, (bottom) a single band is observed in potassium for all sequences. Its fast migration is in excellent agreement with the formation of intramolecular complexes, as judged by molecular size markers such as double-stranded sequences or oligothymidylate repeats (left lanes). Migration was not affected by the addition of unlabelled oligonucleotide, indicating that the predominant species was the same at 50 nM or 4 μM strand concentration, in full agreement with intramolecular structures. In sodium, one also observed a single major
observed in Na+ for some of the sequences with an ANN
plexes predominated in all cases, minor bands correspond-
also in agreement with intramolecular quadruplexes
band, for which concentration-independent migration was
in Na+ for some of the sequences with an ANN
cases, minor bands corresponding
to form species of higher molecularity). Interestingly, sig-
ences were found in sodium, while migration was nearly insensitive
sequence in potassium (compare top and bottom gels). Analysis of all 36 mers in sodium was also performed at
a higher strand concentration (30 μM) by UV-shadow
alysis (Figure S2); it confirmed the results with radiolabelled oligomers.

Analysis of stability
The \( T_m \)s and \( \Delta G^\circ \) of the 36 different oligonucleotides are
summarized in Table 1. For all sequences, \( T_m \) is higher in
sodium than in potassium, as for nearly all quadruplexes
published so far. In this series, the \( T_m \) difference is
moderate as the average Na\(^+\)–K\(^+\) \( T_m \) difference was 
12.7°C. The formation of quadruplex structures, whatever
is their type, is clearly enthalpy driven (Table 2). Despite a
negative contribution of entropy to stability, all quadruplex
structures studied here are stable at physiological
mperature, especially when potassium is present. One
should also note that the correlation between \( T_m \) (Figure 3A) obtained in sodium and potassium is weak
(correlation coefficient = 0.69; data not shown). This
icates that sequence dependent effects are somewhat differ-
in sodium and potassium, and that results obtained
under a given cation are weakly predictive of what can be
expected under another cation. Differences in \( T_m \) accu-
ately reflect differences in \( \Delta G^\circ \) (Figure 3B). A fair correlation
may be found between these two parameters. A 1°C difference in \( T_m \) roughly translates into a 0.13 (in Na\(^+\))
to 0.15 (in K\(^+\)) kcal/mol difference in \( \Delta G^\circ \).

DSC analysis
Three sequences were re-synthesized at a large scale
(1 μM) in order to perform DSC experiments at 200–
241-μM strand concentration. Representative examples

Table 1. Sequence of the oligonucleotide used (part 1)

| Name | Sequence 5′ = 3′ | \( T_m \) \( \Delta G^\circ \) | \( \Delta G^\circ \) |
|------|-----------------|-----------------|----------------|
| TTT  | GGTTGGTGGTTGGTTGGG | 49.3 | 64.1 | +0.02 |
| TAA  | GGTTGGTGGTTGGTTGGG | 49.9 | 65.5 | −0.07 |
| ATA  | GGTTGGTGGTTGGTTGGG | 46.3 | 62.0 | +0.52 |
| AAT  | GGTTGGTGGTTGGTTGGG | 49.8 | 61.0 | +0.71 |
| TTC  | GGTTGGTGGTTGGTTGGG | 53.9 | 64.2 | −0.4 |
| TCT  | GGTTGGTGGTTGGTTGGG | 47.9 | 63.2 | +0.31 |
| CTG  | GGTTGGTGGTTGGTTGGG | 53.4 | 63.6 | −0.46 |
| CTC  | GGTTGGTGGTTGGTTGGG | 51.8 | 62.2 | −0.21 |
| CTG  | GGTTGGTGGTTGGTTGGG | 53.6 | 64.0 | −0.56 |
| CCT  | GGTTGGTGGTTGGTTGGG | 49.5 | 61.6 | +0.2 |
| ATT  | GGTTGGTGGTTGGTTGGG | 49.8 | 61.2 | +0.65 |
| TAT  | GGTTGGTGGTTGGTTGGG | 49.9 | 64.2 | +0.02 |
| TTA  | GGTTGGTGGTTGGTTGGG | 51.3 | 64.7 | −0.18 |
| AAC  | GGTTGGTGGTTGGTTGGG | 49.3 | 60.5 | +0.06 |
| ACA  | GGTTGGTGGTTGGTTGGG | 45.1 | 60.3 | +0.62 |
| CAA  | GGTTGGTGGTTGGTTGGG | 50.1 | 63.0 | −0.02 |
| ACC  | GGTTGGTGGTTGGTTGGG | 48.6 | 58.1 | +0.27 |
| CAC  | GGTTGGTGGTTGGTTGGG | 53.6 | 65.0 | −0.49 |
| CCA  | GGTTGGTGGTTGGTTGGG | 48.4 | 63.0 | +0.20 |
| AAA  | GGTTGGTGGTTGGTTGGG | 44.8 | 60.1 | +0.74 |
| CCC  | GGTTGGTGGTTGGTTGGG | 49.8 | 61.8 | +0.04 |
| ATC  | GGTTGGTGGTTGGTTGGG | 49.7 | 60.0 | +0.1 |
| TAC  | GGTTGGTGGTTGGTTGGG | 53.3 | 63.7 | −0.38 |
| TCA  | GGTTGGTGGTTGGTTGGG | 50.0 | 63.3 | +0.09 |
| ACT  | GGTTGGTGGTTGGTTGGG | 45.9 | 59.2 | +0.65 |
| CAT  | GGTTGGTGGTTGGTTGGG | 50.8 | 63.5 | −0.09 |
| CTA  | GGTTGGTGGTTGGTTGGG | 50.5 | 63.6 | −0.02 |
| AGA  | GGTTGGTGGTTGGTTGGG | 45.5 | 60.2 | +0.56 |
| AGT  | GGTTGGTGGTTGGTTGGG | 47.3 | 61.2 | +0.39 |
| TGA  | GGTTGGTGGTTGGTTGGG | 50.4 | 64.4 | 0 |
| TGT  | GGTTGGTGGTTGGTTGGG | 52.0 | 66.0 | −0.31 |
| TCG  | GGTTGGTGGTTGGTTGGG | 54.0 | 63.5 | −0.58 |
| CGC  | GGTTGGTGGTTGGTTGGG | 54.9 | 64.5 | −0.55 |
| CGT  | GGTTGGTGGTTGGTTGGG | 53.0 | 65.5 | −0.5 |
| AGC  | GGTTGGTGGTTGGTTGGG | 50.0 | 65.5 | −0.03 |
| CGA  | GGTTGGTGGTTGGTTGGG | 49.8 | 64.3 | +0.03 |

\( ^\text{a}T_m \) in °C, with a ± 0.5°C precision; average of two to four independent values; determined from the analysis of UV melting profiles at 295 nm and/or 240 nm.

\( ^\text{b}\Delta G^\circ \) in kcal/mol, determined at 30°C in sodium, 62°C in potassium from the analysis of UV melting profiles at 295 nm. Negative values mean that the oligonucleotide is predominantly folded at this temperature.
Figure 2. Oligonucleotide migration on a non-denaturing gel. PAGE profiles for a selection of nine oligonucleotides at two different concentrations: 0.05 μM (radio-labelled only) and 4 μM. Samples were prepared in a 100 mM NaCl (A) or KCl (B) buffer and loaded on a non-denaturing 15% acrylamide gel supplemented with 20 mM of the corresponding salt and run at 20°C. Migration markers are double-stranded sequences (Dx12 and Dx 9, forming 12 and 9 bp) and ‘single-stranded’ dTn oligomers (15, 21 or 30 nt long).

Table 2. Model-dependent versus model-independent thermodynamic parameters

| Salt | Oligo (Strand) | UV-melting | DSC: average excess enthalpy/entropy | DSC: deconvolution general model, 1 transition | DSC: deconvolution general model, 1 transition |
|------|----------------|------------|-----------------------------------|-----------------------------------------------|-----------------------------------------------|
|      | μM             |            | Tm (°C)          | ΔH° (kcal mol⁻¹) | ΔS° (cal K⁻¹ mol⁻¹) | ΔH° (kcal mol⁻¹) | ΔS° (cal K⁻¹ mol⁻¹) | Tm (°C) | ΔH° (kcal mol⁻¹) | ΔCp° (cal K⁻¹ mol⁻¹) | Tm (°C) |
| Na⁺  | ACT            | 223        | 45                 | 42.0 ± 1.2       | 132 ± 3           | 30.1 ± 2.2       | 94 ± 7            | 47.3 ± 0.7 | 35.5 ± 0.7       | -0.70 ± 0.14       | 50.8 ± 0.9 |
|      | 5              | 45         | 44.1               | 139              |                   |                   |                   |                   |                   |                   |                   |
|      | TCA            | 241        | 50                 | 43.5 ± 1.8       | 135 ± 5           | 31.0 ± 2.2       | 95 ± 7            | 52.0 ± 0.8 | 39.0 ± 0.6       | -0.53 ± 0.11       | 54.5 ± 0.7 |
|      | 5              | 49         | 44.0               | 136              |                   |                   |                   |                   |                   |                   |                   |
| K⁺   | ACT            | 215        | 59                 | (44)            | (133)           | 36.4 ± 3.8       | 109 ± 11          | 62.1 ± 0.5 | 44.6 ± 1.0       | -0.79 ± 0.21       | 64.9 ± 0.5 |
|      | TCA            | 200        | 63                 | (48)            | (142)           | 39.6 ± 3.5       | 117 ± 10          | 66.7 ± 0.3 | 48.6 ± 0.8       | -0.86 ± 0.19       | 69.0 ± 0.4 |
|      | 5              | 63         | (49)               | (146)           |                   |                   |                   |                   |                   |                   |                   |
|      | CGC            | 233        | 63.5               | (43)            | (127)           | 40.1 ± 3.8       | 118 ± 11          | 67.6 ± 0.4 | 47.9 ± 1.1       | -0.86 ± 0.29       | 69.9 ± 0.6 |
|      | 5              | 63.5       | (53)               | (156)           |                   |                   |                   |                   |                   |                   |                   |

*These values (which correspond to a different series of experiments) are in fair agreement with those presented in Table 1.

**ΔH°,ΔS° and ΔCp° in KCl are provided for illustration only, as lnK versus 1/T graphs significantly deviate from linearity (see Supplementary Figure S1D for an example). Hence, linear fitting of these graphs is inappropriate.

ΔH°,ΔS° and ΔCp° are the excess heat capacity function. Average of six heating and six cooling profiles, respectively.

The general transition model directly fits the molar heat capacity Cp (and not the excess heat capacity Cp°). It is used for transitions with ΔCp ≠ 0. In this model, ΔCp(T) is fitted with a second order polynome: $ΔC_p(T) = a + bT + cT^2 = ΔC_p(T_m) + b(T−T_m) + c(T−T_m)^2$. Average of six heating and six cooling profiles, respectively.

ΔH and ΔCp at $T = T_m$. 
of DSC scans (in sodium and potassium) are shown in Figure 4 and full scans are presented in Figure S5. A comparison of the thermodynamic parameters obtained from DSC experiments with a van’t Hoff analysis derived from UV study can be found in Table 2. Calorimetry experiments confirmed the differences in stability between three sequences chosen for in depth analysis (ACT, TCA and CGC):

- a representative example of a low stability case (ACT)
- a representative example of an intermediate stability case (TCA)
- a representative example of a high stability case (CGC)

Figure 4 illustrates the DSC profiles for these oligonucleotides. The $T_m$ temperatures (maximum of the $C_p$ versus temperature plots) derived from DSC experiments were in fair agreement with the $T_m$ deduced from UV-melting experiments (Table 2). The non-linear behaviour of $\ln(K)$ versus $1/T$ Arrhenius plots for UV-melting experiments in potassium (an example is provided in Supplementary Figure S1D) prevented us from accurately determining $\Delta H$ and $\Delta S$ in KCl. Hence, a comparison of $\Delta H_{\text{cal}}$ and $\Delta H_{\text{VH}}$ (and of $\Delta S_{\text{cal}}$ and $\Delta S_{\text{VH}}$) could only be done in sodium. Model-independent $\Delta H_{\text{cal}}$ values were less negative (by 28–36%) than $\Delta H_{\text{VH}}$ for the three oligonucleotides. This less favourable enthalpy was (of course) compensated by a less negative entropy. Deconvolution of the DSC profiles with a general model for a single transition with a non-zero $C_p$ provided intermediate thermodynamic values (Table 2).

Extension to other contexts

Some rules emerged from the wealth of data collected here. Nevertheless, one may wonder if these rules apply to this sequence context only, or if they may be generalized to various quadruplex types. Quadruplexes are highly polymorphic (40–42): rules that are valid for a given loop type (lateral, diagonal, propeller) do not necessarily apply to other types. To test this hypothesis, we chose two loops starting with an adenine (ACT and ATT) and compared them with TCA and CGC. These loop sequences were tested in six other quadruplex types (Table 3) with a variable number of quartets, different positions (i.e. when present in the first, central or last loops). Finally we analysed the effect of adenines in loops of different lengths (two or four bases). As shown in this table, the ACT and ATT sequences are less stable than the TCA and CGC motifs.
in all cases (seven different sequence contexts in sodium versus six in potassium; no comparison could be made in one case due to high stability), demonstrating that the ‘No A as a first base’ rule is general. This rule also applies to loops consisting of two or four bases, as AT and ATTT loops were less stable than TA and TT TT, respectively. On the other hand, the CGC loop is often, but not always (8/11 cases) more stable than the TCA loop on the "most" side (Table 3).

DISCUSSION

In the present study, we analysed the effects of base substitutions in a 3-base-long loop for intramolecular quadruplexes. Although many oligomers adopt relatively similar conformations, the stabilities of these complexes may significantly vary. The comparison of an important number of sequences (a total of 54) in two ionic conditions allowed to draw general rules. The difference found between potassium and sodium is relatively modest for most of the sequences presented here. In comparison, this difference \( \left( T_{m(K^+)} - T_{m(Na^+)} \right) \) is in the same range (8°C) for the human telomeric motif (26) but can reach 36°C for single-base loops sequences (19). These general observations do not explain why some sequences are more sensitive than others to the nature of the cation.

We demonstrate here that overall base composition of the loops is a poor predictor of quadruplex stability. Whatever the parameter considered (total number of purines, or A, T or C in the loops), the correlation with thermal stability or \( \Delta G' \) is poor, especially in potassium (Supplementary Figures S3 and S4). Nevertheless, some sequence effects may be evidenced: adenine is significantly destabilizing over a pyrimidine base (T or C) in a sodium ionic environment (19,43). However, to our surprise, this destabilizing effect mainly applies to the ‘first’ position of the loop (Figure 5A) and was confirmed in loops consisting of 2, 3 or 4 bases. This is not observed when adenine is present at the central or last position.

Other less obvious but still significant effects may be evidenced as well. A cytosine is slightly detrimental when adenine is present at the central or last position. Other less obvious but still significant effects may be evidenced as well. A cytosine is slightly detrimental when adenine is present at the central or last position.

Table 3. Loop sequence effects in various contexts

| Name | Sequence 5' \( \rightarrow \) 3' | \( T_m^{a} \) Na+ | \( T_m^{b} \) K+ | \( \Delta G^{b} \) Na+ \( 50^\circ C \) | \( \Delta G^{b} \) K+ \( 62^\circ C \) |
|------|-----------------------------|-----------------|-----------------|-----------------|-----------------|
| TT   | GGGTATGTTGGTATGGTTATGGG    | 46.9            | 63.9            | +0.4            | -0.3            |
| AT   | GGGTATGTTGGTATGGTTATGGG    | 43.8            | 59.5            | +0.8            | +0.4            |
| TA   | GGGTATGTTGGTATGGTTATGGG    | 55.2            | 66.2            | -0.7            | -0.7            |
| TTTT | GGGTTATGTTGGTATGGTTATGGG   | 58.0            | 68.2            | -1.3            | -1.1            |
| ATTT | GGGTTATGTTGGTATGGTTATGGG   | 57.5            | 68.2            | -1.1            | -1.3            |
| TT TA| GGGTTATGTTGGTATGGTTATGGG   | 64.0            | 71.5            | -2.3            | -2.1            |
| ACT  | GGGTTATGTTGGTATGGTTATGGG   | 52.9            | 62.7            | -0.3            | -0.1            |
| TCA  | GGGTTATGTTGGTATGGTTATGGG   | 57.5            | 66.1            | -1.0            | -0.7            |
| CGC  | GGGTTATGTTGGTATGGTTATGGG   | 61.4            | 68.5            | -1.4            | -1.0            |
| L3ACT| GGGTATGTTGGTATGGTTATGGG    | 46.8            | 60.5            | +0.5            | +0.2            |
| L3ATT| GGGTATGTTGGTATGGTTATGGG    | 47.5            | 61.9            | +0.4            | 0               |
| L3TCA| GGGTATGTTGGTATGGTTATGGG    | 51.7            | 67.6            | -0.2            | -0.9            |
| L3CGC| GGGTATGTTGGTATGGTTATGGG    | 52.8            | 65.8            | -0.4            | -0.6            |
| L1ACT| GGGTATGTTGGTATGGTTATGGG    | 45.5            | 61.9            | +0.6            | 0               |
| L1ATT| GGGTATGTTGGTATGGTTATGGG    | 46.8            | 62.4            | +0.5            | -0.1            |
| L1TCA| GGGTATGTTGGTATGGTTATGGG    | 51.5            | 66.7            | -0.2            | -0.8            |
| L1CGC| GGGTATGTTGGTATGGTTATGGG    | 51.1            | 65.8            | -0.1            | -0.6            |
| X3ACT| GGGTATGTTGGTATGGTTATGGG    | 39.1            | 51.9            | +1.5            | +1.5            |
| X3TCA| GGGTATGTTGGTATGGTTATGGG    | 51.6            | 63.2            | -0.2            | -0.2            |
| X3CGC| GGGTATGTTGGTATGGTTATGGG    | _c              | _c              | _c              | _c              |
| 17ACT| GGGTATGTTGGTATGGTTATGGG    | 40.2            | >80             | +1.1            | <-3             |
| 17TCA| GGGTATGTTGGTATGGTTATGGG    | 48.1            | >80             | +0.2            | <-3             |
| 17GCC| GGGTATGTTGGTATGGTTATGGG    | 50              | >80             | -0.1            | <-3             |
| 15ACT| GGGTATGTTGGTATGGTTATGGG    | <12             | 35.4            | >3              | >3              |
| 15TCA| GGGTATGTTGGTATGGTTATGGG    | 16              | 40.0            | >3              | >3              |
| 15GTd| GGGTATGTTGGTATGGTTATGGG    | 20              | 50.2            | >3              | +1.6            |
| 15CGC| GGGTATGTTGGTATGGTTATGGG    | 26.7            | 48.6            | >3              | +1.8            |

\( ^a T_m \) in °C, with a ±0.5°C precision; determined from the analysis of UV melting profiles at 295 nm.

\( ^b \Delta G' \) in kcal/mol, determined at 50°C in sodium, 62°C in potassium from the analysis of UV melting profiles at 295 nm. In some instances, values could not be accurately determined (stability of the quadruplex is either too low or too high at this temperature) and minimal/maximal values are provided.

\( ^c \) This GC-rich sequence does not form a quadruplex.

\( ^d \) Thrombin aptamer sequence.

\( ^e \) In this sequence context, CGC is slightly less stable than TCA (in contrast with all other samples).
where $Y = C$ or $T$; $H = A$, $C$ or $T$; $W = A$ or $T$ and $D = A$, $G$ or $T$. (Note that, due to ambiguity problem, we cannot easily determine the effect of a guanine as the first or last base of a loop.)

The stability of a structure at 37°C (directly related to $\Delta G^\circ$) is obviously more useful for biological purposes than a $T_m$. Unfortunately, its determination/extrapolation is more difficult for a number of reasons (to name a few: low reproducibility, baseline artefacts, non-zero $\Delta C_p$, large uncertainty in $\Delta H^\circ$ determination and non-two-state melting behaviour). For these reasons, we propose to experimentally determine the folded fraction at a fixed temperature close to the average $T_m$ for a series of sequences, which may immediately be translated into a $\Delta G^\circ$. We simply chose reference temperatures of 50 and 62°C close to the average $T_m$ in sodium and potassium, respectively, so that one can determine $\theta$ and thus $K$ and $\Delta G$ with maximal accuracy. Differences in stabilities ($\Delta \Delta G^\circ$) may then be determined with good confidence and reproducibility. Obviously, these $\Delta \Delta G^\circ$ are determined at a non-physiological temperature; nevertheless, provided that enthalpies of the various sequences are not too different, one may expect that the conclusions still hold at 37°C. Alternatively, Figure 3B demonstrates a fair correlation between $T_m$ and $\Delta G^\circ$, suggesting that for this series of >50 sequences, a $T_m$ difference of 1°C may be translated into a $\Delta G^\circ$ difference of 0.13–0.15 kcal/mol. As the $T_m$ values in sodium span a >10°C temperature range, sequence effects may affect the $K_d$ by a factor of ≈10, showing that sequence effects play a very significant role which was underestimated before.

Our ultimate goal is to establish rules to predict the stability of intramolecular G-quadruplexes based on primary sequence. This approach is complementary to the recent work by Kumar and Maiti (21), who compared the stability of a number of biologically relevant sequences with various loop size and length (total loop length 5–18 bases). In this report, we decided to explore a limited sequence space (only 3-base-long loops are considered) but in a nearly exhaustive fashion, allowing us to evidence relatively subtle effects. Our results also demonstrate that loop composition affect quadruplex structure and thermodynamics, thus making it difficult to draw generalized correlations between loop length and thermodynamic stability (21). The destabilizing effect of adenine as the first base of the loop has not been reported before. Efforts are now being made to understand the structural basis of this destabilization. Therefore, thanks to the data accumulated by several groups, one may build a set of ‘Santa Lucia-like’ table for quadruplexes, in order to propose a predictive algorithm for G4 stability, which may eventually be incorporated in MFOLD. However, the situation will be more complex than for duplexes, as sequences effects cannot be simplified to nearest neighbour approximation. A first prediction algorithm provides a fair $T_m$ estimate in a number of situations (44). Finally, it will be interesting to check at the genome-wide level if loops starting with an adenine are under or overrepresented in G4 putative sequences. It is also striking that telomeric motifs from different species never correspond to ‘loops’ starting with an A (i.e. GGGTTA, GGGTTTA, GGGGTT, GGGGTTTT, etc.) as if the least stable quadruplex motif was selected against in telomeric motifs (Tran et al., in preparation).

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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