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# Novel DNA Superstructures Formed by Telomere-like Oligomers†

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ABSTRACT: DNA oligomers containing three or more contiguous guanines form tetrastranded parallel complexes, G4-DNA, in the presence of alkali cations. However, oligomers that have a single multi-guanine motif at their 3′ or 5′ end, with a guanine as the terminal base, also form higher order products. Thus, the oligomer $T_8G_3T$ forms a unique G4-DNA product at neutral pH in the presence of $Na^+$, $K^+$, or $Rb^+$; however, its isomeric counterpart $T_9G_3$ in $K^+$ or $Rb^+$ generates an additional ladder of products of substantially lower gel mobility. We show that these larger complexes contain, respectively, 8, 12, or 16 distinct strands of oligomer. The octamer structure formed by $T_9G_3$ assembles in moderate salt at room temperature and melts around 60 °C in 100 mM KCl. Methylation protection experiments suggest a nested head-to-tail superstructure containing two tetraplexes bonded front-to-back via G quartets formed by out-of-register guanines. Naturally occurring chromosomal telomeres, which all have guanines at their 3′ termini, may be able to form these superstructures.

DNA oligomers containing one or more guanine motifs (i.e., stretches of contiguous guanines) form, in the presence of alkali cations, a family of higher order structures bonded by guanine–guanine base pairs and base quartets [reviewed recently by Guschlbauer et al. (1990), Sundquist (1991), and Sen and Gilbert (1991)]. The association of four independent strands results in the formation of G4-DNA, a complex in which the four strands run parallel, as determined by methylation protection (Sen & Gilbert, 1988; Kim et al., 1991). A notable difference appears in the conditions necessary for the formation

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of G4-DNA, depending on whether the input oligomers contain simple or complex guanine motifs. Simple motifs form G4-DNA readily in buffers that contain sodium, potassium, or rubidium ions, the potassium form being the most readily formed and the most stable; complex motifs, however, form G4-DNA in the presence of sodium or rubidium ions but not in the presence of potassium ion alone, which favors the formation of antiparallel structures called G′2-DNA (Williamson et al., 1989; Sundquist and Klug, 1989; Sen & Gilbert, 1990). This differential effect of alkali cations can be attributed to a size-specific binding of these cations (with strongly differing affinities) within cavities formed between successive layers of guanine quartets [see Howard and Miles (1982) and references cited therein] and to the undue stabilization of foldback structures by the potassium ion, which then kinetically prevents the formation of G4 structures (Sen & Gilbert, 1990).

The formation of G4-DNA from monomeric oligomers is not influenced significantly by the precise location (i.e., central vs terminal) of the quartet-forming motif(s) within the oligomer. However, in this paper, we show that the precise location of a simple guanine motif (three contiguous guanines) within a simple oligomer has an unanticipated effect on higher order structure formation. For example, while the oligomer $T_{11}G_3T$ forms a unique G4-DNA product, its isomeric counterpart $T_{12}G_3$ forms in addition a series of further products of lesser gel mobility than the G4-DNA. We use a number of strategies to demonstrate that these higher molecular weight complexes correspond to a novel DNA superstructure, assembled from multiples of four individual oligomer strands. They are probably bonded in a nested head-to-tail arrangement, with the guanines associating in a central, continuous quadruple helix, and the remaining portions of the oligomers displaced and radiating out from "nodes" in the quadruple-helical stem.

## Materials and Methods

*Oligomers.* DNA oligomers were synthesized on a Biosearch Cyclone DNA synthesizer, usng standard cyanoethyl phosphoramidite chemistry. Deprotected oligomers were purified on preparative gels, followed by reverse-phase Spice columns (Rainin), and dissolved in 10 mM Tris, pH 8.0, and 1 mM EDTA (TE buffer) to give stock solutions containing 0.5–1.0 $A_{260}$ unit of oligomer/$\mu$L. Approximately 0.5 OD unit of each oligomer was kinased with [$\gamma$-$^{32}$P]ATP, quenched with 15 mM EDTA, and maintained in stock solutions of 25-$\mu$L volume.

*Higher Order Structure Formation.* One to ten microliters of unlabeled oligomer stock solution was combined with an equal volume of labeled oligomer stock solution, denatured at 100 °C in tightly sealed screw-capped microfuge tubes for 1–2 min, then chilled on ice, and spun briefly. One to two microliters of the label-doped oligomer was then mixed with an equal volume of TE + 2 M KCl (or NaCl or RbCl) and allowed to sit at room temperature in a sealed tube for 30–60 min. The final DNA concentration was 3.8–7.5 $\mu$g/mL (assuming 1 $A_{260} \equiv 30$ $\mu$g) or 750 $\mu$M–1.5 mM for a 15-mer. Where a mixture of two oligomers was used to form "mixed" higher order structures, the DNA samples were combined in the low-salt buffer first, with potassium being added as the final step. Analysis of samples for higher order structure formation was carried out on prerun, 10% nondenaturing polyacrylamide gels in 50 mM TBE + 5 mM KCl at 4 °C (equilibrated and run at 4 °C in the cold room) or in 50 mM TBE + 10 mM KCl at room temperature. Two-microliter samples containing a final salt concentration of 1 M, as described above, were mixed into 10 $\mu$L of ice-cold loading buffer (3.3% glycerol plus loading dyes in TE + 5 mM KCl). Gels were run at low voltage until the bromophenol blue had migrated 12–24 cm. Gels were then either dried onto DEAE paper backed by Whatman 3MM paper or wrapped directly in plastic wrap for autoradiography at −20 °C.

G4-DNAs from the "model" oligomers $T_9G_6T_9$ and $T_{12}G_6T_{12}$ were generated by treating the oligomers at ~1 mM DNA concentration in TE + 1 M NaCl at 60 °C for 8–12 h.

*Methylation Protection.* Two-microliter reaction samples containing DNA in TE + 1 M KCl (see above) were each diluted at room temperature with 18 $\mu$L of 100 mM potassium cacodylate, pH 7.0, freshly made up to 0.5% and 1% dimethyl sulfate (DMS), respectively. Methylation was allowed to proceed for 30 min at room temperature. Three microliters of loading buffer (10% glycerol plus dyes in TE + 5 mM KCl) was added to each sample, and the mixtures were loaded onto a 10% nondenaturing preparatory gel at 4 °C in 50 mM TBE + 5 mM KCl. Bands corresponding to monomer and various higher order structures were cut out; their DNA was eluted and purified, cleaved with piperidine, and analyzed on a 10% sequencing gel.

*Melting Experiments.* Higher order structure of $T_{12}G_3$ were formed in TE + 1 M KCL (as above). The solution was diluted 10-fold with ice-cold TE to give a final solution of TE + 100 mM KCl and final DNA concentration of ~100 $\mu$M. Ten-microliter aliquots of the diluted solution were placed in chilled 0.5-mL microcentrifuge tubes compatible with a Perkin-Elmer Cetus thermal cycler, and stored on ice. The thermal cycler was programmed to provide incubation temperatures of 35–65 °C, at 5 °C intervals. Each sample was held at a given temperature for 7 min, then chilled on ice, spun briefly, and stored in ice. One microliter of ice-cold loading buffer (TE + 5 mM KCl containing loading dyes and 10% glycerol) was added to each sample prior to being loaded on a prerun 10% nondenaturing gel run in 50 mM TBE + 5 mM KCl at 4 °C.

## Results and Discussion

*Additional Higher Order Structures Formed by Oligomers with Terminal Guanines.* Sen and Gilbert (1990) showed that oligomers containing a simple guanine motif of three, four, or five contiguous guanines readily formed G4-DNA in the presence of either sodium or potassium cations (though, in the case of only three guanines, potassium was far more efficacious that sodium). The guanine motifs were generally located at the 3′ end of the oligomer, although only in a few instances were they present at the absolute 3′ terminus. In salt, under conditions that generated higher order structures, the oligomers that did not have a guanine at the 3′ terminus and those that did produced different products. The former class of oligomer invariably gave a clean, well-defined, and unique higher order product, which we demonstrated to be G4-DNA by virtue of its containing four distinct oligomer strands. The latter class of oligomers gave, in general, a more complicated pattern of higher order structures. In the case of oligomers containing four or five guanines in their G motif, the pattern was too complicated for sensible analysis, but in the case of oligomers with three guanines, the pattern was more accessible to analysis.

Figure 1A shows the contrasting patterns of higher order structure formation by isomeric oligomers that differ only in having or not having a terminal guanine (lanes a–c, which use $T_9G_3$ and $T_{12}G_3$, versus lanes d–f, which use $T_8G_3T$ and $T_{11}G_3T$). Oligomers were allowed to form higher order structures both individually (e.g., lanes a and c) and from

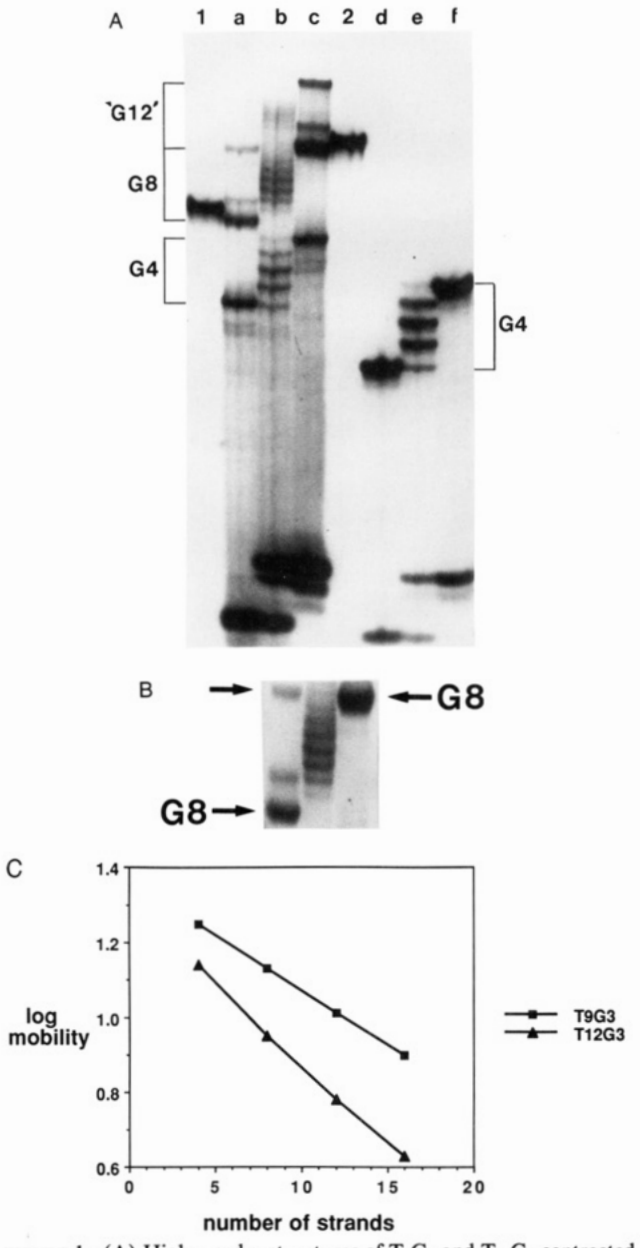


FIGURE 1: (A) Higher order structures of $T_9G_3$ and $T_{12}G_3$ contrasted with those of $T_8G_3T$ and $T_{11}G_3T$. Lane a, $T_9G_3$; lane c, $T_{12}G_3$; lane b, mixture of $T_9G_3$ and $T_{12}G_3$; lane d, $T_8G_3T$; lane f, $T_{11}G_3T$; lane e, mixture of $T_8G_3T$ and $T_{11}G_3T$; lane 1, G4-DNA form of $T_8G_6T_8$; lane 2, G4-DNA form of $T_{12}G_6T_{12}$. (B) The G8-DNA complexes formed by, respectively, $T_9G_3$, a mixture of $T_9G_3$ and $T_{12}G_3$, and $T_{12}G_3$. (C) Semilog plot of gel mobilities of higher complexes with presumed strand stoichiometries of 4, 8, 12, and 16, respectively.

roughly equimolar mixtures of two oligomers (e.g., lane b). Such a disproportionation experiment allows one to quantitate, by analyzing the total number of "mixed" products formed within a particular class of higher order structures, the number of distinct oligomer molecules involved in that structure (Sundquist & Klug, 1989; Sen & Gilbert, 1990; Kim et al., 1991). Lane b shows that the major product from the mixture of the oligomers $T_9G_3$ and $T_{12}G_3$ (shown individually in lanes a and c) is not G4-DNA (defined by its five "mixed" products, corresponding to products $A_4$, $A_3B$, $A_2B_2$, $AB_3$, and $B_4$) but a complex of lesser gel mobility. Figure 1B shows the mixed products of this complex (derived from another experiment using the same oligomers) in greater detail; allowing for very faint bands at either extreme, one counts a total of nine product bands, indicating that the complex contains eight distinct input oligomers. We term this product "G8-DNA". In addition to

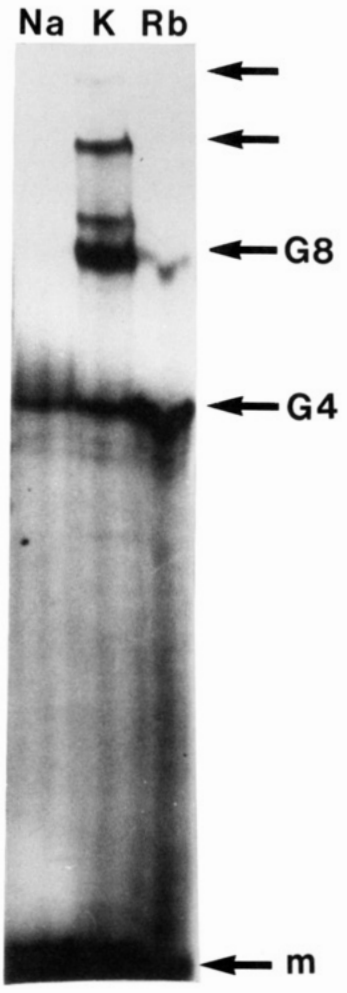


FIGURE 2: Higher order structure formation by the oligomer $T_9G_3$ in the presence of 1 M NaCl, KCl, and RbCl, respectively.

the G8 complex, a G12-DNA and higher order products of even lesser gel mobility appear. Although it is difficult to determine the molecular weights of these further complexes using the mixed-products technique described above, if the logarithms of their gel mobilities (with the single exception of the minor band just above the G8-DNA band) are plotted against putative molecular weights which assume that they contain 12 and 16 oligomers, respectively, an approximately linear dependence is obtained (Figure 1C).

*Oligomers with 5′-Terminal Guanine Motifs Also Form Higher Complexes.* If the terminal guanine motif is located on the 5′ of the molecule, such as in $G_3T_9$ and $G_3T_{11}$, the molecules behave almost identically to $T_9G_3$ and $T_{11}G_3$, if anything showing a more pronounced tendency to form G8 and higher complexes at the expense of G4-DNA under comparable conditions (data not shown). The corresponding complexes from the 5′-motif and 3′-motif oligomers (i.e., G4, G8, or higher) had the same gel mobilities.

*Cation Specificity of G8 Formation.* The cations $Na^+$, $K^+$, and $Rb^+$ show different efficacies in favoring G8-DNA formation. The order of stabilization of G4-DNA by the three cations is K > Rb > Na (Sen & Gilbert, 1990). Figure 2 shows the results of treating the oligomer $T_9G_3$ to 1 M solutions of NaCl, KCl, and RbCl, respectively. While potassium ion favors the formation of G4-DNA as well as all the higher complexes, rubidium chloride shows a faint G8-DNA band, while sodium chloride shows only the G4-DNA. However, a caveat is that if G4-DNA were a pathway intermediate in the formation of G8-DNA, the lack of accumulation of suf-

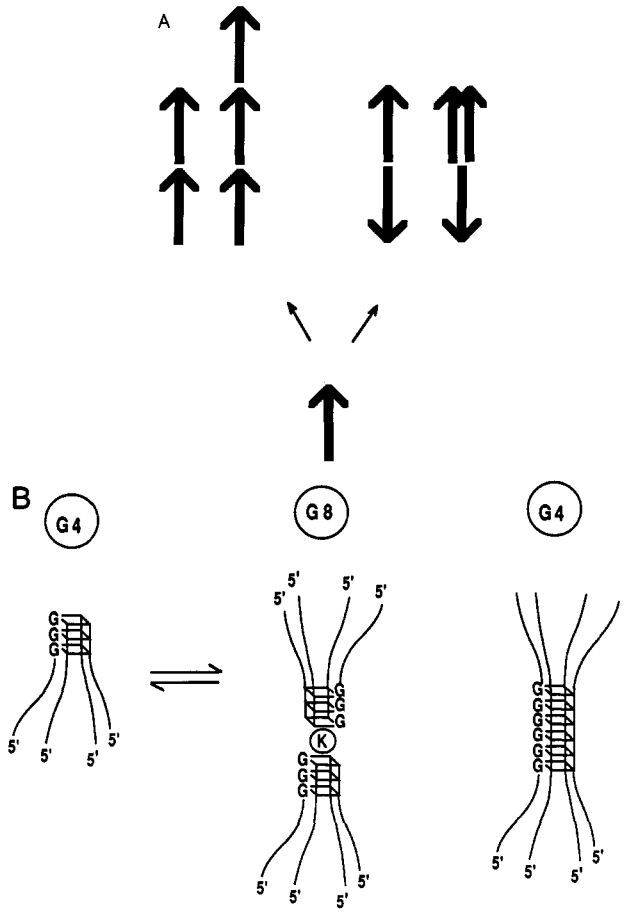


FIGURE 3: (A) Schematic models for the structures G8, G12, etc. Each arrow indicates a G4-DNA complex. The models on the left illustrate a "front-to-back" arrangement of G4-DNA complexes to give the higher structures, and the models on the right illustrate a "back-to-back" arrangement. The precise mode of interaction between the G4-DNA building blocks is not indicated. (B) Schematic arrangement showing a putative back-to-back geometric arrangement of a G8-DNA complex and also the G4-DNA form of an oligomer of twice the molecular weight and a medial guanine motif, that might function as a electrophoretic model for such a back-to-back arrangement of the G8-DNA.

ficient concentrations of G4-DNA in sodium solution (as has been observed with oligomers of the general class $T_nG_3$) might hinder the further formation of G8-DNA. However, a further experiment with the oligomer $T_8G_4$ showed that the facilitation of higher complex formation does indeed follow the order K $\sim$ Rb > Na (data not shown), in agreement with our previous observations on the various alkali cations.

*Model of Orientation of Strands in the G8 Complex.* Two questions immediately arise about the structure of G8-DNA and its homologous complexes: (a) What is the orientation of the individual oligomer strands relative to one another, and (b) how are the strands bonded together? Figure 3A shows a schematic for the relative orientations of G4-like units (shown as large arrows) that could assemble to form G8, G12, and further superstructures (with no assumption being made here as to the nature of the bonding between the units). A front-to-back arrangement could in theory allow a facile and indefinite polymerization of G4-like units, though such an arrangement should involve steric problems for the displaced exiting segments of the input oligomers, the parts not involved in guanine quartet formation [i.e., the oligo(T) stretches]. A back-to-back arrangement would seem sterically ideal for the G8 complex, although less satisfactory for the G12 and higher complexes. However, it might be possible, in the latter complexes, for the terminal G quartets to stack side-by-side and overlap only partially with their corresponding quartets on the opposite "face".

Several G4-like units could be held together by two types of bonds. On one hand, the G4-like units may have the three guanines from the four strands precisely aligned with one another to give three quartets and a "flat" terminal quartet surface at the 3′ end of the complex. Such involvement of all of the guanines in quartet formation would effectively preclude (owing to the presumed structure of the quartet) their participation in any further substantive hydrogen bonding. Thus, a G8 complex would form from two G4 complexes by stacking the 3′-most (or 5′-most and 3′-most) quartets from either. Guanine quartets are expected to have strong stacking energies; in addition, an 8-fold coordination of potassium ions by eight keto oxygens (four from each quartet) might provide an additional factor for stabilization, the potassium ion essentially bridging the two G4-DNA units (see Figure 3B). In such a model, a terminal base other than guanine might block the access of the faces of the quartets to each other, and so prevent the formation of the higher complexes.

The other possibility for bonding necessitates that the G4-like units not have perfectly aligned guanines, thus creating "sticky ends" to join the units together, either in a back-to-back or in a front-to-back arrangement. This would involve hydrogen bonds as well as stacking interactions. Again, the presence of a terminal thymine at the 3′ end might discourage the formation of such a misaligned G4-like precursor, thus explaining the apparent absence of higher complex formation by those oligomers.

Displacing the G motif one base away from the 3′ terminus from $T_9G_3$ to $T_8G_3T$ has a significant effect on the gel mobility of the G4 form. Figure 1 shows that the "X"-like structure of the G4 complex of $T_8G_3T$ moves faster through the gel matrix than the "Y"-like structure of the G4 complex of $T_9G_3$. This sensitivity of the mobility to the disposition of the motifs led us to try to construct a model complex for the back-to-back complex (shown in Figure 3B), essentially a G4-DNA of twice the length and with its (2 times larger) guanine motif located in the center of the molecule: a G4 complex of $T_9G_6T_9$ as an electrophoretic model for a back-to-back G8 complex of $T_9G_3$. Figure 1A shows that when such model G4-DNA complexes are run (lanes 1 and 2) side-by-side with their comparison G8 complexes, they run at almost the same mobility. Originally, we thought this behavior to be a strong argument for the back-to-back orientation. However, as we shall show below, the G8 complexes are not back-to-back. Thus, the safest conclusions from this experiment may only be that the G8 complex has a similar molecular weight to its model G4 or comparable hydrodynamic properties.

*Methylation Protection: A "Slipped" Complex.* Methylation protection experiments can test the nature of the bonding between the two G4-like units in G8-DNA, since a "slipped" G4-like complex with a "sticky" end would necessarily leave some guanines uninvolved in quartet formation and therefore susceptible to methylation, while the properly aligned G4-like complex would show a total protection of the three guanines in each of its strands. Furthermore, as Figure 4A shows, configurations of the "slipped" model for G8-DNA in the front-to-back and back-to-back orientations give rise to distinct methylation patterns. In the front-to-back configuration of the G8-DNA of $T_{12}G_3$, two of the eight G-13's would be uninvolved in quartet formation, as well as two of the eight G-15's. However, in a back-to-back configuration, four of the eight G-13's would be methylatable while all the G-15's would

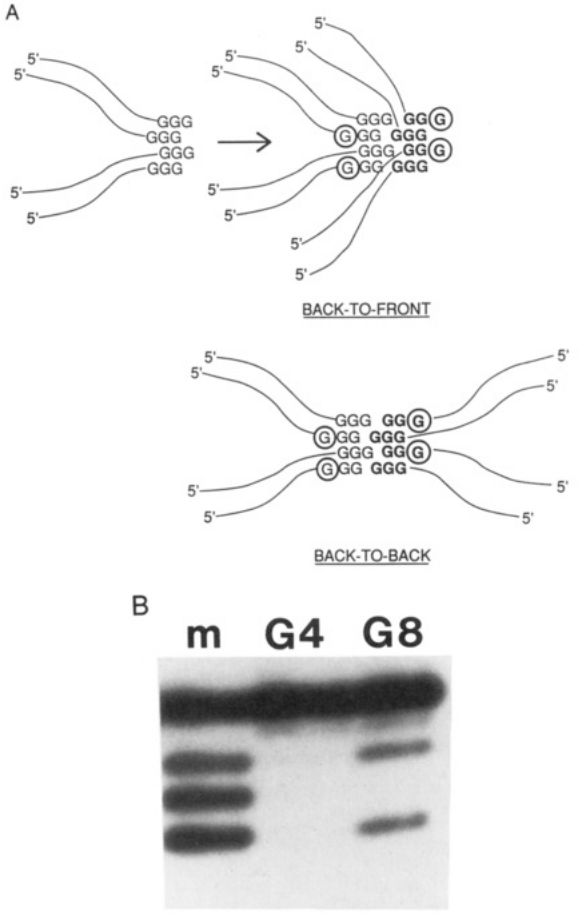


FIGURE 4: (A) Schematic for front-to-back and back-to-back models for G8-DNA which involve bonding of two G4-DNA units via quartet formation by "slipped" guanines from either unit. The unbonded guanines predicted from the two models (distinguishable from one another) are shown circled. (B) Sequencing gel showing methylation protection patterns of guanines from monomeric (m), G4-DNA (G4), and G8-DNA (G8) forms of the oligomer $T_{12}G_3$. Equal amounts of these samples were loaded. While in the G4-DNA form all three guanines are fully protected relative to the m form, in G8-DNA only the central guanine is thus protected, the outer two being accessible to DMS to approximately a quarter of the level as in the monomer.

be protected. Figure 4B shows the results of an experiment for $T_{12}G_3$, in which a mixture of monomers, G4, and higher structures was methylated with dimethyl sulfate, the products were separated on a nondenaturing gel, and the purified DNA from each complex was piperidine-cleaved and analyzed on a sequencing gel. All the guanines in the G4 complex are fully methylation-protected, demonstrating a proper alignment of the four strands of oligomer, while in the G8 complex, only the middle guanine is totally protected and G13 and G15 are reactive to approximately a quarter of the intensity of the same guanines in the monomer form of the oligomer. Thus, this experiment reveals both that (a) the G4-DNA units making up G8-DNA are imperfectly aligned, leaving sticky ends, and that (b) the G4-DNA units are bonded front-to-back in the G8 and hence, presumably, in the G12 and higher complexes.

Figure 5 shows, in highly schematic form, our view of the various kinds of higher order structures that are formed by oligomers with a simple, small guanine motif. Thus, (A) represents the G4-DNA (with aligned strands) of an oligomer with a terminal guanine. Such an oligomer will also form (perhaps transiently) "slipped", improperly aligned G4-like units, which may then associate to give the sugar cane shaped higher complexes [a G12-DNA shown in (C)]. Oligomers with nonterminal guanine motifs, however, will yield only

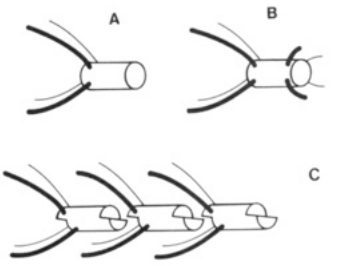


FIGURE 5: Schematics for G4-DNA formed by $T_nG_3$ (A) and $T_{n-1}G_3T$ (B) and the sugar cane model of G8-DNA formed by $T_nG_3$ (C).

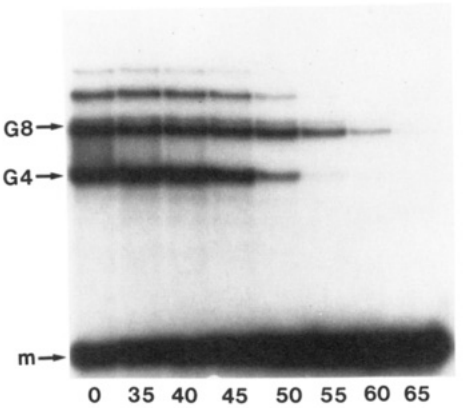


FIGURE 6: Melting point profile of monomer, G4, G8, and higher complexes of $T_{12}G_3$ in TE, pH 8.0, + 100 mM KCl.

properly aligned G4-DNA (B) as a stable product. Although slipped G4-like units may form transiently from these latter oligomers, they probably cannot associate further to give higher structures owing to excessive steric problems involving the clash of the displaced, non-quartet-forming portions of the DNA strands.

*Conditions of Formation and Stability of G8.* The conditions of formation of G8 and higher products closely mirror those promoting the formation of G4-DNA, owing to the essential structural similarity of all of these complexes. G8-DNA will form in as low as 100 mM KCl at room temperature (with starting DNA concentration at 750 $\mu$M–1.5 mM) and is stable when run on nondenaturing gels at room temperature, provided that the gel buffer contains 10 mM KCl (i.e., 50 mM TBE + 10 mM KCl). Curiously, the G8 form of $T_{12}G_3$ is more stable than the G4 in 100 mM KCl. Figure 6 shows that G8 melts around 60 °C while G4 melts around 50 °C. In our model for G8-DNA, the G4-like units are held together by only *one* "mixed" guanine quartet, in which guanines from both G4-like units participate, as well as the stacking interactions. The high stability of both the G4 and G8 complexes formed by oligomers containing only three guanines is consistent with our earlier measurements of the stabilities of G4-DNA complexes (Sen & Gilbert, 1990).

In summary, we note that the higher order structures described in this paper are formed by single-stranded DNA sequences with a 3′- or 5′-terminal guanine motif, in the presence of potassium ion. It is interesting to note that the single-stranded termini of all chromosomal telomeres sequenced to date have a guanine motif at their 3′ termini, with guanine as the terminal base. Given a sufficient local density of telomeres in the potassium-rich environment of the cell, these higher order superstructures may arise. It is well-known from cytological studies of meiosis that telomeres often aggregate to form "bouquets" upon the nuclear envelope [reviewed in Blackburn and Szostak (1984)]. The mechanism for the formation of such structures is not understood and may

well involve nested head-to-tail DNA complexes.

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**Registry No.** $T_9G_3$, 137647-85-9; $T_{12}G_3$, 137741-96-9; $T_8G_3T$, 137647-86-0; $T_{11}G_3T$, 137741-93-6; $T_8G_6T_8$, 137741-94-7; $T_{12}G_6T_{12}$, 137741-95-8; Na, 7440-23-5; K, 7440-09-7; Rb, 7440-17-7.

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# Analysis of Promoter-Specific Repression by Triple-Helical DNA Complexes in a Eukaryotic Cell-Free Transcription System†

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ABSTRACT: A site-specific triple-helical DNA complex has previously been shown to inhibit DNA binding by eukaryotic transcription factor Sp1. To examine the functional consequences of such inhibition, homopurine target sequences for oligonucleotide-directed triple-helix formation were inserted in various configurations relative to Sp1 transcription activator binding sites, upstream of the TATA element of recombinant eukaryotic promoters. The resulting promoters were tested for activity in the presence or absence of recombinant human Sp1 in a *Drosophila* in vitro transcription system lacking endogenous Sp1. When triple-helical complexes were assembled on the promoters by incubation with specific oligodeoxyribonucleotides, promoter-specific repression of basal transcription was observed in the absence of Sp1. Transcriptional repression required the preassembly of triple-helical complexes before addition of nuclear extract. The degree of basal repression was a function of the number and proximity of triple-helical complexes relative to the basal promoter complex. Repression did not result from triple-helix-induced template degradation. Addition of recombinant Sp1 did not cause derepression. These results suggest that triple-helical complexes can repress transcription primarily by blocking promoter DNA assembly into initiation complexes rather than by occluding Sp1 binding. One of several plausible mechanisms for triple-helix-induced repression involves changes in DNA flexibility. Evidence in favor of this model is provided by a permutation-dependent gel mobility assay in which formation of site-specific triple-helical complexes is shown to stiffen double-helical DNA.

Regulation of transcription initiation from specific promoters is a critical step in the spatial and temporal control of eukaryotic gene expression. The rate and precise sequence specificity of transcription initiation by RNA polymerase II result from the interplay of (i) various cis-acting DNA sequence elements located both proximal (basal promoter elements) and distal (upstream activator binding sites, enhancers) relative to the initiation site; (ii) trans-acting transcription activators and repressors, typically proteins, some of which bind cis-acting promoter elements sequence specifically; and (iii) the DNA double helix itself, which provides a context for local and higher order macromolecular interactions. This interplay serves to regulate the organization of a nucleoprotein initiation complex with RNA polymerase II in a process that is, at present, poorly understood (Saltzman & Weinmann, 1989; Mitchell & Tjian, 1989; Levine & Manley, 1989).

Oligonucleotide-directed DNA triple-helix formation, wherein either a pyrimidine (pyrimidine motif) or a purine (purine motif) oligonucleotide binds to a homopurine double-helical DNA target, is a general approach for sequence-specific DNA recognition (Moser & Dervan, 1987; Doan et al., 1987; Cooney et al., 1988; Beal & Dervan, 1991). Intermolecular triple-helical complexes have been shown to inhibit sequence-specific DNA binding proteins (Maher et al., 1989; Hanvey et al., 1989; François et al., 1989). Kinetic and thermodynamic analyses of oligonucleotide-directed DNA

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