The extreme ends of eukaryotic chromosomes contain 3’ extensions in the form of single-stranded G-rich repeats, referred to as telomeric 3’ G-tails or overhangs. Increasing evidence has suggested that telomeric 3’ G-tails can adopt G-quadruplex conformation both in vitro and in vivo. However, the role of G-quadruplexes on the structure and function of telomeric 3’ G-tails remains unclear. In the current study, we showed that the human telomeric 3’ G-tail sequence protected the duplex DNA ends in cis from being recognized as double-strand breaks. This protection is dependent on the G-quadruplex conformation of the 3’ G-tail sequence. These results suggest that the ability of telomeric 3’ G-tails to adopt the end-protecting G-quadruplex conformation may be one of the reasons for the existence of the evolutionarily conserved G-stretch motifs in telomeric DNA sequences.

Telomeres are special protein-DNA complexes at the ends of linear chromosomes in most eukaryotic cells. Interestingly, most telomeric sequences are comprised of similar guanine-rich repeats (1). In human, chromosomal ends contain duplex TTAGGG/AATCCC repeats exhibiting heterogeneity in size ranging from 2 to 30 kb (2,3). At the extreme ends of those duplex repeats are single-stranded 3’ overhangs, termed telomeric 3’ G-tails (4). These 3’ G-tails, which range 130 to 200 bases in size, are extensions of the G-rich strand (TTAGGG) of the duplex repeats (4). Even though the structure and function of telomeric 3’ G-tails are largely unclear, recent studies have suggested that telomeric 3’ G-tails may exist in multiple states in vivo. For example, protein-coated 3’ G-tails have been proposed since proteins (e.g. human POT1 and yeast Cdc13p) that bind specifically to 3’ G-tails have been identified (5,6). In addition, 3’ G-tails have been shown to form a T-loop structure, in which the 3’ G-tail inserts into the preceding duplex region forming a large lariat structure at the chromosomal end (7,8).

In addition to the protein-coated and T-loop structures, increasing evidence has suggested that telomeric 3’ G-tails also exist in the G-quadruplex conformation, a four-stranded nucleic acid structure consisting of stacks of G-quartets (9). It have been well documented that the telomeric 3’ G-tail sequences can form thermodynamically stable G-quadruplex conformation in vitro (10,11). Several studies have also suggested the existence of telomeric G-quadruplexes in vivo. Studies in Stylonychia lemnae have demonstrated the existence of telomeric G-quadruplexes in the macronuclei using indirect immunofluorescence with a G-quadruplex-specific antibody (12). In the same study, G-quadruplexes were not detected in the replication band, suggesting that the G-quadruplex conformation is resolved during DNA replication (12). More recently, using a fluorescent carbazole derivative, a G-quadruplex-specific ligand, the existence of an antiparallel G-quadruplex structure at telomeres of human metaphase chromosomes was detected (13,14). Additionally, another G-quadruplex-specific ligand, telomestatin, has been demonstrated to perturb telomere function in vivo, again suggesting the presence of telomeric G-quadruplexes (15,16). The in vivo significance of G-quadruplexes is also suggested from the presence of many G-quadruplex-specific proteins. For example, the telomere binding/associated proteins such as Rap1p, Stm1p and Mre11p in budding yeast, and the β-subunit of the Oxytricha telomere binding protein, have been shown to bind telomeric G-quadruplexes (17-21).
the *kem1* gene, and the nuclease GQN1 (G quartet nuclease 1) which does not cleave duplex DNA, single-stranded DNA, Holliday junctions, or G4 RNA (22,23). Furthermore, members in the RecQ family including Sgs1p, BLM (gene product of Bloom's syndrome) and WRN (gene product of Werner's syndrome), are apparently unique among cellular helicases in their ability to efficiently disrupt G-quadruplex DNA (24,25). Taken together, these results suggest the G-quadruplexes may have evolved to become biologically significant structures.

One important function of telomeres is to protect the chromosome ends from being recognized as double-strand breaks. Indeed, perturbation of telomeres can efficiently induce DNA damage response characteristic of DNA double-strand breaks (e.g. γ-H2AX, and 53BP1 foci, and ATM autophosphorylation) (26,27). In addition, many DNA damage repair proteins (e.g. Ku70, Ku80 and DNA-PK) are physically associated with telomeres and functionally involved in preventing fusions of deprotected telomeres (28,29). Although the mechanism by which telomeres protect the chromosome ends is still unclear, it has been suggested that the G-quadruplex conformation of telomeric 3' G-tails could protect the otherwise exposed DNA double-strand breaks (1). In the current studies, we have investigated the possible role of the G-quadruplex conformation of the telomeric 3' G-tails in protecting telomeric DNA ends from being recognized as DNA double-strand breaks.

**EXPERIMENTAL PROCEDURES**

*Materials*- VP-16 (etoposide) was purchased from Sigma. Arylmorpholine analog 37(AMA-37) [1-(2-Hydroxy-4-morpholin-4-yl-phenyl)-phenylmethanone], a specific DNA-PK inhibitor, was purchased from Calbiochem. Phospho-Chk2 (Ser-33/35), phospho-Chk1 (Ser-317) and phospho-p53 (Ser-15) antibodies were purchased from Cell Signaling. Anti-Mre11 antibody was purchased from Novus Biologicals. Frozen pellets of HeLa cells were purchased from National Cell Culture Center. 1 Kb DNA Ladder (consisting of up to 12 repeats of a 1018 bp DNA and DNA fragments ranging from 75 to 1636 bp) and 10bp DNA Step Ladder (10 to 100 bp of DNA fragments with 10 bp increments) were purchased from Invitrogen and Promega, respectively. All oligodeoxynucleotides used in this work were synthesized and purified by Integrated DNA Technologies. The sequences of the oligos used in this study are:

36 (a 36-mer duplex DNA): obtained by annealing of single-stranded 36F (GAT GAA GAC TGT GCT CAT GAT GAA GAC TGT GCT CAT) and its complementary single-stranded 36R (ATG AGC ACA GTC TTC ATC ATG AGC ACA GTC TTC ATC). 30: a 30-mer duplex DNA obtained by annealing of single-stranded 30F (GAT GAA GAC TGT GCT CAT GAT GAA GAC TGT) and its complementary single-stranded 30R (ACA GTC TTC ATC ATG AGC ACA GTC TTC ATC). H30: a 30-mer hairpin duplex DNA obtained by self-annelling of a 65-mer single-stranded oligodeoxynucleotide with the sequence CCC TAA TTC ATC ATG AGC ACA GTC TTC ATC CAA GAG ATG ACT TGTC ATG ATG AAT TAG GG (the sequence of hairpin loop is underlined). TG24: a 24-mer single-stranded oligodeoxynucleotide with four tandem repeats of the human telomeric DNA sequence of TTAGGG, mTG24: the same single-stranded 24-mer DNA as TG24 except that the TTAGGG repeat sequence is replayed to TTAGTG. CA24: a 24-mer single-stranded oligodeoxynucleotide with four tandem repeats of CCCTAA. D30: a dumbbell-like duplex DNA with a 30-mer duplex stem, obtained by self-annelling of a 70-mer single-stranded oligodeoxynucleotide with the sequence AGC ACA GTC TTC ATC CAA GAG ATG AAG ACT GTG CTC ATG ATG AAT TAG GGC AAG ACC CTA ATT CAT CAT G (the two hairpin loop sequences are underlined). TEL30, TEL36 and TEL42 are duplex oligos containing 5, 6 and 7 telomeric TTAGGG/AATCCC repeats, respectively. Nuclear extracts preparation-Nuclear extracts were prepared according to the published procedure (30) with slight modifications. Briefly, 5x10^5 ZR-75-1 or HeLa cells were suspended in 5 volumes of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl_2, 10 mM KCl, 0.5 mM DTT, 0.1% Triton X-100 and a protease/phosphatase inhibitor mix which contains 1 µg/ml leupeptin, 2 µg/ml aprotonin, 1 µg/ml pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerophosphate, and 1 mM sodium vanadate).
The nuclei were collected by centrifugation at 1000 xg for 10 min. Isolated nuclei were then extracted with 1 volume of buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and the protease/phosphatase inhibitor mix) on ice for 30 min. Extracts were further centrifuged at 11,000 xg for 30 min. The pellet was designated nuclear pellet (NP). The supernatant, designated nuclear extracts (NE), was aliquoted and stored at -70°C.

For assaying DNA damage signals, reactions (25 µl each), containing 10 µl of nuclear extract, 10 µl of reaction buffer (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 mM EDTA, 0.5 mM PMSF, 10 mM β-glycerophosphate, 2 mM DTT, 2.5 mM ATP, 1 mM sodium vanadate, 25 mM MgCl₂, and 2.5 mM MnCl₂) and 5 µl of a DNA substrate in TE (10 mM Tris, pH 8.0 and 0.1 mM EDTA), were incubated at 32°C for 15 min, and then terminated by 20 µl of 6x Laemmli SDS gel sample buffer.

**Immunoblotting**—The procedures were performed as described before (31). Briefly, protein samples in SDS gel sample buffer were boiled for 10 min and then analyzed by SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane. The membrane was stained with Ponceau-S to confirm protein loading and then immersed in 5% milk for 1 hr. The membrane filter was then incubated with specific antibody overnight, followed by washes with TBST and incubation with secondary antibody for 1 hr. Bound secondary antibody was then detected by X-ray films using ECL reagents (Pierce).

**UV Melting Analysis**—UV melting studies were conducted using a Beckman DU-640 Spectrophotometer equipped with a thermoelectrically controlled cell holder. Quartz cells with a path length of 1 cm were used for the absorbance studies. Temperature-dependent absorption profiles were acquired at 295 nm with a 5 sec averaging time. The temperature was raised in 1.0°C increments and the samples were allowed to equilibrate for 1.0 min at each temperature setting. In these UV melting studies, the DNA concentration was 1.0 (OD₂₆₀), Buffer solutions contained 10 mM HEPES (pH 7.5), 100 mM potassium acetate, and 0.1 mM EDTA. Prior to their use in UV melting experiments, all DNA solutions were preheated at 90°C for 5 min and then slowly cooled to room temperature over a period of 4 hrs.

**RESULTS**

Activation of DNA damage signals in nuclear extracts by exogenous DNA substrates—In order to study the possible protective effect of telomeric 3' G-tails on chromosome ends, an in vitro system using nuclear extracts with exogenous DNA substrates was employed. This nuclear extract system, which was devoid of endogenous chromosomal DNA (see Fig. 1A, right panel, for the lack of any detectable histone H2A), was capable of reproducing many of the DNA damage signals in response to exogenous linear duplex DNA. As shown in Fig. 1A (left panel), duplex DNA oligos containing 5 (TEL30), 6 (TEL36) and 7 (TEL42) human telomeric (TTAGGG/AATCCC) repeats were capable of activating various DNA damage signals including phosphorylations of p53 (Ser-15), Chk2 (Ser-33/35), Chk1 (Ser-317), and c-Abl (Ser-735). Activation of these DNA damage signals was sensitive to PI3 kinase inhibitors, caffeine (Caff.) and wortmannin (Wort.) (Fig. 1B), suggesting the involvement of ATM, DNA-PK and/or ATR. To examine the involvement of DNA-PK, a DNA-PKcs-specific inhibitor (AMA-37) was employed. AMA-37 is an ATP competitive inhibitor of DNA-PKcs with an IC₅₀ value about 270 nM in 0.1 mM ATP (32). Compared to the IC₅₀ value, our results showed that the AMA-37 effectively reduced the phosphorylation signal of p53 (Ser-15) to 50% at 10 µM in 0.5 mM ATP (Fig 1B, upper panel, lane 5) and to less than 10% at 1 µM in 0.1 mM ATP (Fig 1B, lower panel, lane 4) in the nuclear extract. It is worthwhile to note that AMA-37 does not inhibit ATM or ATR even at a concentration as high as 50 µM (32). To test the possible involvement of ATM, the ATM-specific inhibitor (KU 55933) was used (33). As shown in Fig. 1C, KU 55933 at 1 µM did not inhibit p53 phosphorylation in either 0.1 (lower panel, lane 5) or 0.5 mM ATP (upper panel, lane 5) (the IC₅₀ is known to be 12.3 nM in 0.05 mM ATP for ATM inhibition) (33). Only at 10 µM or higher, KU 55933 started to inhibit p53 phosphorylation, possibly due to its non-specific inhibitory effect on DNA-PK. Together, these results suggest that
DNA-PKcs is primarily responsible for the DNA damage signals activated by exogenous DNAs in this extract. Consistent with this interpretation, nuclear extracts prepared from ATM knockout (L3) and wild-type (L40) cells exhibited similar levels of DNA damage signals in response to exogenous DNA substrates (data not shown).

Effect of 3’ overhangs on DNA end-activated DNA damage signals: inhibition by the telomeric 3’ G-tail sequence-In order to study the protective effect of telomeric 3’ G-tails on DNA ends in the in vitro system, a series of DNA substrates were designed and tested for their ability to activate DNA damage signals. As shown in Fig 2A, D30, a 30-mer dumbbell-like duplex DNA substrate with both ends protected by short (5 nucleotides) hairpin loops, was ineffective in stimulating phosphorylation of p53 (Ser-15). By contrast, H30, which was structurally identical to D30 except that one of the hairpin loops was removed resulting in exposure of one duplex DNA end, was highly efficient in activating phosphorylation of p53 (Ser-15) (Fig. 2A, lanes 5-7). This result demonstrates the duplex end requirement for triggering DNA damage signals in this system. Additional studies using circular plasmid DNAs with and without restriction enzymes digestion confirmed this conclusion (data not shown). Next, we tested the effect of 3’ overhangs on the ability of the duplex end to activate DNA damage signals in this extract. As shown in Fig. 2A, H30-TG24 (see the structure in Fig. 2A, right panel), which was structurally identical to H30 except that the duplex end contained a single-stranded 3’ overhang (TG24), was unable to stimulate phosphorylation of p53 (Ser-15) (Fig. 2A, lanes 5-7). This result demonstrates the duplex end requirement for triggering DNA damage signals in this system. Additional studies using circular plasmid DNAs with and without restriction enzymes digestion confirmed this conclusion (data not shown).

The ability of the 3’ overhang to inhibit the duplex DNA end-mediated DNA damage signal appeared to depend on the sequence rather than the duplex-overhang junction since other 3’ overhangs did not exhibit the same inhibitory effect. As shown in Fig. 2A, H30-mTG24, which was structurally similar to H30-TG24 except that TG24 (3’ overhang) was replaced with mTG24 (a mutant form of TG24 with disrupted G-stretches in the repeats), strongly stimulated phosphorylation of p53 (Ser-15) (Fig. 2A, lanes 11-13), suggesting that the G-stretch in the telomeric repeat (TTAGGG) is important for the inhibitory activity of TG24 3’ overhang. This conclusion was also supported by another experiment using an unrelated 24-mer 3’ overhang, CA24. As shown in Fig. 2B, H30-CA24, which was structurally identical to H30-TG24 except that the TG24 3’ overhang was replaced with a 3’ CA24 overhang (24-mer single-stranded oligo with four tandem repeats of the CCCTAA sequence), was highly effective in stimulating phosphorylation of p53 (Ser-15) as well as of Chk2 (Ser-33/35), and Chk1 (Ser-317).

One possible explanation for the above results is that the single-stranded oligos CA24 and mTG24, but not TG24, per se may activate the DNA damage signals. This possibility was ruled out since neither CA24 nor mTG24 alone stimulated p53 (Ser-15) phosphorylation (Fig. 2C, lanes 5-9). Together, these results suggest that the inability of 30H/TG24 to induce DNA damage signals is due to a negative interference from the telomeric TG24 sequence.

The protective effect of the telomeric 3’ G-tail sequence on DNA ends is G-quadruplex conformation-dependent-The failure of mTG24 (four tandem repeats of TTAGTG) to protect the DNA duplex end suggested the importance of the G-stretches in TG24 (four tandem repeats of TTAGGG). It seems possible that the protective effect of TG24 on DNA end-mediated DNA damage signaling is due to its G-quadruplex conformation, which is readily formed with four G-stretches (10,11,34). To test this possibility, UV melting analysis was performed on both TG24 and mTG24 sequences. It has been well documented a transition from G-quadruplex conformation to single-stranded DNA conformation occurs as absorbance increases at 295 nm (35). This is contrary to the transition from duplex DNA to single-stranded DNA conformation, which is characteristic of an increase in absorbance at 260 nm. As shown in Fig. 3A, TG24 underwent a melting transition (a sharp decrease in absorbance at 295 nm as revealed by a temperature derivative plot, dA/dT) at 61°C (Tm) while mTG24 did not show any transition in the temperature employed in this study (15°C to 95°C), suggesting that TG24, but not mTG24, existed in the G-quadruplex conformation under our assay conditions (32°C).
UV melting analysis was also performed on three other substrates, H30, H30-TG24 and H30-mTG24. As shown in Fig. 3B, H30-TG24, but not H30 or H30-mTG24, showed a broad melting transition at 295 nm (Tm about 50 to 65°C), suggesting that TG24 existed in the G-quadruplex conformation in H30-TG24. These results argue that the G-quadruplex conformation of TG24 is responsible for its protective effect on the duplex end in H30-TG24.

The telomeric G-tail sequence protects DNA ends in cis-TG24 could possibly protect DNA ends in trans by titrating (inhibiting) some key signal transducers in the DNA damage signaling pathway. To test this possibility, a competition experiment was performed using TG24 as the competitor. As shown in Fig. 4, increasing amount of TG24 (0.04, 0.2 and 1 µM) had no effect on phosphorylation of p53 (Ser-15) induced by either H30-CA24 (Fig. 4A, lanes 7-9) or H30-mTG24 (Fig. 4A, lanes 10-12). Similarly, TG24 had no inhibitory effect on phosphorylation of p53 (Ser-15) induced by either H30 (Fig. 4B, lanes 7-9) or the 36-mer duplex (Fig. 4B, lanes 10-12). These results demonstrated that the telomeric 3’ G-tail sequence, when introduced in trans, did not block DNA end-mediated DNA damage signaling.

The cis effect of the 3’ G-tail sequence in protecting the DNA ends was also supported by the following experiment. In this experiment, additional substrates, 30 (30-mer duplex DNA), TG24-30 (30 with one 3’ TG24 overhang), and TG24-30-TG24 (30 with 3’ TG24 overhangs on both ends), were designed and tested in the nuclear extract. As shown in Fig. 5A, TG24-30-TG24, but not 30 or TG24-30, was unable to stimulate phosphorylation of p53 (Ser-15). This result suggests that each 3’ TG24 overhang can only protect one DNA end. Complete protection of the duplex DNA requires two 3’ TG24 overhangs, one on each end of the duplex DNA, consistent with the cis inhibition model.

We next tested if the 3’ TG24 overhang could block DNA end-mediated DNA damage signaling at a distance. Two substrates, TG24-30-T15 and TG24-30-T15-TG24 were designed and tested (for structures, see Fig. 5A, upper panel). Interestingly, TG24-30-T15, but not TG24-30-T15-TG24, stimulated p53 (Ser-15) phosphorylation (Fig. 5B), suggesting that the insertion of 15T between the duplex end and the 3’ TG24 overhang did not affect the inhibitory effect of the 3’ TG24 overhang.

**DISCUSSION**

The identification of DNA-PK to be responsible for activating the DNA damage signals by exogenously-added duplex DNA in the nuclear extract is significant since the kinase activity of DNA-PK as well as other proteins involved in NHEJ repair have been shown to be required to protect mammalian telomeres in vivo (29,36). In addition, DNA-PKcs and Ku subunits are known to be physically associated with telomeres in mammalian cells (28). Consequently, the nuclear extract system employed in the current studies is likely to be biologically relevant.

The substrates employed in the current studies were short duplex oligodeoxynucleotides with blunt ends or modified ends (e.g. small single-stranded DNA loops). We found that short linear duplexes with both ends capped by these hairpin loops (e.g. D30) were totally ineffective in activating the DNA damage signals. However, short linear duplexes with only one end capped by the hairpin loop (e.g. H30) were active in inducing the DNA damage signals. These results suggest that double-stranded DNA ends but not hairpin ends can induce DNA damage signals in the nuclear extract. Using one-end hairpin-capped duplex substrates, we were able to study the effect of various 3’ overhangs on the other unprotected end. We showed that one-end-capped linear DNA duplex containing CA24 as the 3’ overhang (i.e. H30-CA24) was highly active in stimulating various DNA damage signals in this extract. Other duplex DNA substrates containing 3’ overhangs were generally quite effective in stimulating the DNA damage signals (data not shown). However, one-end-capped linear DNA duplex containing the telomeric 3’ G-tail (TG24) sequence as the overhang was far less effective (at least 100 fold less) in stimulating the DNA damage signals.

The ability of the telomeric 3’ G-tail sequence, TG24, to protect the duplex end was highly sequence-specific, since a related sequence, mTG24, was completely ineffective in protecting the end. UV melting analysis has confirmed the existence of TG24 (and H30-TG24), but not mTG24 (and H30-mTG24), as a G-quadruplex. These results suggest that the end protective effect
is mediated by the G-quadruplex conformation of the human telomeric 3’ G-tail sequence, TG24.

The mechanism by which TG24 mediates its end-protecting effect was investigated. The possibility that TG24 might titrate (inhibit) a key regulator (e.g. DNA-PK) in the extract was ruled out by a competition experiment in which TG24 was completely ineffective in protecting the ends when introduced in trans. The cis action of TG24 was also supported by the experiment in which complete protection required the presence of TG24 (as 3’ overhangs) at both ends. This experiment further suggests that the end-protecting effect of TG24 is short-ranged since the presence of TG24 3’ overhang on one end does not protect the template which still contains another unprotected end located 30 bp away. Thus, the cis effect of TG24 also excludes the scenario that the inability of the templates (H30TG24, TG24-30-TG24) to induce DNA damage signals was due to degradation by nuclease/helicase or inactivation by template-coating proteins. The simplest explanation for the cis protective action of TG24 is proposed as a steric interference model in which the G-quadruplex conformation of TG24 may block the access of a key regulatory molecule(s) (e.g. DNA-PK, ATM) to the duplex/overhang junction (Fig. 6). To test this model, we inserted a single-stranded 15-mer (15T) in between the duplex end and TG24 3’ overhang. This new construct, TG25-30-15T-TG24, was still completely ineffective in stimulating DNA damage signals, suggesting that TG24 can still protect the end in cis with 15 nucleotides separation between them.

Although these results can still be accommodated by the steric interference model (Fig. 6), other possibilities cannot be ruled out. For example, TG24 in its G-quadruplex conformation may recruit additional protein(s) to form a large protein-DNA complex, restricting the access of DNA-PK to the duplex/overhang junction at least 15 bases away. Alternatively, the presence of the G-quadruplex may block the interaction between the 3’ end and the duplex/overhang junction, which may be necessary for end recognition (e.g. entry of a helicase from the 3’ end). Regardless of the molecular mechanism, the cis protective effect of the human telomeric 3’ G-tail sequence is most likely due to its G-quadruplex conformation.

G-stretch-containing repeats are the fundamental units for most telomeric sequences. However, the reason for this evolutionarily conserved G-stretch motif is unclear (1). Our results could provide an explanation for this conservation. Since the main function of telomeres is to protect the DNA ends of linear chromosomes from being recognized as double-strand breaks, it is likely that the sequence of 3’ G-tails is evolved to protect the chromosome ends due to its intrinsic property to adopt the stable G-quadruplex conformation.

G-quadruplex conformation is likely to be part of the 3’ G-tail structural dynamics (1). During telomere replication in S phase, the telomeric G-quadruplexes need to be resolved for telomere replication by telomerase (12). Many G-quadruplex-specific helicases (e.g. Werner, Bloom, Sgs1) can be involved to facilitate this process (24,25). Indeed, Werner helicase mutant has been shown to be defective in telomere lagging-strand synthesis, suggesting a physical hindrance at the 3’ G-tail (37). The presence of different 3’ G-tail-specific binding proteins (e.g. human POT1 and yeast Cdc13p) may also be necessary to stabilize the single-stranded conformation of the G-tails in S phase. In budding yeast, temperature-sensitive mutant, cdc13-1, results in G2/M phase arrest and contains long telomeres at restricted temperature (38). However, the growth arrest phenotype and abnormal telomere size can be rescued by introduction of a STM1 gene, encoding a G-quadruplex-specific binding protein, in a multicopy vector. (19). Furthermore, overexpression of the SGS1 gene (encoding a G-quadruplex-specific helicase), can abolish the suppressor effect of STM1 on the cdc13-1 phenotype (19). These results suggest that the G-quadruplex conformation at 3’ G-tails is tightly regulated by many factors and actively involved in the telomere maintenance. Clearly, additional studies are necessary to establish the precise role of the G-quadruplex conformation in the structural dynamics of telomeric 3’ G-tails.
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FIGURE LEGENDS

Fig. 1. Duplex DNA oligodeoxynucleotides activate DNA-PK-dependent DNA damage signals in nuclear extracts. (A) DNA damage signals induced by telomeric duplex DNA fragments in nuclear extracts. The nuclear extract (NE) contained p53 and TOP1, but no detectable histone H2A (Western blotting results shown at the right panel). All histone H2A was present in the nuclear pellet (NP) which was removed. TEL30, TEL36 and TEL42 (30-mer, 36-mer and 42-mer telomeric duplex DNA) were added to the nuclear extracts and the DNA damage signals monitored by Western blotting. Phosphorylations of p53 (Ser-15), Chk1 (Ser-317), Chk2 (Ser-33/35) and c-Abl (Ser-735) were detected by their respective phospho-specific antibodies. (B) DNA-PK-mediated DNA damage response. 0.2 μM 36-mer duplex DNA (designated 36) was added to the nuclear extract in the presence or absence of the DNA-PK-specific inhibitor, AMA-37 (lanes 3-6) or other PI3K kinase inhibitors, caffeine (Caff.) and wortmannin (Wort.). The reactions contained either 0.1 mM (middle panel) or 0.5 mM ATP (top panel). (C) 0.2 μM 36-mer duplex DNA (designated 36) was incubated with the nuclear extract in the presence of the ATM-specific inhibitor, KU 55933. The reactions contained either 0.1 mM (middle panel) or 0.5 mM ATP (top panel). For loading controls, reactions shown in A, B, and C were also immunoblotted with anti-Mre11 antibody (lower panels).

Fig 2. A human telomeric 3’ G-tail inhibits DNA end-mediated DNA damage signals. (A) D30, H30, H30-TG24, and H30-mTG24 (see structures in the right panel) were incubated with nuclear extracts and p53 (Ser-15) phosphorylation was measured by immunoblotting with phospho-p53 (Ser-15) antibody. (B) H30-TG24 and H30-CA24 were incubated with nuclear extracts followed by immunoblotting with different phospho-antibody. (C) Single-stranded DNA oligos CA24 and mTG24 do not stimulate DNA damage signals. CA24 and mTG24, as well as H30-CA24 and H30-mTG24, were incubated with nuclear extracts, followed by Western blotting with anti-p53 S15P antibody.

Fig 3. UV melting analysis of 3’ G-tail-containing sequences. (A) UV melting analysis of TG24 and mTG24. The temperature (T) range was 15 to 95°C. The absorbance (A) was measured at 295 nm. 1000xdA/dT was plotted against the temperature (T). (B) UV melting profiles of H30-TG24 and H30-mTG24.

Fig 4. The human telomeric G-tail sequence does not protect the duplex DNA end in trans. TG24 with increasing concentrations (0.04, 0.2 and 1μM) was added to nuclear extracts containing either H30-CA24 (0.2 μM, panel A), H30-mTG24 (0.2 μM, panel A), H30 (0.2 μM, panel B) and 36 (0.2 μM, panel B). After incubation, the reaction mixtures were analyzed by Western blotting using phospho-p53 (Ser-15)
antibody. The same membrane filter was also immunoblotted with anti-Mre11 antibody to assess equal loading.

Fig 5. The telomeric 3’ G-tail sequence protects the duplex DNA end in cis. (A) 30, TG24-30-TG24 and TG24-30 (see structures in upper panel) were incubated with nuclear extracts. After incubation, the reaction mixtures were analyzed by immunoblotting using phospho-p53 (Ser-15) antibody. The same membrane filter was also immunoblotted with anti-Mre11 antibody to assess equal loading. (B) 30, TG24-30-15T, TG24-30-TG24 and TG24-30-15T-TG24 (see structures in the Fig. 5A, upper panel) were incubated with nuclear extracts. After incubation, the reaction mixtures were immunoblotted as described above.

Fig. 6. A schematic model illustrating the role of the G-quadruplex conformation in telomeric 3’ G-tail-mediated protection of DNA ends. In this model, telomeric 3’ G-tail sequences at the ends of chromosomes exist in two dynamic conformational states. In the single-stranded conformation, the chromosome ends can be efficiently recognized by a key DNA damage regulator(s) (e.g. DNA-PK and ATM) leading to activation of various DNA damage signals. In the G-quadruplex conformation, the chromosome ends are protected (e.g. through steric hindrance) from being accessed by the key DNA damage regulator(s).
Figure 1

(A) 

|       | TEL30 | TEL36 | TEL42 |
|-------|-------|-------|-------|
| 0     | 0     | 2     | 10    |
| 2     | 2     | 10    |       |
| 10    |       |       |       |

NP NE

p53 S15P
Chk2 S33/35P
Chk1 S317P
cAbl S735P
Mre11

(B) 

36 - + + + + + + +

AMA-37 Caff. Wort.

|       | 0     | 0.1   | 1     | 10    | 100   | 5000  | 2     |
|-------|-------|-------|-------|-------|-------|-------|-------|

(μM)
p53 S15P (0.5 mM ATP)
p53 S15P (0.1 mM ATP)
Mre11

(C) 

36 - + + + + + + +

KU 55933

|       | 0     | 0.01  | 0.1   | 1     | 10    | 100   |
|-------|-------|-------|-------|-------|-------|-------|

(μM)
p53 S15P (0.5 mM ATP)
p53 S15P (0.1 mM ATP)
Mre11
Figure 2

(A) 

|       | D30 | H30 | H30-TG24 | H30-mTG24 |
|-------|-----|-----|----------|-----------|
| 0     | 0.01| 0.1 | 0.01     | 1         |
| 0.1   | 1   | 1   | 0.1      |           |
| 1     | 1   | 1   | 1        |           |

**p53 S15P**

Mre11

(B) 

|       | H30-TG24 | H30-CA24 |
|-------|----------|----------|
| 0     | 0.01     | 0.1      |
| 0.1   | 1        |          |
| 1     | 1        | 1        |

**p53 S15P**

Chk2 S33/35P

Chk1 S317P

Mre11

D30

H30

TG24

mTG24

H30-TG24

H30-mTG24

CA24

H30-CA24

TG24: (TTAGGG)$_4$

mTG24: (TTAGTG)$_4$

CA24: (CCCTAA)$_4$

(C) 

|       | H30-mTG24 | mTG24 |
|-------|-----------|-------|
| 0     | 0.04      | 0.02  |
| 0.2   | 0.1       | 0.5   |
| 1     | 2.5       | 12.5  |

**p53 S15P**

Mre11

|       | H30-CA24 | CA24 |
|-------|----------|------|
| 0     | 0.04     | 0.02 |
| 0.2   | 0.1      | 0.5  |
| 1     | 2.5      | 12.5 |

**p53 S15P**

Mre11
Figure 3

(A) 

![Graph showing temperature (T) vs. 1000*dA/dT for TG24 and mTG24.]

(B) 

![Graph showing temperature (T) vs. 1000*dA/dT for H30, H30-TG24, and H30-mTG24.]

Figure 4

(A)

|        | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|--------|---|---|---|---|---|---|---|---|---|----|----|----|
| H30-CA24 |   | + |   |   |   | + | + | + |   |   |   |   |
| H30-mTG24 | + |   | + |   |   |   |   | + | + | + |   |   |
| TG24    | + | + |   |   |   |   |   |   |   |   |   |   |

(B)

|        | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|--------|---|---|---|---|---|---|---|---|---|----|----|----|
| H30 36 |   | + |   |   |   | + | + | + |   |   |   |   |
| TG24  |   |   |   |   |   |   |   |   |   |   |   |   |
Figure 5

(A)  

TG24  
TG24  
TG24-30-TG24  
TG24-30  
TG24-30-15T  
TG24-30-15T-TG24  

30  

| 0 | 0.01 | 0.1 | 1 | 0.01 | 0.1 | 1 | 0.01 | 0.1 | 1 |
|---|------|-----|---|------|-----|---|------|-----|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

p53 S15P  
Mre11

(B)  

TG24-30-TG24  
TG24-30-15T-TG24  

30  

| 0 | 0.01 | 0.1 | 1 | 0.01 | 0.1 | 1 | 0.01 | 0.1 | 1 |
|---|------|-----|---|------|-----|---|------|-----|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

p53 S15P  
Mre11

Figure 6

3' G-Tail in Single-Stranded DNA State  3' G-Tail in G-Quadruplex State

DNA-PK, ATM  
DNA Damage Signaling
Protection of DNA Ends by telomeric 3' G-Tail sequences
Yuan-Chin Tsai, Haiyan Qi and Leroy F. Liu

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