

## Ku Binds Telomeric DNA *in Vitro*\*

(Received for publication, March 26, 1999, and in revised form, May 12, 1999)

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**Ku is a heterodimeric protein with high binding affinity for ends, nicks, and gaps in double-stranded DNA. Both in mammalian cells and in budding yeast, Ku plays a role in nonhomologous end joining in the double strand break repair pathway. However, Ku has a more significant role in DNA repair in mammalian cells compared with yeast, in which a homology-dependent pathway is the predominant one. Recently Ku has been shown to be a likely component of the telomeric complex in yeast, suggesting the possibility of a similar role for Ku at mammalian telomeres. However, long single-stranded G-rich overhangs are continuously present at mammalian but not at yeast telomeres. These overhangs have the potential to fold *in vitro* into G-G base-paired conformations, such as G-quartets, that might prevent Ku from recognizing telomeric ends and thus offer a mechanism to sequester the telomere from the prevalent double strand break repair pathway in mammals. We show here that Ku binds to mammalian telomeric DNA ends *in vitro* and that G-quartet conformations are unable to prevent Ku from binding with high affinity to the DNA. Our results indicate that the DNA binding characteristics of Ku are consistent with its direct interaction with telomeric DNA in mammalian cells and its proposed role as a telomere end factor.**

Ku is a heterodimer of 70- and 80-kDa subunits that was originally identified as an autoimmune antigen and subsequently found to be involved in V(D)J recombination and double strand break (DSB)<sup>1</sup> repair in mammalian systems (for review, see Refs. 1 and 2) (3–8). Ku has sequence-independent affinity for double-stranded DNA ends, whether blunt or 3'- or 5'-recessed, and for internal nicks or gaps (9–13). Binding to single-stranded DNA occurs with lower affinity (3, 11). Sequence-specific DNA and RNA binding has also been reported (14–16), but its *in vivo* significance has not been established. The mechanism of Ku binding to DNA remains largely unknown, but actual DNA ends do not seem to be required, because Ku can bind dumbbell-shaped molecules and circular DNA containing single-stranded bubbles (10). Interestingly, Ku is able to slide internally into the duplex DNA after initial binding to the end (11), and internalized Ku molecules are resistant to high salt conditions after DNA circularization, suggesting the possibility of a doughnut-shaped heterodimer (17).

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<sup>1</sup> The abbreviation used is: DSB, double strand break.

Recently, homologs to both Ku70 and Ku80 have been identified in budding yeast (HDF1/YKU70 and HDF2/YKU80, respectively) (18–20). In this organism, contrary to mammalian cells, a RAD52-dependent homologous recombination pathway is responsible for the majority of the DSB repair events (21). However, as revealed by experiments performed in a *rad52* background, yeast also possesses a DNA repair pathway that is HDF1/HDF2-dependent and involves joining of nonhomologous ends (22–26).

In addition to their role in repair, HDF1 and HDF2 play a role at telomeres. Telomeres are the terminal chromosomal elements (27) and are composed of simple tandem repeats, generally bearing short runs of guanines in the strand that runs with 5'-3' polarity toward the tip of the chromosome (*e.g.* 5'-TTAGGG-3' in vertebrates). Telomeres carry out at least two primary functions. First, through the action of the telomerase enzyme, they circumvent the replicative problem of linear DNA ends (28). Second, they protect the ends of the chromosome from being treated as broken ends by the DSB repair pathway (29–32). Both *hdf1*<sup>-</sup> and *hdf2*<sup>-</sup> cells are partially defective in telomere maintenance, with telomeres in mutant cells shrinking to about 70% of their normal length (20, 33, 34).

Hdf1p/Hdf2p may be an integral component of the yeast telomeric complex. First, HDF1 and HDF2 affect telomere position effect, a phenomenon in which genes located near telomeres are transcriptionally repressed. Telomere position effect in yeast telomeres is dependent on a multiprotein complex that includes Sir2p, Sir3p, and Sir4p (for review, see Ref. 35). Loss of HDF1 or HDF2 has a dramatic effect on telomere position effect, equivalent to impairment of SIR2, SIR3, or SIR4 (34, 36). Second, although the nuclear localization of yeast Ku has not been determined directly, in HDF1<sup>-</sup> cells, the subnuclear localization of telomeres appears to be altered (37). Finally, cross-linking studies indicate that Hdf2p is bound to telomeric DNA *in vivo*, and *hdf2*<sup>-</sup> cells experience strand-specific loss of telomeric DNA (38). How Ku in yeast reconciles its dual role as a telomere end factor and as a DSB repair protein remains to be determined.

The DNA binding activity of Ku suggests that the protein may bind directly to telomeric DNA. However, because of the interaction of Hdf1p with Sir4p in yeast two-hybrid assays (39), it is also possible that in yeast, the interaction of Ku with the telomere is mediated by protein-protein interactions. In addition, the effect of single-stranded telomeric tails on Ku binding to double-stranded DNA remains unknown, especially in the light of the recognized ability of telomeric G-rich strands to adopt G-G base-paired conformations *in vitro* (for review, see Ref. 40). Indeed, telomeric G-strand DNA *in vitro* readily forms, under physiological conditions, a structure in which four guanine residues from four different telomeric repeats are arranged in a planar conformation (G-quartet, or G-DNA) that is stabilized by Hoogsteen-type hydrogen bonds. For any particular telomeric sequence, typically three or four of these planar arrangements are stacked onto each other and are stabilized by Na<sup>+</sup> or K<sup>+</sup> ions loosely coordinated at the center. Several types

of related structures, all belonging to the G-quartet family, have been described, with variability residing on the relative orientation of the four strands (parallel or antiparallel) and the loops connecting them (diagonal or lateral) (41–44).

Mammalian telomeres terminate in long (130–270 base pairs) single-stranded G-rich overhangs (45–47), whereas long (>20 base pairs) overhangs occur at yeast telomeres only briefly in late S-phase (48, 49). Thus, differences in the requirements for telomere end binding activities might exist in the two organisms. In particular, the possibility exists that Ku in mammalian cells is limited to a role in DSB repair and that the G-rich overhangs at mammalian telomeres might be sufficient to sequester telomeric ends from this repair pathway. Our results indicate that the ability of Ku to bind to telomeric DNA ends in different conformations is consistent with a direct interaction of Ku with mammalian telomeric DNA and its proposed role as a telomeric protein.

#### EXPERIMENTAL PROCEDURES

**Purification of Ku from HeLa Nuclear Extracts**—Ku heterodimer was purified to apparent homogeneity (as judged from silver staining of SDS-polyacrylamide gels) from nuclear extracts from HeLa cells (50). Four chromatographic steps were used for the purification (11): DEAE-Fractogel 650 (EM Separations), phosphocellulose (Whatman), double-stranded DNA cellulose (Sigma), and single-stranded DNA cellulose (Sigma).

**Oligonucleotides Preparation and Probe Labeling**—Oligonucleotides were synthesized on an Applied Biosystem DNA synthesizer and gel-purified before use. Labeling of 5'-ends was carried out by T4 polynucleotide kinase and [ $\gamma$ - $^{32}$ P]ATP (3000 Ci/mmol). Annealing of double-stranded substrates was achieved by incubation for 2 h at room temperature and was carried out after labeling and folding of overhangs when appropriate. Folding of overhangs was induced by incubating at 90 °C for 3 min and at room temperature for 20 min (41). DMS protection assays were as described in Ref. 41.

**DNA Binding Assays and Gel Electrophoresis**—Binding reactions were carried out at room temperature for 15 min. Reactions described in Fig. 2 were carried out in 20 mM Hepes, pH 7.75, 2 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 0.25 mM dithiothreitol, 200 mM KCl, and 7% glycerol. All other reactions were performed in 10 mM Tris, pH 8.0, 0.1 mM EDTA, with or without added salts, as indicated below in the individual experiments, and glycerol (3% final concentration) was added prior to loading on gel. Electrophoresis was performed in 5% acrylamide (29:1), on 20 cm gels in 1× TBE at 130 V for 2 h at room temperature. Quantitation of experiments was performed by phosphorimaging using ImageQuant software.

#### RESULTS

**Ku Binds to Telomeric DNA Ends *in Vitro***—Given the DNA-end binding activity of Ku and its suggested role at yeast telomeres, it was of interest to investigate the ability of Ku to bind to telomeric DNA *in vitro*. Oligonucleotides were synthesized terminating in the human telomeric TTAGGG repeats in different configurations, both 3' protruding and blunt-ended (Fig. 1, T1, T2, and T3). The affinity of Ku for the various substrates in band-shift assays was then compared with the affinity for an oligonucleotide of similar size but of random sequence (Fig. 1, R1). Two representative binding experiments performed with the control DNA and the three telomeric DNAs as competitors are shown in Fig. 2. In these and in other experiments (data not shown), the telomeric oligonucleotides competed efficiently for Ku binding to either the telomeric DNA T1 (Fig. 2a) or the control DNA R1 (Fig. 2b). The telomeric substrates, in fact, proved to be slightly better competitors than the random sequence DNA. T3 in particular performed as a good competitor. This result is in agreement with the known preference of Ku for the longer double-stranded regions (10). Thus, these results show that DNAs terminating with telomeric sequence are able to bind Ku with an affinity comparable to that displayed by molecules bearing nontelomeric DNA ends.

*Terminally Located G-quartet Structures Do Not Prevent Ku*



FIG. 1. DNA oligonucleotides used in binding and competition experiments with Ku. Telomeric sequences are indicated in boldface.

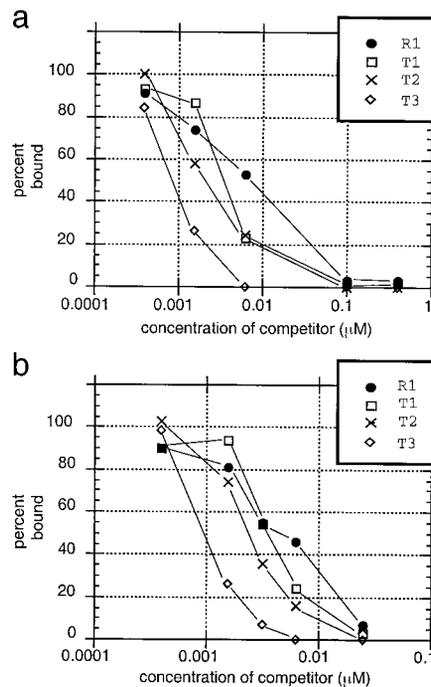
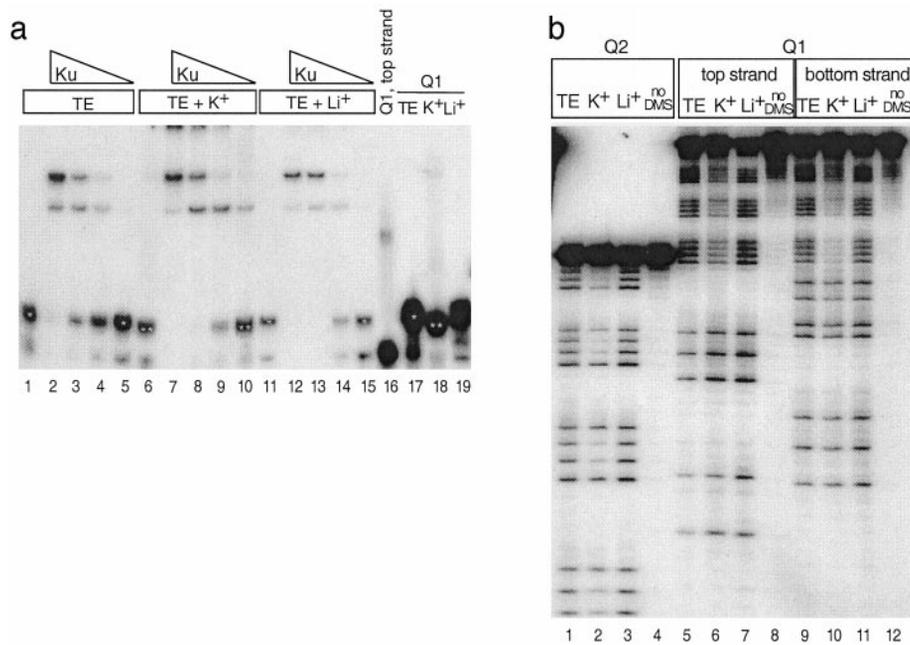


FIG. 2. Competition assays for Ku binding with telomeric and nontelomeric DNA oligonucleotides. Labeled oligonucleotides T1 (a) and R1 (b) were present at concentrations of about 1.5 and 0.5 nM, respectively, and were incubated in the presence of DNA competitors and about 0.2 nM (a) or 0.05 nM Ku (b).

**from Binding to DNA Ends**—Because single-stranded telomeric sequences are known to adopt G-G base-paired structures *in vitro* (for review, see Ref. 40) (41–44), we next determined whether Ku can bind to DNA molecules terminating with telomeric sequences folded in a G-quartet conformation. To this end, we constructed duplex DNA molecules bearing 3.5 copies of the telomeric repeat from *Oxytricha nova* (TTTT-GGGG) in single-stranded form at each DNA end (Fig. 1). Although human telomeric sequences have been shown to fold into G-quartets (51–53), the *Oxytricha* telomeric repeat was chosen because it is the one that more readily adopts an intramolecular G-quartet conformation *in vitro* (41, 44, 54–56). Because intramolecular G-quartets are dependent on the presence of K<sup>+</sup> or Na<sup>+</sup>, but are not formed in the presence of Li<sup>+</sup> (41), the annealed oligonucleotides were incubated either in the



**FIG. 3. Binding of Ku to DNA molecules terminating in G-DNA.** *a*, labeled Q1 at a concentration of about 60  $\mu$ M was incubated in the presence of 0.06 (lanes 5, 10, and 15), 0.2 (lanes 4, 9, and 14), 0.6 (lanes 3, 8, and 13), and 1.7 nM Ku (lanes 2, 7, and 12). Reactions were in TE (Tris, pH 8.0, EDTA 0.1 mM) with no salt (lanes 1–5), 50 mM KCl (lanes 6–10), or 50 mM LiCl (lanes 11–15). No protein and only 3% glycerol (final concentration) were added to samples 16–19 prior to loading. The single asterisks mark the migration rate of the DNA in the absence of salts or in the presence of Li<sup>+</sup>. The double asterisks indicate the faster mobility of the probe observed in the presence of K<sup>+</sup>. *b*, oligonucleotides Q2 (lanes 1–4), Q1 labeled in the top strand (lanes 5–8), and Q1 labeled in the bottom strand (lanes 9–12) at a concentration of 1.5–4 nM were treated with DMS in TE (lanes 1, 5, and 9), TE plus 50 mM KCl (lanes 2, 6, and 10), or TE plus 50 mM LiCl (lanes 3, 7, and 11). Lanes 4, 8, and 12 are controls, with no DMS treatment but with piperidine cleavage.

absence of any cation or in the presence of either K<sup>+</sup> or Li<sup>+</sup>. When K<sup>+</sup> was present, the duplexed oligonucleotides displayed an increased migration rate, consistent with the folding of the two single-stranded tails into a G-quartet conformation (Fig. 3*a*, compare lanes 17 and 18, single asterisk versus double asterisk) (41, 57). Although incubation in Li<sup>+</sup> sometimes gave rise to a double band (Fig. 3*a*, lane 19), the mobility of the oligonucleotide was slower compared with the mobility observed after incubation in K<sup>+</sup> and was comparable to the mobility of the DNA incubated in TE without ions (Fig. 3*a*, lanes 17–19). In addition, when we employed a chemical protection assay to further verify the structure of the terminal tails, both strands showed K<sup>+</sup>-dependent protection of the terminal G<sub>4</sub> arrays (Fig. 3*b*, lanes 5–12), consistent with the fact that the N-7 position in G-G base-paired structures is protected from modification by DMS. Also in agreement with the methylation protection pattern of intramolecular G-quartets was the observation that the protection was slightly greater at the 2 central Gs of each arrays (41).

Having verified the folding of the terminal tails into a G-quartet conformation in the presence of K<sup>+</sup>, we then performed binding assays with the oligonucleotide in different ionic environments to determine whether a change in affinity for Ku existed upon conditions that promote G-quartet formation. Interestingly, Ku showed essentially identical affinity for the DNA, whether it was folded in G-quartet conformation or not (Fig. 3*a*, lanes 1–15). Appropriate control experiments were performed with oligonucleotide R2 to verify that the affinity of Ku for DNA under the different ionic conditions employed did not vary significantly (data not shown).

The K<sup>+</sup>-dependent change in electrophoretic mobility of oligonucleotide Q1 (Fig. 3*a*, lanes 17–19) was still apparent with samples to which Ku had been added (Fig. 3*a*, compare lanes 5 and 6, and lanes 10 and 11), suggesting that the presence of Ku protein preparation had no effect (folding or unfolding) on the structure of the G-tails. Nevertheless, we considered the pos-

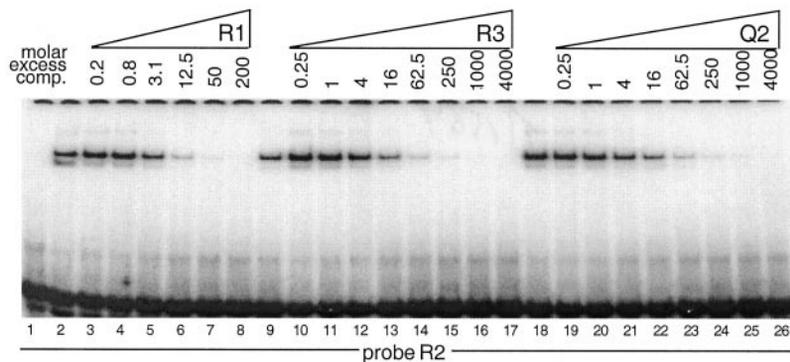
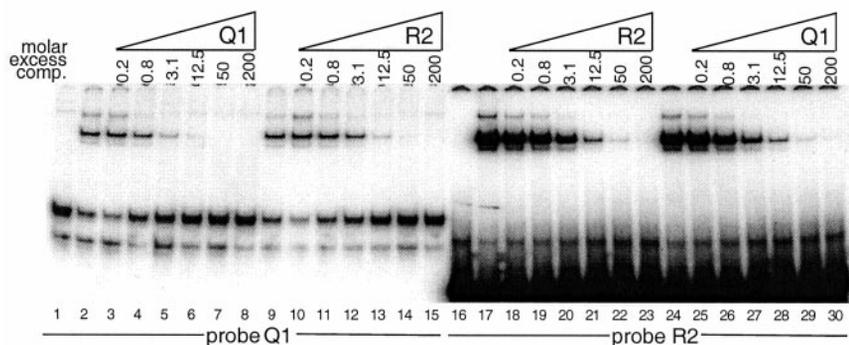
sibility that the 5 mM KCl introduced into the reactions upon addition of Ku protein could have induced G-quartet formation in Q1 DNA, thus hindering our comparison of folded versus unfolded DNA. We therefore performed reciprocal competition experiments with the folded oligonucleotide Q1 and the control oligonucleotide R2 (note that the two molecules have duplex regions of nearly equal length: 33 and 30 nucleotides, respectively). Both probes were incubated with Ku in the presence of K<sup>+</sup> and a molar excess of either cold competitor DNA (Fig. 4). Both DNAs competed to the same extent for Ku binding, indicating that Ku binds to either substrate with very similar affinities, thus supporting our conclusion that terminally located G-quartets do not affect the binding of Ku to the duplex.

*Ku Binds G-quartets with Low Affinity Compared with Double-stranded DNA*—To see whether Ku has the ability to bind to G-quartet structures *per se*, we performed competition experiments with an oligonucleotide entirely composed of four copies of the *Oxytricha* telomeric repeat (Fig. 1, Q2). This molecule displays the expected K<sup>+</sup>-dependent ability to adopt an intramolecular G-quartet conformation as judged by gel electrophoresis (data not shown) and DMS protection (Fig. 3*b*, lanes 1–4). Interestingly, Q2 behaves practically identically to a 33 nucleotides-long single-stranded oligonucleotide of random sequence in its ability to compete for Ku binding (Fig. 5, lanes 10–17, 19–26). Both of these DNAs are about 20 times less effective as competitors compared with the 31-base pair-long double-stranded R2. Thus these results show that Ku binds to G-quartet structures inefficiently, with affinity similar to that of single-stranded DNA.

## DISCUSSION

*Ku as a Component of the Mammalian Telomeric Complex*—The finding that Ku in yeast plays an important role at telomeres raises the obvious possibility that it might exert a similar function in mammalian cells. However, significant differences exist between yeast and mammalian telomeres.

**FIG. 4. Comparison of Ku DNA binding affinities for G-DNA capped DNA and double-stranded DNA.** Labeled oligonucleotides R2 (lanes 16–30) and Q1 (lanes 1–15) were present at a concentration of 250 pM, and Ku was about 200 pM. Binding reactions were carried out in TE plus 50 mM KCl. The amount of competitor (in molar excess) is indicated at the top of each lane.



**FIG. 5. Comparison of Ku DNA binding affinities for G-DNA, double-stranded DNA, and single-stranded DNA.** Labeled oligonucleotide R2 was present at a concentration of 250 pM, and Ku was about 200 pM. Binding reactions were carried out in TE plus 50 mM KCl. The amount of competitor (in molar excess) is indicated at the top of each lane.

Whereas in yeast, no tails are detectable by an in-gel hybridization assay, setting the upper limit for telomeric overhangs at about 20 nucleotides (48, 49), long G-rich overhangs (130–270 nucleotides) are observed at mammalian telomeres (45–47). Thus the biochemical requirements for binding to chromosome ends may be different in the two systems. In particular, the G-rich tails found at mammalian telomeres might adopt G-G base-paired conformations. Although it is not clear whether such DNA structures are formed *in vivo*, G-G base-paired structures are readily adopted *in vitro* by most telomeric repeats under physiological conditions (for review, see Ref. 40). Interestingly, plasmids bearing G-rich overhangs are found to self-associate *in vivo* in yeast, presumably due to the formation of G-G base pairs at the overhangs (49). In addition, the *O. nova* telomeric protein  $\beta$  and Rap1p from *Saccharomyces cerevisiae* promote the formation of G-DNA at telomeric sequences *in vitro* (58, 59). Thus, G-quartet structures may exist at eukaryotic telomeres, possibly in a transient manner in some cell types. Our findings suggest that the long overhangs found at mammalian telomeres, even if folded into G-quartet structures, will not prevent Ku from binding to the DNA. Therefore, the biochemical properties of mammalian Ku are consistent with it being a telomeric protein, like its yeast counterpart.

One of the main unanswered questions in telomere biology is how telomere termini escape becoming a substrate of DNA repair activities. Our data argue against a potential simple solution to this problem: that the binding of Ku to natural chromosome ends is blocked by the unusual structure and sequence of telomere termini. The *in vitro* binding of Ku to a variety of alternate telomeric substrates renders this explanation unlikely and further substantiates the opposite view that Ku may actually be (possibly transiently) bound to telomeres *in vivo*. Clearly, a definitive answer to the role of Ku at mammalian telomeres, if any, requires functional or cytological evidence. Our attempts at identifying Ku at telomeres in human and rodent cell lines have not resulted in proof for co-localization of Ku with the telomeric protein TRF1 (data not shown). However, these experiments are not conclusive, because a signal at the telomere might be masked by the abundance of Ku

throughout the nucleus and because of the expected scarcity of an end factor at the telomere, possibly below the detection limits of immunofluorescence techniques.

The prospect of Ku being positioned at mammalian chromosome ends raises the question of how this telomere bound Ku is prevented from performing its function in the DSB repair. This dilemma also applies to Ku bound at yeast telomeres and to other components of the DNA repair pathway (RAD50, MRE11, and XRS2), for which a role in telomere maintenance and therefore possible binding to telomere termini has recently been demonstrated (34).

**Mechanism of Ku Binding to DNA**—The mechanism of Ku binding to DNA is not known. The search for a common requirement in the various DNA substrates that are bound with high affinity by Ku has led to the suggestion that Ku might recognize the transition from single- to double-stranded DNA (10, 61). This conclusion is based on the fact that free 3'- or 5'-ends are not needed for binding and is supported to some extent by the observation that Ku appears to bind more tightly to oligonucleotides terminating with an AT-rich sequence as opposed to ones terminating in a G-rich sequence, suggesting that possibly the melting of the terminal base pairs provides the substrate for Ku recognition (10). In addition, a Ku DNA-helicase activity that could help in generating such substrate has been reported (60). However, this model for DNA binding is not consistent with our finding that Ku is able to bind with high affinity to interstrand terminally cross-linked (with psoralen) DNA molecules (data not shown).

In the case of the G-DNA substrates used in this work (which all have free 3'- and 5'-ends) it appears likely that the recognition occurs at either the free 3'- or the free 5'-end, or possibly in the T<sub>4</sub>-loop region. With recognition occurring at the 3'-end or in the T<sub>4</sub>-loop only, stabilization of Ku binding on the DNA duplex would appear likely to require unfolding of the G-quartet with lower on-rate and affinity as a likely consequence. Because this was not observed, we favor the hypothesis that it is the free 5'-end that is recognized. Thus, even though our experiments do not address the state of the folded overhang after Ku binding, we suggest that Ku is able to recognize the

junction between double-stranded DNA and G-quartet without the necessity to unfold the structure.

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