Sequence and Environmental Effects on the Self-Assembly of DNA Oligomers Possessing $G_xT_2G_y$ Segments

Stephen P. Marotta,‡ Paul A. Tamburri, and Richard D. Sheardy*

Department of Chemistry, Seton Hall University, South Orange, New Jersey 07079

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ABSTRACT: It is well-known that DNA oligomers possessing contiguous guanine bases can assume non-Watson−Crick type structures through the formation of four-stranded species. The architecture of these four-stranded structures is highly dependent upon the sequence of the DNA and the conditions (e.g., buffer, pH, ionic strength, cations present, and temperature) under which the DNA is prepared. This lab has previously reported the self-assembly of DNA oligomers of sequence $C_4T_4G_4T_{1-4}G_4$ into multistranded high molecular weight species [Dai, T.-Y., Marotta, S. P., & Sheardy, R. D. (1995) *Biochemistry 34*, 3655−3662]. In order to further investigate the sequence and environmental effects on the self-assembly of DNA oligomers possessing $G_xT_2G_y$ (where $x = 1$, 3, or 4 and $y = 2-5$) segments, the synthesis of a number of such oligomers was undertaken. DNA samples were prepared in standard phosphate buffer (10 mM phosphate, pH 7.0) and NaCl, KCl and/or $MgCl_2$ added to different concentrations in order to evaluate the influence of the cations and their concentrations on the self-assembly of the DNA oligomers. The self-assembly of these oligomers was monitored by nondenaturing polyacrylamide gel electrophoresis and circular dichroism studies. Electrophoresis of the oligomers in either 100 mM K^+ or 50 mM Na^+ with 50 mM K^+ indicated the formation of one or two molecular species for these oligomers. In contrast, electrophoresis of these oligomers in the presence of both 100 mM K^+ and 20 mM Mg^{2+} give a ladder of multiple bands of high molecular weight indicative of multistanded DNA structure formation. The results presented here indicate that self-assembly into high molecular weight species is favored by the presence of Mg^{2+} as well as the presence of four or more bases in the terminal G_y segment. These results also suggest that the structure of telomeric DNA, which possesses similar sequences, may be quite unusual.

There has been considerable interest in the development of novel biomaterials for use in nanotechnology applications. For example, the microstructure of these materials allows for their use as scaffolding for attachment of reactive moieties in order to generate synthetic enzymes. The polymorphic nature of DNA oligomers suggests that these molecules can be engineered into useful nanotechnology materials. As a case in point, double-stranded DNA has been used to construct a variety of interesting shapes such as cubes and other polyhedra (Chen & Seeman, 1991; Seeman, 1991; Zhang & Seeman, 1994). This groundbreaking work is based upon enzymatic coupling of branched DNA structures. This group has also been interested in the generation of knotted DNA structures. Interestingly, the presence of a Z-forming segment in the sequence of the oligomer used to generate a knot influences the topology of the resultant knot (trefoil vs figure 8) created by carrying out the enzymatic coupling under Z-forming conditions (Du *et al.,* 1995). This work exemplifies how changing environmental conditions can modulate the types of DNA structures formed.

Indeed, the structures available to DNA oligomers are highly dependent upon both the sequence of the oligomers and the conditions under which they are prepared. The sequence and environmental effects (e.g., buffer conditions including pH, ionic strength, nature of the cations present, and temperature) can then be utilized to "tune" a DNA oligomer into a particular structure. In addition, environmental conditions such as the ionic strength of the medium strength and temperature can be altered to induce reversible conformational transitions between various structures. Such control over the structure leads to control over function and/or activity.

We have recently reported the characterization of DNA oligomers with sequences amenable to quadraplex formation (Dai *et al.,* 1995). These oligomers have the general sequence of $C_4T_4G_4T_{1-4}G_4$ and primarily form intramolecular hairpins in 100 mM Na^+, 100 mM K^+, or 20 mM Mg^{+2} as evident by gel electrophoresis and thermal denaturation studies. However, in the presence of both 100 mM K^+ and 20 mM Mg^{+2}, these oligomers form regular multistranded structures of high molecular weight. Marsh and Henderson (1994) reported similar self-assembly using $G_4T_2G_4$ in the presence of 50 mM Na^+ or K^+. Assembly was most efficient in Na^+, yet the addition of even a small amount of K^+ resulted in a greater degree of stabilization. In addition, phosphorylation of the 5′ terminus inhibited the self-assembly. The structures for the supramolecular assemblies proposed by both us and Marsh and Henderson (depicted in Figure 1) are quite similar and were initially based upon consistency with the electrophoretic results. Marsh and Henderson (1994) designated these structures as "G wires". Later, the wirelike structure of these self-assemblies was confirmed by scanning probe microscopy (Marsh et al., 1995). It was reported here that the presence of Mg^{2+} leads to longer structures in the presence of either Na^+ or K^+, an observation consistent with our results.

* To whom correspondence should be addressed.

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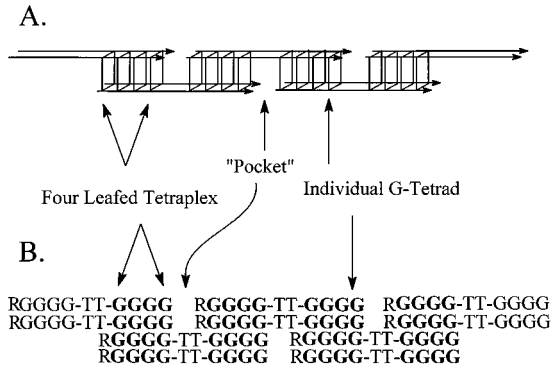

FIGURE 1: Self-assembly of DNA oligomers into multistranded structures. Panel A is a schematic similar to that reported by Marsh and Henderson (1994) for the self-assembly of $G_4T_2G_4$. Panel B is the schematic proposed by Dai *et al.* (1995) for the self-assembly of DNA oligomers of sequence $C_4T_4G_4T_2G_4$ (note, R = C_4T_4). In either representation, contiguous four-leafed tetraplexes, comprised of four G-tetrads, are linked together by two strands of 2 T bases each. Dai *et al.* (1995) also suggested that the linking strands may possess 1−4 T bases and that contiguous tetraplexes could be linked by a single strand possessing 1−4 bases.

Inspection of the proposed structure of the multistranded assembly (Figure 1) suggests its potential as a scaffolding for nanotechnology materials. In order to assess the potential of multistranded assemblies for such applications, a variety of DNA oligomers possessing segments of $G_xT_2G_y$, where x = 1, 3, or 4 and y = 2−5, were designed, synthesized, and subsequently evaluated for their propensity to form multistranded structures. In addition, it was intriguing to us that Marsh and Henderson (1994) observed self-assembly of $G_4T_2G_4$ with just K^+ alone while our oligomers of sequence $C_4T_4G_4T_{1-4}G_4$ did not appreciably self-assemble in K^+ alone but also required Mg^{2+}. Furthermore, a survey of the literature indicates that preparation of quadraplex structures varies from report to report. A study on how sample preparation influences multistranded DNA formation was thus also initiated. The results of these studies are reported here.

MATERIALS AND METHODS

DNA Design, Synthesis, and Purification. Previously reported results (Dai *et al.*, 1995; Marsh & Henderson, 1994) indicated that DNA oligomers possessing $G_4T_{1-4}G_4$ segments self-assemble into multistranded high molecular weight structures (Figure 1) under certain conditions. The DNA oligomers designed for this report, shown in Figure 2, were synthesized in order to evaluate the effect of sequence variations on multistranded structure formation. All oligomers possess a segment of $G_xT_2G_y$ where x = 1, 3, or 4 and y = 2, 3, 4, or 5. The sequences of the abutting segments were also varied.

DNA oligomers were synthesized on an Applied Biosystems 380B DNA synthesizer (Foster City, CA) and purified via trityl select reverse-phase HPLC. After detritylation with 0.2 M acetic acid, a second purification via reverse-phase HPLC was carried out. The samples were then exhaustively dialyzed vs water in Spectra/por MWCO 1000 dialysis bags (Spectrum, Houston, TX). The aqueous solutions were then lyophilized to dryness and the resultant residues were stored in the freezer until ready for use. The typical sample preparation involved reconstitution of the sample in standard TBE buffer (100 mM Tris−borate−EDTA, pH 8.0)

DNA Oligomers

SET I:	***SET III:***
$C_4T_4G_4T_2G_4$	$C_3T_4G_3T_2G_3$
$CATGT_4CATGT_2G_4$	$C_3T_4G_3T_2G_4$
$T_8G_4T_2G_4$	$C_4T_4G_3T_2G_4$
$CATGT_4G_4T_2G_4$	$C_4T_4G_4T_2G_2$
$C_4T_4G_4T_2G_4T_4C_4$	$C_4T_4G_4T_2G_3$
	$C_4T_4G_4T_2G_4$
SET II:	$C_4T_4G_4T_2G_5$
$C_4T_4G_4T_2G_4$	
$C_4T_4G_4T_2G_4T$	***SET IV:***
$CATGT_4G_4T_2G_4$	$C_4T_4G_4T_2G_4$
$CATGT_4G_4T_2G_4T$	$C_4T_4G_4T_2G_2$
$T_8G_4T_2G_4$	
$T_8G_4T_2G_4T$	

FIGURE 2: DNA oligomers under consideration. In order to address certain issues with respect to the self-assembly of DNA oligomers containing $G_xT_2G_y$ segments (highlighted), these oligomers are grouped into sets: set 1 oligomers were used to address sample preparation; set II were used to study the influence of a 3′-terminal T base and temperature effects; set III were used to address the magnitude of x and y; and set IV were studies to address the influence of repeated heating and cooling of samples prior to electrophoresis.

with NaCl, KCl, and/or $MgCl_2$ added to the concentration designated in the figure legends for the gels or CD studies. The samples were then heated to 90 °C for 2 min followed by slow cooling to 4 °C and storage for 48 h at 4 °C prior to electrophoresis. (This is our standard protocol for preparing samples for gel electrophoresis.) Variations from this protocol are noted in the text and/or figure legends.

Polyacrylamide Gel Electrophoresis. Nondenaturing PAGE experiments were carried out using the DNA oligomers prepared as described above in standard TBE buffer with NaCl, KCl, and/or $MgCl_2$ added as noted above. The same buffer was used in the polymerization of the acrylamide for the gels as well for running buffer. Electrophoresis was carried out using 16% total acrylamide [19:1 acrylamide: bis(acrylamide)] at 8 V/cm and 4 °C (unless otherwise noted). Visualization of the DNA bands was carried out by treatment with Stainsall (Bio-Rad, Richmond, CA). Permanent records of the gels were made using Kodak electrophoresis duplicating paper (Eastman, Rochester, NY). Tracking dye consisted of xylene cyanol FF and bromphenol blue (Bio-Rad, Richmond, CA). Typically, 10−15 μg of DNA oligomer was loaded onto the gel.

Circular Dichroism Studies. CD spectra were obtained with an Aviv 62A DS circular dichroism spectropolarimeter (Aviv Associates, Lakewood, NJ) at 25 °C. Samples were prepared by reconstituting lyophilized DNA oligomer into the standard buffer with NaCl, KCl, and/or $MgCl_2$ added to the concentrations noted in the figure legend for the CD results, heating at 90 °C for 2 min, slow cooling back to room temperature, and then equilibrating at 4 °C for 48 h prior to the determination.

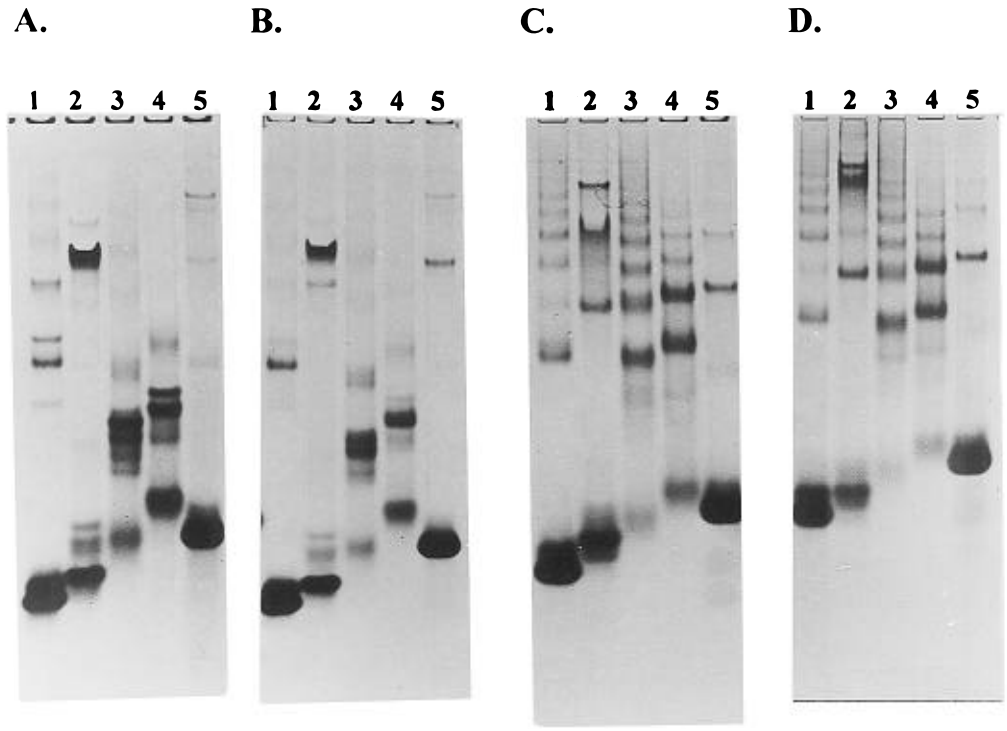

FIGURE 3: Influence of sample preparation on the self-assembly of set I oligomers in standard TBE buffer at 4 °C with (A) 50 mM Na^+/50 mM K^+, (B) 100 mM K^+, (C) 50 mM K^+/20 mM Mg^{2+}, and (D) 100 mM K^+/20 mM Mg^{2+}. Lane 1, $C_4T_4G_4T_2G_4$; lane 2, $CATGT_4$-$CATGT_2G_4$; lane 3, $T_8G_4T_2G_4$; lane 4, $CATGT_4G_4T_2G_4$; lane 5, $C_4T_4G_4T_2G_4T_4C_4$. Each sample was reconstituted in the same buffer as the running buffer specified, heated for 2 min at 90 °C, allowed to slowly cool to 4 °C, and stored at 4 °C for 48 h before electrophoresis. Running dye was added to each sample just prior to being loaded into the well.

RESULTS

The DNA oligomers designed for this study are shown in Figure 2 and are grouped into four different sets. Set I oligomers typically possess one segment of $G_4T_2G_4$, with the exception of $CATGT_4CATGT_2G_4$. This set was generated to study the effects of sample preparation on the self-assembly. Set II oligomer all possess a single $G_4T_2G_4$. The sequence of the segment abutting the 5′ terminus of the $G_4T_2G_4$ is either C_4T_4, $CATGT_4$, or T_8 . An additional T base is present on the 3′ termini of the $G_4T_2G_4$ segment on three of the oligomers. This set was included to study the effect of the presence of the 3′-terminal T base and temperature on the self-assembly. Set III oligomers have the general sequence of $C_mT_4G_xT_2G_y$ (where $m = 3$ or 4, $x = 3$ or 4, and $y = 2-5$) and were designed to address how variations in *m*, *x*, and *y* influence the self-assembly. Set IV oligomers ($C_4T_4G_4T_2G_4$ and $C_4T_4G_4T_2G_2$) were used to study the influence of repeated heating and cooling on the extent of self-assembly. Common to all sets is $C_4T_4G_4T_2G_4$ which was designated as HP1-T2G4 in Dai *et al.* (1995).

The electrophoretic gels of set I oligomers prepared and run under different conditions are shown in Figure 3. As evidenced by the similarities of the number of bands, their mobilities, and intensities in the the electrophoretic patterns obtained in 50 mM Na^+/50 mM K^+ (panel A), 100 mM K^+ (panel B), and 100 mM Na^+ (data not shown), the types of species generated under these three different conditions are quite similar. Similar electrophoretic patterns are also obtained when the gels are run in the presence of 20 mM Mg^{2+} with either 50 mM K^+ (panel C) or 100 mM K^+ (panel D). However, the electrophoretic patterns obtained in the absence of Mg^{2+} are quite different from those obtained in its presence. In the absence of Mg^{2+}, only a few major species are observed for each oligomer of the set although a number of minor species are detected. In the presence of Mg^{2+}, regular ladders of bands are observed for those oligomers with $G_4T_2G_4$ segments (lanes 1, 3, 4, and 5) similar to that observed in the self-assembly of oligomers of sequence $C_4T_4G_4T_{1-4}G_4$ in 20 mM Mg^{2+} and 100 mM K^+ (Dai *et al.*, 1995). The one oligomer with a single G_4 segment (lane 2) does not form a ladder of bands and thus does not self-assemble in the same fashion as those with two G_4 segments. For the oligomers where two G_4 segments are present, Mg^{2+} apparently facilitates the self-assembly in some fashion.

Circular dichroism (CD) determinations have been used to distinguish the various conformations of quadraplex-forming DNA oligomers (Balagurumoorthy *et al.*, 1992; Guo *et al.*, 1993; Lu *et al.*, 1993; Giraldo *et al.*, 1994; Chen, 1995; Dai *et al.*, 1995). Inspection of the CD spectra of $C_4T_4G_4T_2G_4$, $T_8G_4T_2G_4$, and, for comparison, $G_4T_2G_4$ shown in Figure 4 indicate that each oligomer has a unique CD spectrum in the presence of Na^+ alone but similar spectra in either K^+ alone or K^+ with Mg^{2+}. The CD spectra of $C_4T_4G_4T_2G_4$ under all three conditions are quite similar with shallow troughs at 238 nm, peaks at 259 nm, and slight shoulders at 289 nm, all of similar ellipticities. The CD spectrum of $T_8G_4T_2G_4$ in Na^+ is characterized by a peak at 250 nm, a trough at 264 nm, and a peak at 282 nm with a shoulder at 298 nm. However, in the presence of either K^+ alone or K^+ with Mg^{2+}, each spectrum has a trough at 242 nm and a peak at 266 nm. In addition, the ellipticities of the troughs and peaks are nearly identical under the two salt conditions with K^+. Finally, the CD spectrum of $G_4T_2G_4$ in Na^+ is characterized by a peak at 256 nm, a shallow trough at 275 nm, and a peak at 294 nm. In either K^+ alone or K^+ with

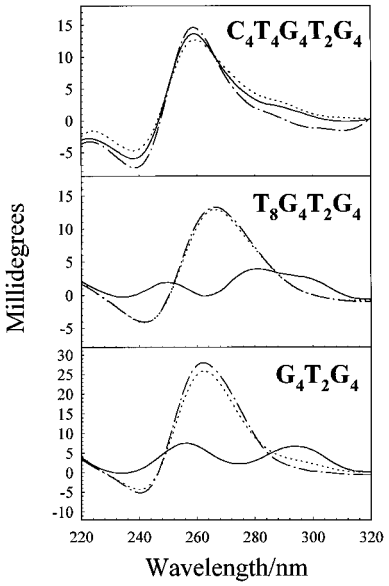

FIGURE 4: Circular dichroism spectra of of select set I oligomers, as well as $G_4T_2G_4$ for comparison, prepared as described under Materials and Methods under various salt conditions. Samples were prepared in standard phosphate buffer plus 100 mM Na^+ (solid line); 100 mM K^+ (dotted line), or 100 mM K^+ and 20 mM Mg^{+2} (dashed−dotted line).

Mg^{2+}, the spectra are characterized by shallow troughs at 240 nm and peaks at 262 nm, all of similar ellipticities. Hence, the CD are unique for each oligomer under all conditions in terms of the exact wavelength values for the peaks. In addition, the ellipticities for the peaks in the K^+ alone or K^+ with Mg^{2+} are much greater for $G_4T_2G_4$ than for either $C_4T_4G_4T_2G_4$ or $T_8G_4T_2G_4$. Finally, the ellipticity of the peak in the K^+ with Mg^{2+} slightly decreases over time for $C_4T_4G_4T_2G_4$ and $T_8G_4T_2G_4$ (4−6%) but dramatically decreases for $G_4T_2G_4$ (ca. 21%).

The electrophoretic gels of oligomers from set II are shown in Figure 5. It should be noted that set II contains three members from set I plus analogues possessing an additional 3′-T base. As can be seen, self-assembly occurs at 4, 20, and 37 °C (panels A, B, and C, respectively). Self-assembly of these oligomers has also been observed at temperatures up to 65 °C (data not shown). Thus, these assemblies are quite stable. The presence of the 3′-T base apparently does not inhibit self-assembly of $C_4T_4G_4T_2G_4$ at either 4 or 20 °C (compare lanes 1 and 4, panels A and B) but does inhibit at 37 °C (lanes 1 and 4, panel C). The presence of the 3′-T base on $T_8G_4T_2G_4$ or $CATGT_4G_4T_2G_4$ results in two effects: (1) the appearance of an additional band with mobility higher than the other bands (compare lanes 2 and 5 and lanes 3 and 6, all panels) and (2) inhibition of the self-assembly at the higher temperatures (same lanes, panels B and C).

Electrophoresis of the set III oligomers was carried out to primarily study the influence of the number of G bases in both the internal and terminal G-runs on the extent of self-assembly. Electrophoresis was carried out in either 100 mM K^+ alone or 100 mM K^+ with 20 mM Mg^{2+} (Figure 6, panels A and B, respectively). Consistent with the observations with the set I oligomers, the presence of Mg^{2+} facilitates self-assembly for all oligomers. For the oligomer $C_4T_4G_4T_2G_5$ (lane 7), self-assembly occurs under both conditions. However, close examination of the gel reveals slight differences in the patterns obtained under the two different conditions. The densitometer scans of Lane 7 under both conditions (Figure 7) clearly shows that the bands have different mobilities and intensities under the two conditions. Also evident is the nearly complete conversion of the hairpin form to the self-assembled form in the presence of Mg^{2+}.

For all oligomers of this set, self-assembly seems to depend more on the number of G bases in the terminal segment than the internal G segment. The self-assembly is more extensive with a G_4 or G_5 terminal segment (lanes 2, 3, 6, and 7, panel B). The oligomers with internal G_3 segments self-assemble to a greater extent when they possess a terminal G_4 (lanes 1−3, panel B), and those oligomers with an internal G_4 segment self-assemble to a lesser extent when they possess a terminal G_2 or G_3 segment (lanes 4−7, panel B). The number of C bases in the 5′- terminal C_m segment apparently does not influence the extent of self-assembly (see Discussion).

In the experiments described above for the oligomers of sets I and III, the same protocol for preparing samples for electrophoresis was followed: the lyophilized sample was dissolved in the buffer of choice, heated at 90 °C for 2 min, slowly cooled to 4 °C, and then allowed to equilibrate at 4 °C for 24 h prior to loading on the gel. The same protocol was applied to the set II oligomers; however, some were also incubated at 20 or 37 °C prior to electrophoresis at those temperatures. A modified protocol was used in the preparation of set IV oligomers. Here, the samples were reconstituted in buffer containing either 100 mM K^+ alone or 100 mM K^+ and 20 mM Mg^{2+}. One sample from each buffer condition was then placed directly at 4 °C for 48 h without heating. The other samples were heated to 90 °C for 2 min and allowed to slowly cool to room temperature either one, two, four, or eight times prior to equilibrating at 4 °C for 48 h. The electrophoretic gels obtained from the samples prepared in this fashion are shown in Figure 8. The sample that was not heated displayed a substantial amount of self-assembly in both buffers (lane 1, all gels, panels A and B). However, as the number of repeats of the heat−cool cycle increases, the extent of self-assembly in both buffers decreases (lanes 2−5, all gels, panels A and B). This is particularly evident for $C_4T_4G_4T_2G_2$ (left gels, panels A and B). Again, close comparison of the 100 mM K^+ gels (panel A) to those of the 100 mM K^+ and 20 mM Mg^{2+} (panel B) reveals slight differences in the electrophoretic patterns, particularly for $C_4T_4G_4T_2G_4$.

DISCUSSION

There has been considerable interest from both a structural and a biological point of view in DNA oligomers that possess contiguous G bases and form four-stranded structures. The molecular architecture of the resulting structures is highly dependent upon the sequence of the DNA and the conditions under which it has been prepared. For example, T_4G_4 and T_2G_4 form simple parallel-stranded quadraplexes (Sen & Gilbert, 1988; Lu *et al.*, 1993; Wang & Patel, 1992; Gupta *et al.*, 1993; Williamson, 1994). An early study on $G_4T_xG_4$ oligomers indicated a four-stranded quadraplex when $x = 1$, a quadraplex formed from the association of two foldback structures when $x = 3$ or 4, and a mixture of the two possibilities when $x = 2$ (Balagurumoorthy *et al.*, 1992). More recently, NMR studies have shown that the preferred conformation for $G_4T_4G_4$ is the bimolecular foldback struc-

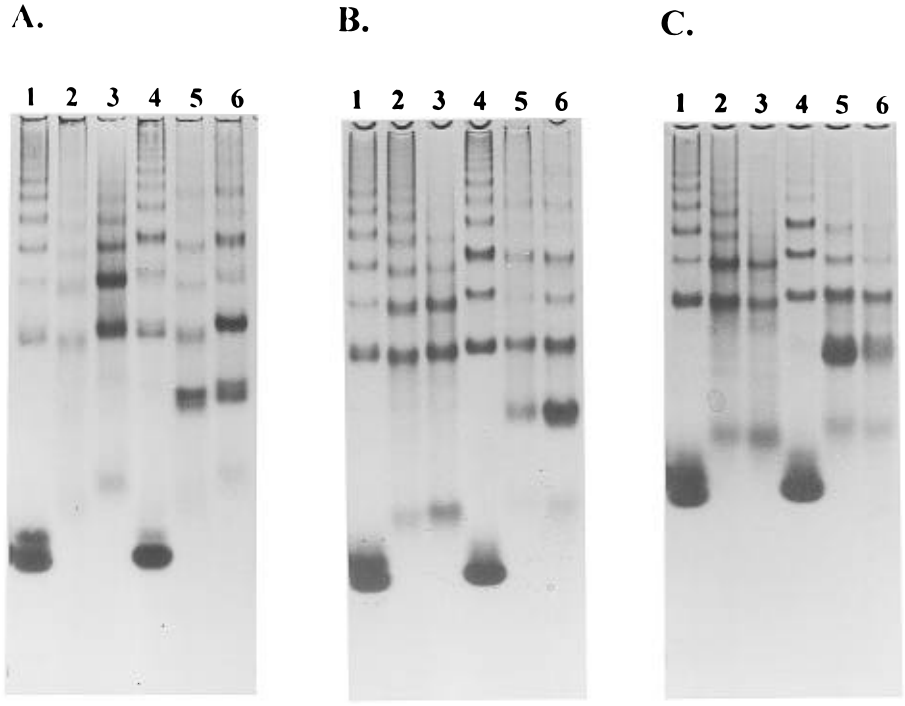

FIGURE 5: Influence of 3′-T base and temperature on the self-assembly of set II oligomers in standard buffer with 100 mM K^+ and 20 mM Mg^{2+} at (A) 4 °C, (B) 20 °C, and (C) 37 °C. Lane 1, $C_4T_4G_4T_2G_4$; lane 2, $CATGT_4G_4T_2G_4$; lane 3, $T_8G_4T_2G_4$; lane 4, $C_4T_4G_4T_2G_4T$; lane 5, $CATGT_4G_4T_2G_4T$; lane 6, $T_8G_4T_2G_4T$. Each sample was reconstituted in the running buffer, heated for 2 min at 90 °C, allowed to slowly cool to 4 °C, and stored at 4 °C for 48 h before electrophoresis. The samples were then incubated for 2 h at the gel running temperature followed by addition of running dye just prior to being loaded into the well.

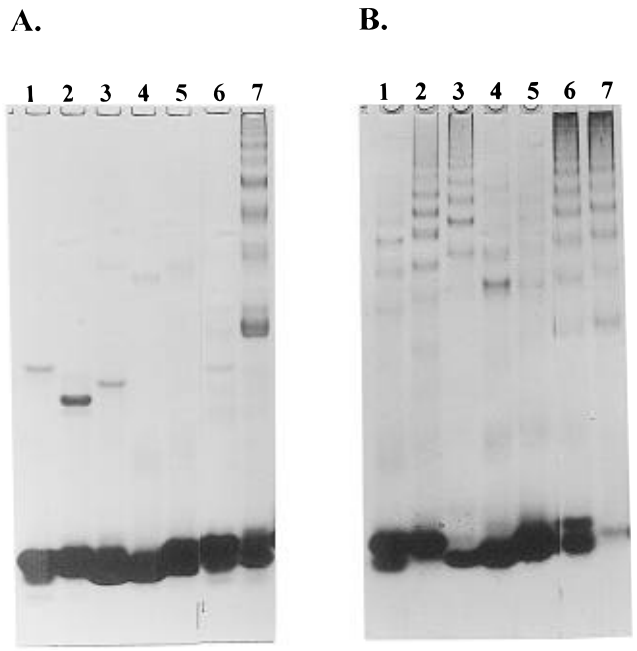

FIGURE 6: Influence of length of the interior G_x ($x = 3$ or 4) and terminal G_y ($y = 2-5$) segments on the self-assembly of set III oligomers. Electrophoresis was carried out at 4 °C in standard buffer with (A) 100 mM K^+ or (B) 100 mM K^+ and 20 mM Mg^{2+}. Lane 1, $C_3T_4G_3T_2G_3$; lane 2, $C_3T_4G_3T_2G_4$; lane 3, $C_4T_4G_3T_2G_4$; lane 4, $C_4T_4G_4T_2G_2$; lane 5, $C_4T_4G_4T_2G_3$; lane 6, $C_4T_4G_4T_2G_4$; and lane 7, $C_4T_4G_4T_2G_5$. Each sample was reconstituted in the same buffer as the running buffer specified, heated for 2 min at 90 °C, allowed to slowly cool to 4 °C, and stored at 4 °C for 48 h before electrophoresis. Running dye was added to each sample just prior to being loaded into the well.

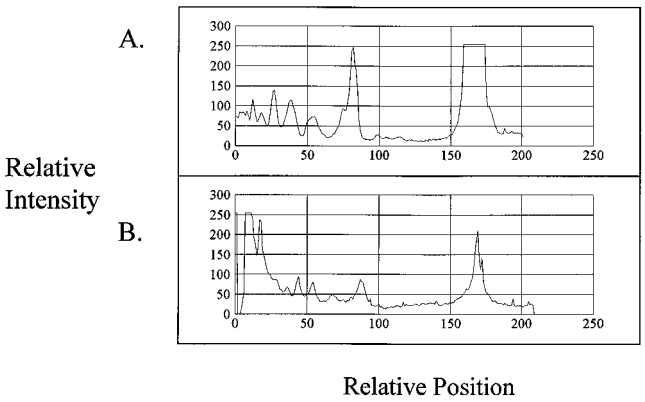

FIGURE 7: Densitometer scans of the electrophoretic gel of $C_4T_4G_4T_2G_5$ (lane 7, panels A and B from Figure 6) prepared and run in (A) 100 mM K^+ or (B) 100 mM K^+ with 20 mM Mg^{2+}.

ture. The exact orientation of the T_4 loops is still under debate (Kang *et al.*, 1992; Smith & Feigon, 1992; 1993; Mohanty & Bansal, 1994; Strahan *et al.*, 1994). It is then quite surprising that two independent groups observed multistranded structures for oligomers possessing $G_4T_{1-4}G_4$ sequences (Dai *et al.*, 1995, Marsh & Henderson, 1994).

As indicated above, environmental conditions also play a role in the formation of four-stranded structures. It has been noted that K^+ is more effective in inducing quadraplex formation than Na^+ for simple oligomers with single G_x ($x = 2-4$) segments such as T_4G_4, T_4G_4T, or T_9G_3 and may give rise to different conformations and/or different stabilities (Sen & Gilbert, 1990, 1992; Hardin *et al.*, 1992; Lu *et al.*, 1993; Aboul-ela *et al.*, 1994; Ross & Hardin, 1994; Williamson, 1994). Initially, Marsh and Henderson (1994) reported multistranded DNA formation for $G_4T_2G_4$ induced by both K^+ and Na^+; however, the assemblies formed in the presence of K^+ were much more stable. In addition, Mg^{2+} has not been shown to have a favorable influence on quadraplex formation (Hardin *et al.*, 1992). However, we observed multistranded DNA structures of $C_4T_4G_4T_{1-4}G_4$

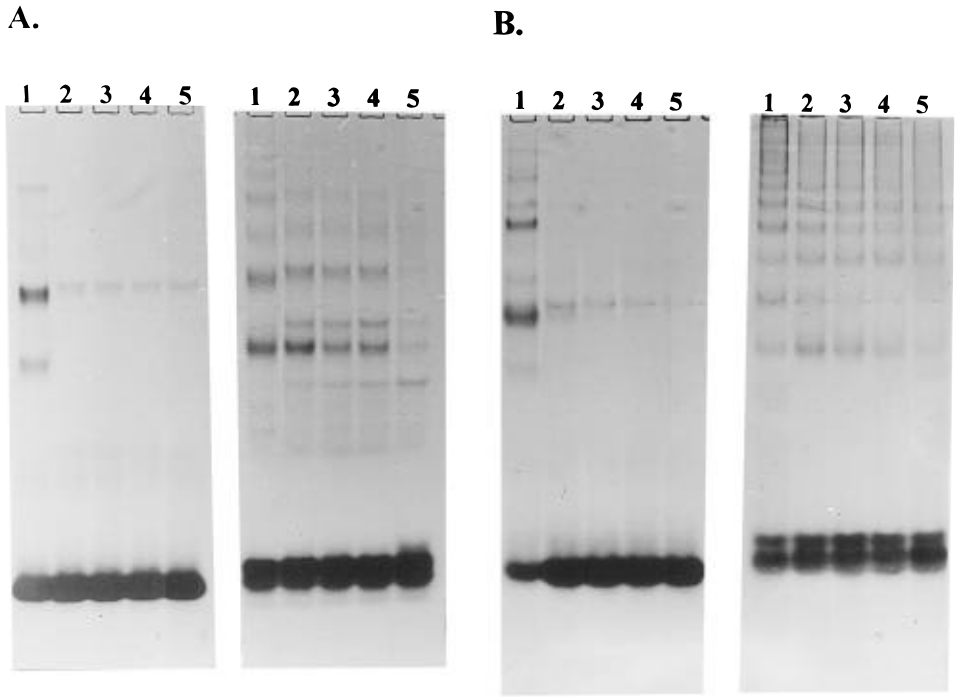

FIGURE 8: Influence of heat cycling of sample on the self-assembly of set IV oligomers. Electrophoresis was carried out at 4 °C with (A) $C_4T_4G_4T_2G_2$ (left gel) or $C_4T_4G_4T_2G_4$ (right gel) in standard buffer and 100 mM K^+ or (B) $C_4T_4G_4T_2G_2$ (left gel) or $C_4T_4G_4T_2G_4$ (right gel) in standard buffer with 100 mM K^+ and 20 mM Mg^{2+}. Each sample was reconstituted in the same buffer as the running buffer specified, heated for 2 min at 90 °C, allowed to slowly cool to 4 °C, and stored at 4 °C for 48. Samples were then reheated to 90 °C and slowly cooled back to 4 °C for, lane 1, zero times; lane 2, one time; lane 3, two times; lane 4, four times; and lane 5, eight times. Running dye was added to each sample just prior to being loaded into the well.

induced by the presence of both K^+ and Mg^{2+} but not with K^+ alone (Dai *et al.*, 1995). This may be due to the observation that our oligomers form quite stable hairpins and, therefore, must first form single-stranded species before forming quadraplexes. It may simply be that the free energy required to denature the hairpin in K^+ alone is not meet by the free energy of quadraplex formation. Thus, Mg^{2+} must be playing a crucial role in the multistranded formation for these oligomers. It has also been observed that the "G-wires" formed from $G_4T_2G_4$ were much longer when formed in the presence of Na^+ and Mg^{2+} or K^+ and Mg^{2+} as indicated by scanning probe microscopy studies (Marsh et al., 1995). This again indicates that Mg^{2+} is an important contributor to the stability and/or formation of these structures. Finally, our initial results showed that such self-assembly was only observed with those DNA oligomers possessing two G_4 segments (Dai *et al.*, 1995). The ability of oligomers with longer or multiple G_x segments to self-assemble has not been reported at this time.

The results presented here indicate that multistranded structure formation is influenced by both the sequence of the DNA oligomer possessing the $G_xT_2G_y$ segments as well as sample preparation. Set I oligomers were studied to test the influence of sample preparation with respect to concentrations and identities of cations present. All set I oligomers form higher order structures under the various experimental conditions. The similarity of the gel patterns for electrophoresis in either 50 mM Na^+ with 50 mM K^+ or 100 mM K^+ alone indicates that the species generated under these conditions are similar. The band intensities suggest that only certain species are favored under these conditions. Unfortunately, such gel electrophoresis studies cannot reveal the structure of these species. It is likely that the species that give rise to the low-mobility bands that are at least four-stranded.

With the exception of $CATGT_4CATGT_2G_4$, the presence of Mg^{2+} results in a well-defined pattern of low-mobility bands for the other set I oligomers. These gel patterns are consistent with multistranded structure formation. The similarities of the patterns obtained under the two different sets of conditions with Mg^{2+} also indicates that higher order structure formation is influenced by the cations present, not their individual ionic strengths nor the total ionic strength.

The band of highest mobility for $C_4T_4G_4T_2G_4$ has previously been assigned as arising from an intramolecular hairpin formed from the Watson−Crick association of the C_4 segment to the internal G_4 segment (Dai *et al.*, 1995). Apparently, $CATGT_4CATGT_2G_4$ also forms a similar intramolecular hairpin as evidenced by the band of similar mobility to that assigned to the hairpin form of $C_4T_4G_4T_2G_4$. However, $CATGT_4CATGT_2G_4$ does not form the same type of higher order structures that the other members of the set do in the presence of Mg^{2+}. The other members of the set each possess a $G_4T_2G_4$ segment at the 3′-terminus, whereas $CATGT_4CATGT_2G_4$ possess a GT_2G_4 segment. Having only one interior G may be insufficient to allow multistrand structure formation. This conclusion is supported by the results obtained with the set III oligomers (see below). The absence of a high-mobility band for $T_8G_4T_2G_4$ is consistent with its inability to form a Watson−Crick hairpin. The faint high-mobility band observed for $CATGT_4G_4T_2G_4$ is most likely due to single-stranded structure since this oligomer is also incapable of forming such a hairpin. Finally, the high-mobility band of high intensity for $C_4T_4G_4T_2G_4T_4C_4$ is most likely due to formation of a double hairpin arising from the association of both terminal C_4 segments with their nearest internal G_4 segment. Although this oligomer does self-assemble in the presence of Mg^{2+}, the degree of multistranded structure formation is noticeable less than for the

other oligomers possessing a $G_4T_2G_4$ segment. Higher order formation for this oligomer would require the denaturation of two intramolecular hairpins prior to association. The bands arising from those oligomers which can form intramolecular hairpins are quite intense, indicating a large population of this species.

It is surprising that the CD spectra of $C_4T_4G_4T_2G_4$ are similar under the three different conditions noted in Figure 4. We previously reported that this oligomer behaves like a hairpinned duplex in the presence of 100 mM Na^+ or 100 mM K^+ as evidenced by DNA concentration-independent thermal denaturation profiles (Dai *et al.,* 1995). However, the CD spectra under these conditions are similar to those obtained in the presence of both K^+ and Mg^{2+}, conditions which favor self-assembly. Even under these conditions, however, a substantial amount of the DNA oligomer exists as the Watson−Crick hairpin as noted above. Hence the resulting CD spectra represent the weighted average of all molecular species. All oligomers tested in either K^+ or K^+ with Mg^{2+} in the CD study gave spectra similar to those expected for a parallel-stranded quadraplex (Balagurumoorthy *et al.,* 1992; Lu *et al.,* 1993; Guo *et al.,* 1993; Giraldo *et al., 1994;* Dai *et al.,* 1995). The CD spectra of $T_8G_4T_2G_4$ and $G_4T_2G_4$ are similar to that expected for a quadraplex formed by the bimolecular association of two foldback species (Balagurumoorthy *et al.,* 1992). The shoulder observed for $T_8G_4T_2G_4$ at 298 nm may be due to the single-stranded T_8 segment. Unfortunately, it appears then that CD cannot distinguish between the various forms of $C_4T_4G_4T_2G_4$, nor between a simple parallel four-stranded tetraplex and the multistranded assemblies observed on the gels.

The multistranded structures arising from the set II oligomers are quite stable at temperatures ranging from 4 to 37 °C. Marsh and Henderson (1994) reported that the "G-wires" produced by $G_4T_2G_4$ were maximal at 37 °C. We have even seen multistranded DNA structure at 65 °C but did not show the gel due to its poor resolution. Marsh and Henderson (1994) also reported that phosphorylation of the 3′ terminus of $G_4T_2G_4$ resuilted in diminished "wire" formation. The presence of a T base on the 3′-terminus of members of the set II oligomers also inhibits, but does not completely block, multistranded structure formation. Sen and Gilbert (1992) observed aggregation of the quadraplexes formed from T_9G_3 in the presence of K^+ or Rb^+. They attributed this formation to species arising from the end-on-end association of "slipped" quadraplexes. The presence of a T base on the 3′ terminus of their oligomer completely inhibited aggregation. The formation of multistranded structures with our oligomers possessing 3′-T bases, although inhibited, suggests that these oligomers are not forming similar "slipped" structures.

The bands of highest mobility in lanes 1 and 4 (Figure 5) are again due to the intramolecular hairpin. These bands are fairly intense, indicating a large population of this structure. The bands of highest mobility for the other oligomers of the set are most likely due to the unassociated single strands. These bands are quite faint, suggesting that most of the DNA is involved in multistranded structures. The presence of an additional T base on the 3′ terminus of $CATGT_4G_4T_2G_4$ and $T_8G_4T_2G_4$ gives rise to an additional band of high intensity. The mobilities of these additional bands would suggest that they arise from the quadraplex generated by the association of two foldback hairpins. The analogues without the 3′-T base could also form such structures, yet the absence of bands of similar mobilities indicates that this is not the case unless the 3′-T base plays a dramatic role in the stability of those quadraplexes. At this point, we cannot say with any certainty what species is responsible for those particular high-intensity bands.

The oligomers in set III were studied to assess sequence effects on self-assembly. Specifically, what is the minimum number of G bases in the interior G-segment and/or the terminal G-segment required for higher order structure formation? We previously reported that two G_4 segments, separated by one to four T bases was necessary for self-assembly into high molecular weight species (Dai *et al.,* 1995). In addition, the abilities of these molecules to form hairpins may limit their higher order structure formation, as noted for $C_4T_4G_4T_2G_4$. In order for this oligomer to form any higher order structure, the hairpin must denature at some point in time. This process would be energetically unfavorable at the temperatures used for the equilibration and subsequent gel electrophoresis experiments and therefore must be offset by a favorable free energy of quadraplex formation (Dai *et al.,* 1995). The intense band of highest mobility observed for each oligomer under both salt conditions indicates a high degree of hairpin formation.

In 100 mM K^+ alone, each oligomer with three interior G-bases gives rise to an additional, moderately mobile band of moderate intensity. The mobilities of these bands are similar to those observed with sequences of $C_4T_4G_4T_{1-6}$, which can form simple four-stranded quadraplexes (Dai *et al.,* 1995). The species responsible for the set III oligomers with three interior G-bases may be similar. For the set III oligomers with four interior bases, only $C_4T_4G_4T_2G_4$ self-assembles to any extent in 100 mM K^+. In 100 mM K^+ plus 20 mM Mg^{2+}, all oligomers form higher order structure as indicated by the ladders of bands observed. It is also interesting to note that those oligomers with four terminal G-bases apparently self-assemble to a greater extent. In addition, the set of species generated by $C_4T_4G_4T_2G_5$ in the presence of K^+ alone are different from those generated with Mg^{2+} also present.

The stabilities of the higher order structures will certainly depend upon the number of G-tetrad leaflets per quadraplex. As seen in Figure 1, the self-assembly of an oligomer with a $G_4T_2G_4$ segment could give rise to multistranded structure comprised of quadraplexes possessing four G-tetrad leaflets linked by two strands of T_2. Any oligomer with less than four G bases in either the internal or terminal segments would generate quadraplexes with less than four G-tetrad leaflets. Hence the failure of $C_4T_4G_4T_2G_2$ to self-assemble to an appreciable extent may simply be due to the lower stability of quadraplexes possessing only two G-tetrad leaflets. However, quadraplexes possessing one and two G-tetrad leaflets have been observed for oligomers such as $CGCG_4CGC$ (Hardin *et al.,* 1992) and $d(CGG)_n$, where $n = 4$, 5, or 6 (Fry & Loeb, 1994; Chen, 1995). In addition, quadraplexes possessing three G-tetrad leaflets are quite stable (Jin *et al.,* 1992). Hence set III oligomers with three G bases in the internal segment, the terminal segment, or both should form quite stable three-leafed quadraplexes. Visual inspection of the gel depicted in panel B of Figure 7 indicates that only those oligomers with three interior G bases and four terminal G bases self-assemble to any appreciable extent. Even the

oligomer with four internal G bases and three terminal G bases does not self-assemble to a high degree.

These observations suggest that the number of G bases in the termini is crucial for self-assembly. All of the set III oligomers form intramolecular hairpins. These hairpins arise from the Watson−Crick association of the 5′-terminal C-segment with the interior G-segment and thus are composed of a 3- or 4-base paired stem, a T_4 loop, and a single-stranded segment (T_2G_{2-5}) at the 3′-terminal of the stem. [Complexation of the C segment to the terminal G-segment would give rise to a less stable hairpin (Dai *et al.*, 1995).] The results are consistent with the following mechanism: association of the terminal G-bases of 2−4 strands followed by hairpin denaturation, and possibly strand exchange, prior to further self-assembly. Hence, those oligomers with four or five terminal G-bases would readily self-assemble and generate enough free energy to induce hairpin denaturation. Those oligomers with three or fewer G-bases may still self-assemble but may not generate enough free energy to denature the hairpins. Previously, we reported that the free energy of denaturation of a hairpin with four G−C base pairs and an T_4 loop to be *ca.* 4.0 kcal/mol in 200 mM NaCl. Jin *et al.* (1992) reported a free energy of quadraplex formation for TG_3T to be −6.9 kcal/mol in 1.0 M KCl or −2.4 kcal/mol per tetrad. Hence, under our self-assembly conditions (i.e., 100 mM K^+/20 mM Mg^{2+}), the free energy arguments above are quite reasonable. Certainly, further investigations into the mechanism of the self-assembly are warranted.

The data presented in Figure 8 are also quite interesting. The normal protocol for preparing samples calls for reconstitution in the standard phosphate buffer with NaCl, KCl, and/or $MgCl_2$ added, followed by heating at 90 °C for 2 min, slow cooling to 4 °C, and equilibration at 4 °C for 48 h before loading the sample onto the gel. In lane 1 of all gels, the heating step was omitted and resulted in a substantial amount of self-assembly for both oligomers under both buffer conditions. Lane 2 in each gel contains samples prepared by the typical protocol. As can be seen, just one heating cycle substantially eliminates the self-assembly for $C_4T_4G_4T_2G_2$ and slightly decreases self-assembly for $C_4T_4G_4T_2G_4$ under both buffer conditions. Samples in lanes 3−5 were subjected to cyclic heating and cooling prior to the 48-h, 4 °C equilibration period. It appears that as the number of heating and cooling cycles increases, the extent of self-assembly decreases even further for $C_4T_4G_4T_2G_2$ and noticeably for $C_4T_4G_4T_2G_5$ in K^+. Only a slight inhibition of self-assembly is observed with this oligomer in K^+/Mg^{2+}.

These data suggest that self-assembly is quite fast and may occur before the heating step is carried out. The heating step is carried out to ensure that all DNA oligomers are single-stranded prior to any association. In the case of $C_4T_4G_4T_2G_2$, the denaturation of the self-assembled structures is nearly complete after only one heating. The hairpin form is thus apparently the most stable form of this oligomer. $C_4T_4G_4T_2G_4$ also readily self-assembles to a quite stable structure. Repeated heating and cooling is insufficient to ensure full denaturation to single strands. The data are also consistent with the suggestion that the self-assembled structures are more stable in K^+/Mg^{2+} than in K^+ alone.

SUMMARY

The results presented and discussed above indicate that higher order formation arising from the self-assembly of DNA oligomers with $G_xT_2G_y$ segments depends upon both the sequence of the DNA oligomer and the conditions under which the samples are prepared. Self-assembly into multi-stranded structures is favored by (1) the presence of Mg^{2+} in the equilibration buffer, (2) the presence of four or more G bases at the 3′-terminus (i.e., $y \geq 4$), and (3) limited heating of the sample after reconstitution in buffer. From a biological point of view, these results indicate that telomeric DNA, which possesses G-rich segments, may have very unusual structures. From a nanotechnology point of view, these results can be used to design DNA oligomers with the potential of forming reversible nanostructures.

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