Mycobacterium smegmatis Ku binds DNA without free ends

Ambuj K. KUSHWAHA* and Anne GROVE*1

*Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, U.S.A.

INTRODUCTION

Ku is a ‘hallmark’ protein of the NHEJ (non-homologous end-joining) repair pathway of DSB (double strand break) repair, and homologues have been identified in all three major domains of life. It is present throughout eukaryotes, sporadically distributed in phylogenetically diverse prokaryotes, and it has also been documented in two archaeal species, Archeoglobus fulgidus and Methanococilla paludicola [1–7]. Eukaryotic Ku proteins are heterodimers consisting of two subunits, Ku70 and Ku80 that together form a functional unit [8]. The crystal structure of human Ku shows that it has a tripartite organization consisting of an N-terminal α/β von Willebrand factor A domain, a central β-barrel domain and a subunit-specific C-terminal SAP domain that together form a pseudosymmetrical ring-like channel that can accommodate a DNA duplex [9]. In contrast, the prokaryotic Ku proteins are homodimers [1,4,5] and much smaller (30–40 kDa), consisting of the conserved central ‘ring-shaped’ core domain of eukaryotic Ku and lacking the N- and C-terminal domains present in Ku70/80, except for homologues from a few bacteria such as Streptomyces coelicolor that contain a SAP-like domain [1,4,5]. Also, Ku proteins from free-living mycobacterial species found in soil and natural reservoirs contain at their C-terminus low complexity repeats containing the amino acids lysine, alanine, and proline that promote DNA end-joining [10].

The role of Ku in NHEJ-mediated DNA repair has been extensively studied in eukaryotes and in selected prokaryotes, such as Bacillus, Mycobacterium and Pseudomonas [4,5,11–16]. Despite its importance in DSB repair, Ku is not ubiquitous among prokaryotes and many of the prokaryotes that encode a Ku homologue spend much of their life cycle in stationary phase during which no cell division occurs and cells may be exposed to desiccation and genotoxic agents that lead to DSBs [4,17–20]. Eukaryotic Ku is multifunctional as, in addition to its central role in repair of DSBs [21–24], it has also been reported to be involved in cellular processes such as antigen–receptor gene rearrangements, apoptosis, transcription, mobile genetic element biology, telomere maintenance, aging/senescence, cell adhesion and cell/microenvironment interaction (for a review, see [8]). In comparison with eukaryotic Ku, which is functionally diverse due to its N- and C-terminal domains, prokaryotic Ku proteins have been shown to play a role in NHEJ only [9,25].

As a participant in NHEJ-mediated DSB repair, Ku is a sequence-independent DNA end-binding protein as shown by in vitro and in vivo analyses [26,27]. In apparent contradiction to this binding mode, reports have implicated eukaryotic Ku in regulation of gene expression, which would imply an interaction with intact chromosomes [28]. Such binding to internal sites could perhaps involve association with local secondary structure in genomic DNA. Also, in vitro binding studies with circular single-stranded DNA and DNA containing hairpin loops at both ends demonstrated that eukaryotic Ku could bind to a ‘closed’ substrate lacking free ends with no significant difference in binding affinity compared with DNA with free ends [27,29]. Recently, a homologue of eukaryotic Ku from Deinococcus radiodurans was shown to bind supercoiled DNA with 67-fold higher affinity as compared with linear DNA, also supporting the notion that DNA binding by Ku is probably more complex than originally thought, and consistent with the possibility that Ku may associate with local secondary structures [30]. Furthermore, we recently demonstrated that the lysine-rich C-terminal repeats of M. smegmatis Ku confer on the protein the ability to promote DNA end-joining, consistent with its role in NHEJ [10]. Although a contribution to DNA end-joining and interaction with short DNA duplexes suggested that the positively charged C-terminal extension may contact DNA directly, its removal enhances DNA-binding affinity of the truncated protein, indicating that extensions may modulate DNA binding by the core domain [10]. To address the role of the C-terminal extension in DNA binding, we explored the binding of M. smegmatis Ku to diverse DNA substrates. With properties such as the ability to translocate to internal DNA sites as well as direct binding to DNA without free ends, M. smegmatis Ku

Key words: DNA binding, Ku protein, low complexity repeat, supercoiled DNA, thermal stability, tryptophan fluorescence.
differs significantly from other prokaryotic Ku homologues and is instead more similar to eukaryotic Ku in its association with DNA. The observed binding mode also suggests that extensions beyond the shared core domain may have independently evolved to expand Ku function.

**MATERIALS AND METHODS**

**EMSA**

*M. smegmatis* Ku and TKu (truncated Ku) lacking the C-terminal repeats were purified as described previously [10]. Oligodeoxyribonucleotides (50 bp) used to generate duplex DNA constructs were purchased and purified by denaturing PAGE. The top strand (5′-TTCAA TCCCCGTCTGTC-CCCCGA TCCCCTGCTCGTAGGCGTGCTTGACCG-3′) was ³²P-labelled at the 5′-end with phage T4 polynucleotide kinase. Equimolar amounts of complementary oligonucleotides were mixed, heated to 90°C and cooled slowly to room temperature (23°C) to form duplex DNA. ³²P-Labelled DNA (5 nM) was titrated with TKu in a total reaction volume of 10 μl in reaction buffer [25 mM Tris/HCl (pH 8), 50 mM NaCl, 0.1 mM Na₂EDTA, 0.05% Triton X-100, 5 mM DTT and 2% (v/v) glycerol]. Reaction mixtures were pre-incubated at room temperature for 1 h. A non-denaturing 8% polyacrylamide gel was pre-run for 30 min at 175 V in 0.5 × TBE buffer [45 mM Tris borate (pH 8.3) and 1 mM EDTA], and samples were loaded with the power on. After electrophoresis, gels were dried and visualized by phosphorimaging.

**Exonuclease III protection assay**

A 50 ng amount of NheI-digested 1 kb DNA fragment was incubated with TKu for 40 min at room temperature. To each reaction, 1 μl of exonuclease III (100 units/μl) was added and incubated at room temperature for 1 h. Reactions were terminated by the addition of 1 μl of stop buffer (5 mM Na₂EDTA, 1.1% glycerol and 0.2 mg/ml proteinase K) and 1 μl of 10% SDS. Samples were run on 0.8% TBE agarose gels. Gels were stained with Ethidium Bromide. All reactions were performed in duplicate.

**Protein melting temperature**

Melting temperatures for proteins were determined according to protocols described by Ericsson et al. [31]. Protein was diluted to 5 μM in a buffer containing 50 mM Tris (pH 8.0), 100 mM NaCl and 5 × SYPRO Orange (Invitrogen) as the reference fluorescent dye. Triplicate 50 μl samples were analysed in a 96-well reaction plate. The fluorescence emission was measured over a temperature range of 5–90°C in 1°C increments for 40 s using an Applied Biosystems 7500 Real-Time PCR System (filter, SYBR green). The total fluorescence yield measured was corrected using reaction mixtures without protein. The resulting data were analysed with SigmaPlot 12 and the sigmoidal part of the curve was averaged for each triplicate. The averaged curves were subsequently fit to a four-parameter sigmoidal equation and the $T_m$ values were determined.

**Agarose gel retardation**

Linear DNA was obtained by digesting pUC18 with EcoRI; nicked DNA was obtained by digesting pUC18 with Nt.BstNBI; and relaxed closed circular DNA was obtained by treating pUC18 with Topoisomerase I (Epicentre). Reaction mixtures were incubated at room temperature in 10 μl of reaction buffer containing 50 ng of supercoiled, linear, nicked or relaxed closed circular pUC18 and various amounts of Ku or TKu. Complexes were resolved on 0.7% 1× TAE-agarose gels and electrophoresed at 2.5 V/cm for 3 h in 1× TAE buffer [40 mM Tris acetate and 1 mM EDTA (pH 8.0)]. Gels were stained with Ethidium Bromide after electrophoresis.

**Tryptophan fluorescence**

Intrinsic fluorescence of Ku alone and in the presence of different molar ratios of linear and supercoiled plasmid DNA (pUC18) were
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Figure 2 TKu protects DNA from exonucleolytic cleavage

Lane 1, 50 ng of 1 kb DNA only; lane 2, DNA and exonuclease III; lanes 3, 5 and 7 contains DNA and 50, 100 or 200 nM of TKu; lanes 4, 6 and 8 contains DNA, exonuclease III and 50, 100 or 200 nM TKu.

recorded from 310 to 410 nm with an excitation of 295 nm on a PTI QuantaMaster4/2006SE spectrofluorimeter at 25 °C using a 0.3 cm pathlength cuvette. All experiments were performed with 1.25 μM Ku and TKu in FL buffer [40 mM Tris/HCl (pH 8.0), 0.2 mM EDTA, 0.1 % Brij 58, 100 mM NaCl and 10 mM MgCl₂]. The protein–DNA reaction mixtures were equilibrated for 15 min before fluorescence was measured. The absorbance values of each sample were recorded on a Varian Cary 50 spectrophotometer from 200 to 450 nm to correct for the inner filter effect. The corrections were performed as described previously [32].

Competition assay

A competition assay to determine the binding preference of Ku and TKu for supercoiled and linear DNA was performed using 32P-labelled 190 bp DNA. Supercoiled and linear DNA were gel-purified to eliminate the presence of other DNA species. Reaction mixtures were incubated at room temperature in 10 μl of reaction buffer containing 2 nM 190 bp DNA and a fixed concentration of proteins (400 nM Ku and 50 nM TKu, considering the molecular mass of the monomer) and various amounts of either linear pUC18 (linearized with EcoRI) or supercoiled pUC18. The reaction mixture was incubated at room temperature for 1 h. Samples were resolved on pre-run 8 % polyacrylamide gels at room temperature with 0.5 % TBE running buffer. Complexes were visualized by phosphorimaging.

For competition assays with biotinylated DNA, 5 nM 32P-labelled 37 bp DNA was mixed with increasing concentrations of either doubly biotinylated DNA or unlabelled 37 bp DNA and incubated with 600 nM of streptavidin at 37 °C for 30 min to ensure the binding of streptavidin to biotinylated DNA. The reactions were cooled to room temperature and 10 nM Ku or 3 nM TKu was added to the reaction mixture, followed by incubation at room temperature for 1 h. Samples were resolved and visualized as described above. The sequence of the top strand of 37 bp DNA is 5′-CCTAGGCTACACCTACTCTTTGTAAGAATTAAGCTTC-3′.

RESULTS AND DISCUSSION

The C-terminal extension of M. smegmatis Ku is not required for binding to internal DNA sites

M. tuberculosis Ku, which has no C-terminal extension, binds DNA ends [15]. In contrast, P. aeruginosa Ku, which contains a C-terminal extension whose sequence is unrelated to the low complexity lysine-rich sequence characteristic of M. smegmatis Ku (Figure 1B), forms multiple complexes with long DNA, interpreted to be due to electrostatic interactions with the C-terminal extensions [12]. Deletion of this extension results in two complexes, inferred to correspond to Ku bound to each DNA end. We previously reported that full-length M. smegmatis Ku can form multiple complexes with 37 bp DNA, whereas protein truncated for the C-terminal extension forms two complexes only, seemingly in agreement with the properties of P. aeruginosa Ku [10]. That truncated P. aeruginosa Ku forms only two complexes also implies that this protein cannot translocate from DNA ends to internal DNA sites, a property previously ascribed to eukaryotic Ku [12,33]. To assess whether the same pertains to truncated M. smegmatis Ku (TKu), we incubated TKu with 50 bp DNA that would be of sufficient length to accommodate three TKu molecules given the estimated site size of 15–18 bp [10]. As seen in Figure 1(A), TKu does form three discrete complexes with this DNA; assuming initial binding of TKu only to DNA ends, this suggests that TKu translocates readily to internal DNA sites and that truncation of the C-terminal extension does not prevent such movement. We also incubated linear plasmid DNA with increasing concentrations of TKu under stoichiometric conditions ([DNA] >> Kₐ), following which exonuclease III was added to assess whether DNA ends were protected by TKu. With 7.8 nM
DNA (15.6 nM DNA ends), 50 nM TKu should be more than sufficient to saturate DNA ends, yet even 200 nM TKu was insufficient to provide full protection against exonuclease III. This indicates that DNA protection may be achieved only on saturation of the entire DNA with TKu (Figure 2). This observation also suggests that TKu is not stably or preferably bound at DNA ends, and it is consistent with the inference that TKu translocates to internal DNA sites upon association with DNA ends.

Electrostatic interactions between DNA and the lysine-rich C-terminal extension of Ku may form the basis for both its ability to promote DNA end-joining as well as the formation of additional protein–DNA complexes compared with TKu [10]. Indeed, computational analyses of disordered extensions in DNA-binding proteins have suggested that such appendages may function to interact non-specifically with DNA, thereby enhancing DNA-binding affinity and promoting intersegment transfer from one DNA to another [34]. However, the increased DNA-binding affinity of TKu compared with full-length Ku suggests that the C-terminal extension interferes with optimal interaction between the ring-shaped core domain of Ku and DNA. Such interference could either be the result of the C-terminus contacting DNA ends, thus preventing Ku from engaging DNA via its core domain, or it could be due to the C-terminus partly obscuring the core domain and its access to DNA by protein–protein contacts. The highly charged C-terminus is reminiscent of the lysine-rich repeats in eukaryotic histone H1, which were previously shown to fold only on interaction with DNA [35–37]. By inference, the C-terminal extension of Ku would be expected to be unfolded in solution unless charges are neutralized; that the C-terminus is probably disordered is also a significant prediction rendered by the DISOPRED2 Disorder Prediction Server [38]. If the charge neutralization required for folding derives from interaction with the core domain, a change in protein stability might result. We therefore measured the thermal stability of both full-length Ku and TKu using SYPRO Orange as a reporter of protein unfolding [31]. As shown in Figure 3, the $T_{m}$ for full-length Ku of 44.3 ± 0.3°C is reduced to 41.8 ± 0.1°C on removal of the C-terminus. We therefore infer that the C-terminus is not a separate unstructured domain, but that it interacts with the protein core.

Both Ku and TKu bind DNA without free ends

While the core domain of Ku is expected to associate with DNA ends, there is no a priori reason to suspect that the C-terminal extension binds preferred DNA structures. To assess the binding of the C-terminal domain to different DNA conformations, DNA binding by Ku and TKu was compared using agarose gel retardation assays. Similar to M. tuberculosis Ku [4], both Ku and TKu bound linear and nicked plasmid DNA (Figures 4 and 5). However, whereas the observed interaction of full-length Ku with supercoiled DNA was not unexpected, titration of supercoiled DNA with TKu also resulted in a marked reduction in electrophoretic mobility (Figure 5A). Since DNA supercoiling might lead to extrusion of hairpin structures, TKu was also incubated with relaxed closed circular DNA and again seen to form a complex (Figure 5C). By comparison, M. tuberculosis Ku, which lacks the C-terminal low complexity sequence repeats, preferentially binds to linear DNA compared with circular DNA or single-stranded DNA [4]. To make sure that binding to supercoiled DNA was not an artefact of a nicking activity, both Ku (Supplementary Figure S1 at http://www.biochemj.org/bj/456/bj4560275add.htm) and TKu (results not shown) were tested for the absence of nuclease and nicking activities. Although complexes between full-length Ku and supercoiled DNA may at least in part be due to interaction with the charged C-terminus, the absence of this domain in TKu implies an alternative mode of binding, perhaps involving association with local secondary structure elements in circular DNA.

M. smegmatis Ku can be modelled on the structure of human Ku70/Ku80 (Figure 6). Each monomer contains two tryptophan residues at positions 45 and 208 in the primary amino acid sequence that both are predicted to reside at the base of the central DNA-binding core domain, hence changes in the intrinsic fluorescence of Ku would be expected on DNA binding, as such binding would alter the environment of these fluorophores. In addition, the JCVI (J. Craig Venter Institute) annotation of M. smegmatis Ku, which was used in designing the cloning strategy [10], reflects an N-terminal extension in which an additional tryptophan residue is found. Using an excitation wavelength of 295 nm, the measured emission maximum for Ku and TKu was observed at 336 nm, consistent with an emission signal predominantly due to tryptophan (Figure 7). The significant blue shift compared with the 340–350 nm emission maximum for tryptophan that is fully exposed to an aqueous environment suggests a less polar environment. With linear DNA, a significant increase in fluorescence was observed for both Ku and TKu at a linear DNA/protein molar ratio of 1:1 (considering the molecular mass of dimeric Ku; corresponding to a 2:1 ratio of free DNA ends to Ku) with an additional increase in fluorescence for a molar ratio

![Figure 4](Image)

**Figure 4** Binding of Ku to linear, supercoiled and nicked DNA

(A) Ku binding to linear and supercoiled DNA. Lane 1, 50 ng of linear DNA only; lanes 2–5, linear DNA with increasing concentrations (100, 200, 400 and 800 nM) of Ku; lane 6, 50 ng of supercoiled DNA only; lanes 7–10, supercoiled DNA with increasing concentrations (100, 200, 400 and 800 nM) of Ku. (B) Ku binding to nicked DNA. Lane 1, nicked DNA only; lanes 2–5, nicked DNA with increasing concentrations (100, 200, 400 and 800 nM) of Ku.
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Figure 5  Binding of TKu to linear, supercoiled, nicked and covalently closed relaxed DNA

(A) TKu binding to linear and supercoiled DNA. Lane 1, 50 ng of linear DNA only; lanes 2–7, linear DNA with increasing concentrations (500, 1000, 1500, 2000, 2500 or 3000 nM) of TKu; lane 8, 50 ng of supercoiled DNA only; lanes 9–14, supercoiled DNA with increasing concentrations (500, 1000, 1500, 2000, 2500 or 3000 nM) of TKu. (B) TKu binding to nicked DNA. Lane 1, nicked DNA only; lanes 2–4, nicked DNA with increasing concentrations (400, 800 or 1600 nM) of TKu. (C) TKu binding to relaxed closed circular DNA. Lane 1, relaxed closed circular DNA only; lanes 2–4, relaxed closed circular DNA with increasing concentrations (400, 800 or 1600 nM) of TKu.

Figure 6  Model of M. smegmatis Ku

Predicted structure of the M. smegmatis Ku homodimer. Each monomer (in light and dark grey) is modelled on template strands 1JEY-A and 1JEY-B (PDB codes) with sequence identities of 11 and 13% respectively. Tryptophan residues are shown in black and marked by an arrow. Double-stranded DNA is shown in grey. The image was prepared with PyMOL (http://www.pymol.org).

Several amino acid side chains (and water molecules) quench the fluorescence from the indole ring of tryptophan; the increase in fluorescence intensity on DNA binding, irrespective of DNA conformation, might be due to steric shielding of tryptophan residues resulting from a change in protein conformation that alters the environment of the fluorophores [39]. For both proteins, addition of DNA ultimately results in a significant increase in fluorescence intensity, consistent with the inferred location of tryptophan residues at the base of the DNA-binding core. The co-crystal structure of human Ku and DNA revealed that the non-sequence-specific binding of Ku to DNA is due to the interaction of basic amino acids with the negatively charged DNA phosphate backbone, whereas no direct contact with the nucleobases were seen [9]. M. smegmatis Ku also binds DNA without sequence specificity; interaction between positively charged residues near tryptophan and the negatively charged DNA phosphate backbone might shield tryptophan residues from solvent due to closer proximity to the apolar base pairs, thereby resulting in the observed increase in fluorescence. Also, if the C-terminal extension contacts the protein core, as inferred from both its ability to change protein stability and DNA-binding affinity, then association with DNA may disrupt or alter such interactions and contribute to changes in the environment of tryptophan residues. We also note that the fluorescence yield of Ku in the absence of DNA is significantly greater than that of TKu (Figure 7), consistent with the interpretation that the Ku C-terminal domain contacts the protein core and alters the environment of tryptophan residues. The data also show that the increase in fluorescence of Ku and TKu is comparable on interaction with linear and supercoiled DNA, indicating that both proteins bind both linear and supercoiled DNA.

To validate this observation further, a competition assay was performed to compare the binding of M. smegmatis Ku to linear and supercoiled DNA. Full-length Ku bound linear and supercoiled DNA with similar preference as evidenced by the observation that both supercoiled and linear DNA competed for binding to 190 bp DNA at equivalent concentrations, as indicated by the disappearance of the preformed complex (Figure 8). A competition assay with TKu similarly showed no preferential binding to either linear or supercoiled DNA (Supplementary Figures S2 and S3 at http://www.biochemj.org/bj/456/bj4560275add.htm). Considering that the core DNA-binding domain of Ku is threaded...
on to DNA, these data suggest that Ku can bind local secondary structures that can form in circular DNA.

A competition assay was also performed using 37 bp biotinylated DNA blocked at both ends by binding to streptavidin. The preformed complex formed between full-length Ku and 190 bp DNA was competed out by both biotinylated DNA bound to streptavidin and 37 bp unmodified DNA duplex, as shown by both a decrease in the amount of complex and an increase in free DNA (Figure 9A). Unmodified DNA was more efficient at competing for binding to the 190 bp DNA, suggesting that Ku can bind the ends of unmodified DNA through its core domain in addition to associating via its C-terminal extension. By comparison, the preformed complexes with TKu were not efficiently competed out by biotinylated DNA bound to streptavidin, but only by unmodified 37 bp duplex (Figure 9B). TKu has been shown to require a minimum of 14 bp for DNA binding, whereas Ku can bind to 7 bp of DNA [10]. Hence, to rule out the possibility that doubly biotinylated DNA has insufficient space for TKu binding at internal sites as a possible reason for the inefficient competition, a competition assay using Dps-1 protein, which requires a minimum of 22 bp of DNA to bind, was performed. This experiment showed efficient binding of Dps-1 to doubly biotinylated DNA, indicating that at least 22 bp duplex is available for protein binding (results not shown) [40]. We therefore conclude that TKu requires free DNA ends for binding to linear DNA, whereas full-length Ku does not, a property conferred by the C-terminal extension. The 37 bp DNA would be too short to adopt the secondary structure elements that might occur in circular DNA, thus imposing a requirement for free DNA ends for binding by TKu, whereas full-length Ku can bind via its C-terminal extension.

Conclusion

Consistent with a circular DNA-binding motif, Ku preferentially associates with the ends of linear DNA. However, additional properties ascribed to eukaryotic Ku include the ability to translocate inwardly from DNA ends and the ability to bind DNA whose ends are closed with hairpin loops [27,33]. Ku
from *P. aeruginosa* and *M. tuberculosis* differ; *P. aeruginosa* Ku deleted for its C-terminal extension forms two complexes with linear DNA, implying that it cannot translocate from DNA ends [12]. *M. tuberculosis* Ku, which does not have a C-terminal extension, does not bind supercoiled DNA, and it forms only two complexes with 66 bp DNA, suggesting that it cannot translocate to internal DNA sites either, since 66 bp DNA should be of sufficient length to accommodate at least three Ku molecules [4,12]. *M. smegmatis* Ku lacking its C-terminal extension appears to be more similar to eukaryotic homologues in its interaction with DNA; it requires free DNA ends for binding to short duplexes, but it can readily translocate to internal DNA sites (Figures 1 and 9). Such movement would be expected to facilitate association of the cognate ligase with DNA ends.

TKu can also bind internal DNA sites in plasmid DNA. This mode of binding, which is most probably due to association with local secondary structure elements, is reminiscent of the reported ability of eukaryotic Ku to bind DNA whose ends are closed with hairpin loops [27,33]. The C-terminal extension also contacts internal DNA sites as shown by binding to shorter duplexes and the formation of additional complexes compared with TKu (Figure 9A) [10]. This extension does not appear to bind preferred DNA structures; considering the similarity to the C-terminus of eukaryotic histone H1, we imagine that this extension likewise folds into a helical segment upon association with DNA and contacts the DNA major groove [37]. Such contacts with DNA may impose a 2D search and facilitate recruitment to sites of DNA damage.

**AUTHOR CONTRIBUTION**

Ambuj Kushwaha performed experiments. Ambuj Kushwaha and Anne Grove contributed to experimental design and data analysis, and to writing the paper.

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SUPPLEMENTARY ONLINE DATA

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Ambuj K. KUSHWAHA* and Anne GROVE*1

*Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, U.S.A.

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**Figure S1 Test for nuclease or nicking activity**

(A) Ku binding to supercoiled and linear DNA. DNA and protein were incubated as described in the Materials and methods section of the main text. Lane 1, 100 nM supercoiled DNA only; lanes 2–4, supercoiled DNA with increasing concentrations (700, 1400 or 2100 nM) of Ku; lane 5, 100 nM linear DNA only; lanes 6–8, linear DNA with increasing concentrations (700, 1400 or 2100 nM) of Ku. (B) Phenol-extracted supercoiled and linear DNA after incubation with Ku. Lane 1, supercoiled DNA only; lanes 2–4, purified supercoiled DNA after incubation with increasing concentrations (700, 1400 or 2100 nM) of Ku; lane 5, linear DNA only; lanes 6–8, purified linear DNA after incubation with increasing concentrations (700, 1400, 2100 or 2800 nM) of Ku; lane 10, supercoiled DNA only; lane 11, linear DNA only; nicked DNA (supercoiled pUC18 treated with Nt.BstNBI).

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**Figure S2 DNA competition assay**

TKu binds to supercoiled DNA. Reaction mixtures contain 2 nM DNA (190 bp) and 50 nM TKu. Lane 1, DNA only; lane 2, DNA and TKu (50 nM); lanes 3–12, DNA and TKu with increasing concentrations (2, 4, 6, 8, 10, 12, 14, 16, 18 or 20 nM) of supercoiled pUC18.

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**Figure S3 DNA competition assay**

TKu binds to linear DNA. Reaction mixtures contain 2 nM DNA (190 bp) and 50 nM TKu. Lane 1, DNA only; lane 2, DNA and TKu (50 nM); lanes 3–12, DNA and TKu with increasing concentrations (2, 4, 6, 8, 10, 12, 14, 16, 18 or 20 nM) of linear pUC18.

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1 To whom correspondence should be addressed (email agrove@lsu.edu).

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