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Telomeric nucleic acids: C-strand structure and a telomerase RNA mutant

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Telomeric nucleic acids: C-strand structure and a telomerase RNA mutant

Ahmed, Shawn Cameron, Ph.D.
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Telomeric nucleic acids: C-strand structure and a telomerase RNA mutant

by

Shawn Cameron Ahmed

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For the Graduate College

Iowa State University
Ames, Iowa

1994
Dedicated to my parents, Mohammed and Shirley
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ABSTRACT

Telomeres, the ends of linear chromosomes, are composed of simple tandem repeats which are usually G-C rich. Telomeres are essential for chromosome stability, organizing the nuclear architecture and ensuring complete replication of the chromosomal terminus. To understand how telomeres carry out these fundamental cellular roles, one must understand the structural and dynamic properties of telomeric repeat sequences. Structural and genetic approaches were taken to learn more about telomeric nucleic acids.

The structural portion of my research concerned an unusual DNA structure formed by the C-rich strand of telomeric DNA. Telomeric C-strand sequences form non-Watson-Crick structures in supercoiled plasmids at low pH. Absorbance thermal denaturation, chemical modification and non-denaturing gel electrophoresis showed that telomeric C-strand oligonucleotides form stable structures at low pH. H1'-H1' nuclear Overhauser effects indicated that these structures were four-stranded. In addition, these four-stranded C-structures were shown to mediate recognition and binding of identical nucleic acid sequences. Thus, a novel nucleic acid dimerization motif was discovered.

The genetic portion of my research concerned analysis of a Tetrahymena mutant with short telomeres. This mutant was heterozygous for a telomerase RNA mutation. Telomerase is a ribonucleoprotein that uses its RNA component as a template for addition of telomeric repeats to chromosome termini. Therefore, telomerase is involved in telomere length regulation, a process that has been implicated in both aging and cancer. The mutant telomerase RNA gene caused telomere shortening when introduced into wildtype cells, and thus identifies a functionally important domain of the telomerase RNA. Although mutant telomerase activity was indistinguishable from wildtype activity in vitro, cells expressing high levels of the mutant telomerase RNA exhibited lethal phenotypes that were due to the presence of very short telomeres.
INTRODUCTION

Thesis Organization. This introduction is meant to provide a broad general perspective of current knowledge in the field of telomeres as it relates to my research. The main body of this thesis is composed of three papers, and a conclusion at the end of this thesis touches on the fruits of my efforts and discusses research topics that may be worthy of further investigation. References for the introduction and conclusion are cited after the conclusion.

The first two papers have been published in peer-reviewed journals. These two papers are the result of my investigations into telomeric C-strand structure. The third paper is a 'manuscript in preparation' and discusses my efforts to understand a telomerase RNA mutation that I discovered. All three papers have different formats because they either have been or will be published in different journals. References are given at the end of each paper.

I am the primary author on each paper and personally completed most or all of the research described. My research was conducted with the guidance of Eric Henderson, the principal investigator. Agustin Kintanar provided invaluable assistance with NMR spectroscopy in paper 2. Mohammed Al-Anazi and Hong Sheng produced several of the mutants described in paper 3.

Telomeres. Telomeres, chromatin domains at the ends of eukaryotic chromosomes, protect chromosomes from degradation and end-to-end fusion, presumably by burying the 'end' in a specialized nucleoprotein complex (Blackburn, 1991; Greider, 1990). Telomeres also help organize a chromosome's architecture within the nucleus and often cluster together at the nuclear periphery (Blackburn and Szostack, 1984). Chromosome ends without telomeres are unstable, and telomere loss often results in chromosome loss (Sandell and Zakian, 1993). Thus, telomeres are essential for genome viability and integrity. Conserved molecular mechanisms by which telomeres accomplish this task probably developed very early in eukaryotic evolution.

Telomeric DNA. In most eukaryotes, telomeric DNA is composed of simple tandem repeats, though the sequence of these repeats can vary from organism to organism (Zakian, 1989). Most telomeric repeats have an asymmetric distribution of guanine and cytosine resulting in one strand being G-rich and the other C-rich. The G-strand always run 5' to 3' towards the end of the chromosome and extends as a single strand by about two repeats (Figure 1) (Henderson and Blackburn, 1989; Wellinger, et al., 1993). Evolutionary conservation of this strand composition bias suggests that it is important to telomere structure and function. Curiously, both strands of telomeric DNA can form their own unusual structures.

\[
\begin{align*}
\text{TTGGGTTGGGTTGGG} & \quad \text{5'} \\
\text{AACCC} & \quad \text{3'}
\end{align*}
\]

Figure 1. The Tetrahymena telomeric terminus has a 2 repeat 3' overhang.
Unusual DNA structures. DNA has the ability to adopt a variety of structures. Most DNA in a cell is double-stranded B-form DNA. However, double-stranded DNA can adopt alternative conformations such as A- and Z-DNA. In addition, three- and four-stranded DNA structures have been shown to exist. These less abundant DNA structures probably have specific biological functions (Rich, 1993). Methods for determining the biological function of an unusual DNA structure vary, but, in general, studies investigate sequences which form unusual structures and proteins that interact with these structures. Ultimately, mutational analysis of sequences which form such structures and of proteins that interact with them will enable us to understand their biological role. Telomeric DNA has been a useful 'model system' for studying unusual DNA structures.

G-strand structures. In the presence of monovalent cations, the G-strand of telomeric DNA was shown to form unusual DNA structures (Henderson, et al., 1987) that were four-stranded (Williamson, et al., 1989; Sen and Gilbert, 1990). Since then, several groups have clearly demonstrated that the ends of chromosomes can associate if their 3' overhangs fold back and dimerize via a four-stranded structure, a G-tetraplex (Figure 2a) (Oka and Thomas, 1987; Williamson, et al., 1989). In this situation, sequences that are identical recognize and bind to each other. G-tetraplexes may be the mechanism by which telomeres cluster together in vivo. In support of this possibility, telomere binding proteins from both yeast and the ciliate Oxytricha facilitate G-tetraplex formation in vitro (Fang and Cech, 1993; Giraldo and Rhodes, 1994). G-structures may also be involved in self-recognition of sister chromosomes during meiosis (Sen and Gilbert, 1988). In vivo evidence of a role for G-structures in meiosis has been provided by deletion mutants of KEM1, a yeast G-tetraplex-specific nuclease, which undergo cell cycle arrest during meiosis (Liu and Gilbert, 1994). Finally, G-tetraplexes have been shown to mediate dimerization of the HIV genome, another process which involves nucleic acid self-recognition (Marquet, et al., 1991; Sundquist and Heaphy, 1993).

Figure 2. a, G-tetraplexes connect two telomeric termini by their 3' overhangs. Monovalent cations are indicated by + symbols. b, A guanine tetrad. c, G-tetraplex structural diversity.
G-tetraplexes. NMR and X-ray crystallography studies have shown that four-stranded G-structures are stabilized by guanine tetrads. The guanines are Hoogsteen-paired to one another in a cyclical manner (Figure 2b) (Smith and Feigon, 1992; Aboul-ela, et al., 1992; Cheong and Moore, 1992; Wang and Patel, 1992; Kang, et al., 1992). Monovalent cations that are essential for G-tetrad formation lie between the planes stacked tetrads and are coordinated by the four carbonyl groups of each tetrad. Depending on the oligonucleotide used, telomeric G-strand structures can be formed by four individual strands of DNA, two strands of DNA which dimerize as hairpins, or a single strand of DNA which folds back on itself (Figure 2c) (Williamson, 1993). The strands of these structures may be parallel (Aboul-ela, et al., 1992; Wang and Patel, 1992; Laughian, et al., 1994) or antiparallel (Smith and Feigon, 1992; Kang, et al., 1992).

C-tetraplexes. C-rich sequences can adopt unusual structures at low pH, and it was recently shown that, like G-structures, C-structures are also four-stranded (Gehring, et al., 1994; Leroy, et al., 1994). However, C-tetraplexes are stabilized by parallel-stranded C-C+ base pairs instead of guanine tetrads (Figure 3a). Low pH is necessary to stabilize C-tetraplexes, because one of the C's of a C-C+ base pair is protonated. Although a C-tetraplex is four-stranded, it consists of two individual parallel-stranded duplexes which are ‘zippered together’ in an antiparallel orientation (Figure 3b) (Gehring, et al., 1994). In a C-tetraplex, base pairs of one duplex alternate with base pairs of the other duplex. One striking characteristic of C-tetraplexes is that H1 sugar protons of adjacent base pairs are very close to one another (Gehring, et al., 1994). This has never been seen for any other DNA structure and is a ‘diagnostic characteristic’ of C-tetraplexes. In vivo, the ability of C-rich sequences to form C-tetraplex structures might facilitate formation of intra- or intermolecular G-tetraplex structures by complementary G-rich sequences. Identification of several binding activities specific for C-rich sequences that form unusual structures indicates that C-tetraplexes could be stabilized with the aid of a binding protein (Edelmann, et al., 1989; Muraiso, et al., 1992; Ito, et al., 1994).

Sequences that form C-structures. Oligopurine-oligopyrimidine sequences, many C-rich on one strand, adopt unusual structures (often at low pH), as revealed by enzymatic and chemical modification assays (reviewed by Wells, et al., 1988). These sequences are found near genes and recombination hot spots. Exactly how these biologically relevant C-rich sequences are

![Figure 3](image)

Figure 3.  

*a*, A C-C+ base pair.  
*b*, A C-tetraplex formed by dTC5.
structured has yet to be determined and could involve either C-G-C+ triplexes or C-tetraplexes. In contrast, the C-rich strand of telomeres has recently been shown to form C-tetraplexes (Ahmed, et al., 1994). When telomeric sequences are put in supercoiled plasmids, the C-rich strand adopts an unusual structure at low pH (Lyamichev, et al., 1989; Belotserkovskii, et al., 1992; Voloshin, et al., 1992). We have examined the structural characteristics of oligonucleotides containing telomeric C-strand sequences. These C-rich oligonucleotides form stable structures at low pH (Ahmed and Henderson, 1992; Ahmed, et al., 1994). Two dimensional NMR spectroscopy showed that these structures were C-tetraplexes (Ahmed, et al., 1994). In addition, electrophoresis experiments demonstrated that C-tetraplexes, like G-tetraplexes, could mediate recognition and binding between identical nucleic acid sequences (Ahmed, et al., 1994).

Telomerase. Telomeres are necessary for the complete replication of chromosome termini. Most DNA polymerases require an RNA primer to initiate synthesis, so it was predicted that chromosome ends would become shorter with each round of DNA replication (Watson, 1972; Olovnikov, 1973). One mechanism that cells use to maintain telomere length involves de novo addition of telomeric repeats to chromosome termini. The enzyme that carries out this process, telomerase, is a ribonucleoprotein that uses its RNA component as a template to add G-strand telomeric repeats to chromosome ends (Greider, 1990). Telomerase was originally identified in the ciliate *Tetrahymena* and has been shown to exist in other ciliates, yeast, frogs, mice and humans (Greider and Blackburn, 1985; Zahler and Prescott, 1988; Morin, 1989; Shippen-Lentz and Blackburn, 1989; Prowse, et al., 1993; Mantell and Greider, 1994; Lingner, et al., 1994; Singer and Gottschling, 1994).

Telomerase RNA structure. Telomerase has an essential RNA moiety which has been cloned from a number of ciliates and yeast (Greider and Blackburn, 1989; Shippen-Lentz and Blackburn, 1989; Lingner, et al., 1994; Singer and Gottschling, 1994). Although there is little primary sequence similarity among these genes, a common secondary structure of ciliate telomerase RNAs has been determined by comparative sequence analysis and confirmed in *Tetrahymena* using enzymatic probes (see Figure 3.1b) (Romero and Blackburn, 1991; ten Dam, et al., 1991; Lingner, et al., 1994). This conserved structure, including the stem-loop III pseudoknot, has been conserved over an evolutionary distance equivalent to that separating the rat from maize or *Chlamydomonas* (Lingner, et al., 1994). Parts of this conserved secondary structure may be important for telomerase RNA functions. The telomerase RNA may participate in catalyzing addition of telomeric repeats, may serve as a scaffold bridging the protein components of telomerase or may be involved in regulation of telomerase activity. Elucidating telomerase RNA structure/function relationships is currently a major task in the telomere community. While most labs addressing this problem are using approaches that involve site-directed mutagenesis, we have chosen to select for ‘random’ telomerase RNA mutations that give interesting phenotypes in vivo (see *Tetrahymena* telomere length mutants, below).

Template mutations. In vivo experiments in both *Tetrahymena* and yeast have shown that telomerase RNA template mutations result in addition of mutant sequences to chromosome termini (Yu, et al., 1990; Singer and Gottschling, 1994). All three *Tetrahymena* template mutants produced cells which were unable to divide properly and died, indicating that healthy telomeres are necessary for cell viability (Yu, et al., 1990). Two of these mutations produced the expected
sequences in telomeric DNA, which resulted in telomere lengthening probably due to disruption of a telomere binding protein's interaction with terminal telomeric DNA (Yu, et al., 1990).

**Human telomere length.** Telomere length in an organism is dynamic and varies from several hundred base pairs in yeast and ciliates to thousands of base pairs in vertebrates (Greider, 1990). Telomeres of human somatic cells shorten with age both *in vitro and in vivo*, although germ line telomeres remain long (Figure 4) (Harley, et al., 1990; Allsopp, et al., 1992). This suggests that telomere shortening may limit a cell's replicative life span, either by influencing gene activity near telomeres or by chromosome instability due to telomere loss (Levy, et al., 1992). Thus, telomere shortening may be a 'genetic clock' which tells a cell how old it is. This clock may be a mechanism developed to guard against immortal tumor cells. If a tumor cell had a limited number of divisions, it would not be able to do much damage. However, immortal cells are able to stop their telomere clocks and stabilize the lengths of their telomeres (Figure 4) (Counter, et al., 1992). In agreement with the observations, telomerase activity has been found in immortal cells but not their somatic precursors (Counter, et al., 1992; Klingelhutz, et al., 1994; Counter, et al., 1994). Thus, a 'suicidal' telomerase RNA could be an effective cancer therapy. Conversely, activating telomerase in somatic cells could be an effective aging therapy.

![Figure 4. Telomere length decreases in somatic but not immortal cells.](image)

**Telomere shortening and senescence in lower eukaryotes.** Telomere length is variable in most organisms and exists in a dynamic equilibrium that can be tilted towards either long or short telomeres (Bernards, et al., 1983; Larson, et al., 1987). As mentioned above, regulation of telomerase activity appears a critical component of human telomere shortening. One *Tetrahymena* telomerase RNA template mutant got short telomeres, presumably as a result of loss of telomerase activity (Yu, et al., 1990). Deletion of the yeast telomerase RNA gene resulted in short telomeres, as did mutants with deletions of the EST1 gene whose function is unknown (Lundblad and Szostak, 1989; Singer and Gottschling, 1994). In both *Tetrahymena* and yeast mutants with short telomere phenotypes, slow growing cultures with many dying cells were observed (Lundblad and Szostack, 1989; Yu, et al., 1990; Singer and Gottschling, 1994). Therefore, telomere shortening has been associated with senescence in both humans and single-celled eukaryotes. The association of these
phenotypes is not unexpected, since telomere loss in yeast leads to chromosome instability and loss (Sandell and Zakian, 1993).

**Regulation of telomere length.** Many genes that regulate telomere length have been characterized in yeast. Some mutations in RAP1, a protein that binds to telomeres *in vitro* and *in vivo*, result in telomere shortening (Buchman et al., 1988; Conrad et al., 1990; Lustig et al., 1990; Sussel and Shore, 1991; Wright et al., 1992). However, overexpression of the RAP1 C-terminus or mutations in RIF1, a protein that interacts with the RAP1 C-terminus, lead to telomere elongation (Hardy et al., 1992). RAP1 and RIF1 may cooperate to sequester telomeres away from telomerase activity, a possibility supported by the fact that mutant telomere sequences, which might disrupt telomere-protein interactions, resulted in telomere elongation in *Tetrahymena* (Yu et al., 1990).

Mutations in the PIF1 DNA helicase also result in telomere elongation, possibly because the helicase is necessary to dissociate telomerase from its telomeric substrate (Schultz and Zakian, 1994). Some mutations in the catalytic subunit of DNA polymerase 1 (CDC17) also result in telomere elongation, while mutations in TEL1 and TEL2 cause telomere shortening (Carson and Hartwell, 1985; Lustig and Petes, 1986). The TEL1 gene codes for an IP3 kinase, indicating that a signal transduction pathway may be involved in telomere length regulation (T. Petes, personal communication).

**Tetrahymena.** The ciliated protozoan, *Tetrahymena thermophila*, has two nuclei, a macronucleus and a micronucleus. The micronucleus is diploid and transcriptionally inactive. The macronucleus develops from a micronucleus which fragments its chromosomes into about 200 - 300 pieces and increases each chromosome's copy number to 45. Thus, there are about 20,000 'minichromosomes' in each macronucleus and about 40,000 telomeres. This makes *Tetrahymena* a good model organism for studying telomeres.

**Tetrahymena telomere length mutants.** If *Tetrahymena* cells are grown in log phase, their telomeres increase in length (Figure 5). After a long period of log phase growth, a spontaneous mutation occurs and a mutant with very short telomeres takes over the culture (Figure 5a) (Larson et al., 1987). The mutation probably occurs in genes that regulate telomere length in response to growth conditions (Figure 5b). The mutation is dominant because mutant cells have less (telomeric) DNA to replicate and thus a shorter S phase than wildtype cells. Therefore, mutant cells divide faster than wildtype cells. Such a phenotype would only be observed in an organism (such as *Tetrahymena*) which has a significant proportion of telomeric DNA relative to total DNA. Once this mutation occurs, the mutant's telomeres never grow again (Larson et al., 1987). Thus, the mutation has been named *tgf* (telomere growth inhibited forever).

**tgf mutations.** Analysis of *tgf* mutations should give insight into aging and cancer, since telomere length regulation is involved in these processes. Unfortunately, *Tetrahymena* does not have a well developed genetic system, and there is currently no way to clone genes identified only by mutation. Therefore, we have used the candidate gene approach to look for *tgf* mutations in the only cloned *Tetrahymena* gene that is involved in telomere length regulation - the telomerase RNA gene. One of the mutants, *tgf1*, was heterozygous for a telomerase RNA mutation. This mutation was lethal when homozygous (Ahmed et al., manuscript in preparation).
Figure 5.  

**a**, Southern blot shows a *tgif* phenotype. This blot was kindly provided by Hong Sheng.

**b**, *tgif* mutants have mutations in genes that regulate telomere length in response to growth conditions.
CHAPTER 1: FORMATION OF NOVEL HAIRPIN STRUCTURES BY TELOMERIC C-STRAND OLIGONUCLEOTIDES

(by permission of Oxford University Press)

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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 (NaH2PO4) (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 NaH2PO4) 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 NaH2PO4 (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.

**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(C4A2)4 samples was ~0.55 (~2.6 mM) and of d(C4A4)4 samples was ~0.43 (~1.4 mM).

**Chemical modification**

1 ng 5' 32P-labelled C-strand in 10 ml of either 50 mM NaH2PO4 (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 (NH2OH) (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. 1.1a, 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. 1.1b). 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(C4A2)4 (Fig. 1.1c). In general, d(C4A2)4 showed greater resistance to G-strand hybridization than d(C4A4)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(C4A2)4 and d(C4A4)4 were performed. As shown in Fig. 1.2, 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(C4A2)4 (Fig. 1.2a), 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. 1.1a). In contrast, thermal denaturation of d(C4A4)4 at pH 7.0 showed a loss of cooperativity (Fig. 1.2b). 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(C4A4)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(C4A2)4, which implies that purine stacking interactions were less substantial in this oligonucleotide than in d(C4A4)4. The apparent pKa's for d(C4A2)4 and d(C4A4)4 hairpin formation were determined based on changes in normalized absorbance as a function of pH (Fig. 1.2e). d(C4A2)4 had a higher apparent pKa (6.37) than d(C4A4)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.

The molecular nature of C-strand structure was further investigated using chemical probes. As demonstrated by a control oligonucleotide, which is predicted to form a partial intramolecular hairpin, NH2OH was most reactive with single-stranded cytosines and had about the same reactivity at pH 7.0 and 5.9, but not at pH 5.0 (21) (Fig. 1.3a). In contrast, reactivity of most C-strand cytosines at pH 5.9 dropped to levels seen with C-G double-stranded cytosines in the control oligonucleotide, indicating that C-C+ (22), C-A+ or A-C+ (23) base pairs stabilize the C-strand hairpins. The most 3' cytosine in each block of cytosines had increased NH2OH susceptibility. This could be due to destabilization of C-C+ pairs adjacent to blocks of purines or in a hairpin loop. DEPC, most reactive
Fig. 1.1. a, b and c, Non-denaturing gel electrophoresis at pH 7, 6 and 5 of 5' 32p-labelled C-strand oligonucleotides with or without non-radio labelled complementary G-strand, d(T2G4)4 or d(T4G4)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 D2, d(C4A2)4-d(T2G4)4, and D4, d(C4A4)4-d(T4G4)4.
Fig. 1.2. Spectrophotometry of C-strand oligonucleotides.

a and b, Absorbance thermal denaturation analysis (260 nm) of d(C4A2)4 and d(C4A4)4 at the indicated pH's.

c, 3 M sodium chloride (NaCl) was included in d(C4A2)4 samples to mimic ionic strength conditions of the hydroxylamine assay (Fig. 3a).

d, C-strand Tm's at pH's tested.

e, C-strand structural changes were monitored as a function of pH by normalizing absorbance at 25°C with absorbance at 85°C. 50 mM NaHPO4 and 50 mM NaOAc buffers were used to span the pH range from 5.0 to 7.8. pKa's of structures formed at low pH are indicated.
**Fig. 1.3. Chemical reactivity of the C-strands at different pH's.**

*a*, NH$_2$OH reactivity with the C-strands and with a control oligonucleotide at different pH's. The predicted intramolecular structure of the control oligonucleotide is shown below the gel. Cytosines of the control oligonucleotide predicted to be single stranded (s) or to form C·G base pairs (d) are indicated. Note differential susceptibility of cytosines at 3' end of each block of 4.

*b*, DEPC reactivity with the C-strands at different pH's with and without 3 M NaCl. Asterisks (*) mark the most reactive block of adenines.
with single stranded and syn purines (A>G) (24, 25), modified d(C4A2)4 at different pH's (Fig. 1.3b) in a pattern similar to that previously observed for tandem d(C4A2)4 repeats in a supercoiled plasmid (19), whose C-strand structure must have been antiparallel. Thus, the intramolecular C-strand structures observed by non-denaturing gel electrophoresis (Fig. 1.1) involve antiparallel base pair interactions. Low DEPC reactivity of C-strand oligonucleotides at pH 7.0 can be attributed to purine stacking interactions reducing the accessibility of the adenines to DEPC (26). Formation of C-strand hairpins resulted in significant increases in DEPC reactivity of all C-strand adenines, except for those at the 3' end of the molecule, indicating that the stacking interactions here are not perturbed by hairpin formation. Adenines were more accessible 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. Tm's of d(C4A4)4 were lower than those of d(C4A2)4 (Fig. 1.2d), 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. 1.4A). 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. 1.3b).

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. 1.3b), although overall reactivity was somewhat diminished. Tm'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. 1.2, a, c and d). However, the trend of an increase in Tm 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. 1.3a) 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. 1.4a. These include complete hairpins with two or three nucleotide loops, partial hairpins and "dumbell" 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
Fig. 1.4. Models.

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 "dumbell" 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.
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 G-strand is "revealed" by C-strand intrastrand hairpin formation, thereby permitting telomere repeat addition by telomerase, an enzyme that does not add telomere repeats to blunt ended telomere duplexes in vitro, but does add repeats in vivo to telomeres that are introduced into cells as blunt ends (28). The recent identification of a binding activity specific for the C-strand of 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


CHAPTER 2: HUMAN TELOMERIC C-STRAND TETRAPLEXES


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Telomeric C-strand sequences form non-Watson-Crick structures in supercoiled plasmids and in oligonucleotides at low pH. Here we examine oligonucleotides composed of 2 or 4 repeats of the human telomeric C-strand sequence d(CCCTAA)n. At low pH, the 2-repeat molecule forms a dimer which exhibits H1'-H1' nuclear Overhauser effects (NOEs) between stacked C-C⁺ base pairs. These NOEs are characteristic of the i-motif, which is a tetraplex composed of two intercalated C-C⁺ duplexes. The 4-repeat molecule forms an intramolecular monomeric structure at low pH, suggesting that four contiguous cytosine tracts fold into a C-C⁺ intercalated tetraplex. These novel structures may be relevant to formation of guanine tetraplexes by complementary G-rich sequences. They may also provide a general mechanism for self-recognition by nucleic acids.

Telomeric DNA is composed of simple tandem repeats which have one G-rich and one C-rich strand¹,². The G-strand of telomeres always runs in a 5' to 3' direction towards the end of a chromosome and extends as a single strand by about two repeats³. In the presence of monovalent cations, telomeric G-strand oligonucleotides form intra- and intermolecular tetraplex structures stabilized by guanine tetrads⁴-¹⁰. Synthetic telomeric single-stranded overhangs have been shown to fold back and dimerize as G-tetraplex structures⁷-⁹. Natural telomeres also self-associate¹¹, presumably by G-tetraplex formation¹². In these situations, a nucleic acid sequence is able to recognize and bind to its twin, rather than its complementary sequence¹³. Evidence of a biological role for G-tetraplexes has been provided by the demonstration that dimerization of HIV genomic RNA is mediated by G-tetraplex formation¹⁴,¹⁵. Moreover, several proteins have been identified that bind to¹⁶-²⁰ or facilitate formation of²¹ G-tetraplexes, suggesting that G-tetraplexes exist in vivo, although the biological significance of these observations is not yet clear.
Recently, C-rich oligomers have been shown to form C-C+ tetraplexes at low pH\textsuperscript{22,23}. These structures consist of two parallel-stranded C-C+ base-paired helices 'zipped together' in an antiparallel orientation. Since the base pairs of one C-C+ duplex are intercalated into the base pairs of the other duplex, the structure is called the "i-motif\textsuperscript{22,24}. It has been suggested that telomeric C-strands may form the i-motif\textsuperscript{22,24} since they are structured at acidic pH\textsuperscript{25,26}. Here we present evidence that this hypothesis is correct. We also show that intercalated C-C+ tetraplexes can mediate self-recognition by nucleic acids.

**pH-dependent C-strand structure**
Telomeric C-strand repeats contain tracts of cytosines separated by A/T tracts. Absorbance melting curves taken at different pH's indicate that the human telomeric C-strand oligonucleotides d(C\textsubscript{3}TA\textsubscript{2})\textsubscript{2} and d(C\textsubscript{3}TA\textsubscript{2})\textsubscript{4} (Hum2 and Hum4, respectively) show cooperative absorbance transitions at low pH (Fig. 2.1) indicating that Hum2 and Hum4 are structured under these conditions. The Hum2 transitions at pH 6.0 and 5.0 are bimodal. 1\textsuperscript{st} derivative analysis indicates the presence of two distinct structural transitions, one of which predominates at low pH (data not shown).

It is clear that structure formation by both Hum2 and Hum4 is pH-dependent (Fig. 2.1). Similar pH-dependent transitions were seen for protozoan telomeric C-strand oligonucleotides\textsuperscript{22,23} and probably would occur for any oligomer that has 'unstructured' cytosine tracts at neutral pH.

**Intra- and intermolecular structures**
Several studies have hypothesized that telomeric C-strands adopt simple hairpin structures stabilized by C-C+ base pairs\textsuperscript{25-28}. Therefore, an oligomer with two tracts of C's, such as Hum2, might be expected to fold back and form a simple C-C+ base-paired hairpin at low pH. However, non-denaturing gel electrophoresis shows that Hum2 forms an intermolecular complex in a concentration-dependent manner at low pH (Fig. 2.2a). In contrast, Hum4's electrophoretic mobility is concentration independent and faster than that of 'unstructured' Hum4 (Fig. 2.2a), indicating that Hum4 forms an intramolecular structure.

**Hum2 stoichiometry**
Hum2 is half as long as Hum4 yet the Hum2 intermolecular complex has a mobility similar to that of Hum4's intramolecular structure (Fig. 2.2a), suggesting that the Hum2 complex is a dimer. In order to determine the stoichiometry of an intermolecular complex, one can mix oligonucleotides which contain 5' tails of different lengths\textsuperscript{7,8}. The number of hybrid species that form indicates the molecularity of the complex.

Oligomers \(X_H\) and \(Y_H\) have the Hum2 sequence at their 3' ends and 5' tails of different lengths (Fig. 2.2b). At low pH, \(X_H\) and \(Y_H\) form intermolecular structures which are concentration dependent (Fig. 2.2b), as seen with Hum2. It is interesting that \(Y_H\) dimerizes more readily than \(X_H\), which may be a non-specific effect of their different 5' tails. When \(\text{\textsuperscript{32}P}\)-labelled \(X_H\) is mixed with a large molar excess of unlabelled \(Y_H\), a single \(X_H/Y_H\) heterodimer is seen (Fig. 2.2b). Longer exposures of the gel do not reveal other \(X_H/Y_H\) hybrid species (data not shown). The presence of a single heterodimeric species indicates that Hum2-mediated intermolecular complexes have a
Fig. 2.1. Absorbance melting curves of Hum2 and Hum4 at the indicated pH values.
Fig. 2.2. Stoichiometry of Hum2 and Hum4 structures.

a, Non-denaturing gel electrophoresis at pH 5 and pH 7 of 5nM $^{32}$P-labelled Hum2 and Hum4 at the indicated concentrations of unlabelled oligomer. Oligomers complementary to Hum2 and Hum4 are labelled as G2, d(T$_2$A$_3$)$_2$, or G4, d(T$_2$A$_3$)$_4$, respectively. Positions of Hum2 and Hum4 Watson-Crick duplexes are indicated by D2, d(C$_3$T$_2$A$_2$)$_2$-d(T$_2$A$_3$)$_2$, and D4, d(C$_3$T$_2$A$_2$)$_4$-d(T$_2$A$_3$)$_4$.

b, pH 5 non-denaturing gel of 5 nM $^{32}$P-labelled X$_H$ and Y$_H$ oligomers mixed with the indicated concentrations of unlabelled X$_H$ or Y$_H$. Monomer as well as homo- and heterodimer species are labelled. Sequences of X$_H$ and Y$_H$ are shown below the gel. At pH 7, only a single species was seen for X$_H$ or Y$_H$ (data not shown).

c, Long exposure of a pH 5 gel with Hum2 at a concentration similar to that used in NMR experiments. The position of a minor species of four stranded Hum 2 is indicated as 'tetramer' and has the same mobility as the Hum4 Watson-Crick duplex, D$_4$. 
stoichiometry of two. At high Hum2 concentrations (used for NMR experiments, see below) a minor species appears that comigrates with a 24-mer duplex, as expected for a four-stranded 12-mer (Fig. 2c). This putative Hum2 tetramer was never more than 2% of the total DNA sample.

**Hum2 NMR**

Total correlation spectroscopy (TOCSY) of Hum2 reveals six dominant cytosine H5-H6 crosspeaks and at least six minor ones (data not shown). This indicates that a single dominant Hum2 species exists, but that other minor species are also present. Since Hum2 forms an intermolecular complex, the equilibrium between its structural species is affected by concentration (Fig. 2.2a,b). Therefore, correlation of Hum2 species observed in NMR experiments with transitions observed in Hum2’s absorbance melting curves (Fig. 2.1) is problematic, due to a 400-fold difference in concentration between the two.

The imino proton NMR spectrum of Hum2 at low pH consists of 6 resonances at 15 to 16 p.p.m., some of which are broadened due to exchange with solvent (Fig. 2.3a). These resonances are characteristic of C+ imino protons. Although there is a single dominant Hum2 conformation, the presence of minor species precludes sequential assignments of Hum2. Therefore, cytosine nucleotide resonances are arbitrarily identified with letters A-E (Fig. 2.3a). A sixth resonance, F, is broad and hidden under the other resonances and is more obvious at lower temperatures (data not shown).

Imino-imino NOEs between protons designated A, B and C indicate that the base pair containing imino proton A is sandwiched between the base pairs of protons B and C (Fig. 2.3b). NOE connectivities between these imino protons and their amino, H5, H6, H1' and H2', H2" protons were established (data not shown). Strong A-B and A-C H1'-H1' NOEs are present (Fig. 2.3c). H1'-H1' NOEs are considered diagnostic for DNA molecules which form the i-motif, therefore the Hum2 dimer is a C-C+ tetraplex.

**Hum2 i-motif structure**

Two types of i-motif Hum2 dimers are possible (Fig. 2.4a). These two dimer tetraplexes are not equivalent. The tetraplex labelled ‘S’ has two fold symmetry, and should only have three imino proton peaks, while an asymmetric tetraplex, ‘A’, would have six imino peaks. Six imino peaks are observed, some of which are broadened due to exchange with solvent (Fig. 2.3a). Fast imino exchange was seen for the two terminal base pairs of dTC5, which forms an i-motif structure. This is consistent with a single asymmetric Hum2 tetraplex whose C-C+ helix contains terminal base pairs that exchange imino protons rapidly and internal base pairs that are protected from imino exchange. Alternatively, the six imino resonances could be due to a mixture of two S tetraplex conformations, A-B-C and D-E-F. The two S tetraplexes should have similar structural properties, since they differ only in the register of their base pairs. However, imino protons D, E and F are exchanging much more rapidly than those of A, B and C. Therefore, we favor the interpretation of a single A tetraplex, although a mixture of two S tetraplexes cannot be ruled out.
Fig. 2.3. Hum2 has stacked C-C+ base pairs with H1'-H1' NOEs.

a, One dimensional $^1$H spectrum of Hum2 C-C+ imino protons.

b, Imino-imino portion of a Hum2 NOESY in 90% H2O/10% D2O. Crosspeaks are seen between imino protons A and B and between imino protons A and C.

c, D2O NOESY shows A-B and A-C H1'-H1' NOEs.
Fig. 2.4. C·C⁺ tetraplex models.

a, Possible folding paths of Hum2. Dimer 1 contains 1 block of C·C⁺ base pairs, whereas dimer 2 contains 2 blocks. Although symmetric (S), asymmetric (A) or tetrameric (T) C·C⁺ tetraplexes are possible, the dominant structured species is probably the A tetraplex. The precise positioning of base pair intercalation may be different than that shown.

b, Putative intramolecular tetraplex formed by Hum4.

c, Intramolecular tetraplexes on both G-rich and C-rich strands complement each other (scenario 1). Intermolecular G- and C-strand tetraplexes may be important for self-recognition between sister chromosomes or for other genomic activities (scenario 2).
Folding pathways

Formation of an intercalated tetraplex structure presents an interesting folding problem. The simplest pathway would involve initial dimerization to form a parallel-stranded helix with 1 or 2 blocks of C:C+ base pairs (Fig. 2.4a). For either dimer 1 or dimer 2, two tracts of C's could fold over and intercalate into a block of C:C+ base pairs to form a C:C+ tetraplex. Tetraplex A might be favored over S if dimer 1 is less stable than dimer 2, by virtue of having fewer base pairs, and conversion of dimer 1 to dimer 2 is fast. Although tetraplex T would also give six imino resonances, only a small amount of a putative four-stranded species of Hum2 was observed by gel electrophoresis (Fig. 2.2c). The quick conversion of dimer 2 to tetraplex A and the slow conversion of dimer 2 to T would explain the low abundance of T.

Oligomers containing four C-strand repeats from Human (Fig 2.2a), Tetrahymena, and Oxytricha25 telomeric sequences form intramolecular structures at low pH. These observations together with the observed dimerization of Hum2 to form a C:C+ tetraplex suggest that four tracts of C's are sufficient for intramolecular tetraplex formation. Therefore, Hum4 probably folds into a structure like the one shown in Fig. 2.4b. Similarly, oligomers with four telomeric G-strand repeats can fold to form intramolecular guanine tetraplexes under physiological conditions9,10,31.

C:C+ and G-tetraplexes

Several C-rich oligomers have been shown to self-associate as tetramers to form the i-motif22,23. Although these structures all had a molecularity of four, it was suggested that four consecutive C-rich tracts could fold to form an intramolecular i-motif structure22,24. Our results demonstrate that a sequence with two tracts of cytosines can dimerize to form the i-motif and suggest that a sequence with four cytosine tracts can fold to form a monomeric i-motif structure. A similar situation exists for G-tetraplexes, where one, two or four tracts of guanines can form tetrameric, dimeric or monomeric G-tetraplex structures, respectively4,7.

Although the i-motif is stabilized by acidic pH, appropriate conditions of superhelical stress or the aid of C-tetraplex binding proteins may enhance C:C+ tetraplex formation, in vivo. Several proteins that bind to or facilitate G-tetraplexes have been identified16-21. Similar proteins may exist for i-motif structures.

It is interesting that G-rich and C-rich telomeric sequences, which are Watson-Crick base-pairing partners, are both able to form tetraplex structures. G-tetraplex and i-motif structures could act in concert to generate stable alternative DNA structures. G/C rich repetitive sequences at telomeres or elsewhere could unwind to form an intramolecular guanine tetraplex on one strand and an intramolecular C:C+ tetraplex on the complementary strand (Fig. 2.4c, scenario 1). A potentially related structure involving a C:C+ hairpin, a guanine tetraplex and a triplex was proposed for human telomeric repeat sequences in a supercoiled plasmid at low pH28. The C:C+ hairpin may have been an intercalated C:C+ tetraplex.

Self-recognition

Finally, the ability of Hum2 to dimerize (Fig. 2.4a) is strikingly similar to dimerization of single-stranded telomeric G-strand tails7,8, where recognition and binding occurs between identical, rather
than complementary oligomers. We propose that C-C⁺ intermolecular tetraplexes may mediate nucleic acid self-recognition in a biological context as has been shown for G-tetraplexes which mediate dimerization of HIV genomic RNA¹⁴,¹⁵. In addition, self-recognition by G-tetrad structures has been implicated in alignment of homologous sequences during meiosis³⁶. Furthermore, telomeric sequences are known to undergo recombination at high frequencies³²-³⁵, a process which may involve alternative DNA structures. Thus, both C-strand and G-strand intermolecular tetraplexes may participate during recombination, meiotic chromosome pairing or other genomic processes involving self-recognition of nucleic acids (Fig. 2.4c, scenario 2).

Methods

Nomenclature. The terms duplex, triplex and tetraplex refer to the number of strands required to form a particular DNA structure without reference to how the strands are connected. The number of oligonucleotide molecules that form a particular structure (e.g. one, two or four) is referred to in terms of subunit stoichiometry (e.g. monomer, dimer or tetramer). Thus, i-motif structures are always tetraplexes, but an i-motif structure may be a monomer, dimer or tetramer. The terms i-motif, C-C⁺ tetraplex and intercalated C-C⁺ tetraplex are used synonymously.

Oligonucleotide synthesis and purification. All oligonucleotides were synthesized using an Applied Biosystems automated DNA synthesizer (B-cyanoethyl phosphoramidite chemistry). For absorbance thermal denaturation and non-denaturing gel electrophoresis, oligomers were purified as described¹⁰ and resuspended in water.

Absorbance thermal denaturation. 17.5 μM Hum2 or 4.6 μM Hum4 were boiled 4 min in either 50 mM sodium phosphate (NaH₂PO₄) (pH 7.0 and 6.0) or 50 mM sodium acetate (NaOAc) (pH 5.0) and slowly cooled at 23°C for 30 min. Absorbance thermal denaturation was performed at 0.5°C steps in a Gilford Response II spectrophotometer with a Thermoset.

Non-denaturing gel electrophoresis. 5' ³²P-labelled oligomers (5 nM) and unlabelled oligomer (concentrations as indicated) were boiled (4 min), incubated at 23°C (30 min) and separated on non-denaturing polyacrylamide gels (12%, 1 mm) at 4°C (5 V cm⁻¹, 15 hrs). Gels were dried and autoradiographed. Gels, gel electrophoresis buffers, samples and loading dyes (50% sucrose, 0.1% bromophenol blue, 0.1% xylene cyanol ff) were either 50 mM NaOAc (pH 5.0) or 50 mM NaH₂PO₄ (pH 7.0). For the 4.5 mM Hum2 sample, 8 μl of 5.2 mM Hum2 was taken directly from an NMR tube (50 mM NaCl, 50 mM NaH₂PO₄, 70% D₂O, pH 5.6) and mixed with 2 μl of 5' ³²P Hum2.

Large scale Hum2 purification. Hum2 (10 mmol) was was ethanol precipitated and purified by G-25 gel filtration as described³⁷. The sample was repurified over a G-10 column to remove a small molecule contaminant, lyophilized, dissolved in 1.5 ml H₂O, lyophilized again and dissolved in 450 ul 50 mM NaCl, 50 mM sodium monophosphate (90%H₂O/10%D₂O) at a concentration of 9.5 mM Hum2, pH 4.1. For the D₂O NOESY, the sample was lyophilized, dissolved in D₂O (600 μl), incubated at
65°C (1 hr) and 42°C (2 hrs), lyophilized again and dissolved in D₂O (450 µl) (final pH of 5.6, not corrected for the deuterium isotope effect).

NMR spectroscopy. Spectra were acquired at 21°C using a Varian Unity-500 spectrometer. One-dimensional spectra in H₂O were acquired using a 1-1 spin-echo pulse sequence with a delay between pulses of 35 µs. The two-dimensional NOESY spectrum in H₂O was obtained using a jump-return pulse sequence as the read pulse, with a 40 µs delay between pulses. The NOESY spectrum in D₂O used presaturation for 1 s to suppress the residual water signal. The two-dimensional TOCSY spectrum in H₂O was obtained using a clean-TOCSY pulse sequence and presaturation to suppress the residual water signal. Two-dimensional spectra were made phase sensitive in the first dimension using the method of States et al. The mixing time was 180 ms for the NOESY and 60 ms for the TOCSY. The spectra were collected with 2048 complex points in t₂ and 600 complex points in t₁. The sweep width was 12000 Hz in each dimension and the recycle delay was 1.8 s. 64 scans were collected per t₁ experiment and the 90° pulse width was 8 µs. The data were apodized with a 60° shifted sine bell in both dimensions.

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CHAPTER 3:  *tgif1*, A TELOMERASE RNA MUTANT WITH SHORT TELOMERES

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Summary

*Tetrahymena* telomere length is dynamic and mutants with short telomeres can be isolated. These *tgif* (telomere growth inhibited forever) mutants identify genes involved in telomere length regulation. One of these mutants, *tgif1*, was heterozygous for a telomerase RNA mutation. Wildtype cells expressing this mutant telomerase RNA acquired short telomeres, which became long again when the mutant RNA was lost. Mutant and wildtype telomerase enzyme activity levels were indistinguishable *in vitro*, suggesting that the mutation’s effect on telomerase activity was subtle. In contrast, the presence of high mutant telomerase RNA levels for a long period of time produced a lethal phenotype that was due to very short telomeres. The *tgif1* mutation may disrupt a conserved telomerase RNA pseudoknot. Thus, *tgif* mutants provide a way to identify functionally important domains of the telomerase RNA *in vivo*.

Introduction

Telomeres, specialized chromatin domains at the ends of chromosomes, are essential for chromosome stabilization, organization of the nuclear architecture and complete replication of the chromosomal terminus (Zakian, 1989; Blackburn, 1991). In most organisms, telomeric DNA is composed of simple tandem repeats which have a G-rich running 5' to 3' towards the end of the chromosome.

Since most DNA polymerases require an RNA primer to initiate DNA synthesis, it is expected that telomeres would become shorter with each round of DNA synthesis (Watson, 1972). One mechanism to compensate for telomere shortening is *de novo* addition of telomeric repeat to...
chromosome termini (Olovnikov, 1973). The enzyme that carries out this mechanism, telomerase, was first identified in *Tetrahymena* (Greider and Blackburn, 1985). Telomerase is a reverse transcriptase that uses its RNA component as a template for addition of telomeric repeats (Greider and Blackburn, 1989). Telomerase RNA genes have been cloned from many ciliates and have a secondary structure that has been conserved over a large evolutionary distance (similar to that separating the rat from maize or *Chlamydomonas*) (Lingner et al., 1994; Romero and Blackburn, 1991). It is likely that telomerase RNA secondary structure was conserved for functional reasons.

Total telomere length can range from tens of base pairs in ciliates to hundreds of kilobases in mammals and is dynamic in many organisms. Telomeres of human somatic cells shorten with age both in vitro and in vivo, although germ line telomeres remain long (Harley, et al., 1990; Allsopp, et al., 1992). This suggests that telomere shortening may limit a cell's replicative life span, either by influencing gene activity near telomeres or by chromosome instability due to telomere loss (Levy, et al., 1992). In contrast to their somatic precursors, immortal cells stop their telomeres from shortening (Counter, et al., 1992; 1994). In agreement with the observations, telomerase activity has been found in immortal cells but not their somatic precursors (Counter, et al., 1992; Klingelhohtz, et al., 1994; Counter, et al., 1994). Thus, a cell may control its telomere length by regulating telomerase activity.

Observations with *Tetrahymena* and yeast telomerase RNA genes suggest that telomerase is necessary for telomere length maintenance. Overexpression of a telomerase RNA template mutant in *Tetrahymena* resulted in short telomeres, presumably due to loss of telomerase activity (Yu, et al., 1990). Deletion of the telomerase RNA gene in yeast also resulted in a short telomere phenotype, as did mutants with deletions of the EST1 gene (Lundblad and Szostak, 1989; Singer and Gottschling, 1994). In both *Tetrahymena* and yeast, the short telomere phenotype correlated with a lethal phenotype which resulted in slowly growing cultures (Lundblad and Szostack, 1989; Yu, et al., 1990; Singer and Gottschling, 1994). Therefore, telomere shortening has been associated with senescence in both humans and single-celled eukaryotes. The association of these phenotypes is not unexpected, since telomere loss in yeast leads to chromosome instability and loss (Sandell and Zakian, 1993).

A number of other yeast genes involved in telomere length regulation have been identified. Mutations in the PIF1 DNA helicase result in telomere lengthening, suggesting that PIF1 may function by dissociating telomerase from its substrate (Schulz and Zakian, 1994). Some mutations of RAP1, a protein which binds yeast telomeres in vivo and in vitro, result in telomere shortening, while mutations in or overexpression of the non-DNA-binding carboxyl terminus cause telomere lengthening (Buchman et al., 1988; Conrad et al., 1990; Lustig et al., 1990; Sussel and Shore, 1991; Wright et al., 1992). The carboxyl terminus of RAP1 interacts with RIF1, and RIF1 mutations also cause telomere lengthening (Hardy et al., 1992). The RAP1 and RIF1 data suggest that telomere binding proteins sequester telomeres away from telomere lengthening enzymes. Addition of mutant sequences to chromosome termini in *Tetrahymena* also results in telomere lengthening, possibly by disrupting interactions with telomere binding proteins (Yu, et al., 1990). Finally, mutations in the yeast TEL1 gene, which codes for an IP3 kinase, result in short telomeres, indicating that a signal transduction pathway may regulate telomere length (T. Petes, personal communication).
If *Tetrahymena* cells are grown in log phase, their telomeres increase in length from 300-400 bp to 700-800 bp. After a long period of time, a population of cells with short telomeres takes over the culture (Larson et al., 1987; for example, see Figure 3.2a). Since each *Tetrahymena* cell has about 40,000 telomeres, cells with less telomeric DNA to replicate (and thus a shorter S phase) may possess a replication advantage when grown under conditions of rapid cell division (i.e. log phase). The short telomere phenotype was hypothesized to arise from a spontaneous somatic macronuclear mutation in a gene that regulates telomere length (Larson et al., 1987). Once cells with a short telomere phenotype take over a culture, their telomeres do not grow again. Therefore, these mutants are designated *tgf* (telomere growth inhibited forever).

Here we analyze a telomerase RNA mutation found in *tgf1*. This mutant telomerase RNA can cause telomere shortening when introduced into wildtype cells, and therefore identifies a functionally important domain of the telomerase RNA component. The original *tgf1* mutant was heterozygous and the mutation was found to be lethal when expressed at high levels. Therefore, *tgf* mutants may produce short telomeres by balancing lethal mutations in a heterozygous condition.

**Results**

**The *tgf1* Mutation**

Several spontaneous *tgf* mutants were generated by growing *Tetrahymena* cells in log phase for long periods of time (data not shown; Larson, et al., 1987). PCR of the telomerase RNA gene from these mutants revealed a mutant/wildtype heteroduplex in *tgf1* (Figure 3.1a). The mutation was sequenced directly from PCR products of *tgf1* genomic DNA and from subclones of *tgf1* PCR products. *tgf1* had a four base-pair (4bp) insertion located within stem III of the proposed telomerase RNA secondary structure (Figure 3.1b) (Romero and Blackburn, 1991).

**Reverse Genetics**

pRD4-1 is a high copy number ribosomal DNA (rDNA) vector which confers resistance to paromomycin (Spangler and Blackburn, 1985). When injected into a *Tetrahymena* macronucleus this vector recombines with the endogenous rDNA chromosome, which is at very high copy number compared with other macronuclear chromosomes (~10,000 copies vs. ~45 copies). Mutant telomerase RNA genes have been introduced into *Tetrahymena* on a pRD4-1 vector and were overexpressed relative to the endogenous wildtype telomerase RNA gene, producing transformants that had high levels of mutant telomerase (Yu, et al., 1990).

The *tgf1* 4bp mutant telomerase RNA gene was subcloned into pRD4-1 to produce pSA34bp. In order to determine if the 4bp mutation could cause a short telomere phenotype, pSA34bp was injected into the *Tetrahymena* strain C3mm1 (Larson, et al., 1986). A C3mm1 parent culture was used for pSA34bp injections, because the *tgf1* mutation was identified in a C3mm1 genetic background. In an initial experiment, one pSA34bp transformant (T2) was obtained. The T2 parent culture grew long telomeres and did not become *tgf* during the life of the T2 culture (data not shown). In a second experiment, four pSA34bp transformants (T3, T4, T5 and T6) were obtained (from cells injected on day 5 of the parent culture). This parent culture also grew long telomeres, but
Fig. 3.1. *tgfl* has a telomerase RNA mutation.

*a*, PCR of the telomerase RNA gene from several wildtype *Tetrahymena* strains and from *tgfl*.

*b*, Predicted secondary structure of the *Tetrahymena* telomerase RNA component. *tgfl*’s four base-pair insertion (shown in bold) is located in stem III. A possible pseudoknot of stem-loop III is indicated by dashed lines. The template used by telomerase is underlined.

Fig. 3.2. rDNA telomere length variation during log phase growth of a C3rmm1 parent (*a*) and its day 5 subculture (*b*, C3rmm1-1, injected but not transformed).

Southern blots were probed for the telomere of the rDNA chromosome.
became *tgif* quickly (by day 39) (Figure 3.2a). However, this mutation was probably not prevalent by day 5 of the culture, since a day 5 subculture (from a cell that was injected but not transformed) grew for 100 days before cells with short telomeres appeared (Figure 3.2b). Thus, all pSA3.4bp transformants came from parent cultures that displayed normal telomere growth and were unlikely to have had pre-existing *tgif* mutations.

Transformants T2, T3, T4, and T5 all obtained the 4bp telomerase RNA gene from pSA34bp, but pSA34bp lost its 4bp telomerase RNA gene during recombination in transformant T6, which served as a transformed control. All transformants were grown in log phase cultures for several months. DNA, RNA and S100 protein extracts were made from these cultures in order to determine how the 4bp gene was affecting telomere metabolism. The 4bp transformants all retained the 4bp telomerase RNA gene at a very high copy numbers for at least one week of log phase growth (Figures 3.3 and 3.4). After one week, the 4bp gene was slowly lost over a period of 30 to 70 days (Figures 3.3 and 3.4). Primer extension showed that the 4bp gene was being transcribed throughout this period in transformants T4 (Figure 3.3a), T3 and T5 (data not shown). The high proportion of 4bp telomerase RNA present in transformants indicated that the 4bp mutant telomerase RNA was assembled into the telomerase enzyme, since previous studies have shown that excess unbound telomerase RNA is degraded (Yu, et al., 1989).

The T6 control had long telomeres for about 3 months before becoming *tgif* (Figure 3.3b). During the time that T6 telomeres were long, a population of cells with short telomeres took over the T4 culture (Figure 3.3a). However, T4 telomeres got long again when the 4bp gene was lost (Figure 3.3a), indicating that the 4bp gene was necessary for the short telomere phenotype. Other 4bp transformants exhibited different telomere length phenotypes. For T2, a population of cells with short telomeres appeared while the 4bp gene was present, but the 4bp gene was lost before the cells with short telomeres could take over the culture (Figure 3.4a). For T3, cells with short telomeres took over when the 4bp gene was present and telomeres got longer as the 4bp gene was lost (Figure 3.4b), as seen with T4 (Figure 3.3a). However, a second population of T3 cells with short telomeres took over shortly after the 4bp gene was lost, and these cells did not retain short telomeres as is normally seen with *tgif* mutants. Perhaps this represents a second class of *tgif* mutation, a class whose effect on telomere length is so severe that the mutation is unstable and quickly lost. For T5, although cells with short telomeres appeared before the 4bp gene was lost, a *tgif* mutant took over the culture at the same time, so one cannot ascertain if the 4bp gene was responsible for any of the telomere shortening observed (Figure 3.4c). Despite these variable phenotypes, the T2, T3 and T4 pSA34bp transformants all acquired a short telomere phenotype when 4bp telomerase RNA was present. This short telomere phenotype reverted once the 4bp telomerase RNA was lost (Figures 3.3 and 3.4).

**4bp Telomerase Activity**

Telomerase assays of S100 extracts from T3, T4 and T5 clearly showed that transformants with high proportions of 4bp telomerase RNA (>80%) had telomerase activity levels that were comparable to telomerase from the T6 wildtype control (Figure 3.5b). Assay conditions were such that dissociation of telomerase from its substrate was rate limiting, and it is clear that 4bp telomerase can be quite processive *in vitro* (Figure 3.5). Although a particular extract consistently yielded the same relative
Fig. 3.3. The *tgif1* 4bp telomerase RNA causes telomere shortening when expressed in wildtype cells.

T4 (a) and T6 (b) pSA3_tg transformants were derived from day 5 cells of the parent C3mm1 culture. For the T6 transformant, the rDNA vector recombined with the endogenous rDNA but did not retain the 4bp telomerase RNA gene. Cultures of T4 and T6 were grown in log phase for several months. Expression of the 4bp telomerase RNA in the T4 culture was tracked by primer extension. In addition, the copy number of the 4bp telomerase RNA gene was tracked by southern blotting using a telomerase RNA probe. Cross-hybridization of the telomerase RNA probe with an rDNA restriction fragment serves as an internal control for DNA concentration. Blots were stripped and reprobed for telomere length using pTre1, a plasmid that contains the end of the rDNA chromosome.
Fig. 3.4. pSA34bp transformants exhibit variable telomere length phenotypes when grown in log phase.

4bp telomerase RNA gene copy number in T2 (a), T3 (b) and T5 (c) was tracked by southern blotting. The blots were stripped and reprobed with pTre1 to determine rDNA telomere length.
Fig. 3.5. Telomerase activity of S100 extracts from pSA34bp transformants during log phase growth.

a. Telomerase RNA was reverse transcribed from total RNA prepared from each extract. 4bp telomerase RNA levels were quantitated using a PhosphorImager.

b. Telomerase assays of S100 extracts. 2.5 ug S100 protein and 2 uM RDG primer were used in each assay under standard telomerase assay conditions.

c. Telomerase activity (in b) was quantitated using a PhosphorImager for extracts of a particular day, activity levels were normalized to the T6 extract of that day (100). Mean variance of relative telomerase activity levels from assay to assay was 10% (error bars).
telomerase activity levels, extracts of wildtype control cultures that were made on different days had activity levels that varied up to 2-fold for a particular culture (data not shown). It is not known if this variation reflected real fluctuations in telomerase activity levels of the cultures. A more detailed study with partially purified 4bp telomerase activity may be necessary to detect subtle changes in telomerase activity.

**Homozygous 4bp Mutants**

Telomerase RNA gene PCR from *tgfl* genomic DNA indicated that both 4bp (58%) and wildtype 42% telomerase RNA genes were present in the mutant culture (Figure 3.6a). This is unusual since *Tetrahymena* cultures usually become homozygous for a macronuclear allele within 50 generations (about 15 days of log phase growth), a process known as phenotypic assortment. Phenotypic assortment occurs because chromosomes are segregated randomly during macronuclear division. Therefore, the heterozygous condition of the *tgfl* telomerase RNA locus appeared to have been selected for.

Stock tube cultures of pSA34bp transformants were found to retain the 4bp gene at very high copy numbers for at least 8 months (Figure 3.6c). Unlike fast-growing log phase cultures of these transformants, stock cultures replicate slowly and have no selective advantage to losing excess DNA, such as the 4 bp gene or long telomeres. Log phase cultures of cells from these stock tube cultures initially grew very slowly in comparison with transformed and untransformed controls. Many single cell isolates from T3, T4 and T5 stock cultures grown at 30°C gave rise to senescent “monster” cells which failed to divide further (Figure 3.6b; Yu, et al., 1990). This senescent phenotype was never observed in wildtype controls that lacked the 4bp gene. T3, T4 and T5 stock cultures all had telomeres with average lengths that were 100 to 250 bp shorter than wildtype or transformed controls. It is interesting that the T6 transformed control had telomeres that were about 100 bp longer than its untransformed brother C1 (Figure 3.6c). We did not determine if either selection with paromomycin or the rDNA vector itself was responsible for this length increase.

Although T3 and T4 had low (5 to 10%) percentages of senescent cells, about 50% of the T5 stock culture was senescent. T5 telomere length was consistently 50 to 70 bp shorter than that of T3 or T4 (Figure 3.6c). However, when T5 cells were grown in log phase for 50 generations, their doubling rate had returned to normal, average telomere length had increased 100 bp and the 4bp mutant telomerase RNA gene that was at high copy number in the stock tube was practically gone from the culture (Figure 3.6c). Taken together, these data indicate that expression of the 4bp gene at nearly homozygous levels for long periods of time had lethal effects that were due to telomere shortening.

**Other *tgfl* Mutants are Heterozygous**

If a *tgfl* mutation is heterozygous because it is lethal when homozygous, then the mutation might be expected to be lost when the mutant is cultured under non-selective conditions of slow growth (such as a stock culture). In agreement with this hypothesis, the original *tgfl* mutant lost its 4bp telomerase RNA gene after 1.5 years in culture (data not shown). The *tgfl* short telomere phenotype might be expected to revert after losing the 4bp mutation, as was seen for the pSA34bp transformants (Figures
Fig. 3.6. The *tgif1* mutation may have been heterozygous.

a, PCR products of the telomerase RNA gene from wt (C3mm1) and *tgif1* genomic DNA separated on a 7.5% polyacrylamide gel.

b, pSA34bp transformants that either did (T5) or did not (T6) have the 4bp telomerase RNA gene were kept under selection in stock tubes for 5 months and then grown in log phase. Cells were fixed with 0.25 M citric acid and stained with 0.5 ug ml⁻¹ DAPI. Phase contrast and UV CCD images were captured with an Olympus BH-2 microscope.

c, Southern blotting analysis of stock cultures, some of which were grown in log phase for 100 generations. The 4.5 kb fragment represents the 4bp telomerase RNA gene present on the rDNA chromosome of transformants while the 4.0 kb fragment represents the corresponding wildtype rDNA fragment. The percentage of senescent cells was determined from 48 single cell isolates taken from a culture on the day that DNA was harvested.
Fig. 3.7. Log phase growth of some tgif mutant stock cultures results in telomere growth. rDNA telomere length was analyzed by southern blotting using the pTre1 probe.

3.3 and 3.4). Curiously, the tgif1 stock culture did not regain the ability for telomere growth (Figure 3.7). This may have been due to some other mutation that occurred in the stock culture or due to a second-site mutation that occurred in the tgif1 log phase culture.

In order to determine if other tgif mutants had lost their mutant genes in stock culture, several of these stock cultures were grown in log phase for 25 days. One of these cultures, tgif3, did not show any telomere growth and thus either had a homozygous tgif mutant genotype or maintained a balanced heterozygous genotype in stock culture (Figure 3.7). In contrast, both tgif2 and tgif4 had regained the ability for telomere growth, indicating that their tgif mutations were heterozygous (Figure 3.7). The tgif2 culture had apparently not completely lost its mutant allele, as telomeres quickly shortened in length once they had gotten long enough for selection of short telomeres to occur.

Discussion

The tgif1 Mutant Telomerase RNA Causes Telomere Shortening

We introduced the tgif1 4bp telomerase RNA gene into wildtype cells and observed a short telomere phenotype when transformants were grown in log phase. In 3 transformants this phenotype reverted when the 4bp gene was lost from the cultures, indicating that the 4bp telomerase RNA was responsible for producing the short telomere phenotype. The fourth 4bp transformant culture (T5) was overgrown by a spontaneous tgif mutant before a specific effect of the 4bp gene could be assessed. Although some tgif mutants may result from multiples genetic changes, these results indicate that a single mutation is sufficient to produce a tgif phenotype.
How Does The 4bp Telomerase RNA Affect Telomere Length?

In a previous study, expression of two Tetrahymena telomerase RNA template mutations caused incorporation of mutant telomere sequences and telomere lengthening (Yu et al., 1990). These transformant cultures grew slowly and exhibited 'monster' phenotypes (due to cell division arrest), and the only cells which survived to grow at normal doubling rates were ones that had completely lost their mutant telomerase RNA gene (Yu et al., 1990). One telomerase RNA template mutation caused rapid telomere shortening, large-scale senescence and cell death, and was so severe that no cultures survived for more than a week (Yu et al., 1990). In contrast, pSA34bp transformant cultures all survived, grew at healthy doubling rates of 3 to 4 hours and took 40 to 70 days to completely lose their mutant telomerase RNA gene. In addition, it took 20 to 60 days for the 4bp mutation to produce a short telomere phenotype in culture. Thus, it is likely that the 4bp mutation had a rather subtle effect on telomere length regulation. In agreement with this possibility, in vitro 4bp and wildtype telomerase activity levels in S100 extracts were indistinguishable. A subtle phenotype might be expected since telomere length needs to be maintained at some minimal level in tgif mutants, and therefore "null" telomerase RNA mutations would probably have lethal effects.

The tgif1 4bp insertion occurs in a position that may disrupt the structure of stem-loop III or affect its ability to form a pseudoknot. The tgif1 mutation identifies a telomerase RNA domain that necessary for proper telomerase function. It has been proposed that stem-loop III's pseudoknot might be involved in a telomerase RNA conformational shift used to reposition the template during processive elongation of a telomeric primer (Lingner et al., 1994). The fact that the 4bp mutation caused telomere shortening in vivo may provide genetic evidence that stem-loop III's pseudoknot is involved in telomerase processivity. However, the telomerase RNA component may be involved in some other aspect of telomere length regulation. For instance, the 4bp telomerase RNA mutation may identify a domain of the RNA component that serves as a regulatory link between telomerase and a cell signalling pathway that controls telomere length (perhaps the TEL1 IP3 kinase pathway; T. Petes, personal communication). It may be possible to select for other telomerase RNA mutations that cause tgif phenotypes from a population of randomly mutated telomerase RNA genes. Such an approach could provide insightful data about in vivo telomerase RNA structure and function.

tgif1 Was a Heterozygote

Although the 4bp mutation's effect on telomere length appeared to be subtle in log phase cultures of transformants, the mutation had more severe effects on stock cultures. Stock cultures of transformants, which retained the 4bp telomerase RNA gene at high copy number for a long period of time, had senescent cells, a phenotype which correlated with the presence of very short telomeres. This lethal mutation was balanced by wildtype telomerase RNA in the tgif1 mutant and was lost in a non-selective stock culture. Other tgif mutants lost their short telomere phenotype in stock cultures, indicating that they possessed heterozygous mutations which may have been lethal if homozygous. Thus, Tetrahymena's ~45 to 60C polyploid genome could be advantageous for identifying lethal mutations in a less severe heterozygous condition.
**Experimental Procedures**

**Strains and Cell Cultures**

The C3rmm1 strain of *Tetrahymena thermophila* was described (Larson, et al., 1986). Stock cultures were kept at 25°C without shaking in 6 ml tubes of either 1% or 2% PPYS complete media (2% proteose peptone, 0.2% yeast extract and 0.003% sequestrene) and were transferred every month. Log phase cultures were grown in 50 ml of 2% PPYS at 30°C in 250 ml erlenmeyer flasks on a cell shaker rotating at 220 rpm. Continuous log phase cultures were maintained by transferring 200 to 400 ul of a culture to a new flask every day (when cell concentrations reached 1 to 5 x 10^5 cells ml^-1). *tgifs* mutants appeared more frequently if cultures were grown to concentrations of 3 x 10^5 cells ml^-1 before transfer (H. Sheng and M. Fan, unpublished), possibly because there was a better chance of propagating a *tgifs* mutation that was initially present in only a few cells.

**DNA Oligonucleotides**

DNA oligonucleotides were gel purified on denaturing polyacrylamide gels as described (Henderson, et al., 1987). PCR of the telomerase RNA gene was carried out using sense primers 9 or 109 and antisense primers 10 or 73, whose sequences are: 9, 5'ATACCGCTTAATTCATTCAGA; 109, 5'ATATATCTTGCTGTCTCTTCT; 10, 5'AAAAATAAGACATCCATTGATAAATAGTGTATCAAATG; 73, 5'GTAGAAGCTTTTTAATAGGATCAATGTCTCATAATA. Telomerase reactions were carried out using RDG, 5'AAAAACTCGACTAGTGCATCGACTTGGGGTTGGGG. For reverse transcription, oligo 10 was 5' end-labelled in a 10 ul reaction: 22 pmol oligo 10 was incubated with 10 units T4 polynucleotide kinase (New England Biolabs), 1x PNK buffer (New England Biolabs), 22 pmol gamma ^32_p-ATP (5000 Ci mmol^-1, Amersham) at 37°C for 30 min, mixed with 10 ul formamide, boiled and gel purified on a 12% denaturing gel as described (Henderson, et al., 1987).

**PCR**

50 ul PCR reactions contained with 2 mM MgCl2, 0.2 mM dNTPs, 0.2 uM primers, 1x PCR buffer (Promega) and 2.5 U Taq polymerase (Promega). Reactions were denatured at 95°C for 10' prior to addition of Taq polymerase and dNTPs. Reactions were then incubated at 95°C 1', 42°C 2' and 74°C 3' for 40 cycles, followed by a 74°C final extension for 10'. 20 ul of each reaction was loaded on a 1.5 mm 5% polyacrylamide gel (PAG), separated at 225V for 40' and stained with ethidium bromide.

To quantitate the level of 4bp telomerase RNA gene in *tgifs*, primer 9 and 5' ^32_p-labelled primer 10 were used in a 20 ul PCR reaction. The products were separated on a 6.5% PAG at 170V for 3 hrs, the gel was dried and exposed to a PhosphorImager screen.

**DNA Sequencing**

PCR products were gel purified for DNA sequencing. 9/73 *tgifs* PCR products were precipitated with 0.1x volume (vol) 3M sodium acetate (NaOAc), 2.5x vol 95% ethanol for 10' at -70°C, microfuged 10' at 4°C, washed twice with 70% ethanol and resuspended in water. Products were digested with Taq1 at 65°C as recommended (New England Biolabs) and restriction fragments were separated on a
7.5% PAG (Figure 3.6a). Mutant and wildtype telomerase RNA products were excised from the gel with a razor blade, eluted by shaking in 10 mM Tris, 1 mM EDTA (pH 8.0) for 12h, precipitated as above with 20 ng glycogen as carrier, resuspended in water and quantitated by gel electrophoresis. In a similar manner, 109/73 tgif1 PCR products were gel purified from a 5% gel without prior restriction digestion. 50 ng gel-purified PCR products were cycle sequenced as recommended (Promega fmol™ DNA sequencing system).

Subcloning

A 550 base pair Ddel telomerase RNA gene fragment of pCG1 (Greider and Blackburn, 1989) with Kpn linkers was subcloned into the multiple cloning site of pBluescript KSII+ (Stratagene) to produce pSA1wt. 180 bp BglII/BsmAl fragments of t gif1 9/73 PCR products containing the tgif1 mutation were subcloned into the BglII/BsmAl telomerase RNA gene sites of pSA1wt to produce pSA24bp, a plasmid with the tgif1 mutation. The 180 bp BglII/BsmAl tgif1 fragments were purified over a Magic™ PCR Preps column (Promega) before subcloning, and the BglII/BsmAl pSA1 vector was produced by complete digestion with BglII (New England Biolabs), partial digestion with BsmAl (New England Biolabs) and purification of the appropriate fragment from a 1% NuSieve GTG agarose gel (Saekem) using a Magic™ PCR Preps column (Promega). pSA1wt or pSA24bp inserts were PCR amplified with SK and RP pBluescript primers, digested with KpnI to produce a 550 bp telomerase RNA gene fragment that was gel purified (as for DNA sequencing, above), Magic™ PCR Preps purified and subcloned into the KpnI site of the pRD4-1 polylinker to produce pSA34bp and pSA4wt. pSA34bp and pSA4wt plasmid DNA for microinjection was made using a Qiagen midiprep kit. Cycle sequencing of these Qiagen preps indicated that no telomerase RNA gene mutations had occurred during the subcloning.

Transformants

Cells from C3mm1 log phase cultures were transformed by microinjection of pSA34bp. Injectees were single cell isolated into microtiter wells and grown in 200 ul 2% PPYS, 250 ug ml⁻¹ penicillin, 250 ug ml⁻¹ streptomycin at 30°C for 3 days. 10 ul of these cultures was put under selection in new wells with 100 ug ml⁻¹ paromomycin (ICN) added. Transformation took 9 to 16 days to appear. 50 ml log phase cultures of transformants were grown under selective conditions (100 ug ml⁻¹ paromomycin) for one week, and then log phase growth was continued in the absence of selection. 100 ug ml⁻¹ paromomycin, 2% PPYS, 6 ml stock cultures were made using 200 ul of cells from day 1 50 ml transformant cultures.

Senescent Single Cell Isolates

Single cell isolates of stock tube cultures were grown at 30°C in microtiter wells with 200 ul of 2% PPYS, 250 ug ml⁻¹ penicillin, 250 ug ml⁻¹ streptomycin. Wells were considered senescent if they failed to reach saturation after 6 days. Most senescent wells had less than 2,000 cells and all senescent wells had cells that exhibited the “monster” phenotype. Of 395 wildtype single cell isolates (150 T6 cells), no slow growing wells or “monster” cells were ever seen.
Preparation of Tetrahymena Genomic DNA

1 to 2 x 10^7 cells were collected by centrifuging a culture for 10' at 4°C. 100 ul of a cell pellet was mixed with 100 ul prewarmed NDS (2% SDS, 0.5 M EDTA, 0.01 M Tris-HCl, pH 9.5) and 200 ul prewarmed pronase, incubated for at least 12 hours at 55°C and then stored at -20°C. Samples were then mixed with 300 ul H₂O, extracted at least 2 times with phenol/chloroform (1:1), precipitated with 1 ml cold 95% ethanol, washed once with 70% ethanol, resuspended in 100 ul H₂O, reprecipitated with 7 ul 3 M NaOAc and 250 ul 95% ethanol, washed three times with 70% ethanol, dried in a speed vac and resuspended in 30 to 50 ul H₂O.

Southern Blots

20 - 30 ug of genomic DNA was digested overnight at 37°C with 10 U Pst1 (Promega) and separated on either 1% (Figures 3.2, 3.3 and 3.4) or 1.5% (Figure 3.6) agarose gels (Saekem LE). The gels were depurinated, denatured, neutralized and blotted to a MagnaGraph nylon membrane as recommended (Micron Separations, Inc). Blots were probed using either pTrel that was digoxigenin-labelled by nick-translation (Boehringer-Manneheim Genius™ kit) or probed using a 9/10 telomerase RNA gene PCR product made in the presence of 0.1 mM digoxigenin dUTP (Boehringer-Manneheim). Blots were hybridized overnight at 65°C, washed twice with either 0.1x SSC, 0.1% SDS at 65C (pTRE1 probe) or 0.2x SSC, 0.1% SDS at 50°C (telomerase RNA probe) and hybridized with alkaline phosphatase-conjugated anti-digoxigenin FAB fragments. Probes were detected using Lumi-Phos™ 530 as recommended (Boehringer-Manneheim) with X-ray film exposures of 5' to 1 day. Membranes were stripped using 0.2 N NaOH, 0.1% SDS at 37°C for 30', washed with 2x SSC, then prehybridized and probed again.

Genomic RNA Preparation

5 x 10⁶ cells of a log phase culture were collected in a clinical centrifuge at 4°C for 10'. Cells were rinsed once in buffer A (10 mM Tric HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA), lysed for 5' at 25°C in 0.5 ml buffer A containing 1% SDS and stored at -20°C. The cell lysate was then extracted once with 0.5 ml of acid phenol, extracted again with 0.5 ml of phenol/chloroform (1:1), precipitated with 0.1x vol of 5 M potassium acetate and 2 vol of 95% ethanol, incubated at -70°C 10', spun in a microfuge at 4°C for 10', washed once with 75% ethanol and resuspended in 100 ul H₂O. Genomic RNA was prepared from S100 extracts by mixing about 1,200 ug S100 (125 to 200 ul) with 0.5 ml buffer A containing 1% SDS and processing as above, except that 20 ng glycogen was used as carrier when precipitating, the pellet was washed twice with 75% ethanol and resuspended in only 10 ul H₂O.

Reverse Transcription of Telomerase RNA

In 10 ul reactions, 0.1 pmol of gel purified 5' ³²P-labelled oligo 10 was incubated with 1 ul genomic RNA (5 to 10 ug), 0.865x telomerase buffer (1x = 50 mM Tric Acetate, pH 8.5, 50 mM potassium acetate, 5 mM B-mercaptoethanol, 1 mM spermidine, 1 mM MgCl₂), 10 mM MgCl₂, 1 mM dNTPs, 2.5 U RNasin (Promega) and 1.25 U AMV reverse transcriptase (Promega) at room temperature for 10' and then at 50°C for 1 hr. Reactions were precipitated with 20 ng glycogen (Boehringer-Manneheim),
15 ul 2.5 M ammonium acetate and 135 ul 95% ethanol at -70°C for 10', washed in a microfuge for 10', washed once with 70% ethanol, dried in a speed vac, resuspended in 3 ul formamide loading buffer (90% formamide, 5% sucrose, 0.01% xylene cyanol and 0.01% bromophenol blue), boiled 4' and separated on a prerun 6%, 7 M urea, 0.6x TBE sequencing gel at 1000 - 1400 V. The gel was dried and exposed to a PhosphorImager screen overnight.

Reverse transcription from S100 RNA preps was done as above, except that 5 ul S100 RNA preps was used instead of genomic RNA.

S100 Extracts
All extracts were made from 50 ml mid-log phase cultures at 0.5 to 3.5 x 10^5 cell ml^{-1}. Cells were spun down, washed twice with Dryls (1.7 mM sodium citrate, 1.2 mM dibasic sodium phosphate, 1 mM monobasic sodium phosphate, 2 mM CaCl2), resuspended in 5 volumes of TMG (10 mM Tris·HCl, pH 8.5, 1 mM MgCl2, 10% glycerol, 5 mM 2-mercaptoethanol, 0.1 mM pepstatin [Boehringer-Manneheim], 10 uM pepstatin [Boehringer-Manneheim]) per volume of packed cells. RNasin (Promega) was added to the TMG at 7 to 30 U/ml before resuspension. TMG with 10 mM Tris·HCl, pH 8.0 and 50 mM 2-mercaptoethanol and without pepstatin was used for day 15 extracts. Cells were lysed in a microfuge tube at 4°C with continuous shaking for 20 min by adding 0.1 vol 2% NP40 in TMG. The cell lysate was then centrifuged at 50,000 rpm for 1 hr at 4°C in a TL-100.3 rotor. The supernatant was designated S100 and was mixed, divided into aliquots, frozen in liquid nitrogen and stored at -70°C until use. Day 15 extracts were thawed twice and day 50 extracts were thawed once before use in assays shown in Figure 3.5. Protein concentrations of S100 extracts were determined by the Bradford method.

Telomerase Assays
Telomerase assays were done essentially as previously described (Greider and Blackburn, 1989). An assay contained 2.5 ug S100 extract in 10 ul TMG and 10 ul 2x reaction mix. The 1x reaction mix was 1x reaction buffer (5 mM Tris acetate [pH 8.5], 5 mM potassium acetate, 1 mM MgCl2, 5 mM 2-mercaptoethanol, 1 mM spermidine), 100 uM TTP, 1.25 uM dGTP, 1.25 uM alpha^32P dGTP (800 Ci mmol^{-1}), 2 uM RDG primer and 0.5 U ul^{-1} RNasin. Assays were incubated at 30°C for 1 hr, stopped with 50 ul 20 mM EDTA, 10 mM Tris·HCl (pH 7.5), extracted with 50 ul phenol/chloroform (1:1), precipitated with 30 ul 2.5 M ammonium acetate, 20 ng glycogen (Boehringer-Manneheim), and 270 ul 95% ethanol at -70°C for 10', spun at 4°C for 10', washed once with 70% ethanol, dried in a speed vac, resuspended in 3 ul formamide loading buffer, boiled 4', separated on a prerun 6%, 0.6x TBE, 7 M urea sequencing gel at 1200V, dried and exposed to a PhosphorImager screen overnight or to X-ray film for 3 days.

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References


GENERAL CONCLUSIONS

Telomeres have been implicated in the processes of aging and cancer (Counter, et al., 1992; Klingelhutz, et al., 1994; Counter, et al., 1994). A better understanding of how telomeres function may give insights into how and why these human conditions occur. I have been studying the structural biology and genetics of telomeric nucleic acids.

**C-strand structure.** A portion of my work has been purely structural. Telomeric DNA sequences have provided a useful 'model system' for studying unusual DNA structures (Henderson, et al., 1987; Sen and Gilbert, 1990; Williamson, et al., 1989). The G-rich strand of telomeres has been shown to form four-stranded structures, which may be involved in chromosome pairing and other nucleic acid self-recognition processes such as dimerization of the HIV genome (Sen and Gilbert, 1988; Marquet, et al., 1991; Sundquist and Heaphy, 1993). When telomeric sequences are put in supercoiled plasmids, the C-rich strand adopts an unusual structure at low pH (Lyamichev, et al., 1989; Belotserkovskii, et al., 1992; Voloshin, et al., 1992). As presented in Chapters 1 and 2, I showed that the C-rich telomeric oligonucleotides could form unusual structures at low pH. As with the G-strand structures, these structures were also four-stranded (as shown by NMR spectroscopy) and can also mediate nucleic acid self-recognition (as shown by non-denaturing gel electrophoresis).

It is interesting that both the G-rich and C-rich strands of telomeric DNA, which are normally Watson-Crick base pairing partners, are both capable of forming four-stranded structures on their own. Perhaps both G- and C- tetraplexes participate in processes such as chromosome pairing at telomeres or fulfill some other telomeric function. One might expect that if these four-stranded DNA structures have important cellular roles, there might be proteins that interact with them. Numerous proteins have been shown to bind G-tetraplex DNA (Fang and Cech, 1993; Giraldo and Rhodes, 1994; Liu and Gilbert, 1994). A few proteins have also been identified which bind to C-rich oligonucleotides that have unusual structures (Edelmann, et al., 1989; Muraiso, et al., 1992; Ito, et al., 1994), but it is unknown if any of these proteins actually recognize C-tetraplexes. Identifying such proteins and determining their functions presents a major challenge for the future of C-tetraplexes.

It is possible RNA may be able to form C-tetraplexes. If this is the case, C-tetraplexes might have important functional roles in RNA-mediated catalysis, since C-tetraplex pKₐ's are close to physiological conditions. In this spirit, the telomerase RNA component might be able to form C-tetrads. Although telomerase RNAs only have a single 'long' 4 nucleotide tract of C's (at the template), a C-C⁺ duplex or a C-tetraplex might occur if the telomerase RNA forms dimers or tetramers when in an active telomerase complex. One might test to see if the telomerase RNA can form C-tetraplexes by analyzing the gel mobility of *in vitro* transcribed telomerase RNA under neutral and acidic conditions. Perhaps adding a protein to the telomerase RNA could facilitate C-tetraplex formation.

**tgif mutants.** Telomere length regulation may be a critical process in onset of both aging and cancer (Counter, et al., 1992; Klingelhutz, et al., 1994; Counter, et al., 1994). I have been studying telomere length regulation in *Tetrahymena* and have identified a telomerase RNA mutation that produces short telomeres if expressed in wildtype cells. Curiously, the mutant telomerase RNA...
appeared to have no effect on telomerase activity in vitro. A control experiment in which transformants are overexpressing the wildtype telomerase RNA gene should be done. Also, we are attempting to produce a tgif mutant by injecting 4bp telomerase RNA gene PCR products that will recombine at the wildtype telomerase RNA locus.

Tetrahymena is well developed for analysis of telomerase and the telomerase RNA both in vitro and in vivo, which nicely complements the ability to identify telomerase RNA mutants like tgif1. We are attempting to identify many telomerase RNA tgif mutations by introducing randomly mutated telomerase RNA genes into wildtype cells. This approach may yield a goldmine of telomerase RNA mutations that are functionally significant.

Although the tgif1 4bp telomerase RNA mutation may affect telomerase activity directly, it is also possible that the mutation interferes with the signalling pathway that regulates telomere length (see introduction, Fig. 6b). Careful analysis of in vitro telomerase activity from purified 4bp mutant telomerase may shed some light on this matter. The 4bp mutation may identify region of the telomerase RNA that interacts with a protein that directly regulates telomerase. It may be possible to identify such a protein using gel mobility retardation assays to the stem-loop III pseudoknot.

We are currently unable to identify most other tgif mutations because Tetrahymena lacks a good genetic system to do complementation. However, it may be possible to pick out other tgif mutations by comparing mRNAs from tgif mutants and their isogenic wildtype parents using a 'differential display' PCR approach (Liang and Pardee, 1992).

An alternative approach to finding telomere length regulation mutations would be to produce tgif mutants in a genetically tractable system such as yeast. We are currently examining the possibility that yeast telomeres change length in different growth conditions.

A final avenue of tgif research that deserves attention is the signalling pathway that controls telomere length (see introduction, Fig. 6b). It is clear that Tetrahymena regulates its telomere length in response to an environmental stimulus and that this stimulus must be propagated into the cell and to the telomere length regulation machinery. This signal may travel through a some sort of signal transduction cascade. In support of this possibility, it has recently been shown that mutations of yeast IP3 kinase cause telomere shortening (T. Petes, personal communication). One might be able to use pharmacological tools to dissect out this problem in Tetrahymena, whereas both pharmacology and genetics could be used in yeast.

The tgif research seems quite stimulating and exciting at this point in time, especially since it may yield a lot of functional information about how telomeres work. However, further pursuit of C-tetraplexes produce hidden treasures that won't become apparent until they are stumbled across.

References.


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