Parallel and Antiparallel G-DNA Structures from a Complex Telomeric Sequence†

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Received February 11, 1993; Revised Manuscript Received March 23, 1993

ABSTRACT: We investigated the formation *in vitro* of higher order structures by a DNA oligomer containing the terminal motif $TGTG_3TGTGTGTG_3$, derived from the *Saccharomyces* telomeric consensus, in order to (a) understand why certain cations favor the formation of parallel-stranded (G4 and G8) G-DNA structures, while others favor foldback, antiparallel structures (G′2) and (b) probe the structures of G-DNAs formed by this telomeric sequence, which is more complex than its well-studied counterparts from the protozoans oxytricha and tetrahymena. We find that dramatic switches in the formation of G4 versus G′2 structures occur in solutions of not only the group Ia cations, Li^+–Cs^+, but also in those of the group IIa cations, Mg^{2+}–Ba^{2+}. These data and the temperature-dependent formation and destruction of the different structures lend support to the kinetic scheme of Sen and Gilbert (1990), by which rapidly forming G′2 structures accumulate in highly stabilizing potassium (and strontium) solutions at the expense of the thermodynamically more stable G4 structures. Both the G4 and the G′2 complexes formed by the *Saccharomyces* sequence show novel structural features. Protection and interference experiments with dimethyl sulfate and potassium permanganate reveal that the core of alternating thymines and guanines within the telomeric motif plays a critical role in the stabilization of the parallel G4 structure, but not of the antiparallel G′2. Very likely, in the G4 complex, this GT core forms a novel higher order arrangement of alternating G and T quartets, the latter possibly comparable to the U quartets described by Cheong and Moore (1992) in their NMR study of the higher order structure formed by rUG_4U.

Single-stranded DNA or RNA molecules that contain one or more guanine motifs (i.e., stretches of contiguous guanines) are able to form a family of higher order structures, termed G-DNA and G-RNA (Cech, 1988). These structures contain noncanonical guanine–guanine base pairs (Henderson et al., 1987) or base quartets, the latter first shown to exist in higher order structures formed by guanosine, GMP, polyG, and polyI (Gellert et al., 1962; Arnott et al., 1974; Zimmerman et al., 1975).

Recently, a large number of reports have described the structure and properties of G-DNA complexes [reviewed by Guschlbauer et al. (1990), Sundquist (1991), and Sen and Gilbert (1991)]. Much of the interest has focused on G-DNAs formed by the repetitive, guanine- and thymine-rich DNA sequences from chromosomal telomeres (Blackburn & Szostak, 1984; Blackburn, 1986), whose termini are single-stranded in living organisms (Klobutcher et al., 1981; Henderson & Blackburn, 1989; Zakian, 1989). Telomeric oligomers used in various *in vitro* experiments can be classified as one-repeat, two-repeat, or four-repeat, depending on how many repeat units of the basic G- and T-rich telomeric sequence that it contains. The G-DNA complexes that are formed by two-repeat sequences [such as $(T_2G_4)_2$] can in turn be classified into two general groups: ones in which four DNA (or RNA) strands hydrogen bond to each other in a parallel orientation (G4-DNA or -RNA) (Sen & Gilbert, 1988, 1990, 1992a,b; Smith et al., 1989; Kim et al., 1991; Hardin et al., 1992; Murchie & Lilley, 1992; Wang & Patel, 1992; Cheong & Moore, 1992; Chen, 1992; Aboul-ela et al., 1992; Guo et al., 1992a,b; Lu et al., 1992) or where two hairpin-shaped strands bond in antiparallel orientations (G′2-DNA) (Oka & Thomas, 1987; Williamson et al., 1989; Sundquist & Klug, 1989; Panyutin et al., 1989, 1990; Sen & Gilbert, 1990; Jin et al., 1990; Hardin et al., 1991; Raghuraman & Cech, 1990; Wang et al., 1991a,b; Kang et al., 1992; Smith & Feigon, 1992; Petracek & Berman, 1992). In addition to these, an entirely novel kind of parallel G-DNA, the "crown" structure, which involves novel T–G interactions, has been proposed for some of the G′2 complexes formed by two-repeat oxytricha telomere sequences (Acevedo et al., 1991). Parallel-stranded G-DNA units can also form superstructures containing eight, twelve, sixteen, or more individual strands (Sen & Gilbert, 1992a; Lu et al., 1992). The smallest and most representative member of this class is a complex composed of eight strands, called G8-DNA.

A number of studies have shown that any given telomeric oligomer is capable of forming both G4 and G′2 complexes *in vitro* and that the formation of the one versus the other structure is dependent on the conditions of incubation of the DNA. Sen and Gilbert (1990) described a sodium–potassium switch for the formation of these two complexes by oligomers containing two-repeat tetrahymena $[(T_2G_4)_2]$ and oxytricha $[(T_4G_4)_2]$ telomeric sequences, by which exclusively G′2-DNA formed in the presence of potassium whereas both G4-DNA and G′2 DNA formed in the presence of sodium. On the basis of their results, the authors proposed a kinetic scheme for the formation of G-DNA complexes from two-repeat telomeres (Figure 1), whereby potassium incubations led to a kinetic trapping of the fast-forming G′2 structures, even though these were thermodynamically less stable than the G4 species (two or three guanine quartets in G′2 versus eight in G4). In the less stabilizing sodium solutions, however, the thermodynamically favored G4 was the predominant end product.

While the remarkably varied effects of individual group Ia cations on G-DNA formation have been examined in some depth (Miles & Frazier, 1972; Howard & Miles, 1982a,b;

† This work was supported by the National Science and Engineering Research Council of Canada. D.S. is a Research Scholar of the British Columbia Health Research Foundation.

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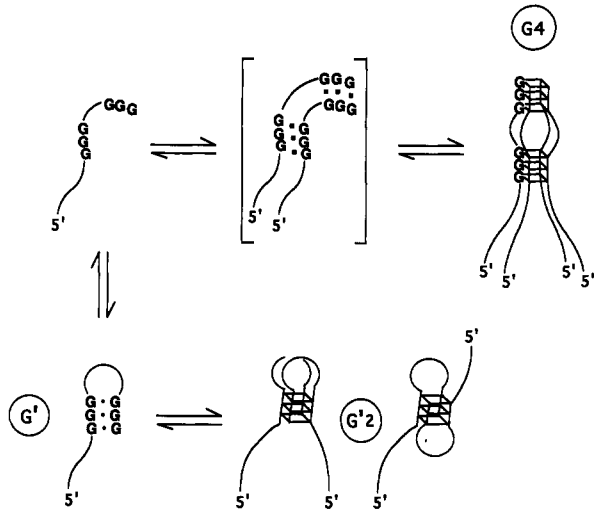


FIGURE 1: Pathways for the formation of different G-DNA complexes.

Oka & Thomas, 1987; Williamson et al., 1989; Sundquist & Klug, 1989; Sen & Gilbert, 1990), the role of divalent cations has been explored to a relatively limited extent. Chantot and Guschlbauer (1969) found that both K^+ and Sr^{2+} stabilized gel formation by 8-bromoguanosine. Lee (1987), working with poly(deoxynucleotides) of the form of $(GA)_n$, $(GAA)_n$, and $(GGA)_n$, showed that G-DNA formation was favored by Mg^{2+} and Ca^{2+}, as well as by Ba^{2+}; Sen and Gilbert (1992b) found that 10 mM of either magnesium and calcium favored the formation of G4-DNA at the expense of G'2 from two-repeat tetrahymena and oxytricha telomeric sequences. Hardin et al. (1992) found that a nonameric oligonucleotide, containing the central motif G_3, formed G-DNA in the presence of Mg^{2+} or Ca^{2+}, the calcium complex being more stable than the magnesium; most recently, Chen (1992) has shown that the Sr^{2+} ion, having an ionic radius (1.3 Å) similar to that of the K^+ ion, is very efficient in promoting the formation and stabilization of G-DNA, particularly the intermolecular complexes formed by the four-repeat telomeric oligomers $(T_2G_4)_4$ and $(T_4G_4)_4$. In this study, we analyze the relative influence of individual cations of the Mg^{2+}–Ba^{2+} series on G-DNA formation, in terms of (a) whether a given ion, at a 10 mM concentration, promotes G-DNA formation at all and (b) whether individual ions preferentially form either the G'2, the G4, or both products, and if indeed such a selectivity exists, how it might be reconciled with Sen and Gilbert's view of the formation of the different G-DNA complexes.

Unlike the regularly repeating telomeric sequences from tetrahymena and oxytricha, telomeres from the yeast *Saccharomyces* show an irregular repeating unit, $[(TG)_{1-6}TG_{2-3}]_n$, first described by Shampay et al. (1984) and Wang and Zakian (1990a). Wellinger et al. (1993) have recently shown that the single-stranded telomeric tails of this sequence on a linear plasmid from *Saccharomyces* are at least 32 bases long. In ciliated protozoans such as oxytricha and tetrahymena, and also in HeLa cells, a ribonucleoprotein complex known as telomerase extends the G-rich telomeric strand in the absence of a template [reviewed in Blackburn (1992)]. Telomerase has not yet been detected in yeast cells; Wang and Zakian (1990b) have proposed that telomere–telomere recombinations, which are known to occur in *Saccharomyces* (Pluta & Zakian, 1989), may play a role in telomere replication and that G-DNA structures may be involved in the recombination process. Recently, evidence of higher order structure (presumably G-DNA) formation *in vitro* by a linear plasmid from *Saccharomyces* has been provided by Wellinger et al. (1993).

The two-repeat *Saccharomyces* telomeric sequence, $TG\text{-}TG_3TGTGTGTG_3$, that we use in this study is more complex than the comparable two-repeat oxytricha and tetrahymena sequences, $(T_4G_4)_2$ and $(T_2G_4)_2$, respectively, which have been the subject of a large number of recent investigations. Oligomers containing these latter motifs form both the G4 and G'2 types of complexes (Sundquist & Klug, 1989; Sen & Gilbert, 1990; Acevedo et al., 1991). A number of G-DNA structures solved recently at high resolution by X-ray crystallography and NMR (Kang et al., 1992; Smith & Feigon, 1992; Wang & Patel, 1992; Aboul-ela et al., 1992) have been derived from the above motifs. However, the propensity of irregularly repeating telomeric sequences, such as from *Saccharomyces*, to form the different G-DNA structures has not been investigated in any detail.

The sequence $TGTG_3TGTGTGTG_3$ can be viewed as containing two distinct elements: two blocks of three contiguous guanines each and regions of alternating thymines and guanines. To date, stable G-DNA structures have been reported with as little as three contiguous guanines (Sen & Gilbert, 1992a) or multiple copies of two contiguous guanines (Jin et al., 1990); it was therefore interesting to determine whether the guanines in the alternating G and T regions of the *Saccharomyces* sequence participated in quartet formation. The conformations of the internal thymines, too, were of interest, particularly in the parallel-stranded G4 complexes. To date, the disposition of non-guanine bases within G-DNA regions has not been probed in detail. An NMR study by Kim and Moore (1992) on G4-RNA formation by ribo-UGGGGU concluded that one set of the terminal uracils hydrogen bonded with each other to form a uridine quartet; Lu et al. (1992) found a stabilizing effect of the adenines within the vertebrate telomere motif $(T_2AG_3)_n$. In this article, we describe the role of the seven-base GT alternating sequence on the formation of the different G-DNA structures.

MATERIALS AND METHODS

DNA. DNA oligomers were synthesized on an ABI 392-08 automated DNA synthesizer, using standard cyanoethyl phosphoramidite chemistry. The oligomers used for these experiments were Sacc1, ACTGTCGTACTTGATAT-GTGGGTGTGTGTGGG, the underlined portion indicating the sequence derived from the *Saccharomyces* telomere consensus, and Sacc2, ATCAAGTACGACAGT, which is complementary to the 5', nontelomeric half of the Sacc1 oligomer. Deprotected oligomers were purified on preparative polyacrylamide gels, followed by reversed-phase C18 Spice columns (Rainin), and dissolved in 10 mM Tris (pH 8.0) and 0.1 mM EDTA (TE buffer) to give stock solutions containing 5–28 μg/μL DNA. Approximately 1 μg of each DNA oligomer was kinased with [γ-^{32}P]ATP and maintained in stocks in TE buffer of 10–15 μL.

Higher Order Structure Formation. 5'-^{32}P-Labeled oligomers in TE solution were denatured by heating for 3–5 min at 90 °C and then chilling on ice. An equal volume of TE + 2 M XCl (where X = Li, Na, K, Rb, or Cs) or TE + 20 mM YCl_2 (where Y = Mg, Ca, Sr, or Ba), as appropriate, was then added to give final solutions of 2–4 μg/μL (0.2–0.4 mM) of DNA in TE + 1 M XCl or TE + 10 mM YCl_2. Samples (typically 2–5 μL) were then sealed in glass capillaries and incubated at 37 C° for 24–72 h. Every 1 μL of recovered sample was diluted with 3 μL of loading buffer (TE + 13 mM KCl + 3.3% glycerol and loading dyes) prior to loading on

nondenaturing gels, which were run in 50 mM TBE + 10 mM KCl at 4 °C.

Complementary Strand Addition for G-DNA Strand Stoichiometry. Sacc1 samples were incubated as above, except that unlabeled complementary strand Sacc2 at molar ratios from 0 to 2.0 was added to the Sacc1 prior to incubation in salt.

Potassium–G4 Complexes and Heat Treatment of G-DNA. G4- and G8-DNA obtained from a standard sodium incubation (vide infra) of Sacc1 were precipitated twice from TE + 300 mM potassium acetate. The final pellet was rinsed with 70% ethanol and dissolved in TE + 1 M KCl, and this was referred to as the Na/K incubation sample. Three 1-μL samples of each of the Na, Na/K, and K incubation samples were incubated in TE + 1 M NaCl or KCl at 22, 37, and 60 °C, respectively, for 5 min prior to cooling on ice and analysis on nondenaturing gels. Methylation protection of samples at the different temperatures was carried out by adding 0.05% (22 °C), 0.0125% (37 °C), and 0.00313% (60 °C) DMS, incubating and treating as below.

Methylation Protection. Three microliters of the incubated oligomer solutions of either Sacc1 or mixtures of Sacc1 and Sacc2 from above were diluted with 50 mM sodium cacodylate, pH 7.0, containing freshly dissolved dimethyl sulfate (DMS) to a final total volume of 11 μL and DMS concentration of 0.05%. The mixtures were incubated at 22 °C for 30 min, ethanol precipitated, and redissolved in 5 μL of TE + 10 mM KCl buffer prior to loading in the gel. The preparative gels were run in 50 mM TBE + 10 mM KCl at 4 °C. Several thin slices of bands corresponding to monomer and the various higher order structures were cut out. For example, three or four separate thin slices of the G′2 complexes were recovered, with the notion of examining possible structural heterogeneity within the G′2 species as a function of slight differences in gel mobility. The DNA from the various complexes was eluted into TE, purified, cleaved with 8% piperidine at 90 °C, and analyzed in 10% denaturing gels run in 50 mM TBE buffer.

Methylation Interference. Samples of labeled oligomers in TE buffer were heated at 95 °C to denature them and then dissolved in 25 μL of 50 mM sodium or potassium cacodylate solution, pH 7.0, containing 0.05% DMS. After incubation at 22 °C for 30 min, the DNA was recovered by ethanol precipitation and used for the saline incubations, as above, to generate higher order structures. As with the methylation-protection experiments, the monomer, G′2, and G4 bands were each recovered in three or more successive thin slices of gel each to probe for structural heterogeneity within these slices. Recovered DNA was treated and analyzed as in the methylation-protection experiments.

Permanganate Reaction. One microliter of each salt-incubated oligomer solution was diluted with 10 μL of TE buffer. Four microliters of a freshly made up solution of 0.254 mM $KMnO_4$ in TE was then added and the mixture incubated at 22 °C for 7 min. Reactions were terminated by the addition of 1 μL of allyl alcohol; complexes were purified, treated, and analyzed as in the methylation-protection experiments.

RESULTS

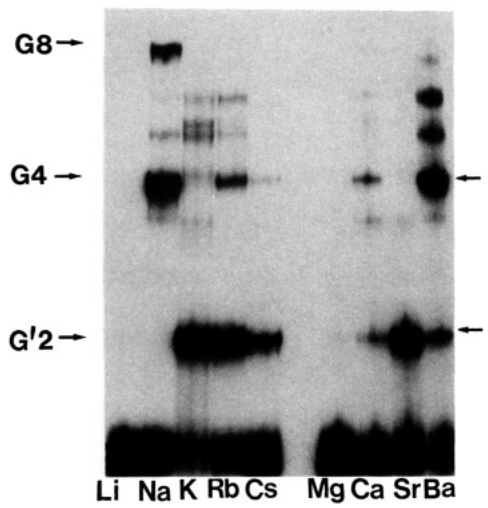
The Roles of Monovalent and Divalent Cations in G-DNA Formation. Figure 2A shows the formation of different G-DNA species when the oligomer Sacc1 was incubated at 22 °C in either TE + 1 M XCl (where X is Li, Na, K, Rb, or Cs) or in TE + 10 mM YCl_2 (where Y is Mg, Ca, Sr, or Ba). The monovalent cation data are in general agreement with previous data on G-DNA formation by oligomers corresponding to tetrahymena and oxytricha telomeres (Sundquist & Klug, 1989; Williamson et al., 1989; Sen & Gilbert, 1990). There is agreement, for example, in the finding that lithium does not support the formation of G-DNA structures at all, while cesium produces mainly G′2. Production of a certain amount of G4, too, is seen in the cesium incubation of Sacc1, in contrast with results from tetrahymena and oxytricha telomeres (Sen & Gilbert, 1990). The difference, however, may be attributed to the presence of a stabilizing 10 mM KCl in the gel shown in Figure 2A, whereas in the earlier report there was none.

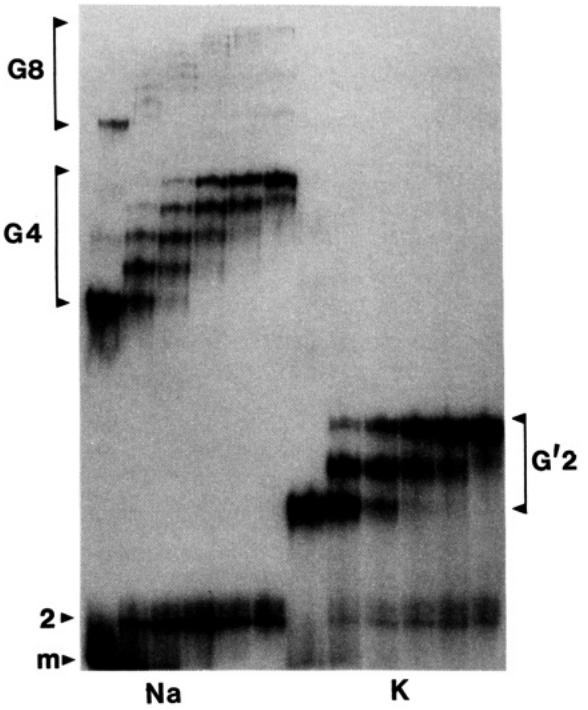
The sodium–potassium switch observed with Sacc1 (Figure 2A,B) is a sharp, all-or-nothing phenomenon, with the sodium ion giving rise exclusively to parallel-stranded G4 and G8 complexes and potassium exclusively giving the antiparallel G′2. In this respect, this oligomer behaves differently from the oxytricha and tetrahymena oligomers, which formed abundant G′2 as well as G4 in sodium solutions. With Sacc1, however, we were able, in a few isolated incubations, to obtain trace amounts of the disallowed G-DNA complex, such as G4 from potassium and G′2 from sodium, and used these for methylation-interference experiments (Figure 5, lane vii).

Incubation of Sacc1 in the presence of 10 mM YCl_2, where Y represents the series Mg–Ba, showed results surprisingly analogous to those from the alkali chloride (XCl) series, although there were some notable differences between the two (Figure 2A). As with the monovalent cations, an individual divalent cation preferentially assisted the formation of a single G-DNA species—either the G′2 or the G4 complex. Magnesium, at the 10 mM ionic concentration used, did not support the formation of G-DNA from Sacc1, whereas the oxytricha and tetrahymena oligomers, with their sets of four contiguous guanines, did form complexes (Sen & Gilbert, 1992b). With Sacc1, calcium formed more G′2 than G4, strontium formed G′2 exclusively, and barium favored G4. None of the divalent ions gave rise to a true G8 complex, a major product of the sodium incubation (Figure 2A,B), although barium did give rise to complexes intermediate in gel mobility between the G4 and the G8.

The question of the involvement of the nontelomeric half of the oligomer Sacc1 in G-DNA structure formation was investigated. Sacc1 was incubated in the different salt solutions in the presence of slightly greater than 1 molar equiv of the oligomer Sacc2, which is complementary to the nontelomeric portion of Sacc1. The results (data not shown) indicated a one-to-one correspondence between the pattern of product formation by the two oligomers together and by Sacc1 alone. It is therefore likely that the nontelomeric portion of Sacc1 plays no significant part in G-DNA formation.

Strand Stoichiometry in G-DNA Complexes. To confirm that the complexes formed by Sacc1 were indeed G′2 (a strand dimer), G4 (a strand tetramer), and G8 (a strand octamer), a fixed concentration of Sacc1 was incubated in the presence of varying molar ratios of the complementary strand, Sacc2. Sen and Gilbert (1988) showed this to be an effective strategy for determining the number of independent strands in any given complex; for example, a G4 complex should bind either zero, one, two, three, or four complementary strands, and each of these species should resolve on a nondenaturing gel. Thus a total of $n + 1$ product bands would be formed by a given complex that was an n-mer. The results of complementary strand binding to the different G-DNA complexes of Sacc1 are shown in Figure 2B. Each set of lanes, for sodium and for potassium complexes, shows, from left to right,





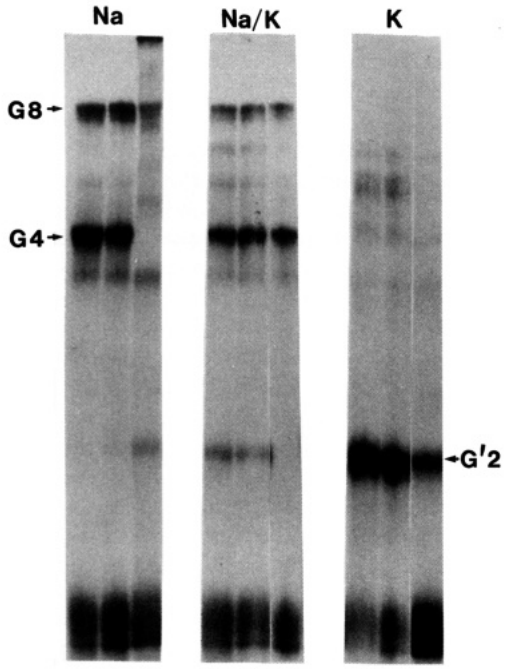


FIGURE 2: (A, top left) Influence of group Ia and IIa cations on G-DNA formation. (B, top right) Complementary strand binding by Sacc1 G-DNA complexes. Complexes formed by Sacc1 in the presence of 0–2 molar equiv of Sacc2 for a sodium incubation (lanes 1–5) and a potassium incubation (lanes 6–10). m and 2 refer to the monomeric Sacc1 and to the Waston–Crick Sacc1–Sacc2 duplex. (C, bottom left) Effect of temperature on the distribution of G-DNA complexes. Panels Na and K show G-DNA formed in 1 M NaCl and KCl, respectively, treated, from left to right, at 22, 37, and 60 °C. The panel Na/K shows a similar treatment of G-DNA formed in 1 M NaCl but heated in 1 M KCl.

progressively greater binding of the complementary strands to the G-DNA complexes. The results indicate three total bands for G'2, five for G4, and nine for G8, confirming that the G'2 formed by Sacc1 is a strand dimer, G4 a tetramer, and G8 an octamer. This is the first demonstration of the formation of a G8 complex by a two-repeat oligomeric sequence. Methylation protection results described below indicate that this G8 complex has a different structure from that obtained from single-repeat oligomers (Sen & Gilbert, 1992b; Lu et al., 1992).

Effect of Ionic Strength. Most of the DNA incubations carried out in this study were done in 1 M salt to accelerate the formation of G-DNA complexes. However, incubation with as little as 125 mM KCl showed the formation of G'2 complexes, whereas G4 was seen at incubation points between 125 and 250 mM NaCl (data not shown).

Effect of Temperature on the Relative Distribution of Complexes. The standard incubation conditions used for generating G-DNA complexes were 1 M salt and 22 °C. Under these conditions, the potassium ion generated the G'2 complex exclusively. We attempted, however, to obtain the kinetically forbidden G4 complex in a potassium solution in order to test its structure and stability in potassium relative to sodium. Borzo et al. (1980) and Detellier and Laszlo (1980) had done

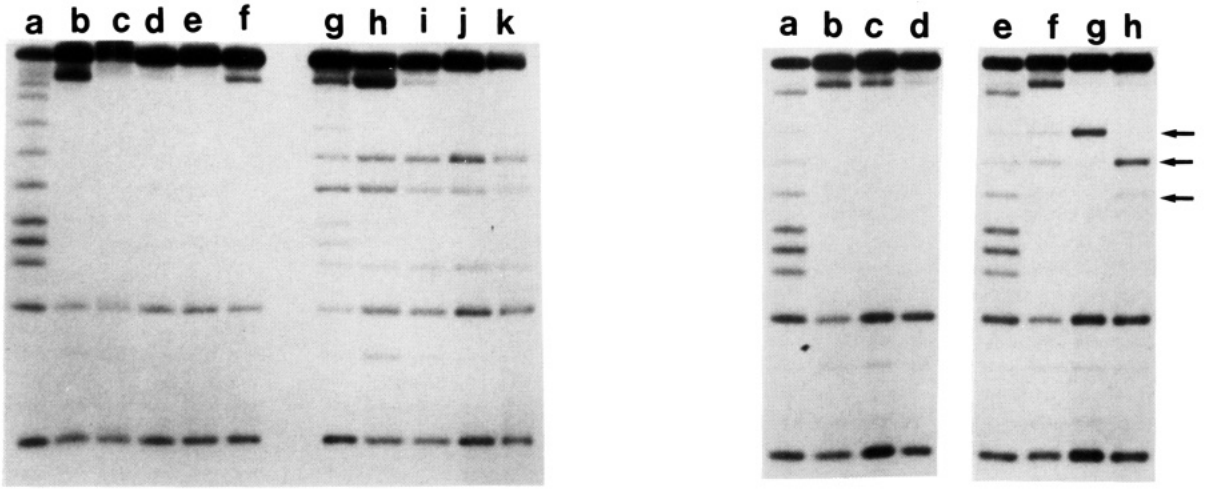


FIGURE 3: (A, left) DMS–methylation protection of G-DNA guanines from incubation in sodium (lanes a–f) and potassium (lanes g–k): (a) monomer; (b–e) G4-DNA; (f) G8-DNA; (g) monomer; (h–k) G′2-DNA. (B, right) DMS–methylation protection of G-DNA guanines from incubation in calcium (lanes a–d) and strontium (lanes e–h): (a) monomer; (b–d) G4-DNA; (e) monomer; (f–h) G′2-DNA. The arrows indicate the positions of the three guanines within the TGTGTGT motif.

elegant ^{23}Na NMR studies on the formation of G-quartet structures by 5′-GMP and estimated the correlation time of sodium ions bound in the interior of the quartet to be ~30 ns at 22 °C. Given that time, it should, in principle, have been possible to replace the sodium ions from Na–G4 comprehensively with potassium ions. G4- and G8-DNA formed in sodium were therefore repeatedly ethanol precipitated from buffers containing 300 mM potassium acetate, and the final pellet was dissolved in TE + 1 M KCl. Figure 2C shows the results of treating, variously, G-DNA formed in 1 M NaCl, 1 M KCl, and after the treatment described above (labeled Na/K) to incubations at three different temperatures (going from left to right within a particular panel), 22, 37, and 60 °C.

A comparison of the Na panel to the Na/K panel reveals the relative stabilities of the different G-DNA complexes. It is apparent that the treatment described above to generate the Na/K samples has been effective in stabilizing the various complexes, as might be expected if we had formed G4 and G8 directly in potassium solutions. In the Na/K panel, the small amount of G′2-DNA (formed presumably during the ethanol precipitation or subsequent storage steps in potassium) breaks down by 60 °C, while the G4 and G8 complexes are stable at 60 °C in 1 M KCl.

The Na panel confirms the unexpected observation (Sen & Gilbert, 1992) that G8 complexes are more stable than G4. The breakdown of the G4 at 60 °C allows a small amount of G′2 to form, presumably not as a direct breakdown product of the G4 but arising from the enlarged pool of free monomeric oligomer.

The K panel shows that the G′2-DNA, which is the sole product of incubation in potassium, is partly broken down at 60 °C. However, the excess monomer generated from this breakdown does not form the thermodynamically preferred G4 complex within the time scale of the experiment.

Methylation Protection. Methylation protection was tested on complexes formed from both Sacc1 alone and from Sacc1–Sacc2 partial duplexes to determine which guanines in Sacc1 participated in G-quartet formation in the different complexes. For these experiments, complex and monomer mixtures direct from incubations were treated with DMS, and the individual species were purified from preparative nondenaturing gels. Bands of both the G′2 and G4 complexes were each recovered as a number of consecutive thin slices, proceeding from the leading edge of the band up to the trailing edge. The goal was to investigate whether the structure of a given complex was homogeneous within a band or whether there was structural heterogeneity. Figure 3A shows methylation-protection patterns for G-DNAs derived from Sacc1 alone from sodium and potassium incubations. Results from the Sacc1–Sacc2 partial duplex (data not shown) were essentially indistinguishable from these. Two striking facts are evident from these results: first, both the G4 and G′2 complexes have some structural heterogeneity; complexes eluted from the leading edge of either complex band have their 3′-most guanine available for methylation, which presumably does not participate in quartet formation, whereas the same complexes of somewhat slower mobility have this terminal guanine protected. The second fact is the difference in protection patterns between the G′2 and the G4: two of the isolated guanines in the TGTGTGT motif are methylated in the G′2 but not in the G4 complex. The G′2 results indicate that these isolated guanines, which are presumably present in hairpin loops, are not participating in Hoogsteen hydrogen bonding (which involves the guanine N-7 position). The total protection of every guanine within the motif $G_3TGTGTGTG_3$ (with the occasional exception of the extreme 3′ guanine mentioned above) in the Sacc1 G4 complex is unexpected; a prior result had shown that the isolated guanine in G4 complexes formed by the motif GGGGGAGCTGGGG was generally methylable (Sen & Gilbert, 1990).

The G8 methylation pattern is indistinguishable from the G4 patterns, and this is a striking departure from the situation of G8- and G4-DNAs derived from single-repeat telomeric sequences, in which each complex has a unique methylation profile (Sen & Gilbert, 1992a). It is therefore clear that the Sacc1 G8 has a novel structure; results presented in this article do not, as yet, allow us to model its structure.

Figure 3B shows methylation patterns of complexes formed in calcium and strontium incubations. The G4 results from calcium essentially parallel the results from sodium. The strontium G′2 complexes, however, are made up of a greater multiplicity of related structures than the potassium G′2; the strontium structures differ primarily in the protection pattern of the three isolated, hairpin loop guanines. Comparison of the strontium and potassium results suggests that, while both cations strongly stabilize G′2 structures, 10 mM Sr^{2+} is

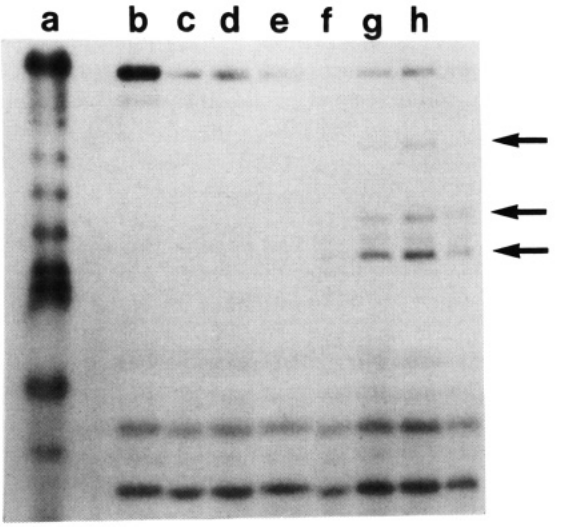


FIGURE 4: $KMnO_4$ oxidation protection of G-DNA thymines: (a) DMS–methylation standard; (b–e) G4-DNA; (f–h) G′2-DNA. The arrows mark thymines 1, 3, and 4 within the motif TGTGTGT, which appear susceptible to oxidation in the G′2 complex.

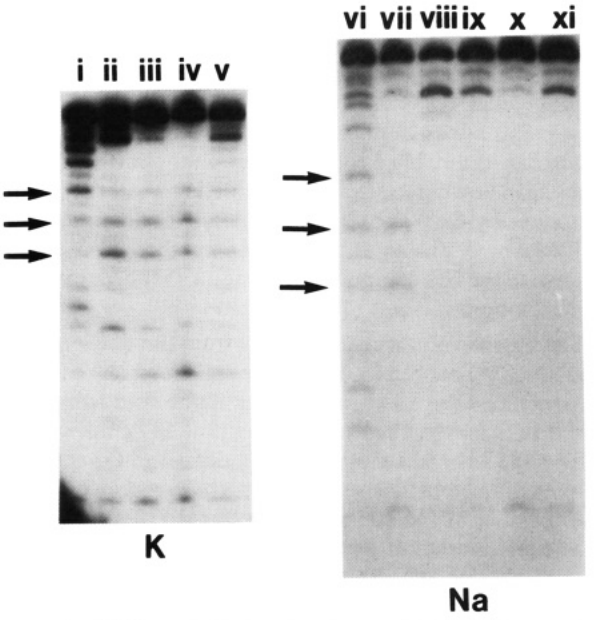


FIGURE 5: DMS–methylation interference in G-DNA complex formation in potassium (lanes i–v) and sodium (lanes vi–xi) incubations: (i) monomer; (ii–v) G′2-DNA; (vi) monomer; (vii) G′2-DNA; (viii–x) G4-DNA; (xi) G8-DNA. The arrows denote the isolated guanines within the motif TGTGTGT.

probably significantly more stabilizing than 1 M K^+, allowing a greater diversity of G′2 structures to co-exist stably.

Reactivity of Thymines with Permanganate. Figure 4 shows the reactivity of the different thymines with potassium permanganate. This reagent discriminates in favor of thymines in single-stranded or disordered regions of DNA relative to thymines in more ordered structures. In G4, none of the thymines in the TGTGTGT motif are modified by permanganate, whereas in G′2, T-1, -2, and -4 are modified while T-3 is protected. The fact of the modification of the three thymines in G′2 is further evidence that this region probably forms part of the looped out structure of G′2 complexes.

Methylation Interference. Two explanations can be offered for the unreactivity of the guanines of the TGTGTGT motif in the sodium and calcium G4 complexes. The first is that these guanines are in fact participating in quartet formation, and the second is that they are not forming quartets but are sterically so constrained in their position between the flanking zones of G-quartets that they are unreactive to DMS. Methylation-interference experiments were therefore carried out with dimethyl sulfate to determine whether the Hoogsteen hydrogen-bonding potential of these particular guanines was in fact required for the formation and stability of the G4 complex. Figure 5 shows the results of methylation interference in the formation of G′2 and G4 complexes. The results mirror those of the methylation-protection experiments shown in Figure 3A,B. Therefore, it appears that the isolated guanines are required for G-quartet formation, which in turn is required for the stability of the G4 complex.

Effect of Temperature on Methylation Protection Patterns. To test whether the isolated guanines in TGTGTGT, implicated by the methylation interference in forming G-quartets in the G4 complex, were somehow looser in their stacking and hydrogen-bonding properties than the consecutive G-quartets of the G_3 motifs, and therefore more easily melted, G4 and G′2 complexes as shown in Figure 2C were treated with DMS at 22, 37, and 60 °C. The results (not shown), however, indicated little change in the methylation-protection patterns at the higher temperatures, suggesting that at least in the G4 complex quartets formed by the isolated guanines were comparably as strong as any others in the complex.

DISCUSSION

Formation of Parallel versus Antiparallel Structures. A major conclusion of this study is that a complex telomeric sequence, such as Sacc1, derived from the *Saccharomyces* telomere, forms the same basic categories of G-DNA complexes *in vitro* as do the more simply repeating telomeres from oxytricha and tetrahymena. Remarkably, the monovalent cation-induced structural switches between G′2 and G4 complexes, observed initially with the two protozoan telomeres (Sen & Gilbert, 1990), occur in a more sharply defined, all-or-nothing manner with Sacc1 under the conditions that we have used. Our experiments with the single-stranded Sacc1 and the partial duplex Sacc1–Sacc2 gave precisely equivalent results in all structure-probing experiments, indicating that it was the telomeric, 3′-half of Sacc1 that was entirely responsible for forming the G-DNA structures.

We have presented data with respect to the specific ionic effects of different group Ia and IIa cations in either favoring or disfavoring G-DNA formation and also favoring the formation of particular G-DNA structures. The divalent cation series Mg^{2+}–Ba^{2+}, at millimolar concentrations, mirrors the behavior of its ionic radius counterparts from the monovalent cation series Li^+–Cs^+, except that 1 M concentrations of the latter were required to provide an effect comparable to 10 mM concentration of the former. The parallels between the effects of potassium and strontium (and to a lesser extent, between those of lithium and magnesium, calcium and sodium, and rubidium and barium) are striking. Both potassium and strontium favor the exclusive formation of G′2-DNA. However, the structural complexity of the G′2 structures is more pronounced in the strontium incubations (Figure 3A,B), suggesting that strontium, of all the cations examined thus far, is the most stabilizing of the G-quartets.

The pattern of G-DNA production by divalent cations is entirely consistent with the kinetic scheme of Sen and Gilbert (1990), derived originally from consideration of monovalent cations. Thus, the stabilizing ability of the divalents follows the order Sr > Ba > Ca > Mg, judging by the G′2/G4 ratio produced and by analogy with the series K > Rb > Na > Cs

> Li. According to the kinetic scheme, in strontium solutions, the rapidly forming G′2 str··ctures are stabilized so strongly that they form a kinetic sink, precluding the formation of G4 and G8 complexes. In the less-stabilizing barium and calcium solutions (as with sodium), we find mainly G4-DNA; the oligomer presumably circulates through the various G-DNA species, and owing to a lesser stabilization of all the G-DNA structures by Ba^{2+} and Ca^{2+}, it is mainly the thermodynamically favored (owing to its greater number of G-quartets relative to the G′2 complex) G4 complex that accumulates in these solutions.

The absence of a true G8 species from the divalent cation incubations (but seen in the sodium lanes of Figure 2A,B) is unexpected. The SacI G8 species is the first from a two-repeat telomeric sequence to be analyzed in detail. Sen and Gilbert (1992) and Lu et al. (1992) described G8 formation by potassium, but not sodium, from one-repeat oligomers. The bonding of the strands in the SacI G8 appears to be different from that of the earlier G8 samples, as determined by methylation-protection experiments (Figures 3A and 5), in that the SacI G4 and G8 have indistinguishable methylation-protection and -interference patterns. A possible structure for this G8 complex might have two G4 complexes stacked back to back, with the 3′-terminal guanine quartet from each G4 stabilized not only by stacking interactions but also by a bridging sodium ion coordinated to four keto oxygens from each of the terminal quartets. Determination of the actual structure of the SacI G8 complex in the future will undoubtedly clarify the ineffectiveness of divalent cations in forming this structure.

Relative Stability of the Structures. The experiments shown in Figure 2C confirmed earlier observations that the order of stability of the G-DNA complexes was G8 > G4 > G′2, reflecting the presumed G-quartet content of the different complexes. We showed further that the kinetic barrier to G4 formation in potassium solutions could be overcome by forming G4-DNA in sodium solutions and simply replacing the sodium with potassium by means of repeated ethanol precipitations.

Lu et al. (1993) have recently made comparative thermodynamic measurements, using differential scanning calorimetry, of parallel and antiparallel G-DNA complexes—hairpin tetraplexes (both strand dimers, to minimize entropic differences) formed by 3′-dG_4T_2-5′—5′-T_2G_4-3′ (the artificial 5′–5′ linkage allowing a hairpin parallel structure to form) and $dG_4T_4G_4$, respectively. They confirm directly, in agreement with our findings, that the parallel complex is the more stable, by a $\Delta\Delta G^\circ$ value of 5 kcal/mol.

Binding Sites of Divalent Cations. It was unexpected that the behavior of the divalent cation series would be so similar to that of the monovalent series, except that the divalents were two orders of magnitude more efficient. An important question remains unanswered as to the actual binding site(s) of divalent cations within the parallel and antiparallel tetraplexes. The enormous efficiency of divalent cations (compared to monovalent cations) in promoting different structural transitions of nucleic acids relates to the polyanionic nature of DNA and RNA. The cationic dependence of such phenomena as the B → Z transition of DNA (Behe & Felsenfeld, 1981), DNA condensation (Widom & Baldwin, 1980; Wilson & Bloomfield, 1979), or chromatin folding (Sen & Crothers, 1986) is satisfactorily explained by the ion-condensation models of Manning (1978) and of Record et al. (1978). However, at a finer level, ion-specific effects are also seen. For example, Ivanov et al. (1973) noted a correlation between the hydrated ionic radii of different alkali cations and their relative ability to promote the B- → C-DNA transition; Behe and Felsenfeld (1981) found an order of magnitude difference between the efficiencies of Mg^{2+} and Ba^{2+} in effecting the B → Z transition of poly(dG–dC)·poly(dG–dC), with $Ba^{2+} > Ca^{2+} > Mg^{2+}$. These ion-specific effects probably relate to the relative ability of different cations to bridge interstrand phosphates (Wang et al., 1979; Drew et al., 1980; Crawford et al., 1980). With regard to the divalent ion-specific effects that we demonstrate, the question remains open as to whether the divalent ions exercise their effect primarily by binding within the G-quartet or along the sugar–phosphate backbone. The similarity of the monovalent and divalent ion patterns and, particularly, the potassium–strontium analogy suggests an intraquartet binding for divalent cations. However, we cannot rule out phosphate–phosphate bridging as a possible mechanism at this stage, in the absence of further structural data on these parallel and antiparallel G-DNA structures.

Structures of the G′2 Complexes. The antiparallel G′2 complexes formed by SacI share key structural features when they are formed in the presence of either potassium or strontium cations (Figure 3A,B), except that the strontium ion seems to stabilize a larger variety of G′2 structures, which vary in the methylation-protection patterns of their TGTGTGT loop regions (Figure 3B). Division of the G′2 bands from methylation- and $KMnO_4$-protection preparatory gels into successive thin slices reveals, in the case of potassium, two closely related complexes which mainly vary in the extent of methylation of the 3′-most guanine. The major product seems to have this terminal guanine uninvolved in quartet formation (and is, therefore, methylable). Models for this structure are shown in Figure 6, panels A–D. The models in each of these panels is consistent with the methylation and $KMnO_4$ data. A and B represent conventional G′2 structures in which the four strands participating in quartet formation run antiparallel, similar to those described for G′2 complexes investigated by X-ray crystallography (Kang et al., 1992) and NMR (Smith & Feigon, 1992); the difference between the two is simply the relative orientation of the two loops. Such models might in theory be distinguished by interstrand T–T cross-linking by ultraviolet light (Williamson et al., 1989). In practice, SacI G′2 complexes gave very poor cross-linking (by comparison with G′2 complexes from the vertebrate telomere). This lack of cross-linking may indicate either that structure B is the correct one or simply that this method cannot be used to distinguish the complexes. A problem with models A and B, however, is the reactivity of the thymines in the loops to potassium permanganate (Figure 5), indicating that they are not present in a substantially ordered conformation, whereas one might expect a tetraloop of TGTG, as shown, to hydrogen bond internally to some extent. An alternative category of structures for the G′2 complexes, therefore, may be those of the crown model, shown in panels C and D of Figure 6 [proposed first by Acevedo et al. (1991) for G′2 complexes of the oxytricha telomere, $T_4G_4T_4G_4$]. A technique such as NMR could distinguish between the two models, for in the antiparallel A and B structures, one would expect an alternation of syn and anti conformations of successive guanosines in a quartet, whereas in the parallel crown model, one might find anti conformations only, similar to those found for all quartet guanosines in G4-DNA.

Structure of the G4 Complexes. DMS–methylation-protection studies (Figure 3A,B) indicate that in the G4 complex, by sharp contrast with the G′2 complexes, every single guanine within the motif $G_3TGTGTGTG_3$ is involved in guanine quartet formation. Previous methylation-protection

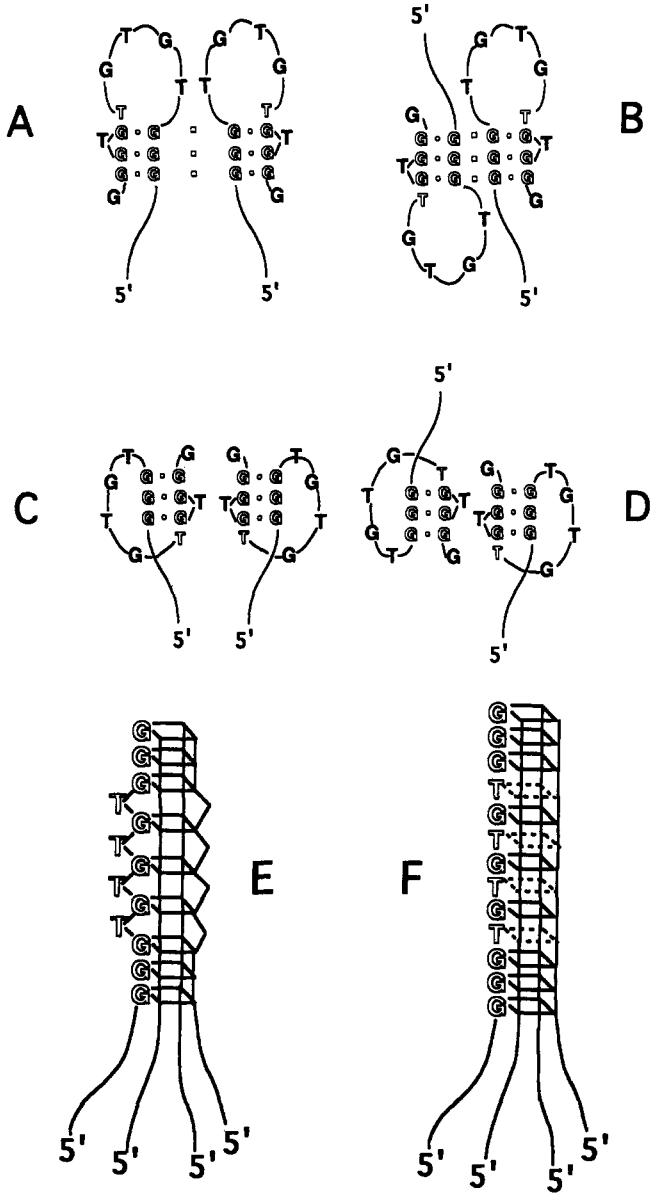


FIGURE 6: Models for the structure of Sacc1 G'2-DNA (panels A–D) and G4-DNA (panels E–F). Panels A and B show an antiparallel model for G'2-DNA; C and D show a parallel, crown model (Acevedo et al., 1991).

experiments on G-DNA complexes had suggested that the method was an exquisitely sensitive probe of the involvement of specific guanines in Hoogsteen hydrogen bonding and that the efficiency of methylation was not strongly affected by the immediate higher order structure context of the guanine (for example, those guanine bases that were stacked upon guanine quartets but were not themselves hydrogen-bonded, as in G8 complexes formed by T_9G_3, were strongly reactive to DMS; Sen & Gilbert, 1992a). In the Sacc1 G4, the case that the guanines within the TGTGTGT region not only form quartets but that their quartet formation is in fact required for the stability of the G4 complex is made by DMS–methylation-interference experiments. Figure 5 shows that the methylation of even one of the guanines within TGTGTGT in a given oligmer strand prevents that strand from participating in a G4 complex.

The role of the alternating central thymines within the quadruplex structure of G4-DNA remains more ambiguous. These thymines are resistant to modification by potassium permanganate (again, in marked contrast to the reactive and presumably looped out thymines in the G'2 complex; Figure 4), which suggests that they exist in an ordered structure within the G4 complex. At this point, we do not have direct evidence of any hydrogen bonding by the thymines, either with each other or with the guanines. Cheong and Moore (1992), in their NMR study of the parallel quadruplex structure formed by rUG_4U, found evidence that one set of terminal uracils in their G4 structure not only stacked well upon their neighboring G-quartet but also hydrogen bonded with each other to give a U-quartet, as evidenced by a stable imino proton resonance that could be attributed to them. In the Sacc1 G4 complex, two possibilities about the disposition of thymines within the quadruplex may be considered. These are shown in Figure 6, panels E and F. In model E, the thymines are forced out of the main body of the quadruple helix so that the intervening guanine quartets may approach and stack upon one another in a more-or-less continuous fashion. The stacking of a pyrimidine quartet upon a purine quartet is likely significantly less stabilizing than that of a purine quartet stacking on purine, in which case, it may be energetically reasonable to push the thymines out of the body of the quadruplex in order to establish highly favorable stacking interactions between G-quartets (comparable to what evidently occurs in G8 complexes; Sen & Gilbert, 1992a). The pushed out but conformationally constrained thymines on the periphery of the quadruplex could still be unreactive to potassium permanganate.

In the other model (panel F), the central portion of the quadruplex would consist of alternating G- and T-quartets, with this potentially labile structure anchored at either end by the well-stacked blocks of three guanine quartets each. The two models, E and F, could be distinguishable by NMR, in that only the latter structure would provide stable imino resonances from T-quartets.

The plausibility of quartet formation by the isolated guanines in the TGTGTGT region raises interesting questions about the coordination of ions by isolated guanine quartets. The first studies on G- and I-quartets suggested that the sodium ion might coordinate in the plane of a quartet, coordinating in a square-planar fashion to the four keto oxygens from four guanines, whereas potassium and cations larger than potassium would sit in the space between successive quartets and coordinate to eight keto oxygens (Howard & Miles, 1982a, b; Miles & Frazier, 1972, 1978). Recent X-ray data by Kang et al. (1992) have, in fact, located a potassium ion precisely at the expected location. Sundquist and Klug (1989) have recently suggested that the coordination of the sodium ion may also be 8-fold and, hence, no different from that of potassium. It is therefore significant that G-quartets from the isolated guanines do seem to form, both in G4-DNA complexed directly in sodium and also in G4 ethanol-precipitated from and resuspended into potassium. However, whether these quartets (whose formation is presumably made facile by the anchoring function of the two blocks of contiguous G-quartets at either end of the $G_3TGTGTGTG_3$ motif) in fact need a central ion for stabilization is an open question. There is a precedent for the formation of G-DNA in the absence of significant concentrations of cations by 20–30 base long continuous stretches of guanines (Panyutin et al., 1989, 1990).

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