

Chlamydomonas reinhardtii telomere repeats form unstable structures involving guanine-guanine base pairs

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ABSTRACT

Unusual DNA structures involving four guanines in a planar formation (guanine tetrads) are formed by guanine-rich (G-rich) telomere DNA and other G-rich sequences (reviewed in (1)) and may be important in the structure and function of telomeres. These structures result from intrastrand and/or interstrand Hoogsteen base pairs between the guanines. We used the telomeric repeat of *Chlamydomonas reinhardtii*, TTTTAGGG, which contains 3 guanines and has a long interguanine A + T tract, to determine whether these sequences can form intrastrand and interstrand guanine tetrads. We have found that ss (TTTTAGGG)₄ can form intrastrand guanine tetrads that are less stable than those formed by more G-rich telomere sequences. They are not only more stable, but also more compact, they are more stable in the presence of K⁺ than they are in the presence of Na⁺. While ds oligonucleotides with ss 3' overhangs of (TTTTAGGG)₂ can be observed to associate as dimers, formation of this interstrand guanine tetrad structure occurs to a very limited extent and requires very high G-strand concentration, high ionic strength, and at least 49 hours of incubation. Our results suggest that, if telomere dimerization occurs *in vivo*, it would require factors in addition to the TTTTAGGG telomere sequence.

INTRODUCTION

Guanine has long been known to form gels *in vitro* at high concentration. To account for these gels, Gellert (1962) proposed a structure involving guanine-guanine hydrogen bonding called guanine tetrads (2). Na⁺ plays a role in the formation of guanine tetrads while K⁺ stabilizes them and allows them to stack (reviewed in (1)). Polyguanine is able to form interstrand and intrastrand guanine tetrad structures (3,4).

In biological systems, G-rich stretches of DNA are found at telomeres, the ends of linear chromosomes. Telomere repeat sequences are organism specific and most conform to the consensus 5' T₍₁₋₄₎A₍₀₋₁₎G₍₁₋₈₎ 3'. The 5' to 3' strand directed toward the end of the chromosome includes frequent repeats of

G_{≥3} and is called the G-strand. The complementary strand directed 3' to 5' toward the end of the chromosome is the C-strand. In species where it has been analyzed, the G-strand has been shown to terminate in a 3' ss overhang of approximately two telomere repeats (5, 6). *In vitro*, the 3' G-strand overhang is used as a primer for the 5' to 3' addition of telomeric DNA sequences by the telomere replication enzyme, telomerase. In addition, the G-strand overhang may function as a template for DNA primase (7) and may be involved in other functions that have been attributed to telomeres. These functions include the protection against exonucleolytic degradation of linear chromosomal DNA, the prevention of fusion between linear DNA molecules, and the association of telomeres both with each other and with the nuclear envelope (reviewed in (8)). Recently it has been proposed that the G-strand overhang may play a role in the initiation of meiotic pairing as well as other DNA recognition steps (7, 9–11).

Models for the structure of the telomeric G-strand overhang have been based on *in vitro* characterization of ss G-rich oligonucleotides. At 37°C and above, these oligonucleotides migrate as linear molecules at their relative molecular weights. At 5°C, oligonucleotides containing 4 consecutive repeats (4-repeat) of the G-strand telomere sequence of G-rich telomeres undergo intramolecular interactions and migrate faster than their C-strand complements (12). Others have shown that Hoogsteen base paired guanine-guanine interactions are present in these structures and that the 4-repeat G-strand telomeric oligonucleotides form antiparallel intrastrand guanine tetrads called G-quartets (12–14). Na⁺, K⁺, and Cs⁺ ions enhance the formation of G-quartets (13), and K⁺ also enhances the stability of the G-quartets (7).

The minichromosomes of *Oxytricha* cohere *in vitro* at high DNA concentrations (1–2mg/ml) without proteins. Coherence of the chromosomes requires the 3' ss overhang and the presence of at least 250 mM NaCl (15). Oligonucleotides containing a ds telomeric region and a 3'-overhang of 2 G-strand telomere repeats with *Oxytricha* telomere sequence cohere as well (15, 16). An oligonucleotide of the *Tetrahymena* telomere sequence (T₂G₄) containing a ds telomeric region and a 3'-overhang of 2 G-strand telomere repeats (Tet1 or TDS) has been tested for guanine tetrad-mediated association. On nondenaturing gels, some of the TDS

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oligonucleotides migrate as dimers (11). Methylation protection analysis of the oligonucleotide dimers demonstrates the involvement of 3'-overhang guanine N7 groups suggesting that the dimers are the result of guanine tetrads formed between hairpins of the 3' ss overhang (11). Dimers form more readily in sodium and are stabilized by potassium.

G-rich telomeric and nontelomeric ss oligonucleotides can also form intermolecular guanine tetrads by the association of four parallel strands (G4 DNA). Unlike intramolecular associations, the formation of G4 DNA is dependent on time and oligonucleotide concentration. Like other guanine tetrads, G4 DNA forms readily in sodium and is stabilized by potassium (9, 10).

G-quartet and guanine tetrad formation have been tested only on those telomeric repeats that are composed of $\geq 50\%$ guanine. To determine whether telomeres that are $< 50\%$ G-rich can form intramolecular G-quartets, we analyzed telomere sequences that contained different guanine composition and sequence. *C. reinhardtii* telomeres (TTTTAGGG)_n are not G-rich. They contain more A+T residues per repeat than other known telomeres (17) and they contain only 3 consecutive guanines per repeat. The *C. reinhardtii* telomeres are similar in sequence to the telomere repeats of higher organisms such as *Arabidopsis* (TTTAGGG)_n and *Homo* (TTAGGG)_n. We find that *Chlamydomonas* telomeric repeat sequences can form intramolecular G-quartets but that these structures are much less stable than similar structures formed by the more G-rich telomeric DNA sequences. In addition, intermolecular guanine tetrads formed by the *C. reinhardtii* telomere repeat are slow to form and are dependent upon very high telomere and Na⁺ concentrations that are unlikely to occur under physiological conditions.

MATERIALS AND METHODS

Oligonucleotide synthesis

Oligonucleotides used are listed in Table 1. Oligonucleotides were synthesized in house on a Pharmacia Geneassembler according to the manufacturer's instructions. Oligonucleotides TG4, TC4, and OG4' were provided by Alan Zahler. Oligonucleotide YG was provided by Eric Henderson. Oligonucleotide OG4 for the melting temperature experiment was provided by Jamie Williamson. Oligo-dT laddar was purchased from BRL, Inc.

Oligonucleotide purification and labeling

0.1–1.0 μ g of oligonucleotide was labeled with γ -³²P-ATP (> 5000 Ci/mM, Amersham) and T4 Polynucleotide Kinase (New England Biolabs) at 37°C for 30 min. Oligonucleotides were gel-purified by denaturing polyacrylamide gel electrophoresis (PAGE) on a 20% 30:1 acrylamide:bis-acrylamide 7 M urea gel in 1×TBE (0.089 M Tris base, 0.089 M Boric acid, 0.002 M EDTA pH8.3). ds oligonucleotides were annealed by heating to 90°C and slow cooling in TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA) followed by gel purification on a 10% nondenaturing polyacrylamide gel.

Analysis of single-stranded oligonucleotides

ss telomeric oligonucleotides were annealed and resolved by nondenaturing PAGE according to Henderson et al. (12) using modifications described by Williamson et al. (13). Briefly, 5' end-labeled gel-purified oligonucleotides were denatured by boiling in TE containing 50 mM NaCl and were equilibrated at the appropriate temperature for 15 min prior to loading on 12%

or 20% nondenaturing gels in 1×TBE/50 mM NaCl which had been prerun for 3 hours. Oligonucleotides were resolved by PAGE at 7.5 volts/cm until the bromophenol blue was at least 16 cm from the origin. Gels were dried on Whatman 3mm paper and exposed to Dupont Cronex single-sided film at -70°C for 3–24 hours.

Melting temperature of ss oligonucleotides in solution

ss oligonucleotides were degassed and boiled in TE containing 50 mM NaCl. Following equilibration of the oligonucleotides for 10 minutes in cuvettes at 17°C, the absorbance of oligonucleotides was determined on a Beckman DU-8 spectrophotometer as the temperature of the solutions was increased 1°C/1.4 min. The absorbance of oligonucleotides was also monitored as the oligonucleotides cooled to ensure the absence of hysteresis.

Methylation interference

Methylation interference was performed as described by Williamson et al. (13). Oligonucleotides were methylated in TE using 2 ng of gel-purified, 5'-end-labeled oligonucleotide and 1 μ l of 1:200 DMS in 20 μ l total volume at 65°C for 2 min. Oligonucleotides were denatured at 90°C for 3 min in 50 mM NaCl or 50 mM KCl and loading dye (bromophenol blue/xylene cyanol/glycerol) followed by equilibration at 4°C for 10 min. Electrophoretically distinct forms were separated by nondenaturing PAGE, excised from the gel, and eluted in 100 μ l of TE for ≥ 6 hours. Methylated guanines were cleaved with 20 μ l of piperidine at 90°C for 1/2 hour, lyophilized 3 times, resuspended in 20 μ l of formamide/bromophenol blue/xylene cyanol, boiled for 5 min and resolved by 20% denaturing PAGE. The gel was dried and exposed to Kodak XAR double-sided film as described above.

UV cross-linking

UV crosslinking of ss oligonucleotides was performed as described by Williamson et al. (13). Briefly, 100 ng of gel-purified oligonucleotide was denatured at 95°C for 5 min in 50 mM NaCl and then incubated at 4°C for 5 min. The oligonucleotide was UV irradiated on ice with a Fotodyne transilluminator at 254 nm for 5 min at a distance of 6 cm. The

Table 1. Oligonucleotides used.

Name	Organism	Oligonucleotide sequence
TC4	<i>Tetrahymena</i>	5'CCCCAACCCCAACCCCAACCCCAA
TG4	<i>Tetrahymena</i>	5'TTGGGGTTGGGGTTGGGGTTGGGG
OG4	<i>Oxytricha</i>	5'TTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG
OG4'	<i>Oxytricha</i>	5'GGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTT
YG	<i>Saccharomyces</i>	5'TGTGTGGGTGTGTGGGTGTGTGGG
AC3	<i>Arabidopsis</i>	5'TCCCTAAACCCCTAAACCCCTAAAGCT
AG3	<i>Arabidopsis</i>	5'TTAGGGTTTAGGGTTTAGGGAAGCT
AG4	<i>Arabidopsis</i>	5'TTTAGGGTTTAGGGTTTAGGGTTTAGGG
HG4	Human	5'TTAGGGTTTAGGGTTTAGGGTTTAGGG
CC3	<i>Chlamydomonas</i>	5'AAAACCCCTAAAACCCCTAAAACCCCT
CG3	<i>Chlamydomonas</i>	5'TTTTAGGGTTTTAGGGTTTTAGGG
CG4	<i>Chlamydomonas</i>	5'TTTTAGGGTTTTAGGGTTTTAGGGTTTTAGGG
TDS	<i>Tetrahymena</i>	5'GTCGACCCGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGG 3'CAGCTGGGCCCAACCCCAACCCCAACCC 5'
CDS	<i>Chlamydomonas</i>	5'GTCGACCCGGGTTTAGGGTTTTAGGGTTTTAGGGTTTTAGGG 3'CAGCTGGGCCCAAAATCCCAAAATCCC 5'

irradiated sample was denatured by the addition of 5 μ l formamide/bromophenol blue/xylene cyanol and incubation at 95°C for 5 min. Crosslinked and uncrosslinked forms were separated on a 10% denaturing polyacrylamide gel (20:1 acrylamide:bisacrylamide). Crosslinked and uncrosslinked forms were eluted in 100 μ l of TE for \geq 16 hours, cleaved with 20 μ l pyrrolidine, for 15 min at 90°C, lyophilized 3 times and resolved by denaturing PAGE.

Analysis of double-stranded/single-stranded overhang oligonucleotides

TDS and CDS oligonucleotide associations were analyzed according to Sundquist and Klug (11). Briefly, annealed oligonucleotides were incubated at 23°C in TE with concentrations of NaCl and KCl as described in the figure legends. Oligonucleotides were resolved by nondenaturing PAGE until the bromophenol blue marker dye migrated \geq 22 cm from the origin. Gels were dried and exposed to double-sided Kodak XAR film for 24–144 hours.

Chlamydomonas cellular K⁺ and Na⁺ determination

Chlamydomonas reinhardtii strain A30 was grown to a density of approximately 6×10^8 cells/ mL in minimal media (18).

Three samples of cells were harvested by centrifugation and washed 3 times with sterile deionized water. The washed *Chlamydomonas* cells were ashed at 500°C for 12 hours and resuspended in 2N HCl. Inductively Coupled Plasma Emission Spectroscopy was performed by the Research Analytical Laboratory at the University of Minnesota.

RESULTS

Intramolecular folding of single-stranded G-strand telomeric oligonucleotides

Since *Chlamydomonas* telomere repeats contain only 3 consecutive guanines and have more interguanine A+T residues than telomeric sequences that have been shown to form G-quartets, we tested the ability of *Chlamydomonas* G-strand telomere repeat oligonucleotides to form G-quartets. We observed the migration patterns of C-strands and G-strands of *Chlamydomonas* (C), *Arabidopsis* (A), *Tetrahymena* (T), Human (H), *Oxytricha* (O), and Yeast (Y) oligonucleotides containing 3 and 4 telomere sequence repeats (Table 1). For each oligonucleotide described, the first letter represents the organism, the second letter represents the strand (G-strand or C-strand), and the number represents the number of telomere repeats in the

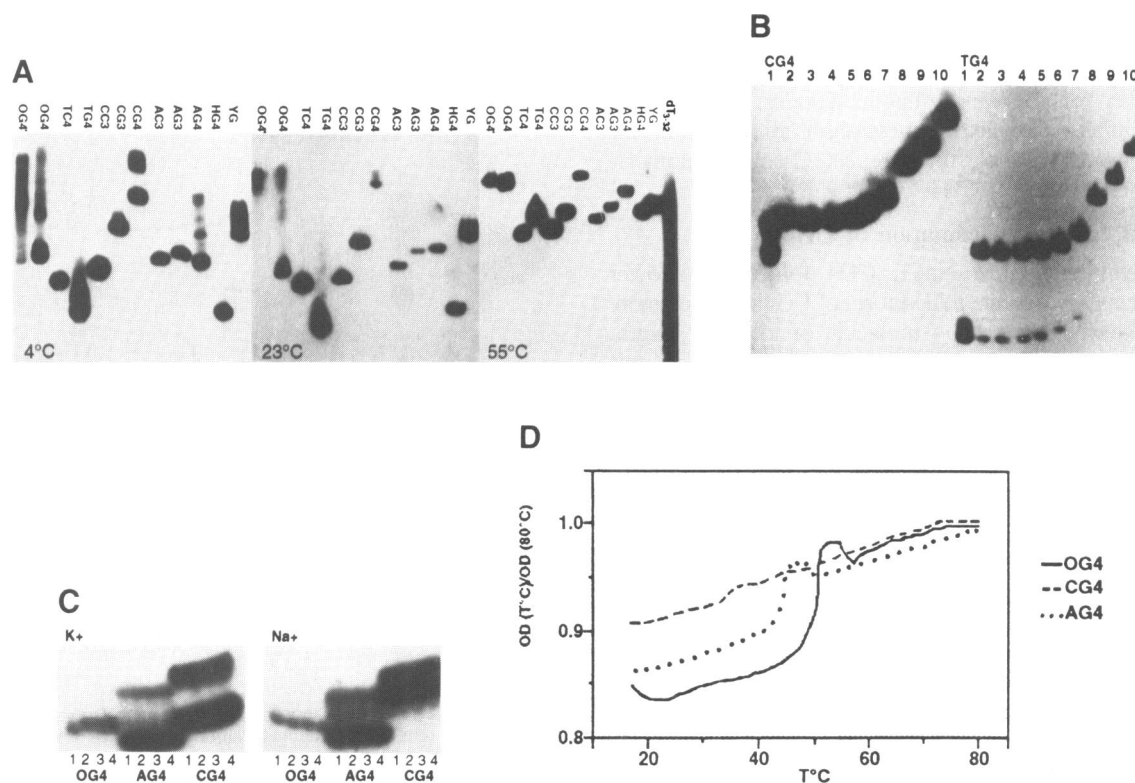


Figure 1. Intramolecular folding of ss G-strand telomeric oligonucleotides. A) Migration of ss oligonucleotides in a nondenaturing gel. Oligonucleotides (listed in Table 1) were denatured, equilibrated and resolved on 20% polyacrylamide gels containing 50 mM NaCl at the temperatures indicated. B) ss oligonucleotide unfolding rates. Aliquots of the equilibrated CG4 or TG4 oligonucleotide were loaded onto a 50 mM NaCl, 12% nondenaturing polyacrylamide gel (lanes 1). A forty-fold molar excess of complementary C-strand oligonucleotide (CC3 or TC4) was added to the equilibrated CG4 or TG4 solutions and subsequent aliquots were loaded onto the gel at 1 min (lanes 2); 2 min (lanes 3); 5 min (lanes 4); 10 min (lanes 5); 20 min (lanes 6); 40 min (lanes 7); 80 min (lanes 8); 120 min (lanes 9); and 160 min (lanes 10) after addition of the complementary oligonucleotide. C) Influence of monovalent cations in solution and in the gels on ss oligonucleotide mobility. 5' end-labeled oligonucleotides OG4, AG4 and CG4 (groups of 4 individual samples) were denatured in TE buffer containing 50 mM LiCl (lanes 1), 50 mM RuCl (lanes 2), 50 mM KCl (lanes 3), or 50 mM NaCl (lanes 4), equilibrated at 4°C and resolved at 4°C on a 12% nondenaturing polyacrylamide gel. The gel contained either 50 mM KCl (K⁺) or 50 mM NaCl (Na⁺). D) Melting temperature of ss telomeric oligonucleotides. Oligonucleotides OG4 (solid line), AG4 (dotted line), and CG4 (dashed line) were denatured at 100°C and then equilibrated at 16°C for 10 minutes. Temperature was raised from 16°C to 80°C at 1°C/1.4 min and results were plotted every 1.4 min. Absorbance was normalized to the absorbance at 80°C.

oligonucleotide. OG4 is oriented 5'(T₄G₄)₄3' while OG4' is oriented 5'(G₄T₄)₄3'. ss oligonucleotides were allowed to equilibrate at 55°C, 23°C, and 4°C and resolved by nondenaturing PAGE (Fig. 1A). At 55°C the oligonucleotides migrated approximately according to relative molecular weight as compared to the migration of an oligonucleotide dT ladder. Oligonucleotides that form intramolecular G-quartets migrate faster at 23°C and 4°C than they migrate at 55°C, presumably due to a more compact conformation.

The electrophoretic mobility of each oligonucleotide at 4°C and 23°C was compared to the mobility of the oligonucleotide at 55°C. As expected, TG4 and OG4 migrated with increased electrophoretic mobility, in 50 mM NaCl gels at both 23°C and 4°C (12). We also observed increased mobility for oligonucleotides CG4, AG4, OG4', and HG4 at 4°C. In addition to increased mobility, CG4, AG4, and OG4' migrated as multiple bands, as did G-quartet forming molecules TG4, OG4, and YG (12), while HG4 migrated as a single species with increased electrophoretic mobility. Hardin et al. have also observed multiple conformations of G-strand oligonucleotides by NMR analysis (19). By analogy to TG4 and OG4, we infer that the faster migrating forms of CG4, AG4, OG4', and HG4 are G-quartet molecules at 4°C. At 23°C, CG4 and OG4' did not migrate as fast as they did at 4°C, although they both displayed a slightly increased mobility relative to their migration at 55°C. We note that, consistent with the observation of Henderson et al. (12), all C-strand oligonucleotides migrated slightly faster at the lower temperatures than they did at 55°C. Finally, we observed no altered mobility of G-strand oligonucleotides containing 3 telomere repeats. Taken together, these observations support the G-quartet model of folding for 4-repeat G-strand telomeric oligonucleotides, including CG4.

Ion effects on G-quartet formation of CG4

In gels containing 50 mM NaCl, CG4 folded at a lower temperature than OG4 and the folded form of CG4 migrated more slowly than the analogous OG4 molecule of identical length. However, when the oligonucleotides were resolved in gels containing 50 mM KCl at 4°C, the CG4 oligonucleotide migrated with a mobility similar to that of the folded OG4 molecule (Fig. 1C). Interestingly, the electrophoretic mobility of either folded OG4 or folded AG4 in Na⁺ or K⁺ gels was not obviously different. While the presence of KCl in the gel affected the mobility of folded CG4, varying the monovalent cations in the folding reactions and in the loaded samples did not influence the mobility of any of the oligonucleotides (Fig. 1C). Despite the altered mobility of folded CG4 in gels containing KCl, the proportion of CG4 present in the folded form was similar in both KCl and NaCl gels. Furthermore, the proportion of folded CG4 was not dependent upon the concentration of CG4 in solution (data not shown).

G-quartet stability

The rates of CG4 and TG4 G-quartet unfolding were compared using a method described previously for determining the stability of the OG4 (Oxy-4) G-quartet (20). The folded oligonucleotides were equilibrated in 50 mM NaCl at 4°C for 1 hour. A 20-fold molar excess of the complementary oligonucleotide was added, and samples were loaded onto a nondenaturing gel at different times after complementary strand addition. The faster migrating G-quartets and the slower migrating ds molecules were resolved (Fig. 1B). Immediately upon addition of TC4, most of the faster migrating TG4 disappeared and the ds TC4/TG4 molecule

became apparent. However, some of the G-quartet form of TG4 was clearly visible for up to 40 min after the addition of TC4. This result is similar to that seen with OG4 (Oxy-4), in which a burst of ds formation is followed by a slow disappearance of the G-quartet form (20). In contrast, the G-quartet form of CG4 disappeared completely after 1 min of incubation with CC3. These results indicate that the *Chlamydomonas* folded telomere structure is much less stable than the *Tetrahymena* folded telomere structure *in vitro*.

The melting temperature (T_m) in solution of CG4 was determined by spectral analysis (Fig. 1D). In 50 mM NaCl, oligonucleotides OG4 and AG4 had sharp melting transitions at approximately 48°C and 43°C, respectively. The CG4 melting was observed at a number of wavelengths between 260 nm and 270 nm and minor changes in the slopes suggested that the melting point is between 25°C and 35°C. The CG4 melting curve in 50 mM NaCl did not have a dramatic two-state transition, possibly because a majority of CG4 does not assume a single conformation (Fig. 1A), making detection of the melting reaction more difficult.

Nucleotide interactions in G-quartets

When TG4 or OG4 G-quartets are irradiated with UV light, thymidine dimers can form between nonadjacent thymidines

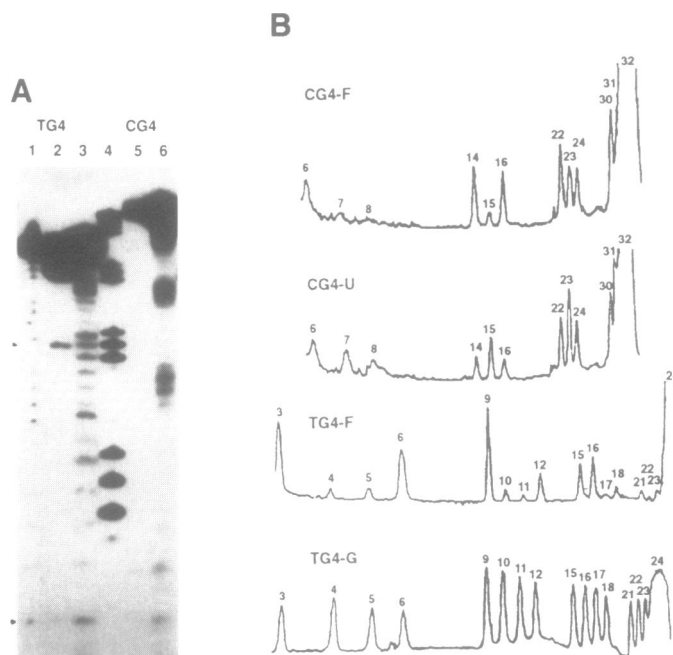


Figure 2. UV crosslinking and methylation interference of intrastrand telomeric oligonucleotide G-quartets. A) UV-Crosslinking. Oligonucleotides were UV-treated, resolved on a 10% denaturing polyacrylamide gel and the faster (crosslinked) and slower (unlinked) forms were eluted from the gel and cleaved with pyrrolidine. The pyrrolidine cleavage products of the faster migrating crosslinked species (lanes 2 and 5) and the slower migrating UV-crosslinked species (lanes 3 and 6) along with G-sequencing reactions of denatured TG4 and CG4 (lanes 1 and 4), were then resolved on a 20% denaturing gel. Pyrrolidine cleaves at thymidine dimers. B) Methylation Interference. 5' end-labeled oligonucleotides CG4 and TG4 were methylated, denatured and allowed to fold. Folded and unfolded forms of the molecule were isolated from nondenaturing gels, cleaved with piperidine, resolved on denaturing polyacrylamide gels and autoradiograms of the gels were scanned using a Biorad, model 620, video densitometer. CG4-F, CG4 faster migrating (folded) molecule; CG4-U, CG4 slower migrating (unfolded) molecule; TG4-F, TG4 faster migrating (folded) molecule; TG4-G, G-sequencing pattern of denatured TG4.

within the G-quartet structure, presumably because these thymidines (in the loops of the structure) are positioned closely together (13). The presence of both adjacent thymidine dimers and these nonadjacent thymidine dimers can thus be detected by cleavage with pyrrolidine. We detected the UV-crosslink reported by Williamson et al. (1989) between two nonadjacent thymidines of TG4 (Fig. 2A, arrows). While a faster migrating UV crosslinked form of CG4 was detected and analyzed, we did not observe any specific crosslinks between nonadjacent thymidines of CG4 (Fig. 2A). When the crosslinked CG4 DNA (Fig. 2A, lane 5) was exposed to film for 200 hours, we observed the same cleavage pattern as seen for the slower migrating form (Fig. 2A, lane 6). We presume that all UV crosslinks in CG4 are non-specific and/or due to the formation of adjacent thymidine dimers, suggesting that the nonadjacent thymidines are not near each other in the folded form of CG4.

Methylation interference of CG4 was performed to determine whether the N7 groups of the guanines were involved in the formation of the faster migrating CG4 molecule (Fig. 2B). Oligonucleotides were methylated with DMS and allowed to fold. The folded and unfolded forms were resolved on a nondenaturing polyacrylamide gel, cleaved with piperidine at the methylated residues, and resolved by denaturing PAGE. The methylation interference patterns for OG4 and TG4 were identical to those previously reported (13, 14). Methylation of guanines 4, 5, 10, 11, 17, 18, 22, and 23 interfered with TG4 folding. Methylation of guanines 12 and 21 may also interfere with TG4 folding to some extent (Fig. 2B). Methylation of the middle two guanine N7s in each repeat of OG4' interfered with folding (data not shown). Although the *Chlamydomonas* and *Oxytricha* telomere repeat sequences differ only by the replacement of the first guanine in each repeat with an adenine (see Table 1), the methylation interference pattern of CG4 was different from the methylation interference pattern of OG4. Methylation of only one guanine in each CG4 repeat (guanines 8, 15, 23, and 31) interfered with folding. Methylation of another guanine in two of the repeats (guanines 7 and 24) only partially interfered with the folding of CG4 (Fig. 2B). The methylation interference patterns of CG4 folded in 50 mM NaCl and in 50 mM KCl were identical (data not shown), implying that the same guanines are involved in G-G base pairs in both folded conformations. Fewer guanine-guanine interactions and the larger interguanine loop size in the CG4 folded molecule may explain the lower T_m of CG4, relative to OG4.

Intermolecular association of *Chlamydomonas* telomere repeats

Since *Tetrahymena* ds telomeric oligonucleotides containing a G-strand overhang associate to form dimers *in vitro* (11), we tested whether *Chlamydomonas* telomeres could form analogous structures. ds oligonucleotides with 3' ss overhangs of both the *Chlamydomonas* (CDS) and *Tetrahymena* (TDS) telomeric sequences (Table 1) were incubated in 275 mM NaCl, 25 mM KCl at concentrations of 0.5–20 μ M for 3 days. TDS forms two species of retarded electrophoretic mobility (11). CDS does not form structures of altered mobility under these conditions (Fig 3A, lane 1). To investigate ion conditions that enhance CDS dimerization, we incubated both TDS and CDS in K^+ , Na^+ , Li^+ , Ru^+ , Mg^{2+} , and spermidine. While TDS associated in the presence of Na^+ and, to a lesser extent, K^+ , CDS dimerization was not observed under any of these ionic conditions (data not shown).

We tested the effect of higher CDS concentration on the dimerization of CDS oligonucleotides. CDS and TDS were incubated at 23°C for 5 days in 275 mM NaCl, 25 mM KCl in the presence of different concentrations of unlabeled telomeric DNA. At 90 μ M (2 μ g/ μ l) total CDS concentration we were able to detect a small amount of CDS with a mobility characteristic of dimer forms. Under these conditions, approximately 50% of TDS was in the dimer forms while approximately 0.5% of CDS was in the analogous forms (Fig. 3A). No dimerization of 5 μ M CDS was observed upon the addition of 2 μ g/ μ l sheared *E. coli* DNA (data not shown), suggesting that dimerization is dependent upon the concentration of ss telomeric DNA ends and not upon the total DNA concentration. With longer exposures, we observed a small amount of TDS migrating slower than the TDS dimer bands (data not shown), which could be a tetrameric G4 DNA form as described by Sen and Gilbert (10). We did not detect any putative G4 DNA with CDS upon longer exposures of the gels, however it is unlikely that we would have been able to detect any CDS molecules with this slower mobility if the ratio of tetramers to dimers was similar to the tetramer to dimer ratio observed for TDS.

In order to determine whether CDS dimers form under ion conditions found *in vivo*, we measured the actual Na^+ and K^+ ion concentrations in whole *Chlamydomonas* cells. Ion concentration in *C. reinhardtii* cells was determined by Inductively Coupled Plasma Emission Spectroscopy. *C. reinhardtii* cells were counted, washed and ashed at 500°C. Three replicate samples were measured. No appreciable concentration of ions was detected in the wash solutions. The apparent measurable cellular ion concentrations were 56 ± 21 mM Na^+ and $138 \text{ mM} \pm 5$ mM K^+ .

The effect of ion concentration on dimerization was tested by incubating 90 μ M TDS or CDS for 5 days in either 275 mM NaCl, 25 mM KCl (shown to permit both putative dimer and

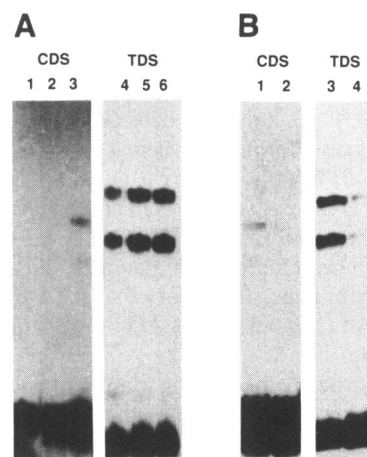


Figure 3. Intramolecular association of *Chlamydomonas* telomere repeats. A) Effect of CDS and TDS concentration. All lanes contain 2.25 μ M ^{32}P labeled ds CDS or TDS plus additional unlabeled ds oligonucleotide of the same type to yield a final concentration of 4.5 μ M (lanes 1 and 4); 25 μ M (lanes 2 and 5); or 90 μ M (lanes 3 and 6). The mixtures were incubated for 4 days at 23°C and the reactions were resolved on 50 mM NaCl, 10% nondenaturing polyacrylamide gels. Lanes 1–3 (CDS) were exposed to film for 120 hours. Lanes 4–6 (TDS) were exposed to film for 16 hours. B) Influence of ionic conditions. 90 μ M CDS or TDS was incubated in 275 mM NaCl, 25 mM KCl (lanes 1 and 3) or 56 mM NaCl, 138 mM KCl for 4 days at 23°C and resolved on 50 mM NaCl, 10% nondenaturing polyacrylamide gels. Lanes 1 and 2 (CDS) were exposed to film for 120 hours. Lanes 3 and 4 (TDS) were exposed to film for 16 hours.

tetramer formation of ss oligonucleotides (10)) or 56 mM NaCl/138 mM KCl. Dimerization of TDS was reduced considerably when the oligonucleotides were incubated in 56 mM NaCl, 138 mM KCl (Fig 3B). No dimerization of CDS was detected when the oligonucleotides were incubated in 56 mM NaCl, 138 mM KCl (Fig 3B) or in 225 mM NaCl, 75 mM KCl (data not shown).

The time dependence of TDS and CDS dimerization was determined in 275 mM NaCl, 25 mM KCl over a 5 day period. The products were simultaneously resolved by nondenaturing PAGE. One hour after resuspension in 275 mM NaCl, 25 mM KCl buffer, TDS dimers were detectable. In contrast, CDS dimerization was not visible until 49 hours after resuspension in 275 mM NaCl, 25 mM KCl buffer (data not shown). As observed previously for G4 structures, the extent of TDS and CDS dimerization increases linearly with time (10).

DISCUSSION

It has been suggested that structures formed *in vitro* by the G-strand overhang of telomeres may play an important role in telomere function. The formation of an intramolecular G-strand structure has been demonstrated for a number of G-rich ss 4-repeat telomere oligonucleotides (12) and most likely assumes a G-quartet structure (13). The CG4 oligonucleotide also appears to form G-quartet structures based upon the increased gel mobility at low temperature, the requirement for 4 telomere repeat units, the independence of folding on oligonucleotide concentration, the stabilization of the structure by K⁺, and the involvement of some of the guanine N7 groups in this structure. Thus all telomere repeats tested are able to form intramolecular G-G base pairs.

However, the G-quartets formed by CG4 are less stable than those formed by G-rich telomere sequences. CG4 requires a lower temperature for intrastrand G-quartet formation, unfolds faster than the *Tetrahymena* TG4, and has a lower T_m in 50 mM NaCl than both the *Oxytricha* OG4 and the *Arabidopsis* AG4. The *Oxytricha* (TTTTGGGG) and *Chlamydomonas* (TTTTAGGG) telomere repeats differ only by the purine adjacent to the thymidines. Only 1 guanine in each CG4 telomeric repeat interferes with G-quartet formation whereas OG4 oligonucleotides have been shown to have at least two guanine N7 groups per repeat involved in G-quartet structures. The *Arabidopsis* (TTTA-GGG) and *Chlamydomonas* (TTTTAGGG) telomere repeat sequences have the same number of guanines and differ only in the number of thymidines. It appears that the increased inter-guanine loop size of CG4 relative to AG4 results in a less stable G-quartet structure.

The G-quartet structure of CG4 is more compact in K⁺ gels than it is in Na⁺ gels as evidenced by the mobility of the CG4 G-quartet relative to the OG4 G-quartet form. Since the proportion of CG4 G-quartet molecules was similar in both Na⁺ and K⁺ gels, and since the methylation interference patterns of CG4 in Na⁺ and K⁺ were indistinguishable, it appears that the same guanines are involved in G-G base pairs in both conformations. It is possible that the *Chlamydomonas* G-strand undergoes G-quartet formation in a manner analogous to G-rich telomere sequences, but that the *Chlamydomonas* G-strand is unusually sensitive to ionic conditions. Perhaps the conformation observed in Na⁺ is an intermediate in the formation of a compact, K⁺-stabilized G-quartet. OG4 and AG4 do not appear to undergo this ion-dependent conformational change.

Interstrand guanine tetrads formed by ds oligonucleotides with a 2-repeat 3'-ss overhang are likely to be more representative of native chromosome ends. We show that dimerization of CDS, like TDS, is dependent upon ion concentration and time. Furthermore, CDS dimerization is dependent on telomere end concentration. Although at high oligonucleotide concentrations, TDS dimerization appears to be independent of oligonucleotide concentration (11), we observe a slight dependence of TDS dimerization on telomere end concentration (Fig 3A, lanes 4–6).

Could *Chlamydomonas* telomere overhangs form dimers *in vivo*? *In vitro*, a small amount of CDS dimerization can be observed when 90 μM CDS is incubated in 275 mM NaCl, 25 mM KCl for at least 49 hours. The inability of carrier DNA to drive CDS to the dimer forms suggests that dimerization of telomeres is dependent on the concentration of telomere ends and not on the concentration of total DNA. In *C. reinhardtii* there are 34 telomeres in a nuclear volume of 3.3 × 10⁻¹⁴ L, giving an effective concentration of 1.7 nM chromosome ends per nucleus. This is approximately 5.3 × 10⁴ times less concentrated than the 90 μM concentration necessary for the observation of some CDS dimerization. Because chromosomes do not diffuse freely in the nucleus, but instead are arranged in a highly organized fashion (21), the effective concentration of telomeres may be several orders of magnitude higher than that which can be calculated based on total nuclear volume. However, in addition to telomere concentration dependence, dimerization of CDS is ion-dependent. Cellular Na⁺ concentrations are generally less than 60 mM and K⁺ concentrations are generally greater than 100 mM in plants and animals (22). In *C. reinhardtii*, the apparent measurable ion concentrations are 56 ± 21 mM Na⁺ and 138 mM ± 5 mM K⁺ (this work). Ion concentrations that favor dimerization of telomeres would need to be provided by local ion sinks (22). In addition, the dimerization of both CDS and TDS is time dependent. Even after 5 days, a very small proportion of the total CDS oligonucleotide adopts the dimer form. It has been proposed that G-rich telomeres may function to bring together chromosomes for pairing (10). The mitotic life cycle of *Chlamydomonas* can be as rapid as 8 hours. This would not be sufficient time for telomere associations to form. The kinetics of CDS association suggests that pairing of other regions of chromosomes may be required to permit telomere dimerization. Alternatively, factors such as proteins may facilitate the telomere dimerization reaction *in vivo*. It is also possible that optimum conditions for *in vitro* dimerization of CDS have not been achieved.

The instability of guanine tetrads may be an important factor in the regulation of chromosome dynamics. In *Oxytricha*, the telomere addition enzyme telomerase cannot recognize ss G-strand oligonucleotides that are in the folded form (7). In addition, Zahler et al. (7) have observed a pausing of telomerase after four repeats of the G-strand have been added onto the oligonucleotide, suggesting that G-quartets may regulate the activity of telomerase and therefore may play a role in the determination of telomere length. Since CG4 can form G-quartets, albeit weaker ones, this mechanism might operate in *Chlamydomonas* as well. It has been proposed that all telomeres share a common structure as evidenced by the ability of telomere sequences from many organisms to function as primers for telomere addition on linear plasmids in *S. cerevisiae* (reviewed in (8)). Although the *Chlamydomonas* telomere sequence can form G-G base pairs, it does not appear to function as a telomere sequence in *S. cerevisiae* (M.E.P., unpublished data). Thus, the ability of a

DNA sequence to form unstable G-G base pairs may not be sufficient to provide telomere function in yeast.

We previously observed an inverse relationship between the extent of G + C richness of an organism's telomeres and its genomic DNA (17). The *Tetrahymena* telomeric repeat is 67% G-rich while the genome has a 25% G+C content. Conversely, the *Chlamydomonas* telomere repeat is 37.5% G-rich while the *Chlamydomonas* genome has a G+C content of 64%. Because G-rich telomere sequences can readily form guanine tetrads, the maintenance of G-rich telomere sequences may be necessary in organisms that have A + T-rich genomes to assure proper meiotic pairing. In contrast, G-rich telomeres and stable guanine tetrad formation may not be necessary for pairing (23) in organisms with G-rich genomes.

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