The Outer Limits: Telomere Maintenance by TRF2 and G-Quadruplex DNA Structures

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THE OUTER LIMITS: TELOMERE MAINTENANCE
BY TRF2 AND G-QUADRUPLEX DNA STRUCTURES

By

Ilene M. Pedroso

A DISSERTATION

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of the University of Miami
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THE OUTER LIMITS: TELOMERE MAINTENANCE
BY TRF2 AND G-QUADRUPLEX DNA STRUCTURES

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Human telomeric DNA consists of tandem repeats of the sequence 5’-d(TTAGGG)-3’ assembled into a nucleoprotein complex that functions to protect the ends of chromosomes. Such guanine-rich DNA is capable of forming a variety of G-quadruplexes, which in turn, can have varying functional consequences on telomere maintenance. G-quadruplex stabilizing ligands have been shown to induce chromosome end-to-end fusions, senescence and apoptosis, effects similar to the expression of a dominant-negative TTAGGG Repeat Factor 2 (TRF2). With this in mind, we analyzed the effect of sequence and length of human telomeric DNA, as well as cation conditions on G-quadruplex formation by native polyacrylamide gel electrophoresis and circular dichroism. Although the structures of short telomeric oligonucleotides have been carefully examined, few studies have focused on longer telomeric DNA, which mimics the very ends of telomeres. We show that K\(^+\) and Sr\(^{2+}\) can induce human telomeric DNA to form both inter- and intramolecular structures. Circular dichroism results suggest that the structures in K\(^+\) were a mix of parallel and antiparallel G-quadruplexes, while Sr\(^{2+}\) induced only parallel-stranded structures. We also found that TRF2, a protein essential for telomere maintenance, affects G-quadruplex structure. These structures serve as useful models to study the effects of G-quadruplexes on the activities of telomeric proteins, like TRF2, from human cells.
The G-strand overhang at the ends of telomeres may periodically adopt at least some of these quadruplex conformations, which could subsequently affect protein binding and telomere function. TRF2, which binds towards the end of the double-strand (ds) telomere region just upstream of the G-strand overhang, is considered one of the key proteins in telomere protection and regulation. While TRF2 is not known to bind single-strand (ss) DNA, work performed in the lab suggested that the type of 3’-overhang in telomeric DNA ss/ds-junctions affects TRF2-binding. Specifically, preventing G-quadruplex formation by changing the overhang sequence from 5’-d(TTAGGG)4-3’, to 5’-dTTAGGG(TTAGAG)2TTAGGG-3’, reduced TRF2 recruitment to the ss/ds-junction from HeLa cell extracts. Using the same techniques as above, we show that the N-terminal basic domain of TRF2 in K+ induces a switch from the mixed parallel/antiparallel-stranded G-quadruplexes usually stabilized by K+ -alone, to parallel-stranded G-quadruplexes. Interestingly, it also promotes intermolecular parallel G-quadruplex formation on non-quadruplex, single-stranded intermediates, but will not induce a switch from an antiparallel to a parallel G-quadruplex in Na+. These results are the first to demonstrate specific TRF2-G-quadruplex interactions. They suggest a novel mechanism for TRF2 recognition of the double-strand/single-strand junction of telomeres, where the myb-like domain binds to the double-strand DNA and the N-terminal basic domain interacts with the overhang, stabilizing the interaction. This model has implications for TRF2 communication with the very terminus of the telomere and for stimulation of strand invasion proposed to stabilize t-loop structures.
DEDICATION

To my parents,
Rosa and Raul Pedroso

To my husband,
Kevin P. O’Neill
ACKNOWLEDGEMENTS

I would like to begin by thanking my advisor, Terace M. Fletcher, Ph.D.; I could not have asked for a better mentor. Tracy was a constant source of wisdom and enthusiasm, the perfect balance of friend and mentor. She gave me a home for my graduate research and inspired me to excel. I cannot thank her enough for making this daunting experience plausible and even pleasant.

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Two labs were instrumental to the success of my research. Our neighbor, Dr. Thomas K. Harris, allowed me to use his UV spectrometer to quantify the oligonucleotides used throughout this dissertation. Dr. James D. Potter generously opened his doors to me, where I spent countless hours using his lab’s spectropolarimter for the circular dichroism experiments. I would also like to take this opportunity to thank the members of the Biochemistry and Molecular Biology Department for all of the technical advice I have received and the reagents I have borrowed.
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Speaking of family, words cannot express the gratitude I feel for mine. Being the youngest in a large Cuban family, often felt like I was in a sitcom. It seems impossible for us to be together without laughing and celebrating. My siblings, Raul, Monica and Albert have inspired and encouraged me throughout my life. They each married wonderful additions to the family that have become like siblings. Together, they created a new generation to amuse and challenge me. My parents are the heart of the clan; they are generous beyond imagination. Their love has guided us through life with courage and determination. Their lives embody the American Dream, and I hope to one day approach the same personal and professional success they achieved. I am who I am because of my family. The newest official addition is my husband, Kevin P. O’Neill. He came into my life when I was elbow-deep in graduate work. Kevin is my perfect partner, balancing my craziness and showing me how wonderful love can be. I cannot thank him enough for coming into my life.
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1 CHAPTER 1: INTRODUCTION TO TELOMERES, G-QUADRUPLEXES AND TRF2

1.1 Telomeres

The preservation and proper duplication of genetic information is essential to the propagation of life. However, the linear nature of eukaryotic chromosomes is inherently problematic. First, the replication problem results in some loss of DNA from the end of the lagging strand with each round of synthesis (Olovnikov, 1996). Additionally, linear chromosomes have ends that could be incorrectly processed as double strand breaks (DSBs). These problems are dealt with by telomeres, long stretches of repeated DNA at the ends of chromosomes, which allow for some DNA loss before genetic information is compromised. In addition to the importance of telomere length, telomeres may adopt protective conformations that prevent chromosome ends from being mistaken as DNA damage (Blackburn, 2000). The telomeric DNA can loop back on itself to form large T-loops (Griffith et al., 1999) which physically sequester the end as well as provide a scaffold for the nucleo-protein telomere maintenance machinery. Additionally, there is growing evidence that G-quadruplex DNA secondary structures may be involved in the protection of the very tip of the telomere. These protective conformations, and their regulation, involve the coordination of DNA sequence and structure with a variety of proteins to maintain the genome. One such protein is TRF2, which binds specifically to telomere repeats and is essential for telomere maintenance (Campisi et al., 2001; Harley et al., 1990; Olovnikov, 1996; Smogorzewska et al., 2000). Dysfunctional telomeres result in the cell entering replicative senescence, undergoing apoptosis or becoming immortal (often malignant in human cells). This regulation has earned telomeres a reputation as the mitotic clock of the cell (Campisi, 1996; Harley, 1991; Hayflick and
Moorhead, 1961; Stanulis-Praeger, 1987), because their shortening limits the possible number of generations.

1.1.1 **Sequence of Telomeres**

Telomeres are guanine-rich repeats at the ends of chromosomes that stretch for 2 to 100 kb depending on species and cell type. In humans, telomeres are typically ~15 kb of double strand 5’-d(TTAGGG)-3’ repeats, ending with a 130-275 base single strand 3’ tail (Makarov et al., 1997; Wright et al., 1997) that is proposed to be involved in the protective conformation of the telomeres.

1.1.2 **Single-Stranded Telomeric Sequences Can Form G-Quadruplex Structures**

Single-stranded guanine-rich DNA, like the 5’-dTTAGGG-3’ repeats of telomeres, can form G-quadruplex secondary structures. G-quadruplexes (Figure 1-1) are formed by stacking planar arrangements of four guanines, Hoogsteen-like base-paired to each other (Balagurumooorthy and Brahmachari, 1994; Kerwin, 2000; Murchie and Lilley, 1994; Wang and Patel, 1993). G-quadruplexes can form spontaneously *in vitro*, but are stabilized by certain cations, including physiological concentrations K⁺ and Na⁺ (Gilbert and Feigon, 1999; Williamson, 1994). (An in depth review of G-quadruplexes concludes this introduction.)

1.1.3 **A Telomeric Loop Sequesters the Ends of Telomeres**

Building in the complexity of telomeric structure, Dr. Elizabeth Blackburn (Blackburn, 2000) describes a model in which telomeres oscillate between the “capped” and “uncapped” states. The “capped” state refers to a telomere configuration that is somehow inaccessible to DNA damage signaling, repair, and replication activities. One possible structural model for this state came from Dr. Jack Griffith’s laboratory, which has shown that telomeric sequences can form T-loops (Griffith et al., 1999), where the
The T( telomeric) loop is considered a protective conformation of telomeric DNA. Jack Griffith’s group isolated these structures from cells after psoralen/UV cross-linking and observed them by electron microscopy.

3’-end loops back to interact with upstream DNA.
(Griffith et al., 1999; Stansel et al., 2001). While the cross-linking indicates DNA-DNA interactions at the junction, the resolution of the images is not high enough to determine the actual configuration. Therefore, the T-loop and G-quadruplexes are not necessarily mutually exclusive. Three models for the junction involve the 3' end invasion of the single-stranded overhang to form (Griffith et al., 1999; Stansel et al., 2001): a pseudo bimolecular G-quadruplex (Figure 1-2 A), a D-loop (Figure 1-2 B), or a 4-way junction (Figure 1-2 C); the latter two resemble recombination-like intermediates. While all three of these are plausible, the actual structure has not been elucidated. In addition to isolating T-loops from cells, the group found that the addition of TRF2 to telomeric DNA was

![Figure 1-2: Possible T-loop junctions. The actual junction of the T-loop is unknown. The 3’-end invasion could result in: (A) a pseudo-bimolecular G-quadruplex, (B) a D-loop, or (C) a 4-way junction.](image-url)
enough to induce T-loop formation in vitro (Griffith et al., 1999; Stansel et al., 2001), possibly by the generation of positive supercoils, thus facilitating proximal strand invasion (Amiard et al., 2007).

1.2 TRF2 is Essential for Telomere Maintenance

Naked DNA does not often persist in cells; telomeres are no exception. Several proteins are involved in telomere maintenance, however, the core complex(es) essential to telomere function is still under debate (de Lange, 2005). One of the proteins considered essential for telomere protection and length regulation is TTAGGG Repeat Factor 2 (TRF2) (Smogorzewska et al., 2000). TRF2 (Figure 1-3) has an N-terminal basic domain, followed by a TRF-homology (TRFH) domain (Broccoli et al., 1996; Broccoli et al., 1997), a linker region and a C-terminal myb-like domain responsible for binding to duplex DNA (Broccoli et al., 1997; Court et al., 2005; Nishikawa et al., 2001).

**Figure 1-3:** Domain structure of TRF2. (L-R) B: aa13-30: N-terminal arginine/glycine rich basic domain, TRFH: aa43-245 Telomere Repeat Factor Homodimerization domain, DBD: C-term myb-like DNA Binding Domain (telobox motif:HTH).

*In vivo*, TRF2 is involved in the ATM-DNA damage pathway (Karlseder et al., 2004; Tanaka et al., 2005). Non-phosphorylated-TRF2 interacts with and blocks the ATM pathway, while exposure to ionizing radiation results in its phosphorylation by ATM (Karlseder et al., 2004; Tanaka et al., 2005). The phosphorylated-TRF2 is found...
localized to dysfunctional telomeres as well as to sites of DNA damage throughout the chromosome (Tanaka et al., 2005). Overexpression of TRF2 results in accelerated telomere shortening with senescence occurring at shorter telomere lengths compared to cells with endogenous TRF2 levels (Karlseder et al., 2002). In contrast, the expression of a dominant-negative form of TRF2, where the basic- and myb-like domains have been deleted, results in the reduction of the total amount of G-strand overhang, end-to-end chromosome fusions, cellular senescence and apoptosis (Karlseder et al., 1999; van Steensel et al., 1998).

TRF2 has previously been shown to bind duplex DNA (Broccoli et al., 1997). However, research in the Fletcher lab indicates that TRF2 recognizes telomeric ss/ds DNA junctions and that the sequence and length of the overhang affect TRF2 binding (Khan et al., 2007; Yanez et al., 2005), suggesting an interaction between the protein and the G-overhang. Specifically, TRF2 affinity to ss/ds DNA junctions is slightly reduced when the central guanine of TTAGGG repeats in the overhang is altered to adenine, suggesting some level of specificity. I have previously shown that the G-strand overhang at telomeric ss/ds DNA junctions is capable of forming a G-quadruplex (Yanez et al., 2005), unless some of the central guanines are altered, which could influence TRF2 binding. In addition to ss/ds DNA junctions, it was recently shown that the N-terminal basic region of TRF2 was capable of recognizing 3-way and 4-way junctions (Fouche et al., 2006a). This same region may be involved in the proximity of TRF2 to the ss/ds junction by interacting with the overhang. This basic region may recognize or induce structures on the overhang, possibly even G-quadruplex structures. Moreover, another recent paper found that TRF2 stimulated strand-invasion on plasmids containing
telomeric sequences (Amiard et al., 2007). The TRFH domain was implicated in TRF2-dependent stimulation of strand invasion. However, lower levels of strand invasion occurred in the absence of TRF2 and were significantly increased under certain cation conditions (Amiard et al., 2007). This is interesting in light of the findings that telomeric DNA within model replication forks have a tendency to experience “slippage” resulting in fork regression (Fouche et al., 2006a; Fouche et al., 2006b). It is interesting that the N-terminal region of TRF2 favors binding to 4-way junctions over 3-way junctions suggesting the possibility that it interacts with regressed forks. One explanation for these results is that repetitive DNA has a tendency to form non-Watson Crick DNA structures and, in the case of telomeres, those structures would be G-quadruplexes. In the study by Amiard et al., addition of Na$^+$ may support supercoiling by stabilizing G-quadruplexes on relaxed portions of the plasmid, thus facilitating strand-invasion. Alternatively, Na$^+$ could be inducing the formation of a TRF2-recognized quadruplex, either on the invading strand or at some bubble in the plasmid. This could strengthen TRF2 interaction with the duplex region, similar to what we believe may be happening at telomeric ss/ds junctions. Our model involves the myb-like domain of TRF2 binding to the double-strand region, while the basic, N-terminal extends to interact with non-duplex DNA such as the G-strand overhang. These dual DNA binding regions in TRF2 along with the homodimerization and oligomerization regions on the protein suggest that TRF2 is an interesting architectural protein that can recognize and/or promote specific telomeric DNA structures.

### 1.3 Many Proteins Participate in Building Higher-Order Telomere Structures

TRF2 is only one of many proteins found at telomeres. The nucleoprotein complex formed at the ends of chromosomes promotes proper telomere function by
regulating length and conformation. According to Titia de Lange (de Lange, 2005) “Shelterin”, the minimum core complex required for telomere regulation, is composed of six proteins: TRF1, TRF2, POT1, TIN2, TPP1 and RAP1. However, even she admits that subcomplexes form within this group. TRF1, like TRF2, binds to double-strand telomeric DNA (Chong et al., 1995; Zhong et al., 1992), but unlike TRF2, TRF1 does not show specificity for the T-loop junction (Griffith et al., 1999). POT1, Protection of Telomeres 1, is a single-strand telomere binding protein with specificity for the 3’-overhang. TIN2 mediates interaction with TPP1/POT1, as well as TRF1-TRF2 interactions. TRF2 can also bind to Rap1. The relationship between these proteins introduces a variety of potential protective conformations. These proteins, together in complexes or acting alone, have been shown to affect various enzymatic activities and telomeric functions such as telomere length maintenance, telomerase activity and recruitment to telomeres, protection from DNA damage signaling, regulation of the telomeric ss/ds DNA junction composition, etc. (de Lange, 2005).

Other proteins found at the telomeres that are believed to contribute to telomere capping function include: telomerase (Zhu et al., 1999), Ku 70/80 (a DSB binding protein), DNA-PKcs (d'Adda di Fagagna et al., 2001), and the RAD50/MRE11/NBS1 complex (Boulton and Jackson, 1996; Espejel et al., 2002; Huang et al., 2001; Samper et al., 2000; Song et al., 2000; Wei et al., 2002; Zhu et al., 2000). Ku70/80 (Kanaar et al., 1998; Lieber, 1998; Ramsden and Gellert, 1998), a heterodimer involved in Non-Homologous End Joining (NHEJ) of the ends of broken chromosomes, and NBS1, part of the Homologous Recombination (HR) complex, interact with TRF2 (Song et al., 2000; Zhu et al., 2000). Defects in Ku70/80, NBS1, TRF2 or DNA-PKcs affect telomere
maintenance in mammalian cells, potentially leading to the loss of the single strand
overhang, which could in turn result in chromosome fusions (Bai and Murnane, 2003;
Bailey et al., 1999; Ranganathan et al., 2001; van Steensel et al., 1998).

The need for proteins involved in HR is interesting with respect to the possible T-
loop junctions; these proteins may be involved in T-loop formation and migration.
Another group of proteins that could facilitate branch-migration and T-loop maintenance
are the RecQ helicases. WRN and BLM have been found to co-localize with TRF2 and
to and stimulates the helicase activity of both proteins on specific templates in vitro
(Opresko et al. 2002). This may be necessary because TRF1 inhibits DNA polymerase α
synthesis, stalling replication (Bianchi et al. 1999). TRF2 may also mediate similar
replication fork stalling, creating a need for the ability of BLM-RPA and WRN-RPA to
unwind TRF2-bound telomeric DNA, and of WRN exonuclease to digest it (Opresko et
al. 2002). Alternatively, BLM and WRN may recruit or be recruited by telomeric factors
(Opresko et al. 2002), such as TRF2, to resolve specific structures at the telomeres (e.g.
G-quadruplexes, D-loops, 4-way junctions, stalled forks or protein-telomere interactions).
Once there, BLM and WRN could assist with replication and repair. As discussed above,
WRN exonuclease activity is stimulated by Ku70/80 (Li & Comai 2001); a protein that
promotes NHEJ throughout the genome, yet helps prevents chromosome end fusions
when bound to the telomeres (d'Adda di Fagagna et al. 2001). The configuration
heterochromatic telomeres adopt to protect the genome must be tightly regulated to allow
appropriate access to the ends of chromosomes, while resisting certain DNA repair
mechanisms. BLM and WRN helicases have been implicated in the manipulation of
telomere higher-order nucleoprotein chromatin structure, which is critical for maintaining genomic integrity.

Even if a minimum group of required proteins can be determined, it is likely that telomere maintenance is executed by a very dynamic array of proteins that associate and disassociate with each other, and with DNA, as needed. We may find that many of the proteins actually serve multiple roles depending on the DNA structure they recognize or their protein partners. This seems especially relevant with respect to proteins like Ku70/80 and the MRE11 complex, which typically process damaged DNA; at telomeres, they protect DNA-ends from being processed as damage.

1.4 Telomeres Are Involved in Chromosome Alignment During Cell Division

In mammalian cells, telomeres are not only relevant to the longevity of a cell line, but are important for proper chromosome and chromatid segregation during mitosis and meiosis, respectively (de La Roche Saint-Andre, 2007). In somatic cells, ATM and other factors (Smilenov et al., 1999) regulate telomere anchoring to the nuclear matrix by telomeric proteins and other ligands (Chikashige and Hiraoka, 2001; Chikashige et al., 2006; Cooper et al., 1998; Okabe et al., 2004). Once attached to the nuclear matrix, telomeres also interact with each other, either directly through DNA-DNA interactions or via DNA-protein/protein-protein interactions (Pandita et al., 2007). During early meiosis, telomeres are attached to the nuclear envelope and migrate to form the evolutionarily conserved bouquet. The clustering of telomeres into the bouquet configuration is believed to facilitate chromosome alignments and crossover events between homologs, both of which are necessary for the proper progression of meiosis (de La Roche Saint-Andre, 2007). Additionally, this stage enables telomere length to be reset by recombination events between telomeres (Joseph et al., 2005). By gathering telomere ends into such
tight quarters, the possibility of forming intermolecular G-quadruplexes increases, in a manner similar to the addition of PEG to a solution (see below). Once formed, G-quadruplexes could hold chromosomes close enough for cross-over events to occur. Alternatively, G-quadruplex structures could be part of the anchoring to the matrix, like they are in ciliates (see TEBP below). Once chromosome exchanges are complete, a number of proteins can destabilize the quadruplexes allowing for separation (As I will discuss shortly, human proteins with potential include: telomerase, hnRNP, MRE11, and Pot1). A strong contender in *S. cerevisiae* is Kem1p, a tetramolecular quadruplex-dependent nuclease, which cleaves single-strand DNA, proximal to the quadruplex. Deleting the KEM1 gene blocks meiosis at the 4N stage (Kim et al., 1990; Liu and Gilbert, 1994), suggesting that it prevents chromosome separation and implicating a need for G-quadruplex elimination for meiotic progression.

1.5 *The Road to Immortalization*

Most somatic cells can undergo a finite number of cell divisions before encountering telomere dysfunction (Harley et al., 1990). Problems with their telomeres arise from gradual shortening, malfunctioning repair mechanisms, and defects in telomere-interacting proteins (Blackburn, 2000). If a cell with such problems does not enter replicative senescence or undergo apoptosis, continuous divisions will result in genomic instability (Campisi et al., 2001). The majority of somatic cells lack substantial telomerase activity and thus lose telomere length with each round of replication (Harley *et al.* 1990). 85-90% of tumor cells avoid telomere disruption-dependent withdrawal from the cell cycle by activating telomerase (Bodnar et al., 1998; Campisi et al., 2001; Counter et al., 1992; Counter et al., 1994; Kim et al., 1994; Ouellette et al., 2000; Vaziri and Benchimol, 1998; Wyllie et al., 2000; Yang et al., 1999) the reverse transcriptase
responsible for extending of telomeres (Biroccio and Leonetti, 2004). Expressing telomerase stabilizes telomere length, affects the persistence of the protective conformation (Blackburn, 2000; Campisi et al., 2001), and can prevent senescence in wild type, WS and BS cells (Oullette et al. 2000, Wyllie et al. 2000).

1.5.1 ALT - The Road Less Traveled

The remaining 10-15% of immortalized cells follow an Alternative Lengthening of Telomeres (ALT) pathway (Stavropoulos et al., 2002). ALT cell lines are characterized by long heterogeneous telomeres that experience dramatic extension and deletion events possibly mediated by homologous recombination between telomeres and the extra-chromosomal telomeric DNA repeats found in ALT cells (Bryan et al., 1995; Murnane et al., 1994; Ogino et al., 1998; Regev et al., 1998; Tokutake et al., 1998). Another common feature of ALT cell lines is the presence of ALT-Associated Promyelocytic Leukemia (AA-PML) nuclear bodies (Yeager et al., 1999). In addition to PML protein, these enlarged, nuclear matrix associated bodies co-localize with telomeric DNA, telomere-specific proteins (TRF1 and TRF2), recombination proteins (hRAD52, hRAD51 and NBS1), and the RecQ homologs (BLM and WRN) (Johnson et al., 2001; Seeler and Dejean, 1999; Stavropoulos et al., 2002; Wu et al., 2000; Yeager et al., 1999; Zhong et al., 1999).

1.6 G-Quadruplex Structures Form on Guanine-Rich Repeated DNA

Telomere regulation is a critical part of cellular health, in addition to the gross configuration of DNA and proteins at chromosome ends, the repetitive guanine-rich sequence of telomeres can potentially form non-Watson-Crick interactions. This could be especially important at times when the 3’-end is not physically sequestered in the T-loop (e.g. during replication, telomerase extension, meiosis and mitosis). While the telomeric
DNA is single-stranded, G-quadruplex secondary structures can form. Their relevance to telomere regulation is still under debate, but evidence for their importance has increased rapidly in recent years.

The guanine-rich repeated DNA, especially within the G-strand overhang has the capacity to form G-quadruplexes. The basic building block of a G-quadruplex is called a G-tetrad, a planar arrangement of 4 guanines reverse-Hoogsteen base-paired to each other. Several G-tetrads stack to form a G-quadruplex (Balagurumoorthy and Brahmachari, 1994; Kerwin, 2000; Murchie and Lilley, 1994; Wang and Patel, 1993). While suitably sequenced DNA can spontaneously form a G-quadruplex, in vitro experiments have shown that near physiological concentrations of appropriately sized cations (e.g. K⁺ and Na⁺) serve to promote and stabilize G-quadruplexes in telomeric sequences (Gilbert and Feigon, 1999; Williamson, 1994). Stable coordination complexes are also formed with other cations, including Sr²⁺ (Han et al., 1999b; Kankia and Marky, 2001; Wang and Patel, 1993).

1.6.1 Characterization of G-Quadruplex Types

The cation serves to induce and stabilize G-quadruplexes, but the sequence, length and concentration of the DNA influence what type of G-quadruplexes can form (Pedroso et al., 2007a; Pedroso et al., 2007b). Four or more TTAGGG repeats on one strand can form an intra- or unimolecular G-quadruplex (Figure 1-1). Multiple strands coming together form intermolecular structures (Figure 1-1). The distinction between parallel and antiparallel G-quadruplex refers to the 3’→5’ orientation of the guanine runs along the four corners (Figure 1-1). While the crystal structure of the telomeric sequence [5’-dAG₃(TTAGGG)₃-3’], which is similar to our T4 (Table 2-1), in K⁺ showed a parallel-stranded quadruplex (Parkinson et al., 2002), solution structures of similar
sequences in K$^+$ and Na$^+$ have shown that T4-like sequences can form parallel, antiparallel and mixed/hybrid-type G-quadruplexes (Li et al., 2005; Ourliac-Garnier et al., 2005; Qi and Shafer, 2005; Wang and Patel, 1993). The sequences connecting these guanine runs lay in the loop regions of the G-quadruplex with loops being lateral, diagonal, or external “propeller” (Figure 1-1). Finally, the orientation of the individual bases may be designated as syn- or anti-. This final distinction is the hardest to determine specifically for the individual bases because it requires high-resolution structural data, but it does affect the CD spectra (Krafft et al., 2002). Parallel-stranded intramolecular quadruplexes only contain bases in the anti-conformation with respect to their glycosidic bonds. Antiparallel G-quadruplexes on the other hand, are composed of a 1:1 ratio of bases in the syn- and anti-conformations (Tang and Shafer, 2006).

1.6.2 Cation Effects on G-Quadruplex Formation

Within the range of possible structures that can form based on DNA sequence, length and concentration, the cation(s) is a very important driving force for stabilizing one type of G-quadruplex over another. While K$^+$ is capable of inducing and stabilizing a variety of quadruplexes (Li et al., 2005; Ourliac-Garnier et al., 2005; Qi and Shafer, 2005), Na$^+$ alone has only been shown to stabilize antiparallel G-quadruplexes (Karimata et al., 2005). In contrast, Sr$^{2+}$, in our hands, only stabilized parallel-stranded quadruplexes. Na$^+$-induced G-quadruplexes have generally been found to be less thermodynamically stable than those stabilized by K$^+$ or Sr$^{2+}$ (Kankia and Marky, 2001; Sen and Gilbert, 1990), this reduced stability is thought to be due to the size of the cation or the coordination chemistry of the Na$^+$-aqua complexes verses Na$^+$-guanine rather than the orientation of the quadruplex strands.
1.6.3 **G-Quadruplexes are Thermodynamically Stable**

G-quadruplexes are highly stable, some with melting temperatures reaching, and possibly exceeding, 95°C (Chen, 1992; Deng and Braunlin, 1996; Salazar et al., 1996; Sen and Gilbert, 1990). The spontaneity and stability of G-quadruplexes in conjunction with the presence of single-stranded G-rich DNA at the 3'‐overhang, and throughout the telomere during DNA synthesis, suggest the structure is relevant to telomere function. G-quadruplexes either exist at telomeres, or are actively prevented.

1.6.4 **G-Quadruplex Transitions May Act as Molecular Switches**

In addition to telomeres, repetitive guanine-rich regions are found throughout the genome; their locations include: some promoter regions, like the c-myc promoter, immunoglobulin heavy chain switch regions, and ribosomal DNA (Huppert and Balasubramanian, 2005; Todd et al., 2005). The sequence and cation-specificity of quadruplex structures could have cellular implications as molecular switches, especially when one considers the dynamic nature of telomere ends. The combination of K⁺ and Na⁺ was shown to increase melting temperatures and, for certain sequences, resulted in a switch from an antiparallel to a four‐stranded parallel quadruplex. This structure could not form in Na⁺, which only stabilizes antiparallel quadruplexes, and it could not form in K⁺ alone because the DNA formed a stable dimer intermediate (Sen and Gilbert, 1990) that could not transition into the four‐stranded G‐quadruplex. Another structural change was promoted by the addition of a nucleic acid analogue to a telomeric DNA sequence. This has been shown to induce the transition from an antiparallel to a parallel‐stranded quadruplex in K⁺ but not in Na⁺ (Dominick and Jarstfer, 2004). Similar switches could occur at chromosome ends as part of the dynamic cycle of telomere maintenance. The
type of quadruplex present could recruit a specific protein or protein complex; alternatively, a protein or protein complex could promote a certain type of G-quadruplex.

1.6.5 Molecular Crowding Effects on G-Quadruplex Structures

In vitro experiments generally have a small reactant-to-volume ratio of less than 1 g/L. This environment, while adequate for reactions to occur, does not necessarily reflect cellular conditions, where macromolecules may occupy 300-400 g/L or 20-40% of the volume (Hall and Minton, 2003; Miyoshi et al., 2006a). To reduce the effective volume of in vitro reactions, a molecular crowder, like the polymer polyethylene glycol (PEG) is added. The addition of PEG to a reaction was found to induce a transition of d(G₄T₄G₄) from an antiparallel to a parallel G-quadruplex. Furthermore, increasing the concentration of PEG to 2M stabilized a human antiparallel G-quadruplex and destabilized duplex DNA (Miyoshi et al., 2006a; Miyoshi et al., 2004; Miyoshi et al., 2002), a result that presents interesting possibilities for in vivo telomeric DNA. Sugimoto’s group suggests that molecular crowding is an important factor in the structure and stability of nucleic acids, and specifically in the formation of G-quadruplexes (Karimata et al., 2005; Miyoshi et al., 2005). PEG is effective at excluding volume from the reaction, which can promote multimeric complex formation (Minton, 2001; Miyoshi et al., 2005; Miyoshi et al., 2006b), but is nonreactive with DNA (Heyes et al., 2006).

Polycations also have the potential to alter DNA conformation by binding electrostatically and neutralizing negative charge. One such polycation is putrescine (Miyoshi et al., 2002). Unlike PEG, the addition of 2 M putrescine does not induce a transition from antiparallel to parallel quadruplex for telomeric oligonucleotides of Tetrahymena sequence. However, putrescine did slightly destabilize the antiparallel G-quadruplex by causing a loss in entropy of the system (Miyoshi et al., 2002). During early
meiosis, telomeres are clustered together in the “bouquet” stage (de La Roche Saint-Andre, 2007; Joseph et al., 2005). It has been proposed that molecular crowding-induced structural transitions to or between quadruplexes may have an important role in chromosome interactions during this phase (Miyoshi et al., 2002; Miyoshi et al., 2001; Sen and Gilbert, 1990).

1.7 Evidence of G-Quadruplex Effects

1.7.1 In Vivo Antibody-Staining of G-Quadruplexes in Nuclei

One can speculate that the role for G-quadruplexes during the bouquet stage is supported by recent evidence linking telomeres and G-quadruplexes to the subnuclear matrix attachment of ciliate chromosomes. G-quadruplex specific antibodies have been used for in situ localization experiments in Stylonychia lemnae, visualizing G-quadruplexes in the macronuclei but not in the replication band. The group raised two antibodies, Sty3 and Sty49; both recognized a 4-stranded parallel quadruplex, but Sty49 could also bind to a bimolecular, antiparallel quadruplex. Only Sty49 localized to the telomeres, indicating that not only were G-quadruplexes present, but that a particular quadruplex was found at telomeres. This suggests an important role for G-quadruplexes in telomere function that must be resolved during replication (Schaffitzel et al., 2001). The disappearance is logical when one considers that G-quadruplexes inhibit both DNA polymerase-δ (Han et al., 1999b; Kamath-Loeb et al., 2001) and telomerase-mediated telomere synthesis (Zahler et al., 1991). These antibodies were later used to show that TEBPα and TEBPβ are required to form these G-quadruplexes on the 3’-overhangs of telomeres (Paeschke et al., 2005). TEBPα attaches telomeres to the subnuclear matrix and recruits TEBPβ. In situ hybridization and antibody experiments show that the
telomeres of these cells are attached to the matrix and that they end in bimolecular antiparallel G-quadruplexes (Paeschke et al., 2005; Schaffitzel et al., 2001). During replication, the phosphorylation of TEBPβ is thought to regulate the necessary disruption of G-quadruplexes. This suggests that G-quadruplexes are involved in chromosome accessibility and positioning.

1.7.2 Other Proteins Interact with G-Quadruplexes

G-quadruplexes have not been visualized in mammalian cells; however, the consequences of introducing quadruplexes or ligands that stabilize them are sometimes severe. This alone suggests that they are involved in at least some cellular processes (even if they are merely being unwound). Recent studies have discovered an increasing number of proteins exhibiting G-quadruplex recognition. In addition to Stylonychia lemnae TEBPα/β, we have also discussed human BLM and WRN helicases; both RecQ helicases are associated with the ALT pathway of telomere maintenance and both show a strong preference for G-quadruplexes over duplex DNA (Mohaghegh et al., 2001; Sun et al., 1998).

Saccharomyces cerevisiae Mre11 is part of a complex that regulates DNA damage, telomere length, cell cycle checkpoint and meiotic recombination (Assenmacher and Hopfner, 2004; D'Amours and Jackson, 2002; Haber, 1998; Petrini, 2000). This nuclease exhibits a significant binding preference for a parallel G-quadruplex over single- or double-strand DNA. In addition to binding the G-quadruplex, it will cleave it, presumably creating an acceptable substrate for telomerase (Larrivee et al., 2004) and other telomere binding proteins. Considering its roles in the cell, if the human homolog
behaves similarly, this presents another possible mechanism for G-quadruplex processing at telomeres and during meiosis.

A structural homolog of TEBPα is human Pot1 (Lei et al., 2003); hPot1 is thought to bind the single-strand overhang of telomeres (Baumann and Cech, 2001; Colgin et al., 2003) and mediate telomerase recruitment (Colgin et al., 2003). An *in vitro* telomerase assay on G-quadruplex DNA restored processivity by the addition of hPot1 (Zaug et al., 2005). In this case, it did not appear as though Pot1 was directly disrupting the quadruplex, rather it was stabilizing and trapping the single-strand (Oganesian and Bryan, 2007; Zaug et al., 2005). As with everything else in the telomere, oscillation between structures is likely to be normal and hPot1 seems to utilize these changes to stabilize an appropriate, non-quadruplex, substrate for telomerase (Fletcher et al., 1998; Zahler et al., 1991). These results propose a role for G-quadruplexes in the protection of telomere ends by preventing telomerase and possibly other proteins from acting on the 3’-end.

Another group of single-strand telomere-binding proteins are the heterogeneous nuclear ribonucleoproteins, hnRNP A1 and D. A previous report had shown hnRNP A1 could destabilize G-quadruplex structures in mini-satellite regions (Fukuda et al., 2002). More recently, hnRNP A1 (Zhang et al., 2006) and D (Enokizono et al., 2005) have been shown to bind and destabilize telomeric G-quadruplexes. It is unclear whether the hnRNPs disrupt quadruplexes passively, as with hPot1, or actively. Regardless, their effects on telomerase extension differ. hnRNP D can recognize and disrupt parallel and antiparallel, intra- and intermolecular quadruplexes (Enokizono et al., 2005). However, once bound, it does not restore telomerase activity (Enokizono et al., 2005), presumably because it remains bound to the single-strand in a protective conformation. On the other
hand, a binding assay showed that hnRNP A1 binds and disrupts G-quadruplexes in a concentration-dependent manner and restores telomerase activity in cell extracts (Zhang et al., 2006).

The idea to target G-quadruplex structures for chemotherapy was based on *in vitro* data showing inhibition of telomerase. While some types of G-quadruplexes, including an intramolecular G-quadruplex, have been shown to inhibit telomerase activity (Fletcher et al., 1998; Oganesian et al., 2006; Sun et al., 1999; Zahler et al., 1991), an important exception is a K⁺-stabilized, 4-stranded, parallel G-quadruplex (Figure 1-1), which is a suitable substrate for *Tetrahymena* telomerase (Oganesian et al., 2006). While this seems counterintuitive, destabilizing intermolecular G-quadruplexes by telomerase-mediated elongation may be a last resort for separating chromosomes and restoring order before a more permanent chromosome fusion occurs. Alternatively, this activity could be part of meiosis if G-quadruplexes are involved in chromosome alignment.

### 1.7.3 The Power of Telomeres in Medicine

The battle with cancer is on the front lines of biomedical pursuits. Regardless of the mechanism for telomere maintenance in tumor cells (telomerase activation or the ALT pathway), the need for cells that become immortalized to maintain their telomeres, introduces many possibilities for cancer treatment. A group of possible anti-cancer drugs includes those that bind to and stabilize G-quadruplexes (Han and Hurley, 2000; Li and Comai, 2001; Mergny and Helene, 1998; Neidle and Read, 2000). Once bound to G-quadruplex or B-DNA, these ligands severely restrict access to the DNA. Many of these ligands can be designed to selectively target G-quadruplex DNA over normal duplex DNA (Hurley, 2001). Such a strategy would inhibit BLM, WRN and telomerase activity at the telomeres. In addition, they have the potential to disrupt the telomeric
nucleoprotein complexes. This strategy is particularly appealing because of the possibility of some ALT-cells existing within any given tumor cell population (Li and Comai, 2001).

1.7.4 G-Quadruplex Ligands as Chemotherapeutic Targets

When G-quadruplexes were found to inhibit telomerase, G-quadruplexes and agents that stabilize them became popular targets for chemotherapeutic treatments (Artandi and DePinho, 2000; Bennett et al., 1993; Berman et al., 1986; Bernards et al., 1983). Several G-quadruplex stabilizing ligands have been identified; among these, a few show particular promise as anti-tumor treatments, including: telomestatin, TMPyP4, and BRACO19 which I will discuss, as well as RHPS4 (Gowan et al., 2001) and 12459 (Riou et al., 2002). (For Reviews see: (Kelland, 2005; Perry and Jenkins, 2001; Rezler et al., 2003; Riou, 2004))

Telomestatin, which binds an intramolecular (Kim et al., 2002), basket-type (Rezler et al., 2005) G-quadruplex, inhibits the proliferation of telomerase-positive cell lines (~90% of tumor cells). In vitro, telomestatin is a potent telomerase inhibitor (Shinya et al., 2001). In cells, telomestatin causes reduction of the G-strand overhang (Gomez et al., 2004) as well as TRF2 (Tahara et al., 2006) and Pot1 (Gomez et al., 2006) dissociation; both proteins interact either directly or indirectly with the overhang (de Lange, 2005). Telomestatin may lock the overhang into an unrecognizable structure, leading to loss of protection and then loss of overhang. It also activates the DNA damage-response pathway (Tauchi et al., 2003), which could recognize and cleave the telomestatin-stabilized structure, leading to loss of protection. While the actual mechanism is unclear, telomestatin’s effects are considered too rapid to be a downstream response to telomerase inhibition (Oganesian and Bryan, 2007; Tahara et al., 2006). The
in vivo effects of introducing telomestatin on tumor cell lines are similar to those of dominant-negative TRF2 (Karlseder et al., 1999; van Steensel et al., 1998); they include: general telomere dysfunction, loss of G-strand overhang, senescence and apoptosis (Gomez et al., 2004; Shammas et al., 2004; Tahara et al., 2006; Tauchi et al., 2006).

TMPyP4 is a porphyrin ligand which facilitates the formation of intermolecular G-quadruplexes (Wheelhouse, 1998) in addition to binding duplex DNA (Ren and Chaires, 1999). As such, it recognizes the c-myc promoter region, stabilizing a quadruplex and down-regulating the oncogene. This subsequently led to a reduction in telomerase transcription (Siddiqui-Jain et al., 2002). TMPyP4 also inhibits telomerase activity in HeLa cell-free extracts and in MCF7 breast cancer cells (Izbicka et al., 1999b). Treatment with the porphyrin increases anaphase bridge formation (Izbicka et al., 1999a), another possible link between telomeres’ role in chromosome alignment during cell division (de La Roche Saint-Andre, 2007) and G-quadruplexes. TMPyP4 could stabilize preexisting structures or form new intermolecular quadruplexes. By preventing their resolution at the proper stage, cell growth is inhibited. While it does not have the singular specificity of telomestatin, TMPyP4 has the added benefit of inhibiting the proliferation of both telomerase-positive and ALT cell lines (Kim et al., 2003).

Another ligand, which acts on both telomerase-positive and ALT cells, is BRACO-19. Its dual activity stems from the down-regulation of the reverse transcriptase subunit of telomerase, hTERT, expression (Burger et al., 2005) as well as by its ability to stabilize intramolecular quadruplexes at the telomere (Incles et al., 2004). Growth inhibition after BRACO-19 treatment has been seen in human uterus carcinoma and prostate cancer cell lines, as well as in early stage tumor xenografts in mice (Burger et al.,
Telomere dysfunction symptoms in these cells include end-to-end fusions and atypical mitosis (Burger et al., 2005; Incles et al., 2004); once again connecting G-quadruplexes to cell division.

1.7.5 Exogenous G-Quadruplexes Induce Apoptosis

In mammalian cells, the introduction of pre-formed telomeric G-quadruplexes induced ATM-dependent apoptosis in various types of tumor cells. The same oligonucleotides introduced as unstructured DNA did not affect cellular viability (Qi et al., 2006). One possible explanation is that the introduction of aberrant G-quadruplexes signaled the DNA-damage response pathway. Alternatively, G-quadruplexes could be recognized by end-capping proteins, and as such, compete proteins away from telomere ends, making the ends of chromosomes susceptible to processing. This effect could be similar to the effects of telomestatin and the expression of the dominant negative form of TRF2, which led to shortened telomeric overhangs, increased chromosome fusions and apoptosis (Karlseder et al., 1999; van Steensel et al., 1998).

1.8 Conclusion

The attachment of telomeres to the nuclear matrix of mammalian cells during early cell division (de La Roche Saint-Andre, 2007) and in non-replicating regions of S. lemnæ macronuclei (Paeschke et al., 2005; Schaffitzel et al., 2001), suggests a possible role for G-quadruplexes at the ends of mammalian telomeres. In S. lemnæ, telomere binding proteins tether the telomeres to the subnuclear matrix and promote bimolecular G-quadruplexes (Paeschke et al., 2005). G-quadruplexes could play a similar role in mammalian cells, stabilizing interactions between telomeres while homologous chromosomes are aligned. Telomere binding proteins, like TRF2, could also be involved in recognizing specific structures and bridging telomeres. While in vivo evidence of G-
quadruplex formation at human telomere ends has not been identified, \textit{in vitro} data suggests that the 3’-overhang can form a variety of intra- and intermolecular quadruplexes under physiological conditions. The ability of G-quadruplexes to switch between forms may be part of the regulation of telomeres by providing a molecular switch between telomeric complexes. Alternatively, G-quadruplexes could temporarily protect the tip of the chromosome when the T-loop is disrupted, as during DNA synthesis or other processes, or actually participate in T-loop formation. The spontaneity and stability of G-quadruplex structures suggests that if they are not part of telomere function, proteins are actively preventing their presence.

Proper telomere maintenance is critical for cellular viability. If telomere length and/or conformation go awry, mechanisms are in place to correct them or sacrifice the cell. When these processes are dysfunctional, cells could become immortal with disastrous consequences for the host organism. Alternatively, cells may enter senescence, resulting in tissue degeneration associated with age-related diseases. In addition to proteins, which regulate telomere dynamics, the DNA structure itself is important.

This dissertation concentrates on G-quadruplexes, DNA secondary structures that are becoming increasingly difficult to ignore when discussing telomere function. We demonstrate the variety of structures that form, \textit{in vitro}, depending on the sequence, length and cation conditions. Then, we investigate how the basic domain of TRF2 interacts with and affects these G-quadruplex structures. This project uses eletrophoretic mobility shift assays, footprinting, model telomere recruitment assays and circular dichroism to investigate interactions between TRF2, an essential telomere protein, and G-quadruplexes, DNA structures potentially involved in telomere function.
2 CHAPTER 2: MATERIALS AND METHODS

2.1 Telomeric DNA Oligonucleotides (Chapters 3 - 5)

HPLC purified oligonucleotides composed of normal or altered human telomeric repeats were ordered from Sigma Genosys. Telomeric sequences (Tn, n=1-9) ranged from one, 5’-d(TTAGGG)-3’ repeat (T1) to nine repeats (T9) as described in Table 2-1. Mutant telomeric sequences (Tn-X) had some or all of the repeats altered to 5’-d(TTAGAG)-3’. The -X denotes which repeat in the 5’→3’ direction was modified. Therefore T4-1 refers to the sequence 5’-d(TTAGAGTTAGGGTTAGGGTTAGGG)-3’, while T8-2,3 refers to 5’-d(TTAGAGTTAGAGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAG
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2.1.1 Native Polyacrylamide Gel Electrophoresis for Samples in KCl, NaCl, LiCl SrCl₂ and Tris-EDTA (Chapters 3 and 4)

Oligonucleotides were labeled with $^{32}$P-$\gamma$ATP. Samples of telomeric DNA were prepared in 1× TE (100 mM Tris pH 8.0, 10 mM Na₂EDTA) and 100 mM KCl, NaCl, LiCl or SrCl₂. Also, reactions in TE without salt were used as negative controls. Oligonucleotide concentrations were 2 µM unless otherwise specified. Samples were heated for 5 minutes at 95°C in a heat block. The block was turned off to allow for slow cooling (2-3 hours) of the samples to room temperature, where they remained overnight. Native 15% polyacrylamide gels were prepared in tris-taurine EDTA (TTE) ± 10mM (SrCl₂) or 100 mM (KCl, NaCl or LiCl) salt. Gels were electrophoresed at 4.2 V/cm for 16-24 h in ambient temperature conditions or in the cold room, then dried. A Storm 840 Phosphorimager (Molecular Dynamics) was used for band detection. Band migration and
intensities were determined using ImageQuant 5.2 software. Experiments were also performed with unlabeled oligonucleotides. Gels were stained with SYBR Gold after a 30 min wash in tris–borate EDTA. No differences were observed between the two methods.

### 2.2 Concentration-Dependent Native Polyacrylamide Gel Electrophoresis Assay in KCl (Chapter 3 and 4)

Reactions were prepared with DNA concentrations (0.1 – 4 μM) indicated in Figures 3-3, 3-4 and 4-2 in TE with KCl or SrCl₂ and treated as described above. Entire samples or aliquots, with an equal number of total moles of oligonucleotide (0.5 pmol), were loaded into each lane. The purpose of loading aliquots instead of entire samples was to better illustrate the changes in relative ratios of structures. Both methods achieved similar results. Native gels were run in the cold room and analyzed as described above. In addition to DNA-concentration, the Sr²⁺ concentration-dependence of intermolecular structures was examined. These experiments were performed as described above, with 1 μM oligonucleotide and 0-100 mM SrCl₂.

### 2.3 Temperature-Dependent Rate of Formation of Intermolecular Structures in KCl (Chapter 3)

Rates of formation of intermolecular structures were performed by preparing DNA samples in TE and 100 mM KCl, heating for 5 minutes at 95°C, then immediately transferring to room temperature, 37, 47, or 57°C. Samples were incubated for indicated lengths of time (0-24 hours) before loading onto native 15% polyacrylamide gels in TTE and 100 mM KCl. Electrophoresis was performed at 4.2 V/cm in a 4°C room. Band migration and intensities were determined using ImageQuant 5.2 software to obtain a plot of the intensity of the slower moving band relative to the total intensity of each lane.
2.4 Temperature Dependent rate of Formation of Intermolecular Structures in SrCl₂ (Chapter 4)

Oligonucleotide T4-1 (2 μM) was prepared in SrCl₂ (100 mM) and TE (100 mM Tris, pH 8.0, 10 mM Na₂EDTA). After 5 minutes at 95 °C, the sample was immediately transferred to 30, 40, 50, 60, or 70 °C for 0.25, 0.5, 1 or 2 h. Samples were loaded onto 15% native polyacrylamide gels in tris–borate Na₂EDTA (no SrCl₂) and run as described above. Band migration and intensities were determined using ImageQuant 5.2 software to obtain a plot of the intensity of the slower moving band relative to the total intensity of each lane.

2.5 UV Absorption Spectroscopy (Chapter 4)

Oligonucleotides (2 μM) in TE alone or with SrCl₂ (100 mM), were heated to 95°C, cooled slowly and incubated overnight at room temperature. Absorption spectra (220–320 nm) of oligonucleotides were obtained in 0.1 nm increments using a Beckman Coulter DU-640 Spectrophotometer with a 100 μl cuvette, 1 cm pathlength. The difference spectra were calculated by subtracting the absorbance in TE alone from the absorbance in TE + SrCl₂.

2.6 Circular Dichroism, CD (Chapters 3 and 4)

Oligonucleotide concentrations were measured using the previously mentioned UV spectrophotometer, before and after CD measurements were taken and the average used as the DNA concentration. Samples were prepared with 2 μM oligonucleotide, 100 mM KF or SrCl₂, and 1 mM Na₂PO₄. Reactions were treated by normal overnight annealing (5 minutes at 95°C, slow cool, overnight at room temperature) or by a 0-24 hour incubation at 47°C immediately after boiling. CD spectra at wavelengths 200-320 nm were obtained with a Jasco J-720 spectropolarimeter in 200-μl cells with a 0.1-cm
pathlength. The resulting CD spectra were the average of 10 consecutively measured scans taken in 0.5-nm increments over 25 min.

2.7 **N-terminal Peptide Binding to Telomeric G-Quadruplex Structures (Chapter 5)**

Reactions (20 µl final) were prepared similarly to circular dichroism experiments in 100 mM* KCl and 13.5 µM* Na₂PO₄ and 4 µM* [γ³²P-ATP]-labeled oligonucleotides (*These are final concentrations, after the addition of peptide). Samples were then boiled for 5 minutes at 95°C, cooled for 5 minutes to room temperature and briefly centrifuged. An equal volume of peptide was added to each reaction, to final concentrations of 0, 2, 4, 8, and 16 µM. These were left for 24 hours at room temperature before the addition of Bromophenol blue/xylene cyanol loading dye. Samples were loaded onto 8% (29:1) native polyacrylamide gels prepared in 1X TTE, 15 mM Na₂PO₄ and 100 mM KCl. Electrophoresis was carried out in a 4°C room at 3 V/cm for 16-20 hours. A Storm 840 Phosphorimager (Molecular Dynamics) was used for band detection. Band migration and intensities were determined using ImageQuant 5.2 software.

2.8 **Circular Dichroism with Basic N-terminal Domain of TRF2 (Chapter 5)**

Structural effects of the N-terminal peptide on telomeric oligonucleotides structures were analyzed by circular dichroism. The basic N-terminal domain of TRF2 (Figure 1-3), containing amino acids 3-33 (N-GGGGSSDGSGRAAGRRASRSSGRARRGRGRH-C) was synthesized by GenScript Corporation. 100 µM stocks were prepared in water and frozen until use. Samples were prepared with 4 µM telomeric oligonucleotide (Table 2-1) in Na₂PO₄ (1* or 20*mM) and KCl (0 or 100 mM). Reactions were boiled for 5 minutes in a 95°C heat block, cooled for 5 minutes at room temperature before the addition of 8 µM peptide (unless otherwise specified) or an equal volume of H₂O. CD
was performed as described above. Alternatively, the oligonucleotides were pre-annealed for 24 hours before the addition of peptide or an equal volume of water. CD was measured at room temperature, as described previously on the Jasco J-720 spectropolarimeter.

2.9 Synthetic Double-Strand/Single-Strand Junctions (Chapter 5)

In addition to the short, telomeric single-strand oligonucleotides, DNA was designed to mimic telomeric double-strand/single-strand junctions (Table 2-1, Figure 2-1). These DNA sequences are as follows: T2Tn, where n=2-4, is 5’-dCCCTAA CCCTAACGTCG [Biotin-TEG]CATCGTCTCATGCGTTAGGG TTAGGG(TTAGGG)n-3’ with complementary (cT2T0) 5’-dCCCTAACCCTAA CGCATGAGACGATGCGACGCTGAGACGTTAGGGTTAGGG-3’. As a negative control, T2T4mut2,3, where the overhang sequence was altered from T4 [5’-d(TTAGGG)4-3’] to T4-2,3 [5’-dTTAGGGTTAGAGTTAGAGTTAGGG-3’] was also purchased. The biotin was used for telomere-recruitment assays with streptavidin that will be described later.

2.10 Electrophoretic Mobility Shift Assay for Double-Strand/Single-Strand Junctions (Chapter 5)

For each biotinylated substrate DNA (T2T2, T2T3, T2T4, T2T4mut2,3), equimolar amounts of the non-biotinylated complementary strand oligonucleotide was annealed at a final concentration of 1 µM, in 5 mM MgCl₂ and TE (40 mM Tris–HCl (pH 8), 1 mM EDTA). The annealing reactions were heated to 95°C, cooled slowly until they reached room temperature, and then placed on ice for 15 minutes. Proper annealing was validated on 12% native polyacrylamide gels where the annealed samples were compared to non-annealed oligonucleotides and size standards. Annealed oligonucleotides (1.5
pmol) were incubated in TE with 100 mM KCl, NaCl or LiCl (10 µl final volume) for 30 minutes at room temperature. Bromophenol blue/xylene cyanol loading dye was added and samples were subjected to 15% (29:1) native polyacrylamide gel electrophoresis in 1XTris-Borate EDTA buffer and ±100 mM salt (KCl, NaCl, or LiCl) at 5 V/cm, until the samples ran 15 cm into the gel (16–24 h). Gels were pre-run for 30 min before loading. The DNA was visualized by staining with SYBR Gold (Molecular Probes).

**Figure 2-1:** Template of oligonucleotides for double-strand/single-strand (ds/ss) junctions. [30 bases of dsDNA designed with a non-telomeric, non-specific sequence (NS).] [Two repeats of ds-telomeric DNA, (TTAGGG)₂. To mimic the G-strand overhang, for T2Tn (n=2,3, or 4), 5'-d(TTAGGG)n-3' or for T2T4mut2,3: 5'-dT TAGGGTTAGAGTTAGAGTTAGGG-3'.

### 2.11 Telomere End Recruitment Assay, TERA (Chapter 5)

Annealed, biotinylated DNA (90 pmol) was incubated with 2 mg Dynal M280 streptavidin-coated paramagnetic beads in binding and washing (B&W) buffer, containing TE, 5 mM MgCl₂, and 0.1% Triton X-100 (final volume 350 µl) for 3–16 h at RT. The beads were washed twice with 350 µl of B&W buffer then stored in 350 µl B&W buffer with 0.1 mg/ml BSA and 0.1% thymersol. To insure that 100% of substrate
DNA was immobilized, an aliquot of the immobilized DNA was eluted from the beads with formamide loading dye, run on a sequencing gel, and the SYBR Gold-stained band intensities of each target DNA were compared to DNA standards. Once the immobilized DNA was quantified, 3 pmol was pre-incubated for 30 min in 20 µl telomere end recruitment assay (TERA) buffer (20 mM Heps, pH 7.3, 100 mM KCl, 10% glycerol, 0.5 mM EDTA, 5 mM MgCl₂, 0.1% Triton X-100, 1 mM DTT, and 1 mM AEBSF, 1 µg/ml of each of aprotinin, pepstatin, and leupeptin, with 2 mg/ml BSA) to allow G-quadruplex formation. The recruitment reaction was initiated by adding 32 µg HeLa-S3 whole cell (Manley et al., 1980) extract, 2 µg calf thymus DNA, and TERA buffer to a final volume of 100 µl. Reactions were incubated at 37°C for 15–30 minutes. Protein recruitment levels were similar between 7.5 and 30 minutes but were reduced at 60 minutes. Immobilized DNA–protein complexes were isolated using a Dynal magnet, and washed once with TERA buffer lacking BSA. Proteins were eluted by heating to 95 °C in SDS loading dye, run on a 7.5% protein gel, and detected by Western blot analysis. The DNA was also released by heating in the SDS buffer allowing for loading precision to be checked by removing the bottom of the protein gel and staining with SYBR Gold. Antibodies used for detection were as follows: TRF2 (mouse monoclonal, Upstate), WRN (rabbit polyclonal, Novus Biologicals), Digital images of the Western blots were quantified using the AlphaImage2000 program to obtain background-corrected integrated intensities of each band. The intensities for each DNA substrate on a particular gel were normalized to T2T4 to allow for comparison between experiments.
3 Chapter 3: Sequence Specificity of Inter- and Intramolecular G-Quadruplex Formation by Human Telomeric DNA Sequences

Human telomeric DNA consists of tandem repeats of the sequence 5’-d(TTAGGG)-3’. Guanine-rich DNA, such as that seen at telomeres, forms G-quadruplex secondary structures. Alternative forms of G-quadruplex structures can have differential effects on activities involved in telomere maintenance such as telomerase activity. With this in mind, we analyzed the effect of sequence and length of human telomeric DNA on G-quadruplex structures by native polyacrylamide gel electrophoresis and circular dichroism. Telomeric oligonucleotides shorter than four, 5’-d(TTAGGG)-3’ repeats formed intermolecular G-quadruplexes. However, longer telomeric repeats formed intramolecular structures. Altering the 5’-d(TTAGGG)-3’ to 5’-d(TTAGA)-3’ in any one of the repeats of 5’-d(TTAGGG)₄-3’ converted an intramolecular structure to intermolecular G-quadruplexes with varying degrees of parallel or antiparallel-stranded character, depending on the length of incubation time and DNA sequence. Intermolecular structures were most abundant in K⁺-containing buffers. Higher-order structures that exhibited ladders on polyacrylamide gels were observed only for oligonucleotides with the first telomeric repeat altered. Altering the sequence of 5’-d(TTAGGG)₈-3’ did not result in substantial formation of intermolecular structures even when the oligonucleotide lacked four consecutive telomeric repeats. However, many of these intramolecular structures shared common features with intermolecular structures formed by the shorter oligonucleotides. The wide variability in structure formed by human telomeric sequence suggests that telomeric DNA structure can be easily modulated by proteins, oxidative damage, or point mutations resulting in conversion from one form of G-quadruplex to
another. This is particularly important since it has been shown that telomerase activity is sensitive to only some forms of G-quadruplex. In addition, intermolecular G-quadruplexes may promote other activities at telomeres such as recombination, t-loop formation, or chromosome-chromosome end clustering or fusions.

3.1 INTRODUCTION

Telomeres are nucleoprotein complexes that function to protect the ends of eukaryotic chromosomes. Telomeric DNA is comprised of repetitive, guanine-rich DNA that forms non-Watson-Crick structures based on reverse Hoogsteen-like base pairing of four guanines in a planar arrangement known as the G-tetrad. Telomeric G-quadruplexes have a variety of structures dependent on DNA sequence, number of telomeric repeats, oligonucleotide concentration and stabilizing cation (Davis, 2004).

The important aspect of G-quadruplexes is that they are stable structures that can form in physiological concentrations of K\(^+\) or Na\(^+\) and likely form in certain environments\textit{ in vivo} (Davis, 2004). Transcription-dependent guanine-loops comprised of human telomeric and other quadruplex-forming DNA are observed in\textit{ E. coli} when the telomeric DNA is the non-template strand (Duquette et al., 2004). Although G-quadruplexes may not be competitive with duplex telomeric DNA under certain conditions (Phan and Mergny, 2002), there are regions in the telomere where the G-strand exists in the absence of the C-strand. For example, free G-strand DNA may be present during lagging strand synthesis. More importantly, the G-strand has been shown to extend several hundred bases beyond the C-strand to create a 3’-overhang (Fletcher, 2003) known as the G-strand overhang. Considering the stability of G-quadruplex DNA, it is highly likely that this G-strand overhang is capable of forming G-quadruplexes.
Indeed, evidence suggests that G-quadruplex structures are associated with telomeric functions. It is generally postulated that G-quadruplexes inhibit DNA polymerization and \textit{in vitro} studies show that these structures inhibit replication (Woodford et al., 1994), polymerase δ (Kamath-Loeb et al., 2001) and the telomere-specific reverse transcriptase, telomerase (Fletcher et al., 1998; Oganesian et al., 2006; Sun et al., 1999; Zahler et al., 1991). Telomerase activity is upregulated in $>$85% of cancer cells (Burger et al., 2005) and the enzyme has been a target for chemotherapeutic agents.

Although G-quadruplexes are all defined by the G-tetrad, they vary in stability, rate of formation and overall structure. These characteristics should have different effects on the cell. For example, it has been shown that intramolecular G-quadruplexes (similar to that shown in Figure 1-1) inhibit telomerase activity (Fletcher et al., 1998; Oganesian et al., 2006; Sun et al., 1999; Zahler et al., 1991). However, a recent study shows that an intermolecular form of G-quadruplex is a suitable substrate for telomerase (Oganesian et al., 2006; Perry et al., 2001). In addition, small molecules designed to interact specifically with different G-quadruplex types (Neidle and Parkinson, 2002; Perry et al., 2001; Rezler et al., 2003) have varying cellular effects. For example, telomestatin has been shown to exclusively stabilize an intramolecular form of G-quadruplex and only affects telomerase positive cells (Kim et al., 2003). However, RHPS4 and TMPyP4, which interact with two different G-quadruplex structures, inhibit the growth of both telomerase positive and negative cancer lines (ALT cells) (Gowan et al., 2001; Kim et al., 2003). Differential activities of TMPyP4 may also be attributed to the fact that it binds well to duplex DNA (Ren and Chaires, 1999).
To better understand the nature of G-quadruplexes formed by human telomeric DNA at replication forks and the G-strand overhang, we studied the structures formed by human telomeric oligonucleotides of various sequence and length by native gel electrophoresis and circular dichroism (CD). As observed by other laboratories, a minimum of four, 5’-d(TTAGGG)-3’ repeats were required to form an intramolecular G-quadruplex. However, altering the central guanine to adenine in at least one of the 5’-d(TTAGGG)$_n$-3’ repeats, resulted in the formation of intermolecular G-quadruplex structures. Some of these oligonucleotides formed increasingly higher-order structures that were observed as ladders in polyacrylamide gels. Longer oligonucleotides such as 5’-d(TTAGGG)$_8$-3’ did not form substantial intermolecular structures when the sequence was altered but did form various intramolecular structures. Interestingly, some oligonucleotides had a CD signature more indicative of antiparallel or parallel-stranded character depending on which 5’-d(TTAGG)-3’ was altered to 5’-d(TTAGA)-3’.

3.1.1 **Key Findings**

Intramolecular G-quadruplex formation induced by Na$^+$ or K$^+$ when 4 or more repeats of 5’-dTTAGGG-3’ are present on a single-strand.

Intermolecular G-quadruplexes will form in K$^+$, but require longer incubation times. They form from shorter oligonucleotides, altered T4 oligonucleotides (where an adenine is inserted into position 5 of a repeat (TTAGA)) and between T7 oligonucleotides.

All structures had mixed hybrid-type antiparallel/parallel structures in K$^+$ except T4-2,3 and T8-2,3,6,7, whose structures were largely parallel stranded.

Time courses showed that many of the oligonucleotides formed a structure with fewer bases in the *anti-* conformation at earlier time points followed by an increase in bases with an *anti-* conformation later. The latter is indicative of more parallel-stranded character, suggesting the conformation is slower to form.
3.2 RESULTS

To investigate the role of DNA length and sequence on secondary structure formation, oligonucleotides T1-T9 denoting the number of 5’-d(TTAGGG)-3’ repeats ranging from 1 to 9 were synthesized (Table 2-1). To study the role of the central guanine in the formation of structures, many of the oligonucleotides had at least one of the 5’-d(TTAGGG)-3’ repeats altered to 5’-d(TTAGA)-3’. For example, T4-1 had four telomeric repeats but the first repeat was converted to 5’-d(TTAGA)-3’. Structures were studied in various salts by native polyacrylamide gel electrophoresis, UV and CD spectroscopy.

3.2.1 Longer Telomeric Oligonucleotides Preferentially Form Compact Structures

Relative mobilities were derived from a standard curve by including non-telomeric, double-stranded size markers in each experiment. Oligonucleotides in KCl, NaCl, LiCl and/or TE were boiled briefly and allowed to cool slowly to allow for the formation of structures (for complete details see Materials and Methods). Native gel electrophoresis of T1-T9 (1-9, 5’-d(TTAGGG)-3’ repeats) in Tris Taurine-EDTA gels demonstrated a decrease in mobility as the size of the oligonucleotides increased, with oligonucleotide T4 running slightly faster than expected compared to the other sizes (Figure 3-1 A, C). The faster migration of T4 was lost when the gel was run at room temperature (data not shown) suggesting that the structure was unstable. T4 had an even greater mobility in gels run at 4°C containing Li⁺ (Figure 3-1 A, C).

A substantial increase in migration rates for T4-T9 was observed in gels containing 100 mM Na⁺ and K⁺ (Figure 3-1 A, C), signifying that they fold into more compact secondary structures. Both T4 and T8 had the greatest migration rates compared
Figure 3-1: Native gel electrophoresis of telomeric oligonucleotides prepared in 1XTE only (None), 100 mM LiCl, 100 mM NaCl or 100 mM KCl as described in Materials and Methods. (A and B) Radiolabeled oligonucleotides were electrophoresed on native 15% polyacrylamide gels run in Tris-Taurine EDTA and salt (as indicated) at 4°C. Lanes are labeled with the number of TTAGGG repeats (n in A or B) or the number of TTAGAG repeats (n* in B). (C) Relative Mobility of oligonucleotides in A and B as a function of the mobility of the 10 bp DNA ladder.
to the other oligonucleotides, indicating that they form the most relatively compact structures. This is consistent with observations of similar oligonucleotides, suggesting that long telomeric oligonucleotides fold into units of 5’-d(TTAGGG)_4-3’ like beads on a string (Vorlickova et al., 2005; Yu et al., 2006).

The presence of K\(^+\), in gels run at 4°C, consistently resulted in the appearance of a significant amount of slower-moving species for T2, T3, and T7 only. T5, T6, and T8, also formed slower-migrating species but these were less abundant. The slower-migrating T2 band had a migration rate equivalent to double the size of the faster moving species relative to double-stranded DNA markers (Figure 3-1 A, C). T3 formed two new species in K\(^+\) with mobilities relative to sizes double and triple that of the faster migrating form.

The mobility of T7 in K\(^+\) is interesting in that it was split between a species that ran much faster than that observed in the absence of salt or in Li\(^+\) and a species that had an apparent size double that of the fast migrating species. Additionally, these larger structures were not observed at room temperature (data not shown), suggesting that their stability was not strong enough to endure electrophoresis. Note that slower-moving structures were also observed in Na\(^+\) but their abundance was much lower than those observed in K\(^+\)-containing buffers (data not shown).

In contrast to the salt-dependent changes in mobility observed with the 5’-d(TTAGGG)-3’ repeat oligonucleotides, the structures of oligonucleotides comprised of 5’-d(TTAGAG)-3’ repeats (Tn-all) were not altered by the addition of K\(^+\) (Figures 3-1 B, C).
3.2.2 Altering the Telomeric Sequence Converted T4 but not T8 into Slower Migrating Structures

To further investigate the role of the central guanine in each 5’-d(TTAGGG)-3’ repeat in the intramolecular T4 structure, oligonucleotides were synthesized in which a single 5’-d(TTAGGG)-3’ was converted to 5’-d(TTAGAG)-3’. Earlier (Yanez et al., 2005), we found that modifying any one of the 5’-d(TTAGGG)-3’ repeats in this manner disrupted the intramolecular G-quadruplex structure in the 3’, G-strand overhang of telomeric single-strand/double-strand DNA junctions (Figure 5-7). In this study we increased the length of incubation time to detect intermolecular G-quadruplex formation. This allowed us to discover structures previously missed.

Alteration of the 5-prime, 5’-d(TTAGGG)-3’ repeat to 5’-d(TTAGAG)-3’ or 5’-d(TTAGTG)-3’ (Figures 3-2) disrupted the formation of a T4-like intramolecular G-quadruplex in K\(^+\) but stimulated the formation of slower migrating species. These structures did not form in Li\(^+\) or Na\(^+\) (Figure 3-2C, data not shown). Similar to those formed by T3, the apparent sizes of these structures were double and triple that of Band 1. In addition, Bands 2 and 3 each represent a cluster of structures. Note that even higher-order oligomers formed for both T4-T1 and T4-1 (Figure 3-2 A, C). T3 formed similar higher-order structures but required longer incubation times (Figure 3-4).

The oligonucleotides with the 5’-d(TTAGAG)-3’ sequence in the 2\(^{nd}\), 3\(^{rd}\), or 4\(^{th}\) telomeric repeats (T4-2, T4-3, and T4-4) also formed species with slower relative mobilities in the gel. Interestingly, Bands 2 and 3 of T4-2, T4-3, and T4-4 had similar mobilities to that of T4-1 and T4-T1. However, no higher-order oligomers were formed even at concentrations up to 4 µM or week-long incubation times (data not shown). Like
The oligonucleotide with 5'-d(TTAGA)G-3' in both the 2nd and 3rd repeats (T4-2,3) had notably different characteristics. T4-2,3 formed a single slower moving species only in the presence of K+, not in Na+ or Li+ (Figure 3-2 A, C). Band 2 of T4-2,3 was a single band with greater mobility than Band 2 of the other T4-derivatives. Additionally,
none of the super-higher-order structures were formed on T4-2,3 even at higher concentrations and weeklong incubation times (data not shown).

There was an observed increase in mobility for all the T8-derived oligonucleotides, except T8-all, with added K\(^+\) (Figure 3-2 B, D). Notably, T8 had the highest mobility in K\(^+\) gels compared to its derivatives, indicative of a more compact structure. Interestingly, T8-2,3,6,7 had the second highest migration rate, comparable to the relative change in T4-2,3 mobility, indicating that it also had a more compact structure than the other T8-derivatives. Note that Na\(^+\) had a similar effect on the T8-derived oligonucleotides except T8-2,3,6,7, which did not exhibit a change in mobility (Figure 3-2 B, D).

3.2.3 Structures With Slower Mobilities Were Concentration-Dependent

Oligonucleotides T4-T9 all formed more compact structures in Na\(^+\) and K\(^+\). With the exception of T7, no structures of slower mobility were abundantly formed, even at a wide range of oligonucleotide concentrations (Figure 3-3, data not shown). This suggests that the species with increased mobility were structures stabilized by intramolecular interactions.

Structures formed in K\(^+\) with slower electrophoretic mobilities showed oligonucleotide concentration dependence. The slower moving structure formed by T2 in KCl was first observed at 50 nM oligonucleotide with a slight increase as the oligonucleotide concentration increased (data not shown). For T3, substantial appearance of Band 2 formed at concentrations as low as 100 nM (Figure 3-3A). Band 3 formed at 300 nM T3 concentration and its intensity did not increase past 500 nM. Bands 2 and 3 together remained at 60% of the total oligonucleotide structures from 500 nM to 4 µM T3 concentrations. Very little of the slower moving structures for both T2 and T3 were
observed at all concentrations tested (0.01-4 µM) when the gels are run at ambient temperature (data not shown).

Structures with slower electrophoretic mobilities formed by the T4-derived oligonucleotides also showed oligonucleotide concentration dependence. Like the T3 oligonucleotide, Bands 2 and 3 for T4-1 (Figure 3-3B), T4-2, and T4-3 (data not shown) increased in intensity as the oligonucleotide concentration increased until its relative ratio remained unaltered from 2 to 4 µM.

The slower moving species formed by T4-2,3 and T7 (Figure 3-3 C, D) also increased in relative amounts with increasing oligonucleotide concentration. In these cases, no significant bands larger than Band 2 were formed. Additionally, it is unclear whether the relative amounts of the two bands have reached a plateau, or if increasing the concentration would increase the relative amount of Band 2.

**Figure 3-3**: Concentration dependent formation of slower moving species. Reactions in 100 mM KCl, 1XTE were prepared with 0.05-4.0 µM γ^32P labeled DNA (A) T3, (B) T4-1, (C) T4-2,3, (D) T7. Species were separated on native 15% polyacrylamide gels with 100 mM KCl/TTE in 4°C room.
The concentration dependence of the slower moving T2, T3, T7, and T4-derived oligonucleotides suggest that these structures were formed by intermolecular interactions. The mobility of T4-4 was also altered with increasing concentration but smears formed instead of defined bands suggesting that these structures are either highly variable or unstable.

Alteration of the T8 sequence also resulted in some structures with slower electrophoretic mobilities (Figure 3-2B). However, these structures were much less abundant relative to those formed by the T4-derived oligonucleotides, regardless of the salt present, even at concentrations up to 8 µM or incubation times up to a week (data not shown).

### 3.2.4 Long Incubation Times Result in Super-Higher-Order Structures

Incubation of guanine-rich oligonucleotides have resulted in significant self-association thought to be G-quadruplex formation (Marsh and Henderson, 1994; Sen and Gilbert, 1992). To determine if human telomeric sequences can assemble into such super-higher-order structures, we incubated T3, T4 and the T4-derivatives at room temperature for two weeks after the initial heating at 95°C. Samples were then analyzed by native gel electrophoresis. T3 and T4-1 formed a ladder in polyacrylamide gels with a defined periodicity in mobility that reached to the very top of the gel (Figure 3-4). Unexpectedly, increasing the concentration of oligonucleotide in these reactions decreased the ratio of super-higher-order structures relative to Bands 2 and 3. Interestingly, T4-2 and T4-3 formed much fewer super-higher-order structures. No super-higher-order structures were observed with T4, T4-4 (Figure 3-4, data not shown) or any of the other oligonucleotides tested in this study (data not shown).
3.2.5 Formation Rates of Slower Moving Structures

Although intramolecular G-quadruplexes often form on a minute timescale, intermolecular G-quadruplex structures generally take much longer to form. To determine the relative rate of formation of the larger structures observed in Figures 3-1 to 3-4, oligonucleotides were incubated under certain conditions and analyzed at various time points. Since the intermolecular structures were sensitive to DNA concentration...
(Figure 3-3), the formation of intermolecular structures could most likely be captured because oligonucleotides are diluted during electrophoresis.

T2 bimolecular structures were slow to form at room temperature with little intermolecular structure appearing until 4 hours (data not shown). The rate of intermolecular structure formation increased with increasing temperature from 37°C to 57°C. However, the relative amounts of intermolecular structure after 24 hours decreased from 37°C to 57°C.

On the other hand, T3 Band 2 formed relatively quickly when incubated at room temperature, but very little Band 3 formed even at 24 hrs of incubation (data not shown). Heating samples to 37°C, 47°C, and 57°C greatly increased the rate of Band 3 formation. The greatest ratio of both Bands 2 and 3 relative to Band 1, at 24 hours, was achieved at 47°C (Figure 3-8). Although incubation at 57°C resulted in the fastest rate of formation of Band 3, it also resulted in less overall intermolecular structure formation, suggesting that 57°C is near the melting point.

The formation of intermolecular structures by the T4-1 (Figure 3-8, data not shown) and T4-3 (data not shown) derivatives followed a similar pattern to T3, with a relatively fast rate of Band 2 formation but very little Band 3 forming at room temperature and 37°C. Heating samples to 47°C and 57°C greatly increased the rate of Band 3 formation. T4-2 (data not shown) also had similar patterns of formation rates as T3, T4-1, and T4-3 except that the overall ratio of intermolecular structures versus Band 1 was much lower.

3.2.6 UV Spectroscopy Suggests These Structures are G-Quadruplexes

The UV spectra of several forms of G-quadruplexes (intramolecular, intermolecular, parallel-stranded, antiparallel stranded, etc.) show that there is a
A reproducible increase in absorbance at 295 nm. In fact, monitoring absorbance at 295 nm produced more precise G-quadruplex melting curves than observing absorbance changes at 260 nm (Mergny et al., 1998). Consequently, we obtained the UV absorption spectra for the oligonucleotides to determine whether the structures described in Figures 3-1 through 3-4 had characteristics of G-quadruplexes. As expected for a G-quadruplex structure, T4 had a higher 295 nm to 260 nm ratio in both KCl- and NaCl-containing solutions.

**Figure 3-5:** The UV absorbance spectra of 2 mM oligonucleotide: T4: (TTAGGG)4, T4-1: TTAGG(TTAGGG)3, T4-3: (TTAGGG)2TTAGG TTAGGG, T4-All: (TTAGG)4, in 100 mM KCl (solid line), LiCl (dotted line) or NaCl (dashed line). 60 µl samples were boiled for 5 minutes then cooled slowly to room temperature and incubated overnight in Tris-EDTA and salt.
buffers as compared to the absorbance spectrum taken in the LiCl buffer (Figure 3-5A). However, T4-all had nearly the same spectrum in all three buffers (Figure 3-5B), consistent with the electrophoresis data and suggesting that this oligonucleotide was unable to form G-quadruplex structures under these conditions. When incubated under conditions that promote intermolecular structures, T4-1 also had a higher 295/260 nm ratio in KCl compared to that in LiCl (Figure 3-5C). However, this was not true in NaCl, congruent with our electrophoresis finding that T4-1 did not form intermolecular structures in NaCl. The results for T4-3 are similar to those with T4-1 (Figure 3-5D). This suggests that the intermolecular structures observed for T4-1 and T4-3 in K⁺ are G-quadruplexes. Circular dichroism was then used to characterize the nature of the G-quadruplexes.

### 3.2.7 CD Spectroscopy Suggests a Variety of Parallel and Mixed Parallel/Antiparallel Structures

Circular dichroism (CD) was used to characterize the nature of the G-quadruplexes. In general, oligonucleotide results are grouped together based on the similarities in spectra (Figure 3-6). In addition, possible structures are in the panels to the right of the spectra. Multiple forms of G-quadruplexes with known solution structures, have been analyzed by CD, which provide a basis for characterizing the structures in this study. Generally, parallel-stranded G-quadruplex structures with an anti-glycosidic bond conformation have a CD spectrum exhibiting a strong positive peak at 260-265 nm and a smaller negative peak at 240 nm (Hardin et al., 1991; Xu et al., 2006). G-quadruplexes typically seen in Na⁺ buffers with lateral and diagonal loops, antiparallel strands, and a mixture of anti/syn-glycosidic bonds have a strong positive peak at 295 nm, a weaker negative peak at 265 nm and a weak positive peak at 245 nm (Balagurumoorthy and
Brahmachari, 1994; Krafft et al., 2002). Hybrid G-quadruplex structures have mixed antiparallel/parallel strands and external (propeller) plus lateral loops (Figure 3-6A, right panel). The CD spectrum of these structures appears to be a hybrid of the two types of quadruplex spectra with a strong positive peak at 290 nm, a shoulder at 270 nm, and a small negative peak at 240 nm (Balagurumoorthy and Brahmachari, 1994; Rujan et al., 2005; Vorlickova et al., 2005; Yu et al., 2006).

In the absence of added K\(^+\), the CD spectra for all tested oligonucleotides were similar, with positive peaks around 250-260 nm and weak negative peaks around 280-290 nm (Figure 3-6, data not shown). The T4 oligonucleotide had a weak positive peak at 255 nm in the absence of K\(^+\) (Figure 3-6A). However, addition of K\(^+\) resulted in a spectrum with a strong positive peak at 290 nm, a shoulder at 270 nm, and a smaller negative peak at 240 nm indicative of the hybrid, parallel/antiparallel form of the G-quadruplex. This has been observed with T4 (Rujan et al., 2005) and similar oligonucleotides such as 5’-d(GGG(TTAGGG)\(_3\))-3’ and 5’-d(AGGG(TTAGGG)\(_3\))-3’ (Balagurumoorthy and Brahmachari, 1994; Rujan et al., 2005; Vorlickova et al., 2005; Yu et al., 2006). The CD spectrum for T8 (Figure 3-6A) was nearly identical to that of T4, in agreement with earlier studies for similar oligonucleotides (Vorlickova, Chladkova et al. 2005), and suggesting that T8 forms a structure comprised of two T4 units. Importantly, addition of 100 mM K\(^+\) to T4-all (Figure 3-6A) and T3-all (data not shown) resulted in no change in the CD spectrum, reaffirming the electrophoresis data, together suggesting that removal of the central guanine in all of the telomeric repeats disrupts the G-quadruplex structures.

T3 in the absence of K\(^+\), had a very similar spectrum to that of T4 with a positive band at 255 nm and a weak negative band 290 nm (Figure 3-6B). Addition of K\(^+\) to T3
resulted in a spectrum with a positive peak at 265 nm that is larger than the one at 290 nm. It is important to note that under the conditions of the experiment, electrophoresis results show that T3 formed more than one intermolecular species. The data in Figures 3-7 and 3-8 will help to delineate the contributions of different structures to the CD spectrum. The T4-2 (data not shown) and T4-3 (Figure 3-6B) oligonucleotides had similar spectra to that of T3.

The CD spectrum of T4-1 was more similar to T3 than to T4 (Figure 3-6B). However, unlike the T3 CD spectrum, the band at 290 nm for T4-1 was similar in intensity to the 265 nm peak. In this respect, the spectrum for T4-1 was similar to what was observed with 5’-d(G3(TTAGGG)4)-3’ (Vorlickova et al., 2005) in K+. In that case, one band was observed in polyacrylamide gels suggesting a single intramolecular structure but T4-1 formed at least two intermolecular structures under the conditions tested.

The oligonucleotides T7, T8-1,5, T8-2,3, and T8-6,7 had strikingly similar spectra (Figure 3-6C) to that of T4-1. Like T4-1, T7 represents the presence of an intermolecular structure in the mixture. However, T8-1,5, T8-2,3, and T8-6,7 were all intramolecular structures. The likeness in spectra is due to the similarity of the G-quadruplexes rather than the number of strands in the structures.

A third type of spectrum was observed for T4-2,3 and T8-2,3,6,7 (Figure 3-6D). Although an intermolecular structure was present in the T4-2,3 mixture and mainly an intramolecular structure existed in T8-2,3,6,7, they both had a strong positive peak at 265 nm. The T4-2,3 spectrum had no peak at 290 nm and the shoulder at 290 nm for T8-2,3,6,7 was relatively weak compared to the peak at 265 nm. This is suggestive of
Figure 3-6: CD spectra of telomeric oligonucleotides (left) and models of the possible G-quadruplexes indicated by the spectra (right). Oligonucleotides (Tn-X) in 1 mM Na$_2$PO$_4$ were prepared without (dashed line) or with 100 mM KF (solid line) as described in Materials and Methods. n= the number of telomeric repeats in the oligonucleotide and X = telomeric repeat(s) in the 5$'$$\rightarrow$3$'$ direction where 5$'$-d(TTAGAG)-3$'$ replaced 5$'$-d(TTAGGG)-3$'$. Molar ellipticity is in deg*cm$^2$/dmol of bases. Model structures on the right show possible interpretations of the CD data (left) for the corresponding oligonucleotide. Each telomeric repeat is represented by an arrow. For intermolecular structures, different colors represent different strands. Each G-tetrad is represented by a square.
parallel-stranded structures with all propeller loops comprised of 5’-d(TTAGAG)₂TTA-3’ repeats with an additional loop of 5’-d(TTA)-3’ for T8-2,3,6,7 (Figure 3-6D, right panel).

The T4-4 spectrum was different than all the other spectra. It had weak positive peaks at 295 and 250 nm (Figure 3-6D). The 250 nm peak was similar to that observed with all the oligonucleotides in the absence of K⁺.

3.2.8 Time-Dependent Formation of Intra- and Intermolecular G-Quadruplex Structures

Since many of the oligonucleotides displayed more than one structure on polyacrylamide gels, the CD spectra can be misleading. Most of these results were obtained after heating the oligonucleotides at 95°C for 5 minutes followed by slow cooling to room temperature (RT). To distinguish the types of structures that may exist in the mixture, CD spectra were obtained at RT, immediately after heating at 95°C (Figure 3-7) and after incubating at 47°C for 1, 4, 8, and 24 h (Figures 3-8 A-C). Although time course experiments at other temperatures were performed (data not shown), 47°C allowed for the most substantial formation of Bands 2 and 3 at the most reproducible time points (Figures 3-8 D-F). This method allowed for isolation of spectral changes attributed to Bands 1, 2, and 3 observed in Figures 3-2 and 3-3.

The spectrum for T4, taken at RT immediately following heating, was similar to that seen in Figure 3-6A, in agreement with other studies that show that the intramolecular G-quadruplex forms quickly compared with intermolecular structures. The spectra for T4-1, T4-2, and T4-3 showed that the 290-nm peak appeared at early time points (Figure 3-7A). However, a peak at 250-255 nm, similar to that seen in the absence of K⁺ in Figure 3-6B, was still observed. The spectra for these oligonucleotides were
similar to that of T4-4 after slow cooling in K\(^+\) (Figure 3-6D). This suggests that these oligonucleotides form some type of structure at early time points in K\(^+\) that is different than that in the absence of salt.

The CD spectra for T3 and T4-2,3 (Figure 3-7B) at the early time point were different than those of T4-1, T4-2, and T4-3. Here the spectra did have a peak at 250 nm but lacked a positive peak at 290 nm, suggesting that these structures for T3 and T4-2,3 are similar to those in the absence of added K\(^+\) (Figure 3-6 B, D). On the other hand, the spectrum for T8-2,3,6,7 already resembled the spectrum observed in Figure 3-6D. Again, this suggests that intramolecular structures form at a much faster rate than intermolecular structures. The only difference is that the ratio of 265:290 nm was smaller in Figure 3-7B than that in Figure 3-6D, suggesting that the parallel-stranded structure required more time to be fully organized.

The CD spectra for the third group of oligonucleotides, T7, T8-1,5, T8-2,3, and T8-6,7, at the early time point had a strong peak at 290 nm (Figure 3-7C). T8-1,5, T8-2,3, and T8-6,7 had a substantial shoulder at 260 nm but it was definitely smaller than that observed in Figure 3-6C suggesting that the structures at early time points have more antiparallel character than later time points. This is even more pronounced with T7 in which the smaller peak centers at 255 nm. Again, this suggests that organization of the parallel-stranded and anti-glycosidic bond features within the structures organized more slowly. In addition, these results indicate that this organization is even slower with T7 because it involves intermolecular interactions.
Figure 3-7: CD spectra of early structures formed by telomeric oligonucleotides in KF. Oligonucleotides (Tn-X) in 100 mM KF and 1 mM Na₂PO₄ were heated for 5 minutes at 95°C and immediately loaded into the RT cuvette. The exception is T4-1 O/N which is the spectrum from Figure 3-6 used as a reference. n=the number of telomeric repeats in the oligonucleotide, whereas X=telomeric repeat(s) in the 5’→3’ direction where 5’-d(TTAGAG)-3’ replaced 5’-d(TTAGGG)-3’. Molar ellipticity is in deg*cm²/dmol of bases.
To further investigate how the structures in Figures 3-6A and 3-6B form, samples were analyzed at several time points by native gel electrophoresis and CD. For T4-1, substantial positive ellipticity was present at 290 nm at the early time points (Figure 3-8A) and the gel demonstrated that only Band 1 was present (Figure 3-8D). As the incubation time increased from 1 to 24 h, T4-1 formed a structure that corresponded with a shift in the 255 nm peak to 265 nm that was completed by 8 h. This corresponded to the appearance of Band 2 in the native gel. By 24 h, both peaks at 265 and 290 nm appeared similar to those in Figure 3-6B. At this point, the ratio of Band 3 to Band 2 increased with little of Band 1 remaining.

The time course CD data for T3 was different than that of T4-1. The 255-nm peak shifted to 265 nm as early as 4 h, followed by an increase in the peak at 290 between 8 and 24 h (Figure 3-8B). These changes corresponded well with the times for the appearances of Bands 2 and 3 in the native gel (Figure 3-8E). This would suggest that the T3 structure that corresponded to Band 2 had more parallel-stranded character than Band 3.

Finally, the T4-2,3 time course demonstrated a simpler path to forming the final structure. The peak at 255 nm shifted to 265 nm at a point between 4 and 8 h (Figure 3-8C). Although a weak peak at 290 nm was present at 1 h, it was not present at later time points when Band 2 appeared in the native gel (Figure 3-8F), suggesting that the intermolecular structure formed by T4-2,3 was parallel-stranded.
Figure 3-8: Temperature-dependent formation of intermolecular structures. Samples were prepared in 1 mM Na$_2$PO$_4$ and 100 mM KF (CD) or Tris-EDTA and 100 mM KCl (electrophoresis), heated for 5 min at 95°C, then immediately transferred to 47°C for the indicated amount of time. Changes in structure over time were analyzed by circular dichroism or by native polyacrylamide (15%) gel electrophoresis (4.2 V/cm) at 4°C. Changes in the CD spectra (left) correspond to the appearance of slower-moving structures on the gels (right).
3.3 DISCUSSION

Our data is consistent with findings that oligonucleotides with four or more 5'-d(TTAGGG)-3' repeats form intramolecular G-quadruplexes (Vorlickova et al., 2005; Yu et al., 2006). It is generally accepted that these structures form in Na\(^+\) or K\(^+\) cations. Interestingly, T4 formed structures with faster migration rates even in Li\(^+\) or TTE alone at 4°C. However, these structures were not present in gels run at room temperature (data not shown), consistent with the findings that intramolecular G-quadruplexes can form in the presence of Li\(^+\) but are less stable than those formed in Na\(^+\) and K\(^+\) (Redon et al., 2001).

3.3.1 Intermolecular G-Quadruplex Formation on Short Telomeric Sequences

As expected, telomeric oligonucleotides shorter than T4 formed intermolecular G-quadruplexes. T2 formed intermolecular G-quadruplexes relatively slowly in K\(^-\) at room temperature consistent with earlier studies with oligonucleotides comprised of two telomeric repeats studied at µM concentrations (Han et al., 1999a). In addition, only a small percentage of the oligonucleotides formed intermolecular G-quadruplexes even when samples were incubated for days (Figures 3-3 and 3-4, data not shown). Both the rate of formation and stability of intermolecular G-quadruplexes can be improved by the presence of quadruplex-recognizing small molecules (Han et al., 1999a) or proteins (Paeschke et al., 2005). Our studies have shown that an additional telomeric repeat (T3 oligonucleotide) slightly increased the rate of formation and substantially increased the percentage of the intermolecular G-quadruplexes formed at longer time points.

While the formation of intermolecular structures was expected, the variety of structures was surprising. The structure of oligonucleotides with three human telomeric repeats has been analyzed by CD (Rujan et al., 2005) and the spectrum is similar to our results with the T3 oligonucleotide. The spectrum suggests a mixture of syn- and anti-
glycosidic bond angles perhaps similar to the hybrid, parallel/antiparallel G-quadruplex structure observed with T4 and other oligonucleotides containing four telomeric repeats (Krafft et al., 2002; Rujan et al., 2005; Vorlickova et al., 2005; Yu et al., 2006). Solution structures of similar human telomeric sequences form the so-called 3 + 1 structure comprised of three parallel strands and one strand oriented in the opposite direction (Luu et al., 2006; Phan et al., 2006; Zhang et al., 2005) (Figures 3-6B and 3-9). Asymmetric bimolecular structures formed by 5'-d(GGGTTAGGGTTAGGGTTA)-3' and 5'-d(GIGTTAGGGTTAGGGT)-3' in Na⁺ were also 3 + 1 structures (Zhang et al., 2005). What complicates the interpretation of the results for the T3 oligonucleotide is that the native gels show three sets of bands suggesting the presence of several structures in the mixture. However, the time course electrophoretic and CD measurements facilitate interpretation of more than one structure. The 3 + 1 structure in Figures 3-6B and 3-9 most likely corresponds to Band 2 and is responsible for the large positive ellipticity at 265 nm that appears at 4 and 8 h. The increase in Band 3 corresponded to an increase in ellipticity at 290 nm, suggesting that this structure may have more antiparallel strands as illustrated in Figure 3-9.

3.3.2 Intramolecular G-Quadruplexes Require Four TTAGGG Repeats

Altering the middle guanine in any of the 5’-d(TTAGGG)-3’ repeats of T4 to an adenine or thymine disrupts the intramolecular G-quadruplex allowing for intermolecular structures to form. Similar to T3, 3 + 1 bimolecular and trimolecular structures are possible with oligonucleotides T4-1, T4-2, and T4-3. Although there are some similarities of T4-1 to T3, the CD spectra are not entirely the same, particularly when observed through time. Taking into account the K⁺-induced increase in mobility of Band 1 and positive ellipticity at 290 nm, it is likely that T4-1 formed some type of intramolecular
structure at early time points that may or may not be a G-quadruplex (Figure 3-2 A-C, compare no salt to Band 1 in K⁺).

T4-2,3 can only form a bimolecular G-quadruplex and CD results suggest that this G-quadruplex is parallel-stranded with external (propeller) loops (Figure 3-6D). A bimolecular, parallel-stranded G-quadruplex has been observed with 5’-d(GGGTTAGGG)-3’ which was in a mixture with an antiparallel-stranded structure (Vorlickova et al., 2005). Heating the mixture or longer incubation times resulted in the mixture being dominated by the parallel-stranded structure. Our results suggest that the longer loops comprised of 5’-d(TTAG4GTAGA4GTGA)-3’ in T4-2,3 stabilize the parallel structure over the antiparallel. Interestingly, T8-2,3,6,7 was more antiparallel at the early time point but, in time, converted to the parallel-stranded structure. This is congruent with findings that antiparallel stranded G-quadruplexes is the more common, innately thermodynamically stable fold (Tang and Shafer, 2006). The parallel-stranded G-quadruplex can be induced and stabilized by a more appropriate cation like K⁺ (Dominick and Jarstfer, 2004) which is a stronger stabilizer than Na⁺.

3.3.3 Longer Oligonucleotides Form Fewer Intermolecular G-Quadruplexes

The larger oligonucleotides formed much less intermolecular structures except for T7. It has been shown previously that 5’-d(GGG(TTAGGG)ₙ)-3’ can form a slower-migrating structure on native polyacrylamide gels (Vorlickova et al., 2005). Our concentration dependence experiment confirms that this is indeed an intermolecular structure (Figure 3-3). Since T7 contains a full T4 + T3 unit, it is possible that its intermolecular structure has characteristics of both smaller oligonucleotides as depicted in Figure 3-6. Early time points suggest that T7 adopts a more antiparallel structure
followed by stabilization of the mixed parallel/antiparallel G-quadruplex observed after several hours.

Since some of the T8-derivatives do not have a string of 4 consecutive telomeric repeats, it was expected that these would also form intermolecular structures. However, substantial amounts were not observed. Like T7, the T8-derivatives, adopted more antiparallel structures before settling on the mixed parallel/antiparallel structures after several hours (Figures 3-6C and 3-7C). This could be due to the findings that intermolecular G-quadruplexes formed by longer oligonucleotides have a reduced thermostability compared to shorter DNA (Vorlickova et al., 2005; Yu et al., 2006). Intermolecular structures formed by longer oligonucleotides may also be too unstable to observe on gels.

3.3.4 Longer Incubation Time Leads to the Formation of Super-Higher-Order Structures for Some Sequences

A final property of these telomeric oligonucleotides is that some of them formed large super-higher-order structures. These may be similar to higher-order structures or G-wires observed by other laboratories (Chen, 1997; Marsh and Henderson, 1994; Sen and Gilbert, 1992). Interestingly, under the conditions of our studies, we were only able to observe substantial super-higher-order structures with the oligonucleotides T3 and T4-1 (Figure 3-4, data not shown) suggesting that these structures need to be stabilized by three consecutive 5’-d(TTAGGG)-3’ repeats.

3.3.5 G-Quadruplex Transitions May Act as Molecular Switches

Switching from intramolecular to intermolecular G-quadruplex forms may have importance in telomere biology, particularly if G-quadruplexes affect the assembly of nucleoprotein complexes at telomere ends (Yanez et al., 2005). The central G in double-strand telomeric DNA is known to be susceptible to oxidation to 8-oxo-dG (von
Zglinicki, 2002). Guanine bases in the G-quadruplex are slightly less susceptible to oxidation than duplex telomeric DNA but more susceptible than single-stranded DNA (Szalai et al., 2002). This could affect telomere structure at the G-strand overhang or during lagging strand synthesis. Favoring of the syn-orientation of the 8-oxo-dG base (Uesugi and Ikehara, 1977) may disrupt certain intramolecular G-quadruplex structures (Szalai et al., 2002; Xu et al., 2006). Alternatively, unrepaired lesions will result in G to T transversions (Besaratinia et al., 2004). In both cases a propensity to form intermolecular instead of intramolecular G-quadruplexes may result in undesirable interchromosomal interactions, which could be facilitated by molecular crowding in the nucleus (Miyoshi et al., 2004).

Our data also suggests that intermolecular G-quadruplexes are less likely to form when the G-strand overhang is sufficiently long. This is interesting in light of observations that telomere dysfunction, which occurs during cellular senescence or by disruption of the telomere nucleoprotein complex, is associated with a reduction of the G-strand overhang (Stewart et al., 2003; van Steensel et al., 1998). Both of these situations result in chromosome end-to-end fusions and intermolecular G-quadruplexes may facilitate chromosome-chromosome interactions.

Finally, it has recently been shown that a parallel, 4-stranded Tetrahymena and Oxytricha telomeric DNA structure is a more efficient substrate for telomerase than an intramolecular structure even though the intramolecular structure has a much faster dissociation rate (Oganesian et al., 2006). This suggests that telomerase may actually recognize certain structures, and stresses the importance of determining the types of G-quadruplexes that may be potential telomerase substrates.
Figure 3-9: Potential G-quadruplex structures formed by T3 and T4-1. Each 5'-d(TTAGGG)-3' repeat is represented by an arrow which points in the 5' to 3' direction. T4-1 would have an additional 5'-d(TTAGAG)-3' repeat that is not expected to be part of a G-tetrad. Each strand contributing to the intermolecular G-quadruplexes is highlighted by a different colored line. Bands 1, 2, and 3 refer to the bands labeled in the gels in Figure 3-2.
4  **CHAPTER 4: INDUCTION OF PARALLEL HUMAN TELOMERIC G-QUADRUPLEX STRUCTURES BY Sr\(^{2+}\)**

Human telomeric DNA forms G-quadruplex secondary structures, which can inhibit telomerase activity and are targets for anti-cancer drugs. Here we show that Sr\(^{2+}\) can induce human telomeric DNA to form both inter- and intramolecular structures having characteristics consistent with G-quadruplexes. Unlike Na\(^+\) or K\(^+\), Sr\(^{2+}\) facilitated intermolecular structure formation for oligonucleotides with 2 to 5 5’-d(TTAGGG)-3’ repeats. Longer 5’-d(TTAGGG)-3’ oligonucleotides formed exclusively intramolecular structures. Altering the 5’-d(TTAGGG)-3’ to 5’-d(TTAGA)-3’ in the 1st, 3rd, or 4th repeats of 5’-d(TTAGGG)\(_4\)-3’ stabilized the formation of intermolecular structures. However, a more compact, intramolecular structure was still observed when the 2nd repeat was altered. Circular dichroism spectroscopy results suggest that the structures were parallel-stranded, distinguishing them from similar DNA sequences in Na\(^+\) and K\(^+\). This study shows that Sr\(^{2+}\) promotes parallel-stranded, inter- and intramolecular G-quadruplexes that can serve as models to study DNA substrate recognition by telomerase, whose ciliate homolog has been shown to favor parallel-stranded intermolecular G-quadruplexes rather than antiparallel, intramolecular G-quadruplexes.

4.1 **INTRODUCTION**

Telomere DNA assembles with specific proteins to form complexes that protect the ends of eukaryotic chromosomes. The guanine-rich sequences of telomeres are capable of adopting a planar, G-tetrad arrangement by reverse Hoogsteen-like base-pairing between four guanines (Figure 1-1). More than one G-tetrad from an oligonucleotide strand(s) are stacked together to form a G-quadruplex (Figure 1-1) which is stabilized by the coordination of a cation with guanine O6 (Davis, 2004).
The specific coordinating cation can stabilize one form of G-quadruplex over another. Physiological concentrations of Na\(^+\) have been shown to stabilize antiparallel quadruplexes, while K\(^+\) can stabilize parallel and antiparallel quadruplexes (Davis, 2004). Divalent cations can induce both intra- and intermolecular quadruplexes that are generally highly stable. For example, the thermostability of a quadruplex complex is increased by Sr\(^{2+}\) more than by Na\(^+\) or K\(^+\), with some melting temperatures surpassing 95°C (Chen, 1992).

Here, we use native polyacrylamide gel analysis, UV absorption and circular dichroism spectroscopy to analyze the types of G-quadruplexes induced by Sr\(^{2+}\) when human telomeric DNA sequence and length are manipulated. We find that Sr\(^{2+}\) preferentially stabilizes parallel-stranded, intermolecular G-quadruplexes for DNA oligonucleotides previously shown to form mixed parallel/antiparallel, intramolecular structures in K\(^+\).

4.1.1 Key Findings

Some Sr\(^{2+}\)-induced structures are stable even in gels run without Sr\(^{2+}\) in the running buffer.

Unlike in K\(^+\), all Sr\(^{2+}\)-stabilized structures appear to be parallel-stranded.

Unlike in K\(^+\), T4 and T5 in Sr\(^{2+}\) formed intermolecular instead of intramolecular structures.

Structures with distinct UV and CD spectra were formed for all oligonucleotides in Sr\(^{2+}\), even if all the TTAGGG repeats were converted to TTAGAG.
4.2 RESULTS

To investigate the role of Sr\(^{2+}\) in the formation of G-quadruplexes of different DNA length and sequence, oligonucleotides T2-T9 denoting the number of 5’-d(TTAGGG)-3’ repeats ranging from 2 to 9 were synthesized. Furthermore, many of the oligonucleotides had at least one of the 5’-d(TTAGGG)-3’ repeats altered to 5’-d(TTAGAG)-3’ to study the role of the middle guanine in formation of these DNA secondary structures (Table 2-1).

4.2.1 Intramolecular and Intermolecular Structures Were Dependent on the Size of the Telomeric Oligonucleotide

To obtain Sr\(^{2+}\)-dependent changes in migration rates, samples for each experiment were electrophoresed in gels with double-stranded DNA size standards to compare relative mobilities. In the absence of Sr\(^{2+}\), the migration rate of T2-T9 (5’-d(TTAGGG)\(_{2-9}\)-3’ repeats) in native polyacrylamide gels decreased as the size of the oligonucleotides increased, with oligonucleotide T4 running slightly faster than expected (Figure 4-1A, no salt). The migrations of oligonucleotides with 2-9 5’-d(TTAGAG)-3’ repeats (T2-all to T9-all, highlighted by asterisks (*) in Figure 4-1A) were very similar to those of T2-T9 except T4-all did not run as fast as T4.

To observe the effect of Sr\(^{2+}\) on oligonucleotide structure, samples were first incubated in the presence of 100 mM SrCl\(_2\) then subjected to electrophoresis in gels without Sr\(^{2+}\) in the gel or running buffer (Figure 4-1A, Sr\(^{2+}\)-in sample only) or in gels with Sr\(^{2+}\) in buffer and gel (Figure 4-1A, Sr\(^{2+}\) sample + gel). Sr\(^{2+}\) present in the sample, but not the gel, caused an increase in migration rate for oligonucleotides T4-T9 (Figure 4-1A, Sr\(^{2+}\) sample only) except T5. The fact that many of these Sr\(^{2+}\)-induced structures were
retained in gels lacking Sr\(^{2+}\) suggests tighter binding of the divalent cation compared to K\(^{+}\) and Na\(^{+}\).

The presence of Sr\(^{2+}\) in the gels and running buffers stabilized additional structures (Figure 4-1A, Sr \(^{2+}\) sample+gel). T2, T3, T4, and T5 formed slower-moving structures. Unexpectedly, Sr\(^{2+}\) caused Tn-all oligonucleotides with 7-9, 5’-d(TTAGAG)-3’ repeats to have slower migration rates and those with 2-5, 5’-d(TTAGAG)-3’ repeats to have faster migration rates.

To further investigate the role of each 5’-d(TTAGG)-3’ repeat in the intramolecular T4 structure, oligonucleotides were synthesized in which at least one 5’-dTTAGG-3’ was converted to 5’-d(TTAGG)-3’ (Table 2-1). In the absence of added cation, T4 and T8 had faster migration rates than all T4- and T8-derived oligonucleotides, respectively (Figure 4-1B, no salt).

Alteration of the 5’-most, 5’-d(TTAGG)-3’ repeat to 5’-d(TTAGG)-3’ or 5’-d(TTAGTG)-3’ (Figure 4-1B, T4-1 and T4-T1, Sr\(^{2+}\) sample only) stabilized a slower-migrating species which most likely represents an intermolecular structure. Slower moving species were also present in T4-derived oligonucleotides with the 3\(^{rd}\) and 4\(^{th}\) repeats altered (T4-3 and T4-4). The mobility of T4-2,3 and T4-all were not substantially altered by Sr\(^{2+}\) in the samples only. Interestingly, Sr\(^{2+}\) caused T4-2 to form a more compact structure with an increased mobility similar to T4. However, T4-2 consistently formed a smeared band suggesting the existence of more than one structure or an unstable structure. Sr\(^{2+}\) caused T8 and its derivatives to have greater migration rates with the exception of T8-2,3,6,7 and T8-all.
Figure 4-1: Native gel electrophoresis of telomeric oligonucleotides in gels with (sample + gel) or without (no salt and sample only) 10 mM SrCl$_2$. Oligonucleotides (2 μM) were prepared ±100 mM SrCl$_2$ as described in Materials and methods. (A) Migration rates of different sized telomeric oligonucleotides with (n) number of telomeric repeats. Tn: [5’-d(TTAGGG)$_n$-3’] and numbers with asterisks (n*) refer to Tn-all [5’-d(TTAGAG)$_{n*}$-3’]. (B) Migration rates of T4-X and T8-X, where X denotes the repeats in the 5’→3’ direction that have been altered to 5’-d(TTAGAG)-3’, and T4-T1 [5’-dTTAG7G(TTAGGG)$_3$-3’]. M refers to a 10 bp DNA Ladder. Samples were electrophoresed on 15% native polyacrylamide gels according to Materials and methods. Bands were visualized using SYBR Gold.
When Sr\(^{2+}\) was present in both the samples and the gel (Figure 4-1B, Sr\(^{2+}\) sample+gel) T4 was converted to a slower moving structure with similar mobility to that of T4-T1, T4-1, T4-3, and T4-4. T4-2 still formed a more compact structure as did T4-2,3 and T4-all. In addition, T8-2,3,6,7 also formed a more compact structure with a similar mobility to T8 and the other T8-derivatives with the exception of T8-all. As shown in Figure 4-1B, T8-all formed a structure that migrated significantly slower compared to that in the absence of salt.

To determine the amount of Sr\(^{2+}\) required to cause structural changes, samples were incubated with increasing SrCl\(_2\) and subjected to electrophoresis in gels lacking Sr\(^{2+}\) (Figure 4-2A). Between 1 and 10 mM Sr\(^{2+}\) was required to induce structure formation regardless if the structures were larger (T4-1 and T5) or more compact (T6). Induction of slower-migrating structures by Sr\(^{2+}\) was also dependent on the oligonucleotide concentration (Figure 4-2B, T4, T4-1, and T5) suggesting that the association of more than one oligonucleotide strand formed them. The Sr\(^{2+}\)-induced, faster-migrating structures formed by oligonucleotides such as T6 were not altered by oligonucleotide concentration, consistent with the formation of intramolecular G-quadruplexes.

Although intramolecular structures formed within a timescale of minutes (data not shown), intermolecular structures were much slower. At room temperature (data not shown) and 30°C, few intermolecular structures were formed by 2 h (Figure 4-2C). However, heating the samples greatly sped up the reaction such that substantial intermolecular structures were present after 1 hour incubation at 40°C.
**Figure 4-2:** Native gel electrophoresis of telomeric oligonucleotides in gels with (sample + gel) or without (no salt and sample only) 10 mM SrCl₂. Oligonucleotides (2 µM) were prepared ±100 mM SrCl₂ as described in Materials and methods. (A) 0-100 mM SrCl₂ concentrations at 2 µM oligonucleotide concentration and (B) 0.1-2 µM DNA concentrations in 100 mM SrCl₂. (C) Time course reactions with T4-1 (2 µM) in SrCl₂ (100 mM). Samples were treated according to Materials and Methods at the indicated temperatures for the indicated times. Samples were electrophoresed on 15% native polyacrylamide gels according to Materials and Methods. Bands were visualized using SYBR Gold.
4.2.2 UV Absorption and CD Spectroscopy Suggests the Formation of G-Quadruplexes

G-quadruplexes and other structures that rely on Hoogsteen-type base pairing have a signature hyperchromicity at 295 nm (Mergny et al., 1998). The UV difference absorption spectra (+Sr²⁺ vs -Sr²⁺) for some of the oligonucleotides in this study possess this characteristic. For example, both T4 (Figure 4-3A) and T8 (Figure 4-3B) have a Sr²⁺-induced hyperchromicity at 295 nm with hypochromicity at 270 nm. The majority of the other oligonucleotides had difference spectra similar to T4 and T8 except that the region of hyperchromicity was less defined and centered around 290 nm instead of 295 nm. The exceptions were T4-2,3, T4-all, and T8-all. T4-2,3 and T4-all (Figure 4-3A) had non-descript difference spectra (Figure 4-3A). This was also true for T3-all (data not shown). T8-all (Figure 4-3B) and T7-all (data not shown) had characteristic spectra but the hyperchromicity was shifted to 285-290 nm and the hypochromicity was shifted to 260 nm.

Circular dichroism spectroscopy was used to characterize the nature of the G-quadruplexes (Figure 4-3 C,D). Parallel-stranded G-quadruplex structures with an anti-glycosidic bond conformation typically have a CD spectrum with a strong positive peak at 260-265 nm and a smaller negative signal at 240 nm (Hardin et al., 1991). Parallel-stranded, intramolecular G-quadruplexes with anti-glycosidic bonds and external or "propeller" loops (Figure 1-1) also have a peak at 260-265 nm with a shoulder around 290 nm (Xu et al., 2006). Antiparallel-stranded G-quadruplexes typically seen in Na⁺ buffers with lateral and diagonal loops and a mixture of anti/syn-glycosidic bonds have a strong positive ellipticity at 295 nm, a weaker negative signal at 265 and a weak positive peak at 245 nm (Balagurumoorthy and Brahmachari, 1994; Krafft et al., 2002). Hybrid
G-quadruplex structures have mixed antiparallel/parallel strands and external (propeller) plus lateral loops with CD spectra having a strong positive peak at 290 nm, a shoulder at 270 nm, and a small negative signal at 240 nm (Balagurumoorthy and Brahmachari, 1994; Rujan et al., 2005; Vorlickova et al., 2005; Yu et al., 2006).

The CD spectra generally mirrored the UV difference absorption spectra in that oligonucleotides with similar UV absorption spectra also had similar CD spectra. In the absence of salt, the CD spectra for all tested oligonucleotides were very similar with positive peaks around 250-260 nm (Figure 4-3C, data not shown). In the presence of K\(^+\), T4 (Figure 4-3C) and T8 (data not shown) had spectra indicative of hybrid, parallel/antiparallel G-quadruplexes. Unlike K\(^+\), addition of Sr\(^{2+}\) created T4 and T8 structures with strong positive peaks at 265 nm (Figure 4-3C, D) indicating parallel-stranded forms of G-quadruplex (Figure 4-3E).

The spectrum for T4-1 was slightly different in that the positive ellipticity centered around 270-275 nm instead of 265 nm. In addition, the negative signal at 240 nm moved to 250 nm. As with the UV difference absorption spectra, the CD spectra for T4-2, T4-3, and T4-4 were very similar to that of T4-1 (data not shown). Also like the UV difference absorption spectra, the CD spectra for T4-2,3 and T4-all were different than the rest of the T4-derived oligonucleotides with a positive ellipticity at 255-260 nm. The CD spectra for the T8-derivatives were nearly identical to T8 (Figure 4-3D, data not shown) with the exception of T8-all. As with the UV absorption difference spectrum, the CD spectrum of T8-all was slightly different. The negative signal at 240 nm was similar to that of T8. However, instead of a positive peak at 265 nm, the peak for T8-all centered around 270 nm. The T7-all spectrum (data not shown) was identical to that of T8-all.
Figure 4-3: UV difference absorption and CD spectroscopy of SrCl$_2$-induced structural formations of telomeric oligonucleotides, [Tn: 5’-d(TTAGGG)$_n$-3’, Tn-all: 5’-d(TTAGAG)$_n$-3’ and Tn-X, where X denotes the repeats in the 5’→3’ direction that have been altered to 5’-d(TTAGAG)-3’]. (A,B) Difference in UV absorption wavelength scans of oligonucleotides incubated overnight in 100 mM SrCl$_2$ (A(S)) and TE alone (A(0)). (C,D) CD spectroscopy of oligonucleotides incubated overnight in Na$_2$PO$_4$ and either 100 mM SrCl$_2$ (default), 100 mM KF or no additional salt (0 salt). Molar ellipticity is in deg * cm$^2$/dmol of bases. (E) Putative intramolecular structures formed by oligonucleotides T4 and T8 with mixed parallel/anti-parallel strand orientation in K$^+$ or all parallel-strand orientation in Sr$^{2+}$. 
4.2.3 DISCUSSION

The results from this study emphasize the diversity of G-quadruplex structures formed by human telomeric DNA in vitro. Although Na\(^+\) typically stabilizes antiparallel structures and K\(^+\) stabilizes mixed parallel/antiparallel structures for sequences similar to our T4 and T8, Sr\(^{2+}\) appears to stabilize exclusively parallel-stranded structures. This has also been shown for the sequence 5’-d(G\(_4\)T\(_4\)G\(_4\)T\(_4\)G\(_4\))-3’ in the presence 2 mM Sr\(^{2+}\) (Chen, 1992) and 5’-d(G\(_4\)T\(_4\)G\(_4\))-3’ in Ca\(^{2+}\) (Miyoshi et al., 2003). However, Sr\(^{2+}\) does not exclusively stabilize parallel-stranded G-quadruplexes for all G-rich DNA since the thrombin binding aptamer (5’-d(GGTTGGTGTGGTTGG)-3’) in Sr\(^{2+}\) forms an antiparallel G-quadruplex (Kankia and Marky, 2001).

In addition to inducing parallel-stranded structures, our data show that the oligonucleotides T4 and T5, previously shown to form intramolecular quadruplexes in K\(^+\) (Vorlickova et al., 2005; Yu et al., 2006), can also form intermolecular structures in Sr\(^{2+}\). This has been observed for the oligonucleotides 5’-d(G\(_4\)T\(_2\)G\(_4\)T\(_2\)G\(_4\))-3’ and 5’-d(G\(_4\)T\(_4\)G\(_4\)T\(_4\)G\(_4\))-3’ (Chen, 1992). However, the longer oligonucleotides, T6-T9 in our study, form only intramolecular structures. Intermolecular structures are also observed when the middle guanine in any of the 5’-d(TTAGGG)-3’ repeats of T4 was altered to an adenine or thymine. Both the UV difference absorption and CD spectra for these structures were only slightly different from those of T4 and T8 suggesting that they are G-quadruplexes.

Unlike the T4-derivatives, altering a middle guanine in two of the 5’-d(TTAGGG)-3’ repeats of T8 resulted in structures that appear to be very similar to T8 even though a stretch of 4, 5’-d(TTAGGG)-3’ repeats are disrupted. However, if all the repeats are altered (T7-all, T8-all, and T9-all) the oligonucleotides adopt structures with
distinct electrophoretic mobilities, UV absorption and CD spectra. It is not clear at this time whether some of these structures with distinct spectra are G-quadruplexes.

G-quadruplex architecture is modified by different cations because of the various coordination complexes formed with guanines in the stacking G-tetrads. Na$^+$ ions have been found to be located within the plane of a G-tetrad (Phillips et al., 1997). However K$^+$ (Haider et al., 2002; Parkinson et al., 2002) and Sr$^{2+}$ (Deng et al., 2001; Shi et al., 2001) are sandwiched between two G-tetrads, coordinating with the carbonyls of eight guanines in a square anti-prism configuration. In the case of K$^+$ complexes, each carbonyl coordinates with a K$^+$ above and below each tetrad (Parkinson et al., 2002) and it has been proposed that this arrangement can have properties of ion channels (Hud et al., 1996). Unlike K$^+$, Sr$^{2+}$ and other divalent cations (Deng et al., 2001; Kotch et al., 2000; Kwan et al., 2007; Shi et al., 2001), are only found between alternating G-tetrad planes because each carbonyl only interacts with one cation. In addition, the divalent cation-carbonyl bond lengths are shorter (Kotch et al., 2000) resulting in a more compressed coordination sphere between the cation and the eight guanines. To accommodate these Sr$^{2+}$-guanine coordination complexes, glycosidic bond angles and consequently strand orientations will likely be different than in K$^+$ complexes.

These studies add to the growing literature demonstrating the high degree of structural polymorphism of telomeric G-quadruplexes. The finding that telomerase preferentially acts upon a parallel-stranded over an antiparallel-stranded quadruplex (Oganesian et al., 2006) suggests that the enzyme may recognize specific telomeric DNA secondary structures. The structures in this study can be used to ascertain telomerase-
DNA substrate interactions and the mechanisms of telomere maintenance disruption caused by G-quadruplex-stabilizing, small molecules.
5  **CHAPTER 5: THE N-TERMINAL DOMAIN OF TRF2 INDUCES PARALLEL-STRANDED G-QUADRUPLEX FORMATION IN K⁺**

Human telomeric DNA consists of tandem repeats of the sequence 5’-d(TTAGGG)-3’. Such guanine-rich DNA is capable of forming a variety of G-quadruplexes, which in turn, could have varying functional consequences on telomere maintenance. G-quadruplex stabilizing ligands have been shown to induce chromosome end-to-end fusions, senescence and apoptosis, effects similar to the expression of a dominant-negative form of TRF2. With this in mind, we initially analyzed the effect of sequence and length of human telomeric DNA, as well as cation conditions on G-quadruplex formation, to find that K⁺ and Na⁺ can induce human telomeric DNA to form G-quadruplex structures. Circular dichroism results suggest that the structures in K⁺ were a mix of parallel and antiparallel G-quadruplexes, while Na⁺ induced only antiparallel-stranded structures under these conditions. These structures serve as useful models to study the effects of G-quadruplexes on the activities of telomeric proteins, like TRF2, from human cells. While TRF2 is not known to bind single-strand DNA, work performed in the lab suggested that the type of overhang affects TRF2-binding. Here we show that the N-terminal basic domain of TRF2 in K⁺ induces a switch from the mixed parallel/antiparallel-stranded G-quadruplexes usually stabilized by K⁺-alone, to parallel-stranded G-quadruplexes. Interestingly, it also promotes intermolecular parallel G-quadruplex formation on non-quadruplex intermediates of appropriate sequences, but will not induce a switch from an antiparallel to a parallel G-quadruplex in Na⁺. These results are the first to demonstrate specific TRF2-G-quadruplex interactions. They suggest a novel mechanism for TRF2 recognition of the double-strand/single-strand junction of
telomeres, where the myb-like domain binds to the double-strand DNA and the N-terminal basic domain interacts with the overhang, stabilizing the interaction.

5.1 INTRODUCTION

Telomeres protect the ends of linear chromosomes from unwanted processing. In humans, telomeres are composed of ~15 kilobase-pairs of 5’-dTTAGGG-3’ repeats followed by a 130-275 base 3’-overhang on the guanine-strand (Makarov et al., 1997; Wright et al., 1997). The length and structure of the DNA is regulated by a variety of proteins (de Lange, 2005) working in conjunction with the telomeric DNA structure to maintain the ends of chromosomes (Blackburn, 2000).

The actual structure of the DNA at telomere ends is unknown. Globally, telomeric DNA is believed to form large T-loops, where the 3’-end loops back to interact with upstream double-stranded repeats (Griffith et al., 1999), however, there is still debate concerning the interaction at the molecular level. One possible arrangement involves the formation of G-quadruplex structures between the invading end and the upstream guanine-strand (Figure 1-2A). Another possible arrangement involves the upstream G-strand oscillating between being duplexed to the C-strand and dissociating from the C-strand to form intramolecular G-quadruplexes. This would free the C-strand for hybridization with the G-strand overhang to form a type of D-loop (Figure 1-2B). G-quadruplexes are formed by stacking planar arrangements of 4-guanines Hoogsteen hydrogen-bonded to each other (Balagurumoorthy and Brahmachari, 1994; Kerwin, 2000; Murchie and Lilley, 1994; Wang and Patel, 1993). They are highly stable structures that form spontaneously on telomeric sequences at physiological concentrations of $K^+$ and $Na^+$. 
5.1.1 **TRF2 is Essential for Telomere Maintenance**

One of the proteins considered essential for telomere protection and length regulation is TTAGGG Repeat Factor 2 (TRF2) (Smogorzewska et al., 2000). TRF2 (Figure 1-3) has an N-terminal basic domain, followed by a TRF-homology (TRFH) domain (Broccoli et al., 1996; Broccoli et al., 1997), a linker region and a C-terminal myb-like domain responsible for binding to duplex DNA (Broccoli et al., 1997; Court et al., 2005; Nishikawa et al., 2001).

TRF2 has previously been shown to bind duplex DNA (Broccoli et al., 1997). The addition of TRF2 to telomeric DNA was enough to induce T-loop formation *in vitro* (Griffith et al., 1999; Stansel et al., 2001), possibly by the generation of positive supercoils facilitating proximal strand invasion (Amiard et al., 2007). Interestingly, in order for TRF2 to stimulate T-loop formation, a small 3’-overhang, on the end of the telomeric DNA, was required (Stansel et al., 2001). Griffith’s group explained the need for single-strand DNA to invade the upstream double-strand region, but the overhang could have also improved TRF2 binding at the ss/ds DNA junction. Research in the Fletcher lab indicates that TRF2 recognizes telomeric ss/ds DNA junctions and that the sequence and length of the overhang affect TRF2 binding (Khan et al., 2007; Yanez et al., 2005), suggesting an interaction between the protein and the G-overhang.

A recent paper found that the N-terminal basic region of TRF2 was capable of recognizing 3-way and 4-way junctions (Fouche et al., 2006a). This same region may be involved in the proximity of TRF2 to the ss/ds junction by interacting with the overhang. This basic region could recognize or induce structures on the overhang, possibly even G-quadruplex structures.
Overexpression of TRF2 results in accelerated telomere shortening, with senescence occurring at shorter telomere lengths compared to cells with endogenous TRF2 levels (Karlseder et al., 2002). In contrast, the expression of a dominant-negative form of TRF2, where the basic- and myb-like domains have been deleted, results in the reduction of the total amount of G-strand overhang, end-to-end chromosome fusions, cellular senescence and apoptosis (Karlseder et al., 1999; van Steensel et al., 1998). Interestingly, expression of a truncated TRF2 that lacks the N-terminal, basic region results in loss of chromosomal telomeric DNA with concomitant formation of extrachromosomal telomeric circles (Wang et al., 2004). This phenomenon requires XRCC3, which suggests that the basic region of TRF2 is involved in suppressing recombination events.

Here, we use native polyacrylamide gel analysis and circular dichroism spectroscopy to analyze the types of G-quadruplexes induced by the N-terminal basic domain of TRF2 when human telomeric DNA sequence and length are manipulated. We find that an N-terminal peptide preferentially stabilizes parallel-stranded G-quadruplexes for DNA oligonucleotides previously shown to form mixed parallel/antiparallel, intramolecular structures in K⁺, but does not flip antiparallel G-quadruplexes stabilized by Na⁺ to parallel quadruplexes. Additionally, we show preliminary evidence of reduced TRF2 recruitment from HeLa cell extracts by mini-telomeric ds/ss junctons when the overhang is altered to prevent quadruplex formation.

5.1.2 Key Findings

The glycine/arginine-rich, N-terminal peptide of TRF2 stimulates intermolecular G-quadruplexes, which do not usually form under rapid-cooling conditions.

The peptide shifts the equilibrium of mixed populations or mixed/hybrid-type structures towards more parallel-character.
The peptide does not induce a shift towards parallel-stranded quadruplexes on fully antiparallel G-quadruplexes in Na\textsuperscript{+}.

Peptide effects are concentration- and time-dependent.

5.2 RESULTS

5.2.1 TRF2 N-terminal Peptide Affects Telomeric Oligonucleotide Mobility

To see changes in mobility induced by the N-terminal peptide of TRF2, 0-16 µM of biotin-labeled TRF2 N-terminal peptide was added to 4 µM of γ\textsuperscript{32}-P-ATP-labeled unstructured telomeric oligonucleotides. Reactions were performed in ~15 mM Na\textsubscript{2}PO\textsubscript{4} and 100 mM KCl, similar to CD reactions below.

Increasing amounts of peptide did not significantly change the mobility of T4, T8 and T8-2,3,6,7 (Figure 5-1). However, the width of the band narrowed, suggesting that the peptide may have decreased the variation of intramolecular G-quadruplexes within the population. Additionally, a slight decrease in lane intensity occurred with 16 µM of peptide. This may have resulted from some DNA-peptide complexes forming large structures that did not enter the gel.

Previous mobility shift assays (Figures 3-1 through 3-4, 4-1, 4-2) allowed structures to form during a slow cooling and overnight incubation. In this experiment, samples were boiled and immediately transferred to room temperature. This explains the absence of a band representing the bimolecular structure in the T4-2,3 sample without peptide. Increasing the amount of peptide results in a shift from the “unstructured” oligonucleotide to a species with a relative mobility of bimolecular complex. The addition of peptide appears to overcome the limitations of rapid-cooling, facilitating the assembly of a two-stranded structure.
In earlier studies (Pedroso et al., 2007b; Vorlickova et al., 2005) (Figures 3-1 and 3-3) T7 was shown to form a compact intramolecular G-quadruplex as well as a larger, bimolecular one. With the rapid cooling of this experiment, the majority of T7 remains in the intramolecular population (Figure 5-1). Addition of peptide begins to shift the population distribution towards the intermolecular structure. Interestingly, at 8 µM peptide, a third band appears with half the mobility of Band 2, possibly representing a four-stranded species. At 16 µM peptide concentration, most of Band 1 has shifted to Bands 2 and 3. This 3rd Band could be explained by the peptide binding to the DNA, however, the addition of streptavidin to the reaction did not change the mobility of any of the species in the experiment, suggesting that the peptide-DNA interactions were either transient or not strong enough to survive electrophoresis.

![Figure 5-1: The effect of increasing concentrations of N-terminal domain of TRF2 (peptide) on the mobility of telomeric oligonucleotides. Peptide concentration ranged from 0-16 µM, as indicated, while oligonucleotide concentration was fixed at 4 µM. M (10, 20, 100) represents the location of a molecular weight marker in 10 bp increments. Details of oligonucleotide sequences can be found in Table 2-1. Briefly, Tn= 5’-(TTAGGG)n-3’ and Tn-X, X denotes which repeats in the 5’→3’ have been altered to 5’-(TTAGAG)-3’.
As expected, T4-all and T8-all showed no change in mobility with respect to peptide concentration (data not shown), suggesting that the peptide does not affect the mutant telomere templates’ structures. This ineffectiveness could be due to a lack of recognition on the part of the peptide or an inability of the DNA to form stable structures.

5.3 TRF2 N-terminal Peptide Does Not Alter the Strand Orientation of Antiparallel G-Quadruplexes

At physiological concentration of Na$^+$ (~15 mM), T4, T7 and T8 show spectra typical of antiparallel G-quadruplexes in with a peak ~295 nm, a negative peak ~265 nm and a smaller positive peak ~ 245 nm (Figure 5-2A). The smaller T7 peak is actually shifted closer to 250 nm, which may be the result of the extra DNA. T4 and T8 can have all of their DNA involved in G-quadruplexes, but T7 in Na$^+$ does not form intermolecular structures, so there is an excess of three 5’-d(TTAGGG)-3’ repeats which could be in the loops or as “unstructured” DNA on the end of the G-quadruplex. The addition of peptide to these samples does not change the position of the peaks, but does affect the amplitude. It is unclear whether these changes are significant, as repeated experiments will exhibit slightly different amplitudes between spectra.

The spectra for T4-2,3, T8-2,3,6,7, T4-all, T7-all and T8-all in ~15 mM Na$^+$ (Figure 5-2 B, C) are close to the corresponding spectra for these oligonucleotides in 1 mM Na$^+$ (Figure 3-6, data not shown)(Pedroso et al., 2007b), suggesting that these oligonucleotides do not form structures in low Na$^+$. Interestingly, in ~15 mM Na$^+$, the spectra T4-23 and T8-2367 look similar to each other (Figure 5-2B), as do the ones for T4-all, T7-all and T8-all (Figure 5-2C). While these spectra are not characteristic of G-quadruplex formation, their respective similarities suggest that these are sequence dependent spectra.
Figure 5-2: CD spectra exhibiting the effects of the N-terminal peptide of TRF2 (8 µM) on telomeric sequences (4 µM) in ~15 mM Na$_2$PO$_4$. (A) The peptide does not induce a change in structure, but does change the amplitude of the peaks. (B,C) No change was seen with non-quadruplex structures. Together, this suggests that the peptide has specificity for antiparallel G-quadruplexes. Tn=5’-(TTAGGG)n-3’ and Tn-X, is similar but X denotes which repeats in the 5’ → 3’ direction have been changed to 5’-(TTAGAG)-3’.
5.3.1 **TRF2 N-terminal Peptide Shifts the Population of Hybrid G-Quadruplex Structures to Parallel-Stranded Structures**

T4 and T8 in ~15 mM Na$_2$PO$_4$, 100 mM KCl have CD spectra (Figure 5-3A) reminiscent of their spectra in 1 mM Na$_2$PO$_4$, 100 mM KF (Figure 3-6A) (Pedroso et al., 2007b). These spectra, with large peaks near 290 nm, humps around 270 nm and small negative peaks near 240 nm, are characteristic of hybrid-type G-quadruplex structures composed of mixed parallel/antiparallel strands. Hybrid quadruplexes have been observed on short telomeric oligonucleotides by other laboratories (Balagurumoorthy and Brahmachari, 1994; Rujan et al., 2005; Vorlickova et al., 2005; Yu et al., 2006). The addition of 8 µM peptide shifts the spectra to one with a slightly smaller peak at 290 nm, a larger defined hump at 265-270 nm and a larger, but still small negative peak near 240 nm. This is indicative of an increase in the parallel character of the structure(s). Hybrid spectra could result from an actual hybrid quadruplex or could be due to a mixed population of G-quadruplexes. Although we cannot distinguish between these two possibilities with CD, the current literature suggests that hybrid-type G-quadruplexes dominate under our experimental conditions (Matsugami et al., 2006). Regardless, the addition of peptide to T4 and T8 in K$^+$ results in more parallel character of the G-quadruplex population, suggesting that the peptide recognizes and affects G-quadruplex structures.

These samples were prepared, boiled and cooled rapidly to room temperature before an overnight incubation. This was in contrast to previous CD experiments in which samples were allowed to cool slowly to room temperature (Figures 3-6 and 4-3) (Pedroso et al., 2007a; Pedroso et al., 2007b). This rapid-cooling presents the possibility of the DNA forming an intermediate non-quadruplex structure that prevents it from
eventually adopting a G-quadruplex conformation. Thus, T4-2,3 and T8-2,3,6,7 in ~15 mM Na$_2$PO$_4$, 100mM KCl (Figure 5-4A, dashed lines) remain “unstructured” with spectra resembling the spectra in 1-15 mM Na$_2$PO$_4$ alone (Figure 5-2B, data not shown). The addition of the TRF2 N-terminal peptide to these samples, overcomes the barrier caused by rapid-cooling. The new spectra (Figure 5-4A, solid lines) resemble the spectra of slow-cooled T4-2,3 and T8-2,3,6,7 (Figure 3-6D). They both had a strong positive peak near 265 nm. The T4-2,3 spectrum had no peak at 290 nm and the shoulder at 290 nm for T8-2,3,6,7 was relatively weak compared to peak at 265 nm. This is suggestive of parallel-stranded structures with all propeller loops comprised of 5’-d(TTAGAG)$_2$TTA-3’ repeats with an additional loop of 5’-d(TTA)-3’ for T8-2,3,6,7 (Figure 3-6D, right panel). This agrees with the mobility shift data (Figure 5-1), which showed that T4-2,3 with 8 µM peptide shifts toward an intermolecular structure, while T8-2,3,6,7 remains unimolecular.

T7 alone in ~15 mM Na$_2$PO$_4$, 100 mM KCl, has a spectrum similar to T7 from earlier studies (Figure 3-6C), with two humps, of similar intensity, at 265 nm and 290 nm and a small negative peak at 240 nm (Figure 5-3B, red dashed line). Interestingly, the addition of 4 µM peptide causes almost no change to the spectrum (Figure 5-3B, black line). These spectra are similar to that observed for 5’-d(GGG(TTAGGG)$_4$)-3’ in K$^+$ (Vorlickova et al., 2005) indicative of a mixed parallel/antiparallel G-quadruplex. However, the addition of 8 µM peptide induces a significant shift at 265 nm (Figure 5-3B, red solid line). The new spectrum has a large peak at 265 nm, a shoulder near 290 nm and a small negative peak near 240 nm, suggesting the transition from a mixed to a parallel-stranded quadruplex. The increase at 265 nm without a corresponding decrease
at 290, suggests that the population is divided between types of quadruplexes. Considering the EMSA data where 8 μM peptide results in a shift away from Band 1 towards Band 2 and a new Band 3, the intermolecular structures may have a tendency to be parallel stranded while the intramolecular G-quadruplex(es) exhibit mixed characteristics.

**Figure 5-3:** CD of peptide with 4 μM telomeric oligonucleotides [Tn=(TTAGGG)n] in ~15 mM Na₂PO₄, 100 mM KCl. Unless specified, 8 μM peptide was added to the DNA at room temperature, 5 minutes after boiling. These results indicate that the N-terminal peptide of TRF2 induces a population shift towards parallel G-quadruplexes. Additionally, (B) 4 μM of peptide (black line) was not enough to induce this shift in T7. (C) Potential model for peptide-mediated transition of T7 in K⁺. The model shows two-hybrid G-quadruplexes joining to form a third, parallel quadruplex.
As expected, T4-all, T7-all and T8-all are not affected by 100 mM KCl (Figure 5-4B). Their spectra show small negative peaks near 240 nm and 280 nm, and small positive peaks between 265-270 nm. The addition of peptide does not change the spectra of T4-all and T7-all. The spectrum for T8-all with peptide exhibits a sharp increase in intensity at 268 nm. While this change may indicate a peptide-stabilized structure, its intensity and shape do not resemble any of the other G-quadruplex spectra we have seen.

*Figure 5-4:* CD of peptide (8 µM) with altered telomeric oligonucleotides (4 µM) in ~15 mM Na$_2$PO$_4$, 100 mM KCl. These results indicate that the N-terminal peptide of TRF2 (A) induces parallel G-quadruplex formation on T4-2,3 and T8-2,3,6,7, (B) but not on templates that have had all their repeats changed to TTAGAG (Tn-All). For detailed sequences see Table 2.1.
5.3.2 The TRF2 N-terminal Peptide Affects G-Quadruplex Structures in a Time-Dependent Manner

The CD spectra for T7 and T8 alone or with peptide were measured at various time points between 0 and 24 hours. As above, the samples were boiled and cooled quickly before the addition of peptide. Time points were measured from the time of peptide (or an equal volume of water) addition.

T8, as two-tandem T4 units, forms intramolecular G-quadruplex structures. These structures form quickly, in less than an hour, and do not seem to be affected by time or annealing conditions. Without peptide, there is no change between 1, 8, and 24 hours for T8 structures (data not shown), nor is there a difference between slow-cooled (data not shown) and quick-cooled samples. In ~15 mM Na\(^+\), the T8 spectra with and without peptide mirror the respective spectra from Figure 5-2A (bright green dashed (T8 alone) and solid (with peptide) lines); the addition of peptide resulted in a more negative peak at 265 nm, as in Figure 5-2A, but this appeared early and did not change significantly with time (data not shown). The spectra for T8 alone at all time points in ~15 mM Na\(^+\), 100 mM KCl look similar to the spectrum in Figure 5-3A (Dark green dashed line). However, the addition of N-terminal peptide to T8 in K\(^+\) resulted in a time-dependent increase in intensity at 270 nm (Figure 5-5). The initial shoulder at 270 nm transitions into a peak by 8 hours and by 24 hours, the peak at 270 nm is larger than the peak at 290 nm.

Previous work (Pedroso et al., 2007b) suggested that T7 acts like T4+T3; it is capable of forming both intramolecular and intermolecular quadruplex structures in K\(^+\). In Na\(^+\) however, T7 remains mostly in the intramolecular configuration (Figure 3-1). As such, the time course in ~15 mM Na\(_2\)PO\(_4\) resulted in a fast forming (<30 minutes).
antiparallel structure, but did not exhibit time-dependent spectral changes (data not shown). The spectra for T7-alone and T7-with peptide at all time points (0-24 hours) in ~15 mM Na$_2$PO$_4$ resembled the spectra from Figure 5-2A (red lines, dashes (T7 alone), solid (T7 peptide)).

The spectra of T7 in ~15 mM Na$_2$PO$_4$, 100 mM KCl did change over time (Figure 5-6). This was expected because intermolecular G-quadruplexes form more slowly than intramolecular ones. Initial time points (< 1 hour) resulted in spectra with a large peak at 290 nm and a large shoulder near 265 nm, indicative of a quadruplex population composed of a mix of structures or a hybrid-type structure. In the absence of peptide or
with 4 µM peptide (1:1 molar ratio of peptide-to-DNA), the shoulder increased in amplitude resulting in bimodal spectra with two peaks, of roughly equivalent size, at 265 nm and 290 nm by 16-24 hours (Figure 5-6, data not shown). This approached, but did not reach the spectrum of T7 cooled slowly to room temperature (Figure 5-6, black dashed line), suggesting that a 1:1 ratio of peptide to DNA is not enough to induce a structural transition beyond what would normally happen over time.

The addition of 8 µM of the N-terminal peptide resulted in a much larger increase at 265 nm by 16 hours (Figure 5-6). This suggests that a molar excess of peptide is required to induce a significant structural transition towards parallel-stranded G-
quadruplexes. Even at 8 µM, the shoulder at 290 nm remained quite large, either more peptide is require to change the entire population to parallel-stranded G-quadruplex, or T7 in K⁺ is incapable of becoming entirely parallel-stranded G-quadruplexes. Considering the mobility shift data, it seems that the intermolecular G-quadruplexes have more parallel character than the intramolecular T7 quadruplex.

5.3.3 G-Quadruplexes Can Form at Telomeric ss/ds-DNA Junctions that Mimic Mini-Telomeres

To test the effect of the overhang on TRF2 binding, we designed oligonucleotides to mimic telomeric ds/ss junctions with varying lengths of single-strand overhangs (Figure 2-1, Table 2-1). These DNA constructs had a non-specific region to ensure proper pairing, surrounded by double-strand 5’-d(TTAGGG)₂-3’/5’-d(CCCTAA)₂-3’, and finished with single-strand 5’-d(TTAGGG)ₙ-3’ on each 3’-end, where n=1-4. Additionally, a mutant which should be unable to form an intramolecular quadruplex was designed with an overhang of 5’-d(TTAGGG(TTAGAG)₂TTAGGG)-3’. After duplex annealing, the DNA was subjected to 100 mM KCl, NaCl or LiCl and separated via native polyacrylamide gel electrophoresis.

A transformation of the mini-telomeres to a more compact structure (Figure 5-7) was observed by a change in mobility in the presence of quadruplex promoting salts (K⁺ and Na⁺), but not in equimolar concentrations of KCl and NaCl. See Table 2.1 for sequences.
other salts (Li⁺). The mobility of most of these oligonucleotides shows size dependent retardation. The two exceptions occur in the mobility of the T2T4 template in quadruplex promoting salts (Figure 5-7). This shift appears to be quadruplex dependent as it is not seen in Li⁺, nor does it occur with the mutant.

To further validate G-quadruplex formation and ensure that structural changes were occurring mainly in the G-strand overhang, dimethylsulfate footprinting experiments were performed by Alexandra Locovei and Dr. Fletcher (Yanez et al., 2005). The results demonstrated that N7 of the G-strand overhang guanines were inaccessible to DMS suggesting that the guanines were involved in reverse Hoogsteen base pairing seen in G-quadruplexes. The footprinting was K⁺ dependent and only occurred in the G-strand overhang, not the duplex region of the DNA construct. In addition, a non-telomeric DNA substrate control showed no K⁺-dependent DMS protection. All together, these data suggest that the G-strand overhang can form a G-quadruplex at mini-telomeres.

5.3.4 3’ Single-Strand Overhang Affects TRF2 Recruitment

The templates were then used in protein recruitment assays. This was possible because of the biotin-label they carried in the double stranded region (Figure 2-1). Streptavidin-immobilized DNA was incubated with HeLa extract in the presence of K⁺. Recruited protein was detected by western blot.

![Figure 5-8: Telomere-End Recruitment Assay with mini-telomere double-strand/single-strand junctions from HeLa cell extracts. Recruitment of both WRN and TRF2 were reduced when the overhang sequence was altered to prevent G-quadruplex formation. See Table 2.1 for sequence detail.](image)
analysis with TRF2 specific antibodies (Figure 5-8). Results showed a decrease in the binding of TRF2 when mini-telomere G-strand overhang was changed from TTAGGG to TTAGAG in the 2nd and 3rd telomeric repeats. At the time of these studies, the dependence of TRF2 binding on a wild type G-strand overhang was not expected. TRF2 has very little affinity for single-stranded telomeric DNA oligonucleotides.

5.4 DISCUSSION

Earlier, work showed the spectra of T4 and T8 in K+ were characteristic of hybrid-type intramolecular G-quadruplexes (Figure 3-6). The resultant hybrid CD spectra could have resulted from actual hybrid-type G-quadruplexes and/or a mixed population or parallel and antiparallel structures. However, the current NMR structural characterizations favor the presence of one or two hybrid G-quadruplexes for sequences and experimental conditions similar to those performed in our studies (Matsugami et al., 2006). At this time, we suggest that the biotinylated peptide does not appear to form a strong interaction T4 or T8 in KCl, as the mobility barely changes even with added streptavidin (Figure 5-1, data not shown). However, the bands for T4 and T8 become tighter with increasing peptide, suggesting that the peptide may be shifting the population distribution towards a specific G-quadruplex. Two types of mixed parallel/antiparallel G-quadruplexes have been observed in K+ in addition to the fully-parallel, propeller loop structure (Antonacci et al., 2007; Matsugami et al., 2006; Rujan et al., 2005; Xue et al., 2007).

The mobility T4-23 in K+ in the absence of peptide shows a mixed population between unstructured and a bimolecular structure with the majority of the DNA in the unimolecular state (Figures 3-2, 3-3 and 5-1). Increasing amounts of peptide shifts more of the population towards the bimolecular products (Figure 5-1), suggesting that the
peptide either facilitates the interaction between strands and/or stabilizes the intermolecular quadruplex. Again, the interaction with the peptide may not be stable enough to survive electrophoresis. The positive charge of the peptide may neutralize the negative charge of the DNA, allowing strands to come together more easily. Additionally, the peptide may be stabilizing the G-quadruplex. Although the EMSA alone is not enough to show that it is an intermolecular G-quadruplex, these results are consistent with data from Chapter 3, stressing the importance of the oligonucleotide concentration dependence studies (Figures 3-3 and 5-1) and CD (Figure 3-6D).

Although two-stranded structures were formed by T4-2,3 in Sr\(^{2+}\), they were unable to form under these rapid cooling conditions (data not shown). Those results were interpreted to mean that a fast-forming structure was formed in Sr\(^{2+}\) that prevented the slower-forming intermolecular structure. Alternatively, the slower-forming structure required higher temperatures during the slow cooling to overcome its activation energy of formation. This is not the case for TRF2 N-terminus (Figure 5-1), demonstrating that although the TRF2 N-terminus promotes intermolecular, parallel-stranded G-quadruplex formation like Sr\(^{2+}\), it is capable of performing this activity at lower temperatures (Figure 5-4A).

The population of T7 in K\(^+\) is also divided between a unimolecular and a bimolecular structure. However, unlike T4-2,3, the unimolecular structure is also a G-quadruplex. When cooled slowly, the majority of the DNA remains in the intramolecular configuration, but a substantial amount resides in the bimolecular conformation. Here, under rapid-cooling conditions, very little is able to form the bimolecular G-quadruplex. Similarly to T4-2,3, it appears that the non-quadruplex region of the oligonucleotide
adopts an intermediate structure, and as such, the activation energy must be overcome. The addition of the peptide seems to facilitate the transition towards intermolecular G-quadruplexes. Increasing the amount of peptide led to an increasing amount of the slower migrating Band 2, the intermolecular structure. At 8 µM peptide (2:1 peptide-to-oligonucleotide) a 3rd band appeared, which migrated slower than Bands 1 and 2. At 16 µM Peptide, Band 1, the intramolecular structure almost disappeared in favor of Bands 2 and 3. This suggests that the peptide is indeed assisting with the formation of intermolecular structures, either by bridging of DNA or neutralizing charges, and/or serving as a molecular crowder allowing DNA strands to come together more easily. Additionally, the last lane is lighter than the other lanes, suggesting that the peptide may also be causing DNA condensation resulting in large structures that cannot enter the gel. We also cannot rule out the possibility that DNA charge neutralization is so great at some peptide concentrations that the DNA can no longer migrate to the cathode. We are using siliconized tubes, so DNA should not stick to the tube, but they could be clumping at the bottom just the same.

The addition of peptide to T4-all and T8-all (data not shown) did not affect the mobility of the DNA. This again distinguishes peptide effects from Sr²⁺. The addition of Sr²⁺ to the Tn-all (TTAGAG)n oligonucleotides resulted in a change in mobility when electrophoresed. Small oligonucleotides (T2-all – T4-all) exhibited an increase in mobility, suggesting they adopted a compact conformation. In contrast, larger oligonucleotides (T6-all – T9-all) experienced retardation in mobility when electrophoresed in the presence of Sr²⁺. While these oligonucleotides apparently formed
structures, it is not clear whether the UV and CD data indicate that they are quadruplex structures.

5.4.1 The N-terminal Region of TRF2 Does Not Stabilize Parallel G-Quadruplexes in Na^+

Sodium stabilizes antiparallel G-quadruplexes resulting in CD spectra similar to those of T4, T8 and T7 in ~15 mM Na_2PO_4, with negative peaks near 265 nm and positive peaks near 295 nm (Figure 5-2A, dashed lines). The addition of peptide to these structures results in a slight increase in the amplitude of the peaks, but does not change their positions, suggesting that the peptide is interacting with the structures. The N-terminal peptide of TRF2 does not however result in a switch from antiparallel to parallel-stranded G-quadruplexes (Figure 5-2A, solid lines). The reason for this could be that the peptide is incapable of flipping a full-antiparallel quadruplex to a parallel one. Alternatively, the peptide may be inducing a change in structure that cannot be stabilized without an appropriate cation (e.g. K^+ or Sr^{2+}), resulting in a reversion to the original structure. Mostly likely, the peptide and cation must coordinate to stabilize and/or induce specific G-quadruplex conformations.

The spectra of T4-2,3 and T8-2,3,6,7 in ~15 mm Na^+ do not significantly change with the addition of peptide (Figure 5-2B). These sequences do not form antiparallel G-quadruplexes, and as such, it would have been surprising if the peptide had induced their formation. As explained above, it does not seem possible to form stable parallel-stranded G-quadruplexes under these conditions, which lack an appropriate cation.

5.4.2 The Peptide Does Stabilize Structural Transitions Towards Parallel G-Quadruplexes in the Presence of K^+

In K^+, T4 and T8 form mixed hybrid-type G-quadruplexes (Figures 3-6A and 5-3A). Addition of peptide increases the parallel character of the CD measurements. As
with any dynamic population, structures may appear transiently, and the peptide may
simply be trapping parallel-stranded G-quadruplexes as they form, ultimately resulting in
a shift in the population. However, since the peptide also affects the spectra of
antiparallel G-quadruplexes, it seems probable that the peptide could bind multiple
structures in the mixture. Such a passive approach would not necessarily result in a
dramatic change in the spectra.

As discussed earlier, the literature supports an actual hybrid-structure for T4 in
solution, not a mixed population of structures (Matsugami et al., 2006). However,
telomeric oligonucleotides similar to T4 can form a fully parallel-stranded structure when
crystallized (Parkinson et al., 2002). The peptide may be inducing the formation of
parallel G-quadruplexes, which are stabilized by K⁺. If so, this could explain, both the
CD results as well as the electrophoresis data. The peptide effects on T8 are more
dramatic than those on T4; this is logical when one considers the two oligonucleotides
(Table 2-1). T4 is the quintessential intramolecular quadruplex substrate (Dai et al.,
2007; Pedroso et al., 2007a; Pedroso et al., 2007b). As such, at a given time, almost all of
T4 will most likely be in an intramolecular quadruplex. T8 should be similar to T4, but
since it is essentially two-T4 units, there are potentially twice as many G-quadruplexes
that can form. Additionally, both units do not have to be in the G-quadruplex
conformation for mobility to increase. With this in mind, the electrophoresis results seem
more logical (Figure 5-1). T4 exhibits some tightening of the band with increasing
peptide, not more because most of T4 is already compact. T8 shows a more dramatic
effect because there are probably more strands of DNA with one stable G-quadruplex and
one set of 4 repeats in some intermediate state. The CD for T4 shows a very slight
reduction at 290 and a moderate increase at 265 nm. T8 however, shows a larger transition because there are twice as many potential parallel G-quadruplexes to form (Figure 5-3A).

The population of T7 is mixed. Even without peptide, it has at least two structures, an intramolecular and an intermolecular G-quadruplex (Figure 5-1). Its spectrum (Figure 5-3B) is consistent with a mix of structures rather than a mixed structure (although a mixed hybrid-quadruplex is a likely component). The addition of an equimolar amount of peptide to DNA does not appreciably change the spectra. However, adding a two-fold excess of peptide leads to a drastic shift in spectra, from two similar-sized peaks at 265 and 290 nm, to a large peak at 265 nm and a large shoulder at 290 nm. Interestingly, the shoulder at 290 nm is roughly the amplitude of the previous peak at 290 nm. This in conjunction with the mobility data suggests that the peptide is facilitating intermolecular G-quadruplex structures. The pool of N-terminal peptides could be simultaneously switching some hybrid-quadruplexes to parallel and inducing new intermolecular parallel G-quadruplexes, expanding the total population and shifting the distribution. Since addition of peptide results in a large increase at 265 nm, it appears that the intermolecular quadruplexes induced by the peptide, which populate the slower migrating bands, are mainly parallel-stranded G-quadruplexes, while the intramolecular structure is a hybrid-type quadruplex.

As we have discussed, T4-2,3 and T8-2,3,6,7 did not form G-quadruplexes when cooled quickly to room temperature (Figure 5-4A). It seems that a rapid cooling results in an intermediate non-quadruplex structure that is stalled in some sort of energy minimum, because quadruplexes do not form on these sequences even with increasing time (data not
shown). Addition of the peptide is sufficient to overcome this energy barrier, inducing both oligonucleotides to form parallel stranded G-quadruplexes (Figure 5-4A) with spectra mirroring their slow-cooled spectra (Figure 3-6D).

5.4.3 Peptide-Induced Structural Transitions Time-Dependent

While T8 adopts an intramolecular G-quadruplex configuration within minutes (data not shown), the actual conformation may continue to change until equilibrium is reached (data not shown). This seems especially likely under rapid-cooling conditions, as the drastic rate of cooling could actually slow the approach to equilibrium. Thus the addition of peptide (2:1) to unstructured T8 exhibits a time-dependent structural transition from a hybrid-type G-quadruplex to a parallel quadruplex (Figure 5-5). After 24 hours, the population remains mixed. This suggests that the hybrid structure is quick to form and that the parallel quadruplex forms more slowly.

As with T8, unstructured T7 also exhibits a time-dependent structural transformation in the presence of peptide. With lower peptide concentrations, the results are similar to T8, with a fast-forming peak at 295 nm, indicative of the hybrid-type quadruplex, and a slower appearing peak at 265 nm. By 24 hours, a 1:1 peptide-DNA ratio leads to a spectrum similar to that of slow-cooled T7 alone (Figure 5-6), however, doubling the amount of peptide dramatically increasing the amount of parallel-stranded character in the population. This supports our interpretation of the T8 data, again suggesting that parallel stranded quadruplexes form more slowly than hybrid structures.

5.4.4 Full-Length TRF2 Binding to Mini-Telomere ds/ss Junctions is Affected by the Structure of the 3’-Overhang

TRF2 is believed to bind to and interact with the double-strand region of telomeres. However, its proximity to the ds/ss-junction suggests that it also interacts with
the single-strand 3’-overhang of telomeres. Studies by Sheik Khan in the laboratory demonstrated that recombinant TRF2 preferentially recognizes telomeric ss/ds DNA junctions with specificity regarding the sequence of the G-strand overhang and the sequence of the duplex region most proximal to the overhang (Khan et al., 2007). The DNA substrate preferences for purified TRF2 binding and recruitment of TRF2-containing complexes from extracts are slightly different. It is likely that these mini-telomeres are recruiting at least part of the shelterin complex since we have observed recruitment of both Pot1 and Rap1 from extracts in addition to TRF2 and these three proteins have the same DNA substrate preferences. Since Pot1 is also a ss-DNA binding protein, it is not too surprising that optimal shelterin assembly would be affected by the nature of the G-strand overhang. In addition, shelterin would have to compete with other proteins in the extract for the DNA junctions as demonstrated by the recruitment of WRN, Ku complex and Mre11 (Yanez et al., 2005). It is possible TRF2 can recognize a particular telomeric end or that another protein in the extract recognizes the G-strand overhang and stabilizes a TRF2-containing multi-protein complex. Evidence suggests that the N-terminal basic region of TRF2 affects DNA binding and TRF2 activity. This region has been found to bind to 4-way junctions and may be the key to TRF2 recognition of the single-strand overhang.

5.4.5 Models for TRF2 Interaction with the 3’-Overhang

Our model involves the myb-like domain of TRF2 binding to the double-strand region, while the basic-N-terminal extends to interact with the overhang.

Two possible models for TRF2 interaction with the overhang are:

1) TRF2 interaction with the 3’-overhang could be transient; it recognizes the end and mediates its transfer into the protective structure. This
could involve the stimulation T-loop formation by first interacting with the overhang, then mediating strand invasion by inducing a G-quadruplex and/or binding to the 4-way or 3-way junction.

2) Alternatively, TRF2 bound to dsDNA via the myb-domain could oligomerize, subsequently inducing G-quadruplex formation in G-strand. If the TRF2 oligomer near a bubble is stabilized, TRF2 could then recognize it, allowing for strand invasion and resulting in the T-loop being stabilized.
6  CHAPTER 6: GENERAL DISCUSSION AND FUTURE DIRECTIONS

Telomeres protect linear chromosomes; the maintenance of their nucleo-protein structure is a critical part of cellular viability. This regulation involves the coordination of DNA sequence, length and structure with a variety of protein complexes. The guanine-rich repetitive sequence of telomeres can form G-quadruplex secondary structures. This dissertation focused on the G-quadruplex structures telomeric DNA of varying lengths and sequence alterations can form under specific cation conditions. Once a variety of structures had been characterized, they became tools to identify G-quadruplex-specific activities of telomere binding proteins. TRF2 is a protein that is essential for telomere maintenance. Its proximity to the double-strand/single-strand junction of telomeres made it the perfect candidate with which to begin this study. While the myb-domain of TRF2 (Figures 1-3 and 6-1) binds specifically to dsTTAGGG repeats, the N-terminal basic domain has been shown to stimulate 4-way junctions.

6.1  Review of Key Findings

6.1.1  Chapter 3

Intramolecular G-quadruplex formation induced by Na$^+$ or K$^+$ when 4 or more repeats of 5'-dTTAGGG-3' are present on a single-strand

Intermolecular G-quadruplexes will form in K$^+$, but require longer incubation times. They form from shorter oligonucleotides, altered T4 oligonucleotides (where an adenine is inserted into position 5 of a repeat (TTAGAG)) and between T7 oligonucleotides.

All structures had mixed hybrid-type antiparallel/parallel structures in K$^+$ except T4-2,3 and T8-2,3,6,7 whose structures were largely parallel stranded.

Time courses showed that many of the oligonucleotides formed a structure with fewer bases in the anti-conformation at earlier time points followed by an increase in bases with an anti-conformation later. The latter is indicative of more parallel-stranded character, suggesting the conformation is slower to form.
6.1.2 Chapter 4

Some Sr\textsuperscript{2+}-induced structures are stable even in gels run without Sr\textsuperscript{2+} in the running buffer.

Unlike in K\textsuperscript{+}, all Sr\textsuperscript{2+}-stabilized structures appear to be parallel-stranded.

Unlike in K\textsuperscript{+}, T4 and T5 in Sr\textsuperscript{2+} formed intermolecular instead of intramolecular structures.

Structures with distinct UV and CD spectra were formed for all oligonucleotides in Sr\textsuperscript{2+}, even if all the TTAGGG repeats were converted to TTAGAG.

6.1.3 Chapter 5

The glycine/arginine-rich, N-terminal peptide of TRF2 stimulates intermolecular G-quadruplexes, which do not usually form under rapid-cooling conditions.

The peptide shifts the equilibrium of mixed populations or mixed/hybrid-type structures towards more parallel-character.

The peptide does not induce a shift towards parallel-stranded quadruplexes on fully antiparallel G-quadruplexes

Peptide effects are concentration- and time-dependent

6.2 Model of TRF2/G-Quadruplex Interaction

This dissertation shows that a synthetic peptide of the N-terminal domain of TRF2 interacts specifically with G-quadruplex structures. Additionally, the peptide can induce a structural transition from non-quadruplex telomeric DNA or a hybrid parallel/antiparallel G-quadruplex to a more parallel structure. These results are the first to identify TRF2 as a potential G-quadruplex binding protein, and as such present a new model for TRF2-telomere interaction, as well as a role potential role for G-quadruplexes at telomeric 3'-overhangs.
Once bound to the dsTTAGGG near the end of the double-strand region of the telomere, the N-terminal basic domain may undergo a conformational change, allowing it to interact with the 3’-overhang (Figure 6-1B). The overhang could then stabilize or stimulate the formation of a G-quadruplex. The TRFH domain would then mediate oligomerization between the end and the upstream telomere, potentially leading to a T-loop. TRF2 protection of the end could also be vital when the end is not looped back and tucked away (e.g. during replication). G-quadruplex structures inhibit a variety of proteins, including telomerase. TRF2 could stabilize G-quadruplex structures on the overhang and prevent telomerase elongation of the telomere, thus regulating unwanted elongation (Figure 6-1C). It is important to note that the effects of a parallel-stranded, intramolecular G-quadruplex on telomerase activity have not been tested. It is possible that TRF2 may actually stimulate telomerase activity by converting the mixed, parallel/antiparallel quadruplex to a parallel quadruplex. Regardless, stabilization of the G-quadruplex is in line with the preliminary N-terminal basic domain data. Alternatively, TRF2 could bind to a G-quadruplex and destabilize it, thus allowing telomerase or other telomere binding proteins to bind. Determining a single role for TRF2 at the overhang may be elusive. The multitude of protein complexes involved in telomere maintenance also leads to multiple roles for a single protein, with its current partners influencing a protein’s function.
basic domain could be protected until the DBD binds dsDNA. Once bound near the junction, the basic domain could reach out to interact with the overhang. Then the TRFH domain could mediate oligomer formation with upstream TRF2, stimulating T-loop formation. (C) Hybrid-intramolecular G-quadruplexes block telomerase activity. TRF2 could recognize the structure and destabilize it, thus allowing telomerase to act. Alternatively, TRF2 could stabilize a parallel G-quadruplex, maintain the end in the protective conformation and block protein activity. It is unclear whether this would be a substrate for human telomerase, as a 4-stranded, parallel Tetrahymena sequence G-quadruplex has been shown as a telomerase substrate.
6.3 FUTURE DIRECTIONS

This data shows that the N-terminal domain of TRF2 has specificity for G-quadruplex structures. In addition to further exploring the influence of the peptide on G-quadruplex DNA, the next step is to investigate the interaction of the full-length TRF2 and the other domains with G-quadruplexes. All these constructs are available in the Fletcher laboratory. Now that we know that the N-terminus of TRF2 can alter G-quadruplex structure, it is important to determine whether these effects are amplified or diminished in the context of the full-length protein. In particular, we will be interested in observing the role of TRF2 oligomerization on stimulation of intermolecular G-quadruplex formation. These pursuits will include electrophoresis binding studies and CD, similar to those in the current study, as well as melting temperature assays. Destabilization of G-quadruplexes can be detected by observing the ability of a G-strand to anneal to added C-strand after the G-quadruplex has been destabilized. Footprinting can be used to investigate protection patterns on telomeric DNA. Also, Isothermal Titration Calorimetry can inform stability and stoichiometry of interactions. NMR could provide detailed structural studies of some of the G-quadruplexes we have characterized, as long as the mixtures are reasonably homogenous in structure.

Once TRF2 has been explored, the same techniques can be applied to investigate other interactions. This project can be repeated with other telomere proteins, like telomerase, or it can be combined to investigate the effects of G-quadruplex and TRF2 on other proteins’ activities. The ultimate goal would be to elucidate a detailed model of the role of G-quadruplexes in telomere-end protection.
7 REFERENCES


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**Vita**

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**Journal Publications**

Pedroso IM and Fletcher TM: TRF2 Interactions with Human Telomeric G-Quadruplex Structures *in preparation*.

