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Formation and structural determinants of multi-stranded guanine-rich DNA complexes ☆

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Abstract

We have investigated the complexes formed by oligonucleotides with the general sequence $d(T_{15}G_n)$, where $n = 4–15$. Two distinct classes of structures are formed, namely, the four-stranded tetraplex and frayed wires. Frayed wires differ from four-stranded tetraplexes in both strand association stoichiometry and the ability of dimethyl sulfate to methylate the N7 position of guanine. Thus, it appears that these two guanine-rich multistranded assemblies are stabilised by different guanine–guanine interactions. The number of contiguous guanine residues determines which of the complexes is favoured. Based on the stoichiometry of the associated species and the accessibility of the N7 position of guanine to methylation we have found that oligonucleotides with smaller number of contiguous guanines; $n = 5–8$, form primarily four-stranded tetraplex. Oligonucleotides with larger numbers of contiguous guanines adapt primarily the frayed wire structure. The stability of the complexes formed by this series of oligonucleotides is determined by the number and arrangement of the guanines within the sequences. We propose that the formation of the two types of complex proceed by a parallel reaction pathways that may share common intermediates.

Keywords: DNA structure; Guanine; Telomere; Tetraplex; Frayed wires; Electrophoresis

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1. Introduction

Guanine can self-associate in aqueous solution to form highly ordered structures such as the tetrameric planar aggregates formed by guanosine-3-phosphate or the helical structures formed by guanosine-5-phosphate [1]. Oligonucleotides with several consecutive terminal guanine bases self-associate via intermolecular interactions between the guanine residues to give rise to a number of different types of DNA structures [2–7,9–11]. The structures exhibit distinguishing chemical [12], spectroscopic [13], and physical properties [14]. The nature of the complex formed by an oligonucleotide depends in part on the base sequence and the fraction of guanine residues. Telomeric DNA sequences containing guanine-rich repeats, for example $d(T_2G_4)_n$, self-associate to form four-stranded tetraplex structures [8]. In these complexes, each guanine is involved in two asymmetric hydrogen bonds with each of the neighbouring guanines, resulting in a four-base G-tetrad. A four-stranded helix arises from the stacking of consecutive G tetrads. One of the characteristics of the four-stranded helix is the presence of metal ions co-ordinated by the guanines within the axis of the helix. These ions further stabilise the four-stranded structure [15]. The dimensions of the cavity between the four guanines restrict the ionic radius, and hence the nature of the cation that can be located within the four-stranded helix [16].

We have reported that oligonucleotides such as $d(T_{15}G_{15})$ form stable multistranded complexes, which we call DNA frayed wires [7,12,17]. DNA frayed wires display cation preference and strand association stoichiometry [12] which are distinct from those of telomeric complexes [12]. Frayed wire formation is favoured by magnesium ions while telomeric complexes are stabilised by potassium ions. Complexes formed by telomeric sequences consist of two, four, or multiples of four DNA strands, whereas frayed wires are complexes consisting of 1,2,3,4,5,...oligonucleotides [12]. Methylation at N7 sites of the guanine of the parent oligonucleotide of DNA frayed wire does not hinder formation of the associated structures [12]. However, methylation of the guanine N7 of the parent telomeric oligonucleotide abolishes its ability to form stable aggregates [12]. This observation supports the notion that the guanine N7 sites of frayed wires do not participate in the stabilisation of the associated complex. This is consistent with the observed differences in the sensitivity of frayed wires and the complexes formed by oligonucleotides with a telomeric sequence toward degradation by nucleases [17].

Thus, despite the apparent similarity of oligonucleotides such as $d(T_{15}G_{15})$ and $d(T_{15}G_4T_2G_4)$, self-aggregated species they form arise from different guanine–guanine interactions. In the present study, we have explored the importance of the number of consecutive guanines in determining the type of aggregate formed by examining the behaviour of a series of parent oligonucleotides $d(T_{15}G_n)$, where $n = 4–15$.

2. Material and methods

2.1. DNA oligonucleotide

The DNA oligonucleotides were purchased from the Centre for Applied Genomics, Hospital for Sick Children, Toronto. The deprotected oligonucleotides were purified by cartridge, and lyophilised.

2.2. High-order structure formation

The oligonucleotides with the general sequence $d(T_{15}G_n)$ (Table 1), $n = 4–15$, were dissolved in 89 mM Tris, 89 mM Boric acid, 2 mM EDTA, pH 8 (TBE). We labelled the oligonucleotides with ^{32}P using [γ-^{32}P] ATP and T4 polynucleotide kinase. Unincorporated ^{32}P was removed and the sample was desalted by means of a P-6 Bio-Spin chromatography column (Bio-Rad, Inc). Formation of high molecular weight structures by $d(T_{15}G_n)$ was induced by the addition of cations to the solution to a final concentration of 20 mM or 1 M $MgCl_2$ or 20 mM KCl, followed by heating at 90°C for 30 min. A similar procedure was used for $d(T_{15}I_{15})$ but with different cations (Na^+, K^+, Mg^{2+} and NH_4^+) at concentrations ranging from 0.0625 to 2 M. The extent of aggregation was

Table 1
Oligonucleotides

$T_{15}G_4$	5′-TTTTTTTTTTTTTTTGGGG-3′
$T_{15}G_5$	5′-TTTTTTTTTTTTTTTGGGGG-3′
$T_{15}G_6$	5′-TTTTTTTTTTTTTTTGGGGGG-3′
$T_{15}G_7$	5′-TTTTTTTTTTTTTTTGGGGGGG-3′
$T_{15}G_8$	5′-TTTTTTTTTTTTTTTGGGGGGGG-3′
$T_{15}G_{10}$	5′-TTTTTTTTTTTTTTTGGGGGGGGGG-3′
$T_{15}G_{11}$	5′-TTTTTTTTTTTTTTTGGGGGGGGGGG-3′
$T_{15}G_{12}$	5′-TTTTTTTTTTTTTTTGGGGGGGGGGGG-3′
$T_{15}G_{15}$	5′-TTTTTTTTTTTTTTTGGGGGGGGGGGGGGG-3′
$T_{15}I_{15}$	5′-TTTTTTTTTTTTTTTIIIIIIIIIIIIIII-3′

analysed using gel electrophoresis in 15% polyacrylamide under native and denaturing (7 M urea, run at 55°C) conditions. In both cases, TBE was used as the running buffer. After electrophoresis, the gels were dried under vacuum and analysed using an Ambis Model 4000 radioanalytic imaging system (Ambis Inc., Billerica, MA, USA).

2.3. Methylation

We used the standard Maxam–Gilbert sequencing procedure to assess the availability of N7 position of guanine to methylation. The procedure used in this study was similar to that used in our previous study [12]. Whenever the N7 site at the guanine base does not engage in hydrogen bond, it can be methylated by dimethyl sulfate and subsequently cleaved by piperidine. The presence of bands in the denaturing gel of the subsequent analysis indicates that the N7 site of the corresponding guanine base does not engage in stable hydrogen bonding. High molecular weight aggregated structures were prepared by dissolving ^{32}P-labelled $d(T_{15}G_n)$ in 1 M $MgCl_2$. After the methylation reaction with dimethyl sulfate, we separated the methylated samples on a native polyacrylamide gel with TBE as the running buffer. The wet gel was then analysed with the Ambis imager for 15 min. With the help of the scanned image, we located and excised the region of the gel containing the high molecular weight structures. These samples were eluted from the gel and cleaved with piperidine [12]. The cleaved samples were analysed by electrophoresis in a denaturing 20% polyacrylamide gel.

In other experiments, the DNA oligomers were methylated prior to exposure to magnesium chloride. In this case the oligonucleotides at 1 μM (strands) were methylated as above. $MgCl_2$ was added to the solution containing the methylated oligomers to a final concentration of 1 M $MgCl_2$ and then heated at 90°C for 30 min. The products were analysed by electrophoresis in a native (non-denaturing) polyacrylamide gel.

3. Results

Fig. 1 shows the result of electrophoresis of $d(T_{15}G_n)$, $n = 4–12$, under denaturing conditions. In this figure, the bands at the bottom of the image, i.e. the fastest migrating bands, correspond to the monomeric forms of the parent oligonucleotide. Bands corresponding to slower migrating species result from aggregates of the parent oligonucleotide. Earlier work in this laboratory showed that the first intense band above the parent oligonucleotide of $d(T_{15}G_{12})$ is the trimer, i.e. $[d(T_{15}G_{12})]_3$, and that the bands above the trimer differ from each other by the successive addition of a single strand of the parent oligonucleotide. The relative fraction of high molecular weight species increases with the number of consecutive guanine residues. Clearly, the number of guanines plays an important role in the stability of the aggregated species.

The stability of the aggregates under denaturing conditions is a function of the number of

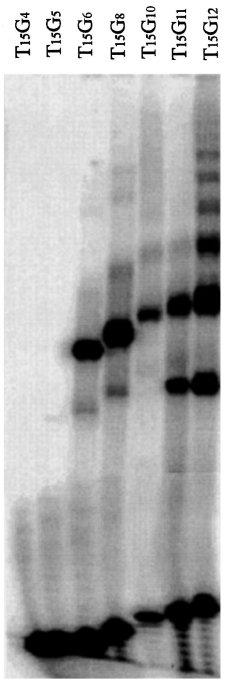

Fig. 1. Oligonucleotides (0.33 μM) were analysed in 15% polyacrylamide gel under denaturing conditions (7 M urea, 55°C): (1) $d(T_{15}G_4)$; (2) $d(T_{15}G_5)$; (3) $d(T_{15}G_6)$; (4) $d(T_{15}G_8)$; (5) $d(T_{15}G_{10})$; (6) $d(T_{15}G_{11})$; and (7) $d(T_{15}G_{12})$.

consecutive guanine residues. The structures formed by the oligonucleotides with the fewest consecutive guanines, $d(T_{15}G_4)$ and $d(T_{15}G_5)$, were not stable under denaturing conditions whereas the aggregates formed by oligonucleotides with larger numbers of contiguous guanines were stable under denaturing conditions. Between these two extremes, the predominant aggregated species formed by $d(T_{15}G_6)$, $d(T_{15}G_8)$ or $d(T_{15}G_{10})$ is a four-stranded complex, e.g. $[d(T_{15}G_8)]_4$. These oligonucleotides also formed a small fraction of higher molecular weight structures (Fig. 1). The oligonucleotide $d(T_{15}G_{11})$ formed stable three-stranded and four-stranded complexes, $d(T_{15}G_{12})$ formed a broad distribution of multistranded species which were stable during electrophoresis in a denaturing gel (Fig. 1). This behaviour is characteristic of DNA frayed wires.

Fig. 2 shows that the type and distribution of aggregated species formed in 20 mM $MgCl_2$ were similar under native and denaturing conditions. We found that $d(T_{15}G_4)$ remained monomeric, whereas $d(T_{15}G_5)$ (data not shown), $d(T_{15}G_6)$ and $d(T_{15}G_8)$ formed predominantly four-stranded complexes. As observed in previous experiments, the oligonucleotides $d(T_{15}G_{12})$ and $d(T_{15}G_{15})$ formed an array of multistranded species [12]. A similar pattern of bands was observed when these oligonucleotides were dissolved in 20 mM KCl (Fig. 2).

Dimethyl sulfate (DMS) can methylate the N7 of guanine only if it is not involved in stable hydrogen bonding. If the N7 is inaccessible to DMS, methylation and subsequent strand cleavage of the oligonucleotide backbone by piperidine does not occur. Fig. 3 shows the result of the reaction of the aggregates of the $d(T_{15}G_n)$ oligonucleotides with dimethyl sulfate. Piperidine cleavage products are evident on denaturing electrophoresis gels as new bands that migrate faster than the parent oligonucleotide, the appearance of these bands implies that guanine N7 was not involved in a stable hydrogen bond.

By studying the extent of methylation protection, we can distinguish two types of complexes formed by $d(T_{15}G_n)$ oligonucleotides. The band

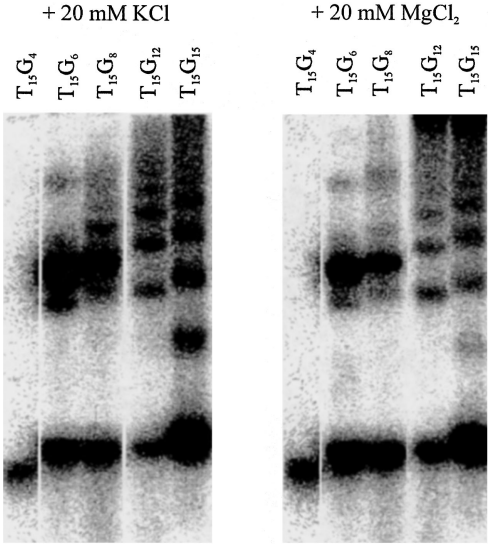

Fig. 2. Oligonucleotides (0.33 μM) were prepared in (A) 20 mM $MgCl_2$ or (B) 20 mM KCl and analysed in 15% native gel: (1) $d(T_{15}G_{15})$; (2) $d(T_{15}G_{12})$; (3) $d(T_{15}G_8)$; (4) $d(T_{15}G_6)$; (5) $d(T_{15}G_4)$.

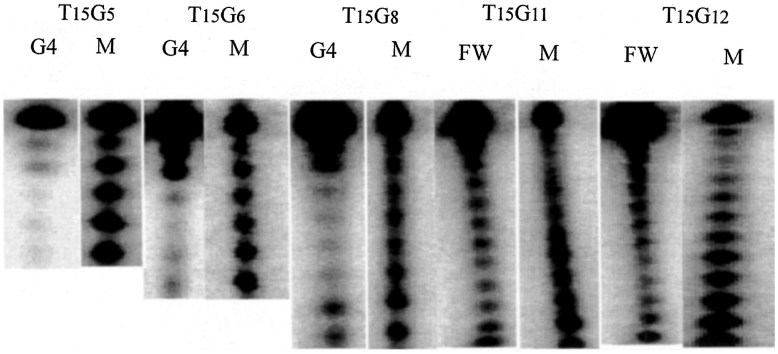

Fig. 3. Methylation protection of guanines. Banding pattern in 20% denaturing gel of piperidine-digested oligonucleotides after methylation at the N7 sites of guanine bases: (A1) Four-stranded complex (G4) of $d(T_{15}G_5)$; (A2) $d(T_{15}G_5)$ monomer; (B1) G4 complex of $d(T_{15}G_6)$; (B2) $d(T_{15}G_6)$ monomer; (C1) G4 complex of $d(T_{15}G_8)$; (C2) $d(T_{15}G_8)$ monomer; (D1) frayed wire (FW) complexes of $d(T_{15}G_{11})$; (D2) $d(T_{15}G_{11})$ monomer; (E1) FW complexes of $d(T_{15}G_{12})$; and (E2) $d(T_{15}G_{12})$ monomer.

pattern shown in Fig. 3 indicates that most of guanines of the four-stranded complexes formed by $d(T_{15}G_5)$, $d(T_{15}G_6)$ and $d(T_{15}G_8)$ were protected from methylation. In general, guanines further from the 3′ end of the oligonucleotide are more protected, while the two guanine bases at the 3′-end were more accessible to methylation. In the case of $d(T_{15}G_8)$, the two guanine bases of the four-stranded complex close to the 5′ thymines were also more accessible to methylation (Fig. 3, C1 and C2). On the other hand, all the guanine bases of the multistranded species formed by $d(T_{15}G_{11})$ and $d(T_{15}G_{12})$ were accessible to methylation. Again, these results are similar to those of the frayed wire formed by $d(T_{15}G_{15})$ [12].

From these results, we conclude that the N7 position of guanine is not necessary for the formation of stable frayed wires. To further probe the role of N7 in stabilising DNA frayed wires, we studied an inosine-containing oligonucleotide. Inosine forms tetrad structures with only the inner hydrogen bonds [18]. We investigated the ability of an inosine-containing oligonucleotide, $d(T_{15}I_{15})$, to form aggregated species in an attempt to understand the role of the inner hydrogen bonds in their stabilisation. The ^{32}P-labelled inosine-containing oligonucleotide was dissolved in a buffer containing 1 M $MgCl_2$ and held at 90°C for 30 min. Fig. 4 shows the result of electrophoresis of $d(T_{15}I_{15})$ in a native gel, there is no evidence of the formation of aggregates formed

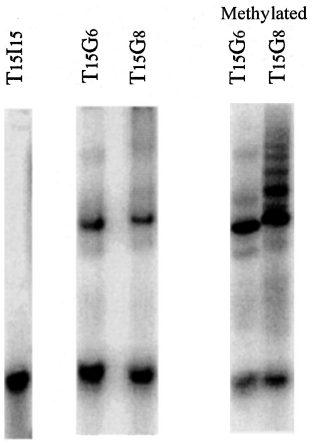

Fig. 4. Oligonucleotides (0.33 μM) analysed in 15% native gel: (1) $d(T_{15}I_{15})$ in 1 M $MgCl_2$; (2) $d(T_{15}G_6)$ in 1 M $MgCl_2$; (3) $d(T_{15}G_8)$ in 1 M $MgCl_2$; (4) methylated $d(T_{15}G_6)$ in 1 M $MgCl_2$; and (5) methylated $d(T_{15}G_8)$ in 1 M $MgCl_2$.

by $d(T_{15}I_{15})$ under either condition. The inosine-containing oligonucleotide also remained monomeric in solutions containing other cations (Na^+, K^+, Mg^{2+} and NH_4^+) over a range of concentrations (from 0.0625 to 2 M, data not shown). The results contrasted to that of $d(T_{15}G_{15})$ under the comparable conditions [12]. It appears that the interior hydrogen bonds that stabilised inosine tetrads are not responsible, or not solely responsible, for the stabilisation of frayed wires.

As mentioned above, $d(T_{15}G_6)$ and $d(T_{15}G_8)$ formed predominantly the four-stranded com-

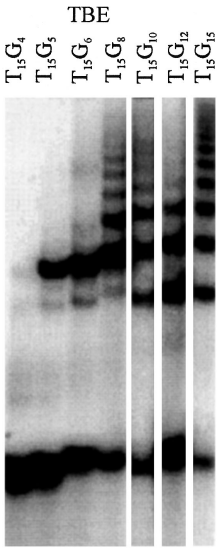

Fig. 5. Oligonucleotides (0.33 μM) in TBE buffer analysed in 15% native gel: (1) $d(T_{15}G_{15})$; (2) $d(T_{15}G_{12})$; (3) $d(T_{15}G_{10})$; (4) $d(T_{15}G_8)$; (5) $d(T_{15}G_6)$; (6) $d(T_{15}G_5)$; and (7) $d(T_{15}G_4)$.

plexes. The same behaviour was observed in 1 M $MgCl_2$ (Fig. 4). When $d(T_{15}G_8)$ was methylated and incubated in 1 M $MgCl_2$ at 90°C for 30 min, the banding pattern in the native gel changed significantly. Methylated $d(T_{15}G_8)$ formed an array of multistranded complexes instead of primarily the four-stranded complex (Fig. 4). We did not observe this change for $d(T_{15}G_6)$ (Fig. 4).

We also analysed the complexes formed by $d(T_{15}G_n)$ in TBE buffer without any additional cation. With the exception of $d(T_{15}G_8)$, the banding patterns of these oligonucleotides were similar to those in 20 mM $MgCl_2$ (Figs. 2 and 5). The similarity of the results in TBE and Mg^{2+}-containing buffer arises from the exposure of the oligonucleotides to magnesium cations during the ^{32}P labelling procedure. The oligonucleotide $d(T_{15}G_8)$ incubated in TBE buffer without additional cations behaved differently. Instead of forming the four-stranded complexes observed in 20 mM $MgCl_2$ (Fig. 2), $d(T_{15}G_8)$ formed an array of multistranded complexes in TBE buffer similar to those of $d(T_{15}G_{10})$, $d(T_{15}G_{12})$, and $d(T_{15}G_{15})$ (Fig. 5).

A similar change in complex formation was observed for $d(T_{15}G_8)$ if we extended the equilibration at room temperature after incubating in 20 mM $MgCl_2$ at 90°C (Fig. 6). The four-stranded complex of $d(T_{15}G_8)$ predominated after incubation in 20 mM $MgCl_2$ at 90°C for 30 min (Fig. 2). By extending the equilibration time at room tem-

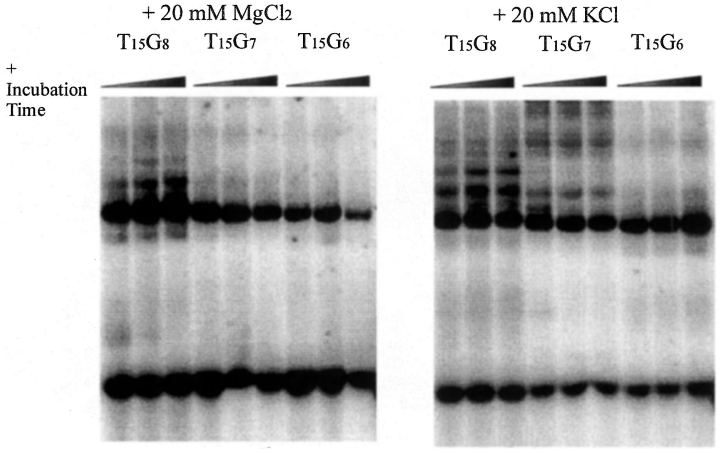

Fig. 6. High molecular weight structures of oligonucleotides (3.33 μM) formed after 30 min at 90°C in either 20 mM $MgCl_2$ (A) or 20 mM KCl (B). After heating, the oligonucleotides were equilibrated at room temperature for different times (5 h, 1 day or 2 days) before analysing in 15% native gel. (A) 20 mM $MgCl_2$: (1) $d(T_{15}G_8)$ for 5 h; (2) $d(T_{15}G_8)$ for 1 day; (3) $d(T_{15}G_8)$ for 2 days; (4) $d(T_{15}G_7)$ for 5 h; (5) $d(T_{15}G_7)$ for 1 day; (6) $d(T_{15}G_7)$ for 2 days; (7) $d(T_{15}G_6)$ for 5 h; (8) $d(T_{15}G_6)$ for 1 day; and (9) $d(T_{15}G_6)$ for 2 days. (B) 20 mM KCl: (1) $d(T_{15}G_8)$ for 5 h; (2) $d(T_{15}G_8)$ for 1 day; (3) $d(T_{15}G_8)$ for 2 days; (4) $d(T_{15}G_7)$ for 5 h; (5) $d(T_{15}G_7)$ for 1 day; (6) $d(T_{15}G_7)$ for 2 days; (7) $d(T_{15}G_6)$ for 5 h; (8) $d(T_{15}G_6)$ for 1 day; and (9) $d(T_{15}G_6)$ for 2 days.

perature, we observed the slow formation of other multistranded species (Fig. 6). We did not observe a similar conversion by $d(T_{15}G_7)$ or $d(T_{15}G_6)$ (Fig. 6). We found that when potassium was the cation, multistranded species of $d(T_{15}G_7)$ and $d(T_{15}G_8)$ were formed; however, $d(T_{15}G_6)$ did not convert to multistranded species under these conditions (Fig. 6). The band pattern of $d(T_{15}G_7)$ was distinct from that of $d(T_{15}G_8)$ (Fig. 6).

In Fig. 7, we have graphed the percentage of multistranded aggregates formed by $d(T_{15}G_n)$ vs. the number of contiguous guanine bases in the oligonucleotide. In 20 mM $MgCl_2$, the fraction of tetraplex increases with the number of contiguous guanines at the 3′-end (Fig. 7). When the $d(T_{15}G_n)$ oligonucleotides were incubated with 20 mM KCl instead of $MgCl_2$, the proportion of tetraplex among the aggregated species was not simply correlated to the number of contiguous guanine bases. Of the $d(T_{15}G_n)$ oligonucleotides, we found that $d(T_{15}G_6)$ in KCl formed the greatest proportion of tetraplex aggregates while larger numbers of guanines led to a decrease in the fraction of tetraplex (Fig. 7). Thus, the formation of tetraplex by $d(T_{15}G_n)$ oligonucleotides can be controlled by altering the solution conditions. For example, $d(T_{15}G_6)$ formed a greater fraction of tetraplex in KCl than $MgCl_2$, whereas $d(T_{15}G_8)$ formed more tetraplex in $MgCl_2$ than KCl. The oligonucleotides $d(T_{15}G_5)$ and $d(T_{15}G_7)$ formed comparable percentage of tetraplex in both KCl and $MgCl_2$ (Fig. 7).

For aggregated structures other than the four-stranded tetraplex, there was a direct relation between the number of contiguous guanine residues and the extent of aggregation. The larger the number of contiguous guanines the greater the fraction of high molecular weight structures (other than the four-stranded tetraplex) formed. The effect was more pronounced in 20 mM KCl than in 20 mM $MgCl_2$.

4. Discussion

4.1. Two types of DNA complexes are formed by $d(T_{15}G_n)$ oligonucleotides

In this study, we have shown that at least two types of DNA complexes are formed by $d(T_{15}G_n)$

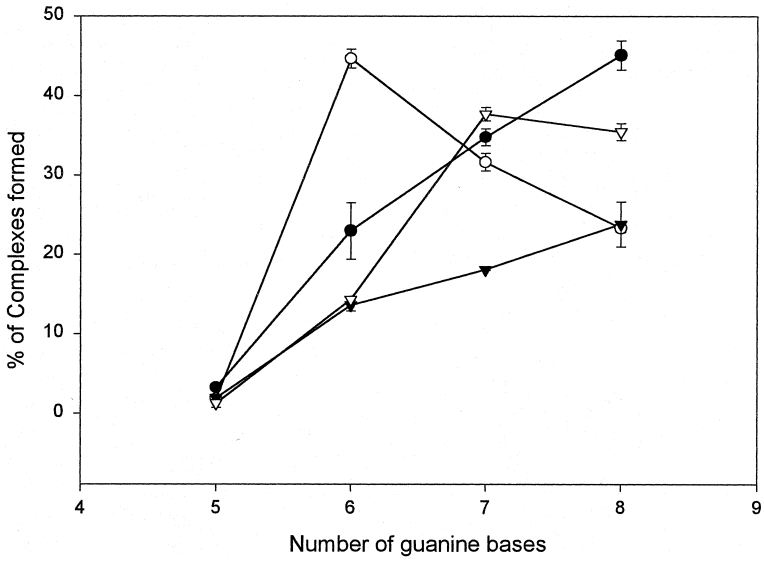

Fig. 7. A plot of percentage of high molecular weight structures at 12.4°C vs. oligonucleotide of $d(T_{15}G_n)$ ($n = 5–8$) (3.33 μM) containing different number of contiguous guanine bases. ● Four-stranded complex (G4) formation vs. oligonucleotides in 20 mM $MgCl_2$; ○ G4 complex formation vs. oligonucleotides in 20 mM KCl; ▼ other high molecular weight structures vs. oligonucleotides in 20 mM $MgCl_2$; ∇ other high molecular weight structures vs. oligonucleotides in 20 mM KCl.

oligonucleotides. We have differentiated these complexes by their susceptibility to chemical modification and strand association stoichiometry. One type of complex exhibits properties similar to those of G-tetrads, whereas the other type is similar to that of frayed wires. The guanine N7 sites of the DNA tetrad complexes are protected from methylation, whereas frayed wires do not exhibit the same protection. The strand association stoichiometry between these two complexes is also different. The tetrad complexes formed by $d(T_{15}G_n)$ oligonucleotides consist of predominantly four DNA strands, whereas frayed wires arise from the association of apparently any number of DNA molecules.

The pattern of bands resolved on electrophoresis gels is a function of the number of contiguous guanine bases in the oligonucleotide. Those with eight or less contiguous guanines form predominantly four-stranded complexes that are presumably tetrads. However, when the number of contiguous guanines increases to 12 and 15, the formation of four-stranded species decreases significantly. Instead of forming primarily a four-stranded complex, $d(T_{15}G_{12})$ and $d(T_{15}G_{15})$ form multistranded complexes called frayed wires.

The four-stranded species formed by the oligonucleotides with eight or less contiguous guanines were not methylated under normal conditions by dimethyl sulfate. However, the two terminal 3′ guanines were accessible to methylation. This implies that the N7 sites at most of the guanine bases in $d(T_{15}G_6)$ and $d(T_{15}G_8)$ engage in hydrogen bonding similar to that seen in tetraplex. The accessibility of the last two guanines to methylation is consistent with fraying at the ends, this has been observed previously in tetraplexes formed by DNA with telomeric sequences [17].

Increasing the number of consecutive guanines to 12 and 15 led to the formation of DNA frayed wires. All of the guanines in these aggregates are accessible to methylation. This is one of the characteristics that distinguish frayed wires from the aggregates formed by oligonucleotides with telomeric sequences. In addition, the frayed wire structures formed by $d(T_{15}G_{12})$ are stable under denaturing conditions; while the frayed wires arising from oligonucleotides with fewer contiguous guanines are not. Since we cannot distinguish the four-stranded tetraplex from the four-stranded frayed wire in an electrophoresis gel, the four-stranded complex that we observed might be a mixture of both tetraplex and frayed wire. Our results only allow us to conclude that there is an increase in the frayed wire formation with the number of contiguous guanines; however, the relative proportion of the G4 tetraplex in the complexes formed by these oligonucleotides remains unknown.

The guanine–guanine interaction observed in the frayed wire complex also differs from that of the inosine tetrad. Although the covalent structure of inosine is similar to that of guanine, inosine does not have a C2 amino group. Therefore, it cannot form the outer hydrogen bonds with another bases via the N7 sites and the C2 amino group as seen in the canonical tetraplex. Rather the inosine tetrad observed in poly(I) is stabilised only by the inner hydrogen bonding [18]. We did not observe the formation of high molecular weight structures by $d(T_{15}I_{15})$ under the conditions used for $d(T_{15}G_{15})$. Therefore, it appears that the frayed wire complexes are not stabilised solely by single inner hydrogen bonds. However, we do not exclude the possibility that frayed wire complexes may be stabilised by a combination of different guanine–guanine interactions. Although the guanine N7 site is not involved in the stabilisation of frayed wires, the C2 amino group plays an important role in stabilising the structure of frayed wires.

The two types of complexes formed by $d(T_{15}G_n)$ oligonucleotides can interconvert. We find that the frayed wire complex can transform to the four-stranded tetraplex or vice versa by varying the solution conditions. This phenomenon is clearly observed for $d(T_{15}G_8)$, which forms both four-stranded tetraplex and the frayed wire. In TBE, without additional cation, $d(T_{15}G_8)$ exists in the frayed wire form, but the complex transforms to a four-stranded (presumably tetraplex) form upon incubation at 90°C for 30 min in the presence of magnesium ion. Although the four-stranded tetraplex is the predominant form of $d(T_{15}G_8)$ immediately following this treatment, a

small amount of another complex, possibly frayed wires, is formed after 1 day of equilibration. If $d(T_{15}G_8)$ is methylated, the methylated form aggregates as a frayed wire instead of the four-stranded tetraplex in the presence of magnesium ions. Formation of a standard tetraplex requires the N7 sites of guanine bases to form hydrogen bonds, methylation of these sites abolishes the capability to form the outer shell of hydrogen bond in the tetraplex. Inhibiting tetraplex formation by methylation favours the formation of frayed wires by $d(T_{15}G_8)$.

4.2. Properties of $d(T_{15}G_n)$ DNA complexes

The physical and chemical properties of the complexes formed by oligonucleotides with sequence domains similar to telomeric sequences, such as $d(T_{15}G_4T_2G_4)$, differ from the properties of the $d(T_{15}G_n)$ oligonucleotides with regard to cation requirement for stabilisation, strand association stoichiometry, reactivity of the guanine N7 with dimethyl sulfate, and thermal stability. The $d(T_{15}G_n)$ oligonucleotides form complexes in solutions containing either magnesium or potassium ions, whereas the complexes formed by $d(T_{15}G_4T_2G_4)$, are stabilised by potassium ions but not magnesium ions [12].

Both potassium and magnesium ions stabilise the four-stranded complexes formed by $d(T_{15}G_6)$ or $d(T_{15}G_8)$. In the presence of magnesium ions, these complexes are stable in solution and in denaturing polyacrylamide electrophoresis gels (Fig. 1). This is in contrast to the structure formed by $d(T_{15}G_4T_2G_4)$ in the presence of potassium ions described in our previous study [12]. The extent of formation of tetraplex and higher molecular weight non-tetraplex species by $d(T_{15}G_6)$, $d(T_{15}G_7)$ and $d(T_{15}G_8)$ increases with the number of contiguous guanines in solutions containing magnesium ions, this was not observed in the presence of potassium ions (Fig. 7). In the solutions containing potassium ions, the oligonucleotides containing six contiguous guanines formed mostly the four-stranded tetraplex. The fraction of tetraplex decreased, and the fraction of higher molecular weight structures increased, with larger numbers of contiguous guanines (Fig. 7).

We found that the thermal stability of the DNA complexes is influenced by various factors. For example, the thermal stability of the structures formed by the $d(T_{15}G_n)$ oligonucleotides, is dependent upon the number of consecutive guanines. For oligonucleotides with fewer contiguous guanines, the frayed wire complex is less thermally stable than the tetraplex form. Thus, only the four-stranded species formed by $d(T_{15}G_8)$ and $d(T_{15}G_{10})$ are stable under denaturing conditions (Figs. 1 and 5). Oligonucleotides with a larger number of contiguous guanines aggregate to form predominantly frayed wire structures, which are stable under denaturing conditions. However, because these oligonucleotides form predominately frayed wires, we can assess only the stability of the frayed wires.

The second factor affecting the thermal stability of the DNA complexes is the arrangement of guanine bases within the sequence, a larger number of contiguous guanines enhances stabilisation. Thus, complexes formed by $d(T_{15}G_5)$ are unstable under denaturing conditions, whereas those formed by $d(T_{15}G_6)$ are stable under these conditions. However, examination of the stability of the aggregates formed by oligonucleotides with telomere-like sequence domains showed that the thermal stability of the complexes is not simply correlated with the total number of guanine bases. The six guanine bases in $d(T_{15}G_6)$ stabilise the DNA complexes to a greater extent than the eight guanine bases in $d(T_{15}G_4T_2G_4)$. Complexes formed by $d(T_{15}G_6)$ are stable in a denaturing gel, whereas those formed by $d(T_{15}G_4T_2G_4)$ are not. Thus, the sequence of the guanine residues contributes to the type and stability of the complex formed. The effect of adding an additional guanine on the thermal stability of the complexes is greater when the added base is contiguous with other guanines.

A third factor contributing to the thermal stability of the DNA complexes is the balance between the stabilisation force of guanine-rich region vs. the unfavourable contribution of the non-guanine portion. In this study, we observed

that $d(T_{15}G_4)$ cannot form aggregated structures in solutions containing either potassium or magnesium ions (Figs. 1 and 2). This is in contrast to the behaviour of other $d(T_{15}G_n)$ oligonucleotides containing a greater number of contiguous guanines (Figs. 1 and 2) and those with shorter non-guanine portions, for example $d(T_4G_4)$ [19]. The inability of $d(T_{15}G_4)$ to form high molecular weight complexes is presumably due to destabilisation by the 15 thymines at the 5′ end. We find that a minimum of five contiguous guanine bases at the 3′-end is required to form a stable structure with the 15 thymines, and at least six contiguous guanines are necessary for stability under denaturing conditions. This is consistent with the notion that the overall stability of the complex is a balance between the favorable guanine–guanine interactions and an unfavourable energetic contribution due to the 5′-extension [12,20].

4.3. Proposed mechanism for $d(T_{15}G_n)$ complex formation

In this study, we report the formation of at least two types of DNA complexes by $d(T_{15}G_n)$ oligonucleotides: the four-stranded tetraplex and frayed wires. The type of complex formed is influenced by the number of contiguous guanines within the $d(T_{15}G_n)$ sequences and the experimental conditions. We hypothesise that formation of these two species proceeds via parallel reactions which may occur simultaneously and which may share common intermediates. The kinetics and energetics of one reaction pathway influence the relative concentration of species in the other reaction path. Blocking one of the reaction paths may lead to an increase in the fraction of products from the other path. The predominance of the frayed wire or tetraplex form is due to external conditions, salt type or concentration, heating, etc., or to factors related to the structure or sequence of the oligonucleotide (e.g. a large number of consecutive guanines, methylation). Methylation of the guanine N7 position blocked the formation of tetraplex by $d(T_{15}G_8)$; however, frayed wire structures were still formed. Thus, the tetraplex form is not an intermediate in the reaction leading to frayed wires. Although it is more equivocal, the data do not indicate that the frayed wire form an intermediate in the path leading to tetraplex structures. There was no evidence of the formation of frayed wires prior to the formation of the four-stranded tetraplex during incubation.

The data for $d(T_{15}G_8)$ indicate that frayed wire structures form much more slowly than tetraplexes. At room temperature, tetraplexes were observed to form first and frayed wires appeared only after incubation for 1 day. Although the formation of the tetraplex precedes the frayed wire, the data imply that this is a consequence of the different rates of formation and not due to a sequential reaction pathway.

In Fig. 8a, we have outlined a possible mechanism leading to the formation of these structures. This scheme is adapted from the mechanism presented by Hardin et al. [21] In their original proposal, the formation of guanine aggregates occurs via an initial ‘open’ base paired structure [22]. As suggested by Hardin et al. [21], the vacant base pairs in these open structures could allow base pairing with other single-stranded oligonucleotides. This in turn leads to the formation of multistranded species in which methylation at the N7 site of guanine base is possible at least at the terminal strand (Fig. 8a). It is necessary to pass through a large energetic barrier to convert the open structures to a closed tetraplex form and vice versa. Factors that increase the kinetic barrier would likely favour formation of multistranded species (Fig. 8a). For example, methylation at the N7 site of guanine prevents the formation of tetraplex, this results in a shift in the equilibrium towards the reaction path leading to frayed wires. This is consistent with our observation that methylation of the oligonucleotide favours formation of frayed wires.

In our model of the growth of aggregated species, hydrogen-bonding interactions take place between the guanines in adjacent strands of the open structure (Fig. 8a). When three or more strands interact, there are two classes of strands: interior strands, i.e. oligonucleotides engaged in hydrogen bonds with two neighbouring strands; and exterior strands, oligonucleotides at the edges of the aggregated complex. Unoccupied hydrogen binding sites present in the exterior DNA strands

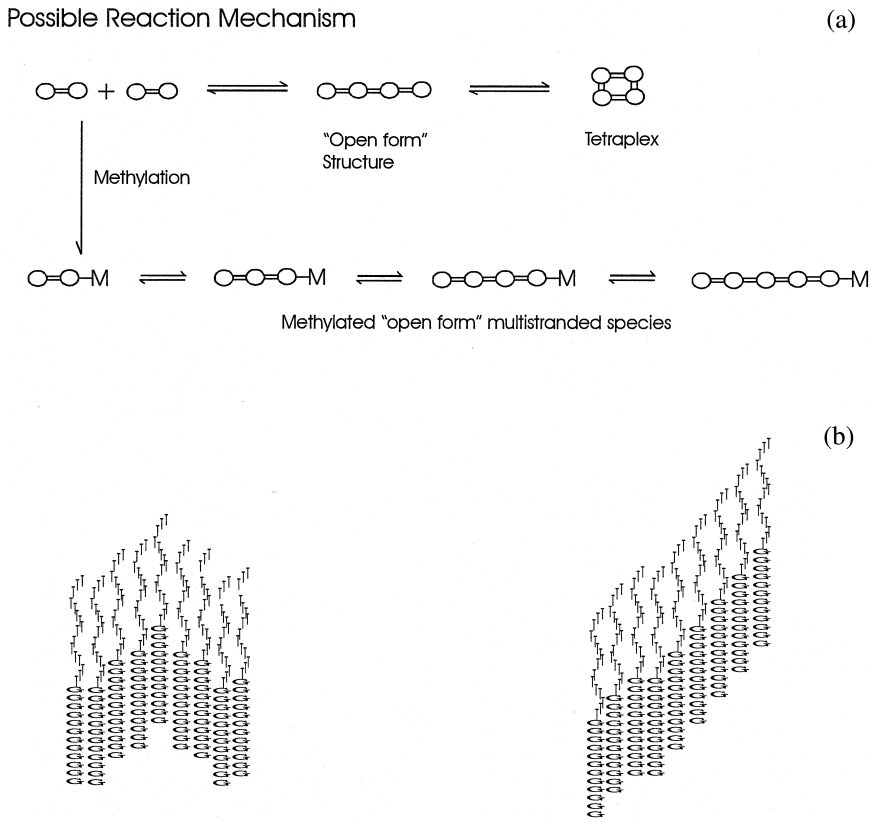

Fig. 8. (a) Schematic representation of the reaction scheme proposed for the formation of aggregates by d($T_{15}G_n$) oligonucleotides. In this schematic, ○: a vertical view of a guanine-rich DNA oligonucleotide; =: guanine–guanine interaction between intermolecular DNA strand. (b) Schematic representation of two possible frayed wire structures. The interactions between the strands may be out of register with each other in the 5′ or the 3′ direction by several base pairs. In this drawing, the two structures are represented as planar; however, the structures are almost certainly helical.

would allow the binding of additional DNA strands leading to the further growth of the multistranded species (frayed wires). Oligonucleotides with larger number of contiguous guanines can adopt a greater number of possible structures, facilitating the formation of frayed wire complexes. Many structures in which parallel guanine-rich DNA oligonucleotides have different degrees of alignment with each other are possible (Fig. 8b). This is consistent with the data that oligonucleotide with larger number of contiguous guanine form predominantly frayed wire species. Additional factors also stabilise the open form and favour the formation of frayed wires. For example, methylation of the N7 site prevents the transition to a closed form, or G-tetrad, and may provide a method to discriminate between the two exterior strands.

In summary, d($T_{15}G_n$) oligonucleotides can form two types of multistranded complexes. One type is stabilised by G-tetrads, the other type is stabilised by guanine–guanine interactions distinct from the canonical tetrad. These latter aggregates are called frayed wires. The formation of aggregates by d($T_{15}G_n$) oligonucleotides is determined by the number of contiguous guanines and the availability of the guanine N7. The thermal stability of the complexes is influenced by the type of complex (tetrad or frayed wire), the number and arrangement of contiguous guanines within the sequences, and the balance between the stabilisation force of the guanine bases vs. the

unfavourable effect of the non-guanine portion within the sequences.

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