

Formation of novel hairpin structures by telomeric C-strand oligonucleotides

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ABSTRACT

Telomeres are specialized structures at the ends of chromosomes that are required for long term chromosome stability and replication of the chromosomal terminus. Telomeric DNA consists of simple repetitive sequences with one strand G-rich relative to the other, C-rich, strand. Evolutionary conservation of this feature of telomeric repeat sequences suggests that they have specific structural characteristics involved in telomere function. Absorbance thermal denaturation, chemical modification and non-denaturing gel electrophoretic analyses showed that telomeric C-strand oligonucleotides form stable non-Watson-Crick hairpin structures containing C·C⁺ base pairs. Formation of such hairpins may facilitate previously reported G-strand exclusive interactions.

INTRODUCTION

Telomeres are specialized chromatin domains at the ends of chromosomes that contain simple repetitive elements (1–3). All telomeric repeats have an asymmetric distribution of guanine and cytosine resulting in one strand being relatively G-rich in comparison with the other. These are referred to as the G-strand and the C-strand, respectively. Telomeres are involved in stabilizing the chromosome mechanically and genetically, ensuring complete replication of the chromosomal terminus and organization of the nuclear architecture (4, 5). To understand how telomeres carry out these fundamental cellular roles one must understand the structural and dynamic properties of telomeric repeat sequences.

Both duplex and 'single-stranded' telomeric sequences have unusual structural properties. Telomeric duplexes cloned in bacterial plasmids show unusual susceptibility to a single strand nuclease, S1 (6), and both natural and cloned telomeric duplexes are extraordinarily good templates for primer elongation by Klenow fragment of DNA polymerase 1 from *E. coli*, a 5'-3' exonuclease deficient polymerase (7). Thus, telomeric duplexes share some characteristics with single-stranded DNA. Telomeric G-strand oligonucleotides form structures stabilized by G·G base pairs or quartets (8–14). It has been proposed that these structures may

be involved in telomere function (8–13, 15, 16), meiotic chromosome pairing (17) and the control of gene expression (18). Tandem repeats of the *Tetrahymena* telomeric sequence, d(C₄A₂), in a supercoiled plasmid, adopted an unusual structure at acidic pH that appeared to be due to formation of a C-strand hairpin stabilized by C·C⁺ and A·A⁺ base pairs (19). In this study we examined the structural properties of oligonucleotides containing telomeric C-strand repeats of the ciliated protozoans *Tetrahymena*, d(C₄A₂)₄, and *Oxytricha*, d(C₄A₄)₄, in the absence of superhelical torsion and near neutral pH.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides were synthesized using an Applied Biosystems automated DNA synthesizer, purified by polyacrylamide gel electrophoresis as previously described (8), and resuspended in either water, d(C₄A₂)₄ and d(C₄A₄)₄, or TE, d(T₂G₄)₄ and d(T₄G₄)₄. 5' ³²P-labelled oligonucleotides were purified by polyacrylamide gel electrophoresis (8).

Non-denaturing gel electrophoresis

Polyacrylamide gels, gel electrophoresis buffers, samples and loading dyes (50% sucrose, 0.1% bromophenol blue, 0.1% xylene cyanol ff) were either 50 mM sodium phosphate (NaHPO₄) (pH 7 and 6) or 50 mM sodium acetate (NaOAc) (pH 5). 10 ml samples were mixed with 3 ml loading dye and electrophoresed on 12% polyacrylamide gels at 2–5°C (20 h, 9 V cm⁻¹). pH 6 (50 mM NaHPO₄) running buffer was recirculated during electrophoresis. After electrophoresis, all gel electrophoresis buffers were within 0.5 pH units of their initial pH. Gels were dried and autoradiographed.

Absorbance thermal denaturation

Buffer was either 50 mM NaHPO₄ (pH 7.0 and 6.0) or 50 mM NaOAc (pH 5.0). Oligonucleotides were incubated in the appropriate buffer at 90°C for 3 min and slowly cooled to 23°C. Absorbance thermal denaturation was performed using a Gilford Response II spectrophotometer with a Thermoset.

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Normalized absorbance

Samples were prepared as for absorbance thermal denaturation. Absorbance was monitored at 25°C and then at 85°C. Absolute absorbance of $d(C_4A_2)_4$ samples was ~ 0.55 (~ 2.6 mM) and of $d(C_4A_4)_4$ samples was ~ 0.43 (~ 1.4 mM).

Chemical modification

1 ng 5' ^{32}P -labelled C-strand in 10 ml of either 50 mM $NaHPO_4$ (pH 7.0 and 5.9) or 50 mM $NaOAc$ (pH 5.0) was boiled 2 min and slowly cooled to 23°C. 20 ml of ~ 4 M hydroxylamine (NH_2OH) (titrated to the appropriate pH with diethylamine) was added and the mixture incubated 10 min at 23°C. In 50 mM salt at the appropriate pH, 20% diethyl pyrocarbonate (DEPC) was shaken at 23°C for ~ 30 min. 10 ml of the 20% DEPC was then added to a sample and incubated 10 min at 23°C. DEPC reactions were also performed with 3 M $NaCl$ in all buffers. After chemical treatment, samples were ethanol precipitated, reprecipitated, washed twice with 70% ethanol, cleaved with piperidine and separated on 7 M urea 20% polyacrylamide gels.

RESULTS

Non-denaturing gel electrophoresis has proven to be an informative probe of telomeric oligonucleotide structure (8–11, 17). As shown in Fig. 1A, C-strand oligonucleotides migrated as single bands on a non-denaturing gel at pH 7 and readily hybridized to the complementary G-strand oligonucleotides. Their migration rates were slightly faster than expected for completely unstructured molecules. This suggests the presence of some structure at neutral pH and low temperature although this structure was of insufficient stability to preclude hybridization to the complementary strand. In contrast, at pH 6, both C-strand oligonucleotides formed more compact structures that migrated as two bands (arrows) and did not readily hybridize to the complementary G-strands (Fig. 1B). At pH 5, the resistance of the C-strand oligonucleotides to hybridization with

complementary G-strand was even more pronounced, and formation of a third band was observed for $d(C_4A_2)_4$ (Fig. 1C). In general, $d(C_4A_2)_4$ showed greater resistance to G-strand hybridization than $d(C_4A_4)_4$. The fact that the C-strand oligonucleotides migrated faster than their Watson-Crick duplexes suggests that they are compact intramolecular structures, presumably hairpins. Further, the formation of these structures was concentration independent (data not shown), supporting the idea that the structures formed are intramolecular.

To further investigate this possibility, absorbance thermal denaturation analyses of $d(C_4A_2)_4$ and $d(C_4A_4)_4$ were performed. As shown in Fig. 2, A and B, large cooperative hyperchromic shifts at pH 5.0 and 6.0 were observed for both oligonucleotides, indicative of disruption of base pairs as temperature increases. A small but significant shift was present at pH 7.0 for $d(C_4A_2)_4$ (Fig. 2A), suggesting that C-strand structure formation can occur near physiological pH (7.1–7.4) (20). However, the melting temperature (T_m) was quite low (14°C) at this pH, consistent with the ability of this molecule to hybridize readily with its complement due to instability of the non-Watson-Crick hairpin form (Fig. 1A). In contrast, thermal denaturation of $d(C_4A_4)_4$ at pH 7.0 showed a loss of cooperativity (Fig. 2B). This suggests that longer blocks of purines interfere with the C-strand base pairing interactions involved in structure stabilization. Moreover, disruption of purine stacking interactions was observed as a linear increase in absorbance for $d(C_4A_4)_4$ at pH 7.0, and above and below its cooperative transitions at pH 5.0 and 6.0. This effect was not seen for $d(C_4A_2)_4$, which implies that purine stacking interactions were less substantial in this oligonucleotide than in $d(C_4A_4)_4$. The apparent pK_a 's for $d(C_4A_2)_4$ and $d(C_4A_4)_4$ hairpin formation were determined based on changes in normalized absorbance as a function of pH (Fig. 2E). $d(C_4A_2)_4$ had a higher apparent pK_a (6.37) than $d(C_4A_4)_4$ (5.92). This lends support to the notion that larger blocks of purines interfere with hairpin formation, possibly because of decreased flexibility of stacked purine domains.

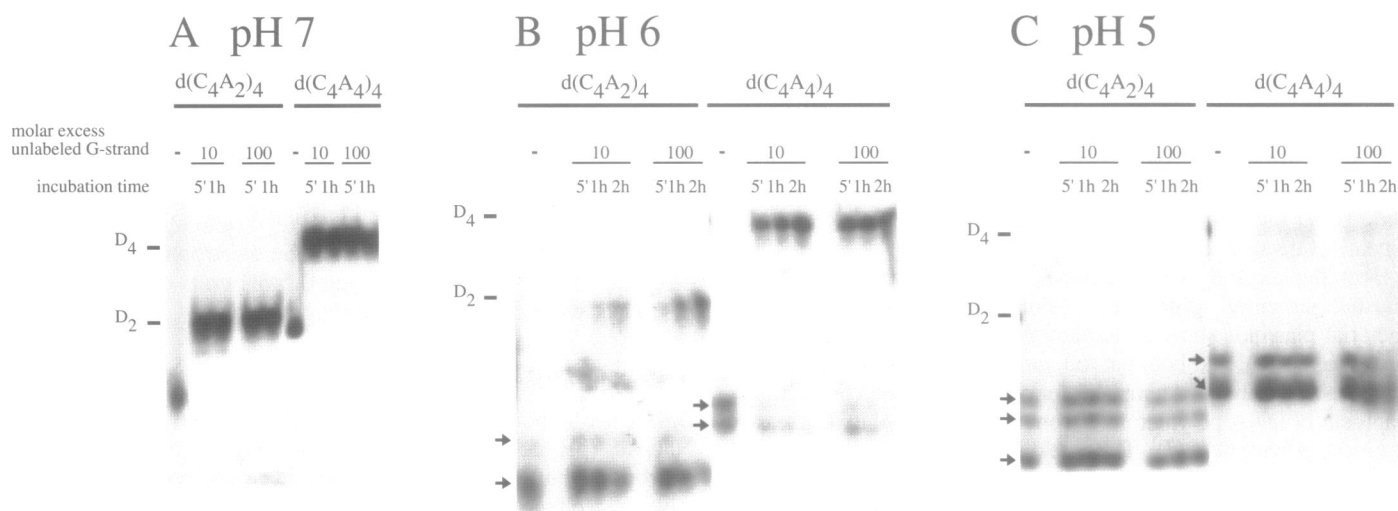


Fig. 1. (A, B and C) Non-denaturing gel electrophoresis at pH 7, 6 and 5 of 5' ^{32}P -labelled C-strand oligonucleotides with or without non-radiolabelled complementary G-strand, $d(T_2G_4)_4$ or $d(T_4G_4)_4$, added. C-strand oligonucleotide samples (1 ng) at the appropriate pH were boiled, slowly cooled to 23°C, mixed with the indicated molar excess of G-strand oligonucleotide and incubated at 23°C for the indicated time before electrophoresis. Positions of C-strand/G-strand duplexes are indicated by D_2 , $d(C_4A_2)_4$. $d(T_2G_4)_4$, and D_4 , $d(C_4A_4)_4$. $d(T_4G_4)_4$.

to DEPC at low pH, as seen by the increase in purine reactivity with the reagent, either due to participation in non-Watson-Crick base pairs (19, 23) or due to altered geometry (i.e., unstacking) as a consequence of C-strand hairpin formation stabilized by C·C⁺ base pairs. T_m's of d(C₄A₄)₄ were lower than those of d(C₄A₂)₄ (Fig. 2D), suggesting that A·A⁺ (and probably A·C⁺ and C·A⁺) base pairing is not involved in hairpin formation, since one would expect additional adenines to stabilize rather than destabilize the hairpin if this were the case. The block of adenines most reactive with DEPC (*) was at a center of symmetry between blocks of cytosines. We suggest that the high reactivity

of this group of adenines is a consequence of its participation in a hairpin loop (Fig. 4A). Previous work indicated that the innate chemical activity of DEPC was not pH dependent in the pH range used in our studies (24), and this is corroborated by the similarity of reactivity of the 3' block of A residues at all pH values tested in the study presented here (Fig. 3B).

In order to assess the effect of the high ionic strength of the NH₂OH reactions on C-strand structure, DEPC reactivities and thermal denaturation profiles in 3 M NaCl were determined and compared to results obtained at low ionic strength (24). The pH dependence of C-strand DEPC reactivity in 3 M NaCl was similar to that at low salt concentration (Fig. 3B), although overall reactivity was somewhat diminished. T_m's of d(C₄A₂)₄ in 3 M NaCl indicate that the hairpins formed by d(C₄A₂)₄ are more stable at high ionic strength (Fig. 2, A, C and D). However, the trend of an increase in T_m with a decrease in pH was still observed, suggesting that similar structural transitions occur in high and low ionic strength environments. Thus, although the NH₂OH reactions (Fig. 3A) were at high ionic strength, their results should reflect pH-dependent structural transitions occurring at low ionic strength.

DISCUSSION

We have shown that synthetic oligonucleotides containing telomeric C-strand repeat sequences are capable of forming hairpins near neutral pH. These hairpins are stabilized by non-Watson-Crick interactions, primarily C·C⁺ base pairs, although contributions by A·A⁺, A·C⁺ and C·A⁺ base pairs can not be rule out. Once formed, these structures do not readily hybridize with their Watson-Crick complements. Our data do not allow us to discriminate between several likely structures. The structures that we feel are most consistent with the data are presented in Fig. 4A. These include complete hairpins with two or three nucleotide loops, partial hairpins and 'dumbbell' structures. Increased loop size necessitates a reduction in the number of C·C⁺ pairs by the loss of hydrogen bonds may be compensated by reduced strain in the loop. An NMR investigation of these structures, currently underway, should help eliminate those that are incorrect.

What are the potential biological roles for these and related structures? Several recent reports have suggested that unusual structures formed by guanine rich sequences could be important for telomere function (8-13, 15, 16), meiotic chromosome pairing (17), and control of gene expression (18). These are extremely stable structures but are generally slow to form (11, 15, 17, 27). In a Watson-Crick environment it is unlikely that, in the absence of other factors to facilitate the process, the two strands would ever be separated long enough to permit G-strand structure formation. The phenomenon reported here provides, in principle, a mechanism that would stabilize this type of strand separation, which is diagrammed in two scenarios in Fig. 4B. In the first scenario, separation of the G/C duplex by intrastrand C-strand hairpin formation permits inter- or intramolecular G-strand interactions. In the second scenario, a telomeric duplex is converted to a molecule with a G-strand overhang by formation of an intrastrand C-strand hairpin. This molecule can then serve as a substrate for telomere repeat addition by telomerase.

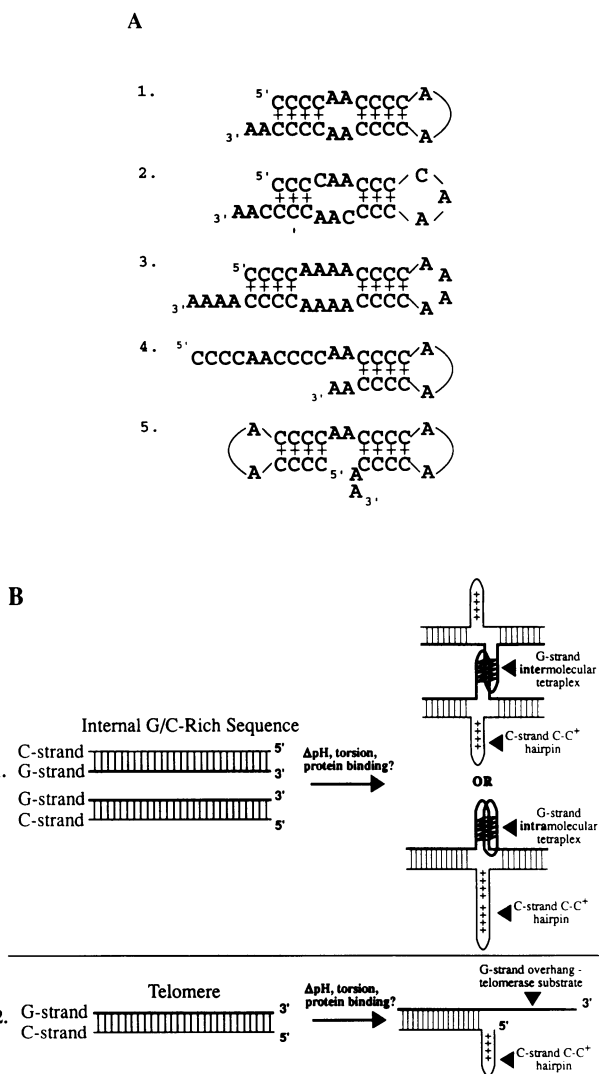


Fig. 4. (A) C-strand oligonucleotide hairpin structures are stabilized by C·C⁺ base pairs and contain an unpaired 3' end. Of the possible hairpin structures formed by these molecules, those shown here are most consistent with the data presented in this report. Structures 1, 2 and 3 are complete hairpins stabilized by C·C⁺ interactions for d(C₄A₂)₄ (1 and 2) and d(C₄A₄)₄ (3). In structure 2 the loop size is increased to three nucleotides. Structures 4 and 5 are a partial hairpin and a 'dumbbell' structure respectively. Similar structures can be drawn for d(C₄A₄)₄. **(B)** Two scenarios illustrating possible biological roles for C-strand hairpins. In the first scenario, formation of intrastrand C-strand hairpins facilitates formation of inter- or intraduplex G-strand structures. In the second scenario, a blunt ended telomeric duplex is converted to a molecule with a G-strand overhang by formation of an intrastrand C-strand hairpin. This molecule can then serve as a substrate for telomere repeat addition by telomerase.

an oligopyrimidine·oligopurine tract with unusual structure suggests that C-strand hairpin structures could be stabilized with the aid of a specific binding protein *in vivo* (29).

Finally, we note that oligopyrimidine·oligopurine sequences, many C-rich on one strand, form unusual structures that are usually facilitated by low pH and torsional strain (30). These sequences are found near genes and recombination hot spots. Thus, C·C⁺-stabilized hairpins could be involved in the regulation of gene expression and recombination.

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REFERENCES

- Zakian, V. A. (1989) *A. Rev. Genet.*, **23**, 579–604.
- Greider, C. W. (1990) *BioEssays*, **12**, 363–369.
- Blackburn, E. H. (1990) *J. Biol. Chem.*, **265**, 5919–5921.
- Yu, G. L., Bradley, J. D., Attardi, L. D. and Blackburn, E. H. (1990) *Nature*, **344**, 126–132.
- Blackburn, E. H. (1991) *Nature*, **350**, 569–573.
- Budarf, M. L. and Blackburn, E. H. (1987) *Nucleic Acids Res.*, **15**, 6273–6292.
- Henderson, E. H., Larson, D., Melton, W., Shampay, J., Spangler, E., Greider, C., Ryan, T. and Blackburn, E. H. (1988) *Cancer Cells*, **6**, 453–461.
- Henderson, E., Hardin, C. C., Walk, S. K., Tinoco, I. J. and Blackburn, E. H. (1987) *Cell*, **51**, 899–908.
- Williamson, J. R., Raghuraman, M. K. and Cech, T. R. (1989) *Cell*, **59**, 871–880.
- Sundquist, W. I. and Klug, A. (1989) *Nature*, **342**, 825–829.
- Sen, D. and Gilbert, W. (1990) *Nature*, **344**, 410–414.
- Panyutin, I. G., Kovalsky, O. I., Budowsky, E. I., Dickerson, R. E., Rikhirev, M. E. and Lipanov, A. A. (1990) *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 867–870.
- Sundquist, W. I. (1991) In 'The structures of telomeric DNA.' Eckstein, F. and Lilley, D. M. J. (ed.), *Nucleic Acids and Molecular Biology*. Springer-Verlag, Berlin, Vol. 5.
- Acevedo, O. L., Dickinson, L. A., Macke, T. J. and Thomas, C. A. (1991) *Nucleic Acids Res.*, **19**, 3409–3419.
- Raghuraman, M. K. and Cech, T. R. (1990) *Nucleic Acids Res.*, **18**, 4543–4552.
- Zahler, A. M., Williamson, J. R., Cech, T. R. and Prescott, D. M. (1991) *Nature*, **350**, 718–720.
- Sen, D. and Gilbert, W. (1988) *Nature*, **334**, 364–366.
- Smith, S. S., Baker, D. J. and Jardines, L. A. (1989) *Biochem. Biophys. Res. Com.*, **160**, 1397–1402.
- Lyamichev, V. I., Mirkin, S. M., Danilevskaya, O. N., Voloshin, O. N., Balatskaya, S. V., Dobrynin, V. N., Filippov, S. A. and Frank-Kamenetskii, M. D. (1989) *Nature*, **339**, 634–637.
- Bright, G. R., Fisher, G. W., Rogowska, J. and Taylor, D. L. (1987) *J. Cell Biol.*, **104**, 1019–1033.
- Johnston, B. H. (1988) *Science*, **241**, 1800–1804.
- Gray, D. M. and Cui, T. (1984) *Nucleic Acids Res.*, **12**, 7565–7580.
- Hunter, W. N., Brown, T., Anand, N. N. and Kennard, O. (1986) *Nature*, **320**, 552–555.
- Johnston, B. H. and Rich, A. (1985) *Cell*, **42**, 713–724.
- Herr, W. (1985) *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 8009–8013.
- McCarthy, J. G., Williams, L. D. and Rich, A. (1990) *Biochemistry*, **29**, 6071–6081.
- Hardin, C. C., Henderson, E. R., Watson, T. and Prosser, J.K. (1991) *Biochemistry*, **30**, 4460–4472.
- Greider, C. W. and Blackburn, E. H. (1988) *Symposium On The Molecular Biology Of RNA Held At The 17th Annual UCLA (University Of California Los Angeles) Symposia On Molecular And Cellular Biology, Keystone, Colorado, USA.*
- Edelmann, W., Kroger, B., Goller, M. and Horak, I. (1989) *Cell*, **57**, 937–946.
- Wells, R. D., Collier, D. A., Hanvey, J. C., Shimizu, M. and Wohlrab, F. (1988) *FASEB J.*, **2**, 2939–2949.