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Telomeric repeat mutagenicity in human somatic cells is modulated by repeat orientation and G-quadruplex stability

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ABSTRACT

Telomeres consisting of tandem guanine-rich repeats can form secondary DNA structures called G-quadruplexes that represent potential targets for DNA repair enzymes. While G-quadruplexes interfere with DNA synthesis *in vitro*, the impact of G-quadruplex formation on telomeric repeat replication in human cells is not clear. We investigated the mutagenicity of telomeric repeats as a function of G-quadruplex folding opportunity and thermal stability using a shuttle vector mutagenesis assay. Since single-stranded DNA during lagging strand replication increases the opportunity for G-quadruplex folding, we tested vectors with G-rich sequences on the lagging versus the leading strand. Contrary to our prediction, vectors containing human $[TTAGGG]_{10}$ repeats with a G-rich lagging strand were significantly less mutagenic than vectors with a G-rich leading strand, after replication in normal human cells. We show by UV melting experiments that G-quadruplexes from ciliates $[TTGGGG]_4$ and $[TTTTGGGG]_4$ are thermally more stable compared to human $[TTAGGG]_4$. Consistent with this, replication of vectors with ciliate $[TTGGGG]_{10}$ repeats yielded a 3-fold higher mutant rate compared to the human $[TTAGGG]_{10}$ vectors. Furthermore, we observed significantly more mutagenic events in the ciliate repeats compared to the human repeats. Our data demonstrate that increased G-quadruplex opportunity (repeat orientation) in human telomeric repeats decreased mutagenicity, while increased thermal stability of telomeric G-quadruplexes was associated with increased mutagenicity.

1. Introduction

Telomeres are nucleoprotein structures at chromosome ends that critically impact lifespan and health, as well as cell viability and genome stability [1–3]. Progress in recent years indicates that the inability to completely replicate chromosome ends is not the only source of telomere attrition, and that inappropriate processing by DNA repair enzymes or failures in telomere replication can cause rapid telomere loss (reviewed in [4]). Telomeres consist of an array of repeat sequences that interact with specific proteins to prevent the chromosome ends from being recognized as double strand breaks [5,6]. Mammalian telomeres comprise of TTAGGG repeats, and human telomere lengths vary from 5 to 15 kb and terminate in a 3′ ssDNA tail that is 50–500 nt long [7]. The 3′ tails can invade preceding telomeric repeats to form a lariat like t-loop/D-loop structure that is further stabilized by the shelterin protein complex [8,9]. Shelterin proteins TRF2 and TRF1 bind duplex telomeric DNA and POT1 binds to single strand TTAGGG repeats [10,11], and together they recruit the remaining shelterin proteins TIN2, RAP1, and TPP1 [4]. How these proteins influence the fundamental processes of DNA repair and replication in telomeric repeats has yet to be fully realized.

Cellular evidence indicates that telomeres are fraught with potential obstacles to DNA replication and require specific proteins to prevent stalling. In *Saccharomyces cerevisiae* DNA replication fork stalling is greatly increased at telomeres in the absence of the Rrm3p helicase [12]. In *Schizosaccharomyces pombe* and humans the telomeric proteins Taz1 and TRF1, respectively, are required to prevent replication fork stalling at telomeres [13,14]. The precise mechanism is not known, but some evidence suggests that TRF1 recruits helicases BLM and RTEL to dissociate alternate DNA structures [15]. The consequences of fork stalling in the telomeres can be loss of telomeric DNA or aberrant telomere structures including doublets that resemble broken telomeres [14–16]. Telomere doublets are induced by aphidicolin treatment which stalls replication

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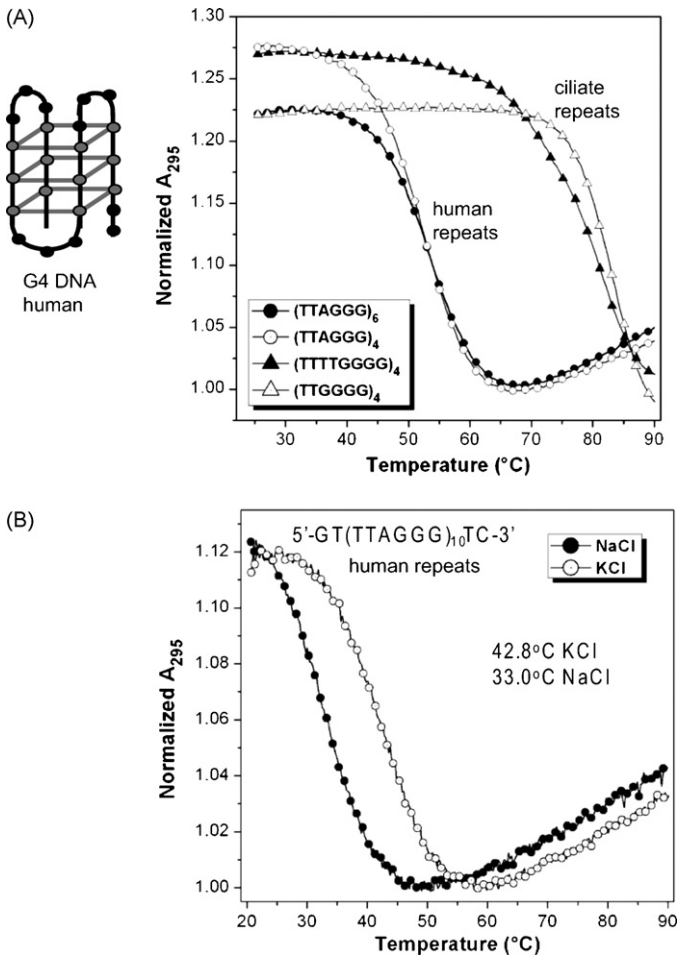

Fig. 1. Ciliate telomeric repeats form G4 DNA structures of greater thermal stability compared to human telomeric repeats. (A) UV Melting curves showing intramolecular G-quadruplex formation in telomeric ssDNA with flanking sequence. Melting curves for oligonucleotides GT-$(TTAGGG)_4$-TC, GT-$(TTAGGG)_6$-TC, GT-$(TTGGGG)_4$-TC and GT-$(TTTTGGGG)_4$-TC recorded at 295 nm in solutions containing 100 mM KCl. A schematic of one possible G4 conformation for human telomere repeats is shown. Grey balls indicates Gs, and grey lines indicate a quartet formed by base pairing between four Gs. (B) UV melting curves for the oligonucleotide GT-$(TTTAGGG)_{10}$-TC in two different salt solutions, 100 mM KCl and 100 mM NaCl.

forks and induces breaks at fragile sites [14]. The mechanistic models of mutagenesis in repetitive sequences involve stalling and/or dissociation of the DNA replication fork due to road blocks [17]. Studies in yeast and bacteria demonstrate that sites of stalled replication forks are susceptible to chromosomal breakage [12,18,19]. Thus, replication-mediated breaks in telomeres may represent an important source of telomeric loss.

Possible sources of replication fork stalling at telomeres include oxidative DNA damage which preferentially occurs at G runs [20], or alternate DNA structures including the t-loop/D-loop or G-quadruplex (G4) DNA which can form in ssDNA with tandem guanines. Telomeric DNA forms G4 structures spontaneously *in vitro* and *in vivo* [21–26] that block DNA polymerase progression *in vitro* [27]. G4 structures consist of planar arrays of quartets, and each quartet is formed by four guanines interacting through Hoogsteen base pairing [28] (Fig. 1A). The number of quartets in a quadruplex influences the stability of the structure and depends on the number of guanine residues [29]. The potential for G4 formation in the telomeres exists either in the 3′ overhang, displaced DNA in the D-loop, or in the G-rich sequences present on the lagging strand. Okazaki fragment processing during lagging strand DNA synthesis is expected to produce transient regions of ssDNA, and G4 DNA folds in ssDNA regions [26,30]. Cells deficient in the Werner syndrome protein (WRN), POT1 or FEN1 exhibit preferential loss of telomeres replicated from the G-rich lagging strand [15,31,32], suggesting these proteins may function in preventing and/or dissociating G4 structures. Furthermore, an agent that stabilizes G4 DNA induces defects in telomere replication and causes telomeric aberrations [33]. Whether G4 structures can interfere with telomere replication in normal cells has yet to be established.

Previous work indicates that sequences with the ability to form various alternate structures exhibit increased mutagenic potential (reviewed in [34]). In these studies shuttle vectors with mutation reporter genes have been invaluable. The insertion of sequences with the potential to form H-DNA and Z-DNA adjacent to a reporter gene induced breaks and large deletions in the shuttle vector after transfection into normal mammalian cells [35,36]. The impact of G4 DNA on shuttle vector stability is unknown, but studies in yeast and worms suggest that G4 structures can be mutagenic. Loss of DOG-1 helicase in *Caenorhabditis elegans* leads to deletions in genes containing G-runs [37], and loss of Pif1 helicase in *S. cerevisiea* promotes instability in an artificial human G-rich minisatellite in the yeast genome [38]. However, the fidelity of telomeric repeat replication and the impact of G4 potential on the mutagenicity of telomeric repeats in human cells are largely unexamined.

Studies of ciliated protozoa provide evidence for G4 formation at telomeres and G4 resolution during replication. Ciliates contain a macronucleus consisting of up to 10^8 small DNA molecules that are terminated by telomeres consisting of about 20 bp of duplex DNA and a 16 nucleotide 3′ G-rich ssDNA tail (reviewed in [39]). This high concentration of telomeres allowed for the detection of G4 DNA by immuno-staining with antibodies raised against G4 structures [40]. DNA replication occurs exclusively in a distinct replication band [41] in which G4 DNA is not detected [40]. G4 formation is regulated by telomere-binding proteins TEBP-α and TEBP-β [23,25]. These studies suggest that G4 DNA is resolved during telomere replication in ciliates.

In this study, our goal was to test the mutagenic potential of telomeric repeat sequences and their ability to induce breaks and deletions upon replication in normal human cells, using a well-established shuttle vector mutagenesis assay. We hypothesized that the mutagenicity of telomeric repeats correlates with G4 forming potential and thermal stability. To test this, we examined various telomeric repeats that differ in G-quartet numbers and compared repeats with the G-rich sequence on the lagging strand versus the leading strand. We show that the ciliate repeats from *Tetrahymena thermophila* (TTGGGG) and *Oxytricha nova* (TTTTGGGG) form more stable G4 DNA than human repeats (TTAGGG) *in vitro*. We demonstrate that while all of the vectors with various telomeric repeats exhibited low mutant rates after replication in human cells, the orientation of the human telomeric repeats (G-rich lagging versus leading strand) and the stability of the potential G4 structures significantly affected the vector mutant rates. We also observed an increase in mutagenic events in the ciliate telomeric repeats compared to the human repeats. However, in contrast to H-DNA and Z-DNA forming sequences, our data indicate that normal human cells possess the ability to effectively manage G4 forming sequences, particularly human telomeric repeats, during replication.

2. Materials and methods

2.1. Reagents

Oligonucleotides containing telomeric repeat sequences and primers used in sequencing reactions were ordered from Inte-