Mechanics of DNA bridging by bacterial condensin MukBEF *in vitro* and *in singulo*

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Structural maintenance of chromosome (SMC) proteins comprise the core of several specialized complexes that stabilize the global architecture of the chromosomes by dynamically linking distant DNA fragments. This reaction however remains poorly understood giving rise to numerous proposed mechanisms of the proteins. Using two novel assays, we investigated real-time formation of DNA bridges by bacterial condensin MukBEF. We report that MukBEF can efficiently bridge two DNAs and that this reaction involves multiple steps. The reaction begins with the formation of a stable MukB–DNA complex, which can further capture another protein-free DNA fragment. The initial tether is unstable but is quickly strengthened by additional MukBs. DNA bridging is modulated but is not strictly dependent on ATP and MukEF. The reaction revealed high preference for right-handed DNA crossings indicating that bridging involves physical association of MukB with both DNAs. Our data establish a comprehensive view of DNA bridging by MukBEF, which could explain how SMCs establish both intra- and interchromosomal links inside the cell and indicate that DNA binding and bridging could be separately regulated.

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Introduction

Chromosomes of all living cells are folded into a highly ordered structure with multilevel organization. The global folding of the chromosome is mediated by structural maintenance of chromosome (SMC) proteins, which stabilize the higher-order chromatin architecture by bringing distant DNA segments together (Holmes and Cozzarelli, 2000; Huang *et al*, 2005; Nasmyth and Haering, 2005; Hirano, 2006; Rybenkov, 2009). SMCs are highly conserved across all kingdoms of life (Cobbe and Heck, 2004) and form in solution the distinctive V-shaped structure, wherein two globular ATP-binding domains are connected through long coiled coils joint at the

hinge (Melby *et al*, 1998; Anderson *et al*, 2002; Matoba *et al*, 2005). SMC proteins comprise the core of several multi-subunit complexes with specialized functions (Huang *et al*, 2005; Nasmyth and Haering, 2005; Hirano, 2006). In particular, condensins and cohesins have central roles in supporting, respectively, chromosome condensation and sister chromatid cohesion before cell division (Huang *et al*, 2005; Nasmyth and Haering, 2005; Hirano, 2006).

How SMCs control DNA architecture remains unknown. SMC complexes are widely believed to act by trapping DNAs within a large protein ring. Accordingly, isolated yeast minichromosomes were found topologically entrapped within cohesin rings (Haering *et al*, 2008). In contrast, biochemical studies strongly argue that condensins physically associate with DNA (Hirano and Hirano, 2004; Strick *et al*, 2004; Stray *et al*, 2005; Petrushenko *et al*, 2006a, b; Cui *et al*, 2008). The apparent disparity between these two lines of enquiry led to the suggestion that condensins and cohesins might use fundamentally different mechanisms to organize DNA. However, the high structural and functional similarity between the proteins prompts the search for a common mechanism.

This search is hampered by difficulties in reconstitution of DNA organization *in vitro*. Purified condensins displayed a number of DNA reconfiguring activities, including DNA supercoiling, looping, chiral knotting and condensation (Kimura *et al*, 1999; Strick *et al*, 2004; Stray *et al*, 2005; Petrushenko *et al*, 2006a; Cui *et al*, 2008). Given these activities, however, it is not immediately clear how the proteins carry out their presumed intracellular task of controlled DNA bridging. And although several studies clearly demonstrated the ability of condensins to bring distant DNA fragments together, the observed DNA bridging was invariably overshadowed by other DNA reshaping activities of the complex.

We examined here DNA bridging by MukBEF. MukBEF plays a key role in organizing the chromosome of *Escherichia coli* (Niki *et al*, 1992; Yamanaka *et al*, 1996; Sawitzke and Austin, 2000; Danilova *et al*, 2007; She *et al*, 2007) and is a bacterial prototype of eukaryotic condensins and cohesins. The DNA remodelling activities of the complex reside in its SMC subunit MukB (Petrushenko *et al*, 2006a, b), whereas the kleisin MukF (Fennell-Fezzie *et al*, 2005) and MukE form a stable complex, MukEF, which dynamically interacts with the head domain of MukB (Yamazoe *et al*, 1999; Matoba *et al*, 2005; Petrushenko *et al*, 2006b; Woo *et al*, 2009) and modulates its activity *in vitro* and *in vivo* (Petrushenko *et al*, 2006b; Wang *et al*, 2006; She *et al*, 2007).

Composition of the functional MukBEF remains unclear. Reconstitution and structural studies identified two distinct complexes with stoichiometries $B_2(E_2F)_2$ and $B_2(E_2F)_1$ (Petrushenko *et al*, 2006b; Woo *et al*, 2009). On the basis of symmetry argument, $B_2(E_2F)_2$ is often assumed to be the physiological form of MukBEF. Several observations, however, challenge this straightforward interpretation. Some of

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the key findings of these studies are (i) the asymmetric affinity of the dimeric MukB towards its two MukEF units (Petrushenko *et al*, 2006b); (ii) the finding that ATP or even removal of magnesium displaces one of MukEFs from MukBEF, yielding the $B_2(E_2F)_1$ complex (Petrushenko *et al*, 2006b; Woo *et al*, 2009); and (iii) dramatic decline in DNA-binding activities of the complex upon $B_2(E_2F)_1$ to $B_2(E_2F)_2$ transition *in vitro* (Petrushenko *et al*, 2006b) and, apparently, *in vivo* (She *et al*, 2007). These data indicate that functional MukBEF has stoichiometry $B_2(E_2F)_1$, perhaps formed only transiently during operation of the complex, or, at least, that assembly of MukBEF–DNA complex involves disruption of MukB–MukEF interface.

MukB can condense purified DNA (Petrushenko *et al*, 2006a; Cui *et al*, 2008) and entire chromosomes (Wang *et al*, 2006) and, similar to other condensins (Kimura *et al*, 1999; Strick *et al*, 2004; Stray *et al*, 2005), traps large DNA loops (Petrushenko *et al*, 2006a). A recent magnetic tweezers study revealed that MukB binds DNA in a highly cooperative manner producing multi-molecular force-resilient clusters (Cui *et al*, 2008). This finding led to the suggestion that the protein may act as a web of macromolecular clamps that stabilize the giant-loops architecture of the chromosome (Cui *et al*, 2008; Rybenkov, 2009). Indeed, purified MukBEF is highly prone to the formation of macromolecular assemblies (Matoba *et al*, 2005; Petrushenko *et al*, 2006b; Woo *et al*, 2009). However, the ability of MukB to bridge DNAs has never been clearly established.

In this study, we report two novel assays that directly assess DNA bridging. Using magnetic tweezers and the magnetic bead pull-down assay, we show that MukB can indeed establish bridges between two DNAs. Further characterization of this activity revealed a novel, multistep mechanism that could explain the dynamic formation of both intra- and interchromosomal links inside the cell.

Results

MukB supports bridging between linear DNAs

We assessed DNA bridging using the 8.4 kb linear pBIO DNA, which was attached to streptavidin-coated magnetic beads by its biotin-labeled terminus (Cui *et al*, 2008), and the 4.0 kb linear pUC40 DNA. The two DNAs were incubated with MukB, after which the pBIO-bound pUC40 DNA was recovered using magnetic separator (Figure 1A). In this system, MukB-mediated DNA bridging was detected directly and with high specificity (Figure 1B; lanes 1–4).

We observed even greater bridging when we removed unbound MukB from the reaction before the addition of pUC40 DNA (Figure 1B, lanes 4, 5). Similarly, the addition of fresh MukB inhibited the reaction (Figure 1B, lane 6). Thus, the bridge is established between DNA-bound MukB and a free DNA rather than between two MukB filaments. Bridging developed slowly, over several minutes and was essentially complete after 30 min incubation. Only 16% greater bridging was observed when fresh protein-free pBR322 DNA was supplied into reaction after 30 min incubation (compare lanes 7 and 8, Figure 1B). However, the extent of secondary bridging markedly increased when the captured pUC40 DNA was treated with MukB before the addition of pBR322 DNA (lanes 9, 10, Figure 1B). We conclude therefore that a large fraction of the captured pUC40 DNA remained

protein-free and, therefore, suitable for secondary bridging. This, in turn, indicates that the bridge is established between two relatively short stretches of DNA while leaving the rest of the molecule protein-free.

This view was supported in our next experiment, when we compared DNAs of different length and topological state. We found that MukB does not distinguish between linear, supercoiled and nicked circular DNA but is more efficient with the shorter, 443 bp DNA (Figure 1C). Although the total mass of captured DNA decreased almost two-fold, the number of captured 443-bp molecules was five-fold greater than for the 4.0 kb pUC40 DNA. Apparently, more bridges are established with the longer pUC40 DNA, whereas even one stable bridge would suffice for detection in our assay.

Notably, reversing mixing order resulted in a markedly reduced bridging. When pUC40 DNA was treated in bulk with MukB before its addition to the bead-tethered pBIO DNA, robust bridging could only be detected for the protein-coated (Figure 1D, bottom panel), but not protein-free pBIO DNA (top panel). Even in this case, however, excessive MukB inhibited bridging reaction (Figure 1D, bottom panel). Importantly, we again observed robust bridging (0.22 ± 0.04 fmol) when we immobilized 4 ng biotinylated pUC40 DNA on beads, treated it with MukB and then incubated the resulting filament with 1.8 fmol unbiotinylated pBIO DNA. Thus, the efficiency of bridging is only modestly affected, if at all, by the length and sequence of the used DNA but depends on prebinding the protein to immobilized rather than freely diffusible DNA.

This result underscores our earlier findings of the multi-step mechanism of bridging and the high stability of MukB–DNA bridges. Indeed, once the reaction was complete, very little additional bridging could occur with freshly supplied DNA (Figure 1B, lane 8). Apparently, binding of MukB to DNA in bulk is quickly followed by the bridging step, and the resulting bridges remain stable throughout the experiment. In contrast, the bead-tethered DNAs are far apart, which precludes immediate bridging. As a result, pBIO-bound MukBs remain bridging-proficient until presented with the freely diffusing pUC40 DNA (Figure 1B and D, bottom panel).

MukEF modulates DNA bridging

In agreement with previous studies (Petrushenko *et al*, 2006b), preassembled MukBEF (stoichiometry $B_2(E_2F)_2$) was unable to bind the bead-tethered DNA (Figure 2A; lanes 1, 2). When mixing order was reversed, MukEF readily associated with DNA-bound MukB producing MukB₂–MukE₂F₁ complex (lane 3). Further increase in MukEF concentration did not alter the resulting stoichiometry (lane 4), indicating saturating binding of MukEF to MukB.

The resulting complex supported DNA bridging, albeit with much lower efficiency than MukB alone (Figure 2B; lanes 1, 5, 6). Including extra MukEF into the reaction further suppressed subsequent bridging (lanes 3, 4), indicating that DNA bridging and binding involve the same moieties on MukB. Accordingly, incubation of MukEF with a preformed bridge reduced the amount of captured DNA (Figure 2B; lanes 1, 7), although a similar competition with another DNA did not have any adverse effects on bridging (Figure 1B; lanes 5, 8). Taken together, these data demonstrate that MukEF is expendable for DNA bridging but, similar to its role in DNA

Figure 1 MukB promotes DNA bridging. **(A)** Illustration of magnetic bead pull-down assay. The 8.4 kb pBB10 DNA with one biotinylated terminus (pBIO) (Cui *et al*, 2008) was attached to streptavidin-coated magnetic beads (Invitrogen), incubated with MukB and non-biotinylated 4.0 kb pUC40 DNA and separated from unbound protein and DNA using magnetic separator. Reactions were usually performed in a multistage format with components often being removed before the addition of the next component. The bottom panel illustrates mixing order for a typical reaction. **(B)** Gel electrophoretic analysis of DNA recovered from the beads. Several multistep mixing protocols were used. Magnetic beads, with or without attached pBIO DNA, were resuspended in 12 μ l of Reaction buffer containing 10 ng linear pUC40 DNA (pU), or 10 ng pBR322 DNA (pR) or 1.2 μ g MukB **(B)** or mock reaction mixture (m) and subjected to upto four 30 min treatments as indicated above the gel. After some treatments (marked with the arrow), the unbound components were removed from the beads before the addition of the next component. The bead-associated pUC40 or pBR322 DNA was then recovered from the beads and quantified using gel electrophoresis. The average (4 to 12 experiments) amount of recovered DNA ($\pm$ s.d.) is indicated below the gel. **(C)** Efficient DNA bridging with linear and circular DNA. MukB-pBIO complex was assembled as described in panel B, rinsed to remove unbound MukB, and incubated with 10 ng linear (L), supercoiled (SC) or nicked circular (NC) pUC40 DNA or linear 443 bp (L.4) DNA. The DNA recovered after bridging was electrophoresed next to serially diluted mixture of 10 ng each DNA. NA, not applied. **(D)** Low bridging efficiency of MukB-DNA complex assembled in bulk. 10 ng pUC40 DNA was incubated with increasing amounts of MukB (as indicated above the gels) for 30 min and then applied to the bead-tethered pBIO DNA, which was either coated with 3.5 pmol MukB (bottom panel) or not (top panel). DNA was then recovered from the beads and analysed as described in panel B.

binding (Cui *et al*, 2008), is likely to have a regulatory role in chromatin rearrangements.

Bridging a single pair of DNAs

We next examined DNA bridging using magnetic tweezers. In these experiments, we stretched pBB10 DNA between a magnetic bead and the surface of a glass capillary with a constant 10 pN force and focused on beads with two attached DNAs (Figure 3A). Preparation of beads with two DNAs is described in greater detail in Materials and methods section. The reaction was initiated by the introduction of 3 nM MukB into the capillary. As before (Cui *et al.*, 2008), no intramolecular DNA condensation occurred under such high force. The bead was then rotated by one turn to cross the attached DNAs and, after a brief incubation, rotated back to the zero rotation. Owing to DNA entanglement, DNA extension declines after rotation (Charvin *et al.*, 2005) but is immediately restored once the molecules are untwisted unless a protein holds them together. In the presence of MukB, the restoration of the DNA length was often delayed

indicating the formation of the protein bridge (Figure 3B). We quantified the delay times (marked as t_{life} in Figure 3B) to evaluate the frequency and stability of MukB bridges.

The bridging reaction occurred on a sub-minute time scale and was accelerated, by about three-fold, in the presence of ATP (Figure 3C). This result mirrors our previous finding that ATP activates MukB for DNA binding (Cui *et al*, 2008). Crossing DNAs for 60 s in the presence of ATP, resulted in a 0.69 ± 0.28 ($\pm$ s.d.; $n = 329$) probability of producing a bridge.

Notably, the frequency of bridging markedly declined when we rotated magnets in the opposite direction to cross DNAs (Figure 3D). Only 2 out of 75 left-handed crossing events, when the two DNAs were twisted by one turn, resulted in the formation of a bridge. MukB retained its high preference for positively twisted DNAs when we included wheat germ topoisomerase I into reaction (Figure 3E). This enzyme efficiently removes both positive and negative supercoils from DNA and ensured complete DNA relaxation at concentrations as low as 0.1 nM (Cui *et al.*, 2008).

The high sensitivity of MukB to the chirality of DNA crossings is especially striking when compared with the lactose operon repressor LacI. LacI acts as a tetramer that brings two distant DNA segments together. Although the protein prefers its operator sequence, it also demonstrates robust activity on nonspecific DNA as well. Crystallographic studies revealed that the two LacI-bound DNA segments form

Purified K40DS41G was indistinguishable from the wild-type protein in binding either supercoiled (Figure 3G) or linear (data not shown) DNA, as judged by the gel-shift assay. In contrast, the DNA bridging activity of the mutant was markedly impaired. The rate of bridging of the braided DNAs was about three-fold lower for the mutant than for the wild-type protein (Figure 3E), whereas virtually no bridging activity could be detected in the magnetic bead pull-down assay (Figure 3H). Taken together with the previously found stimulation of DNA bridging by ATP (Figure 3C), these data strongly argue that DNA binding and bridging are distinct activities of MukB and that ATP hydrolysis has a role in modulating DNA bridging.

Both these models give rise to double-exponential kinetics but can be distinguished by examining how the rates change in response to varied bridging times. In particular, the isomerization model predicts that longer reactions could increase the fraction of the more stable species but would not affect dissociation rate constants. In contrast, the sequential model predicts that both rates and the abundance of stable

Figure 3 Magnetic tweezers analysis of DNA bridging. pBB10 DNA with biotin- and digoxigenin-labeled termini was stretched between the surface of an anti-digoxigenin-coated glass capillary and a streptavidin-coated magnetic bead using 10 pN magnetic force (Cui *et al*, 2008). Beads with two attached DNAs were selected by identifying the beads that produce symmetric DNA extension versus rotation curves both at high and low stretching force (Strick *et al*, 1998; Charvin *et al*, 2005). DNA bridging reaction was initiated by rotating the bead by one turn to cross the attached DNAs in the presence of 3 nM MukB. DNAs were untwisted to the zero rotation to initiate unbridging. (A) Diagram of the experiment. (B) Time course of an experiment. DNA extension (red symbols) declines once the bead is rotated (blue line) but, in the absence of protein-mediated bridging (double arrow), immediately recovers once the rotation is reduced to zero. Virtually identical time courses were observed in the absence of the protein (data not shown). In contrast, when bridges are formed (arrows), a delay between the changes in rotation and DNA extension is observed. The lifetime of the bridges was measured as the length of the delay, t_{life} , as a function of bridging time, t_{rxn} . Note the greater stability of the bridge produced after the longer bridging time (when DNAs were kept crossed). The shortest measurable delay time was limited by the speed of our CCD camera (25 frames per second). A delay was scored only when it lasted at least two frames (0.08 s). We never observed such events in the absence of the protein. (C) ATP stimulates bridging rate. Bridging probability was measured as the fraction of DNA crossing events that resulted in a bridge. The average probabilities ($\pm$ s.d.) measured for six beads (between 290 and 800 bridging events for each data point) are shown. (D) Bridging probability (60 s bridging time, in the presence of 1 mM MgATP) as a function of the bead rotation angle. (E) Chirality-sensitive DNA bridging by wild-type and mutant MukB and by LacI. Bridging reactions were carried for 30 s in the presence of 1 mM MgATP and 6 nM MukB (wt or mutant) and the indicated amounts of wheat germ topoisomerase I or for 8 s in the presence of 60 nM LacI. Shown are mean values of bridging probability ($\pm$ s.e.; between 58 and 220 trials per data point) observed after rotating the bead by plus or minus one turn. (F) Temperature sensitive phenotype of K40DS41G mutant. Shown are colony-forming units (CFU; $\pm$ s.d.; $n = 3$) for the $\Delta\text{mukB}::\text{kan}$ strain GC7528 (Niki *et al*, 1991), which was transformed with either p15sp-B03a or p15sp-B03a-K40DS41G. (G) Purified wild type and mutant (MukB*) were reacted for 30 min with 10 ng supercoiled (SC) pBR322 DNA at the indicated molar ratios and analysed by gel shift assay as previously described (Petrushenko *et al*, 2006a). (H) Magnetic bead pull-down assay with wild-type (B) and mutant (B*) MukB, carried out as described in Figure 1.

species would increase (see Materials and methods section for greater detail).

We found no significant difference between stabilities of the complexes that formed in the presence of ATP (10 s incubation; bridging probability 0.24) and its absence (60 s incubation; bridging probability 0.38), indicating that formation of the stable species is not strictly dependent on ATP (Figure 4A and B). In both cases, the majority of the

complexes were unstable, with the decay rates of $1.2 \pm 0.2 \text{ s}^{-1}$ (plus ATP) and $1.7 \pm 0.4 \text{ s}^{-1}$ (no ATP).

Stability of the bridges markedly increased after longer reaction times. When we prepared bridges by keeping DNAs crossed for 60 s in the presence of ATP, the average lifetime of the bridges was 7.4 s, and longer yet incubations often produced bridges that were stable throughout the experiment (data not shown). The fast exponent in the double-exponential

Figure 4 Stability of bridges is affected by the length of reaction and extent of DNA overlap. (A–E) Bridges were formed by incubation for various times or bead rotations, as indicated in the figure, and their lifetime was then measured as outlined in Figure 3. The measured lifetimes were then binned according to their duration and plotted as a histogram (right axis) or cumulative distribution (symbols; left axis). Cumulative distributions were then fit to a double-exponential decay function. In all cases, the squared value of Pearson's correlation coefficient (R^2 ; a goodness-of-fit measure) was greater than 0.99. The best fit rate constants and the fraction of stable complexes (N_2/N_{Σ}) are shown ($\pm$ s.e.) for each plot. (F) The fast and slow rate constants determined from the double-exponential approximation in panels A–E.

approximation appeared unchanged, compared with its value for the 10 s time point, whereas the slow decay rate was significantly lower, 0.13 ± 0.01 versus $0.23 \pm 0.07 s^{-1}$ (Figure 4A and D). Similarly, the proportion of stable complexes increased from 35 ± 3 to $82 \pm 1\%$. Such kinetics strongly argues in favor of the sequential binding model.

In further support of this view, we found that the frequency of multiple bridging events could be altered by varying DNA intertwining during reaction, which in turn limits the number of MukBs that can bind at the crossing. The proportion of stable complexes increased from 72 ± 1 to $89 \pm 1\%$ when the bead rotation during incubation was increased from 0.8 turns to 2 turns (Figure 4C–E). We further found that the average stability of these complexes (as determined from the measured decay rates) also increased at higher rotations as would be expected if more MukBs joined the complex (Figure 4F). We conclude therefore that several MukBs cooperate to enhance stability of the bridge.

Discussion

Our experiments clearly establish that MukB can form a bridge between two DNAs. The reaction is highly efficient and suggests a mechanistic basis for all DNA reshaping activities reported so far for condensins, including DNA

Figure 5 A three-step mechanism of DNA bridging. The first step is loading of MukBEF onto DNA, which produces DNA-bound macromolecular clusters of the protein. Random collisions with appropriately oriented DNA fragments result in transient formation of a protein bridge (second step), which must be further stabilized by additional MukBs (step three). MukBEF contributes to the reaction by modulating formation and dissolution of the bridges. This modulation is likely to be mediated by ATP given that ATP was shown to affect both MukB–DNA and MukB–MukBEF interactions. The multi-step nature of the reaction lays grounds for differential regulation of MukBEF loading onto DNA and its bridging proficiency.

supercoiling, chiral DNA knotting and condensation. These data strongly support the view that DNA bridging is the primary activity of condensins that enables their intracellular function. Furthermore, this activity could explain the emergence of both condensin-stabilized chromosomal loops and the cohesin-mediated interchromosomal links. It is tempting to speculate therefore that various SMC complexes share fundamentally the same mechanism.

The use of two direct, complementary assays helped reveal numerous mechanistic details about bridging. We found that MukB acts as a macromolecular assembly that can form a stable complex with DNA. This complex is bridging-proficient and readily captures another fragment of protein-free DNA that happens nearby. The protein physically associates with both DNAs, perhaps inadvertently producing topological entanglements (Figure 5). The capture begins with the formation of a single unstable link, which is quickly strengthened by additional bridges. This feature could serve as a

safeguard against binding to DNAs that are too short or protected by DNA-binding proteins.

The uncovered mechanism of bridging allows straightforward extrapolation to the intracellular environment and could explain, for example, how chromosome cohesion could be established in the absence of DNA replication (Strom *et al*, 2007; Unal *et al*, 2007). Further studies are needed to determine how the SMCs are recruited to specific loci or how their activity is regulated. Our finding that MukEF can inhibit DNA bridging without displacing MukB from DNA implicates the non-SMC components in such regulatory functions.

Structural organization of MukBEF

MukBEF acts as a complex inside the cell. The evidence for it is overwhelming. Inactivation of MukB, MukF or MukE produces very similar phenotypes (Yamanaka *et al*, 1996). Similarly, correct subcellular localization of the proteins requires coexpression of all three subunits (Ohsumi *et al*, 2001; She *et al*, 2007). Finally, the proteins can be purified from cells as a complex (Yamazoe *et al*, 1999; Petrushenko *et al*, 2006b). It came as a surprise, therefore, that all known DNA reshaping activities reside in MukB, whereas MukEF disrupts the MukB–DNA complex (Petrushenko *et al*, 2006b; Cui *et al*, 2008). As we report here, this is true for DNA bridging as well.

Importantly, the inhibitory effect of MukEF can be reproduced in live cells and is, therefore, physiologically significant. It has been shown that mildly excessive MukEF ($\sim 10 \times$ overproduction) is detrimental for MukB function *in vivo* (She *et al*, 2007), whereas similar ($\sim 10 \times$) overproduction of MukB or MukBEF does not lead to discernable physiological effects (Wang *et al*, 2006; She *et al*, 2007). Moreover, MukB alone induces chromosome condensation in live cells, albeit it acts somewhat better in the presence of MukEF (Wang *et al*, 2006). Taken together, these data strongly argue that the activity of MukBEF is optimal at intermediate MukEF-to-MukB ratios.

Structural basis for this conclusion can be found in recent biochemical and crystallographic studies. Although MukBEF can form a complex with stoichiometry $B_2(E_2F)_2$, only one of the Muk(E_2F) units is bound to MukB with high affinity (Petrushenko *et al*, 2006b). The second Muk(E_2F) is displaced by ATP hydrolysis (Woo *et al*, 2009), the removal of magnesium or even chromatographic separation (Petrushenko *et al*, 2006b). We further show that DNA-bound MukBEF has stoichiometry $B_2(E_2F)_1$ and the ratio perhaps declines further upon formation of a bridge (Figure 2A, lane 5). Taken together with the inability of $B_2(E_2F)_2$ complex to bind or bridge DNA, these data persuasively argue for a regulatory function of MukEF. Perhaps, by modulating composition of MukBEF, whether transiently or for long periods of time, MukEF helps control loading and unloading of MukB onto DNA.

The interplay between MukEF and DNA might seem counter-intuitive given that they bind at the opposite faces of MukB heads (Woo *et al*, 2009). A major clue here is that both MukB–MukEF (Woo *et al*, 2009) and MukB–DNA (Cui *et al*, 2008) interactions are affected by ATP. The stimulatory effect of ATP on MukB–DNA interactions has now been confirmed not only in cofactor mixing experiments but also by the analysis of an ATPase mutant of MukB (Figure 3). Apparently, both MukEF- and DNA-binding interfaces of

MukB are deformed during its cycle of ATP hydrolysis, forcing the protein to alternate between its DNA- and MukEF-binding forms.

Mechanics of DNA bridging

The system of single-DNA braids that we used here was originally developed to study topological constraints in DNA (Strick *et al*, 1998; Charvin *et al*, 2005) and later applied to DNA unlinking by topoisomerases (Charvin *et al*, 2003; Stone *et al*, 2003; Neuman *et al*, 2009). In this study, this system proved its power for studies of protein-induced DNA bridging as well. Its main advantages include unparalleled control over chirality, extent and duration of DNA overlap. A different approach, which involves simultaneous manipulation of four beads within an optical trap, the Q-trap, was applied to characterize DNA bridging by H-NS (Dame *et al*, 2006). While lacking in a control over chirality and extent of DNA crossings, Q-trap offers advantages in studies of multimolecular, repeated structures and complexes, in which little DNA intertwining occurs during the formation of the bridges.

Although micromechanical studies of DNA bridging are still in their infancy, first trends are beginning to emerge. Both H-NS and MukB showed high propensity for DNA bridging only when presented with naked, but not with protein-coated DNA. Both proteins exhibited cooperativity in their interaction with DNA. In both cases, the lifetime of a single-protein bridge was on the order of 1 s but was strengthened by additional molecules. This is in stark contrast to the highly stable nucleosome–DNA complex (Cui and Bustamante, 2000; Brower-Toland *et al*, 2002) and might reflect a general distinction between chromatin organization in prokaryotes and eukaryotes.

Topological selectivity of condensins

The finding that MukBEF efficiently supports DNA bridging might seem counterintuitive given the high preference of condensins for intramolecular DNA segments. Indeed, in contrast to cohesins, condensins preferentially support intramolecular DNA condensation, which points to the ability to select DNA segments from the same DNA. (Kimura *et al*, 1999; Losada and Hirano, 2001; Stray *et al*, 2005; Petrushenko *et al*, 2006a). The mechanism of such topological selectivity remains unknown. It has been argued that the high cooperativity of MukB–DNA interaction could be responsible for such activity (Cui *et al*, 2008).

Another mechanism could be proposed on the basis of comparison with type 2 DNA topoisomerases (Wang, 1996; Schoeffler and Berger, 2008). These enzymes are also able to select either intra- or intermolecular DNA crossings, as appropriate for their catalysed reactions (Rybenkov *et al*, 1997). It has been recently shown that topoisomerases achieve this remarkable feat by sharply bending DNA at the binding site (Vologodskii *et al*, 2001; Dong and Berger, 2007). It is not impossible that SMCs use a similar mechanism to select DNA segments. The marked preference of MukB for right-handed DNA crossings (Figure 3D) is a strong indication that MukB does not merely capture randomly colliding DNA segments but alters their shape in the process, which could serve as a basis for topology recognition. Moreover, a recent crystallographic study revealed a sloping DNA-binding site on MukB, which offers a structural basis for the expected DNA bending (Rybenkov, 2009; Woo *et al*, 2009). A comparison

between condensins and cohesins in DNA bridging should offer more mechanistic clues to this intriguing ability of SMCs.

Materials and methods

Proteins and DNA

MukB, MukEF and MukBEF were purified and reconstituted as previously described (Petrushenko *et al*, 2006a, b). pBB10 DNA with multiple biotin and digoxigenin modifications at its termini was produced as previously described (Cui *et al*, 2008). pBIO DNA was the same, except it contains only the biotin-labeled terminus. To construct K40DS41G mutant of MukB, the 5'-end of *mukB* (encoded within pBB10) was amplified by PCR, with a mutagenic primer, and then replaced with this amplified fragment. The resulting plasmid, pBB10m5, encodes C-terminally His-tagged K40DS41G mutant MukB and was used for its expression and purification as described for the wild-type protein (Petrushenko *et al*, 2006a). For complementation analysis, the mutant and wild-type *mukB-His10* genes were transferred under the control of its native promoter onto the pACYC184-derived p15sp-E02a plasmid (She *et al*, 2007), yielding plasmids p15sp-B03a and p15sp-B03a-K40DS41G. LacI (Zhan *et al*, 2006) was a generous gift from Drs Hongli Zhan and Kathleen Matthews.

Magnetic bead pull-down assay

Streptavidin-coated Dynabeads C1 (Invitrogen) were washed twice with PBS and once with TENG buffer (10 mM Tris-Cl, pH 7.9, 1 mM EDTA, 1 M NaCl, 2 μ M poly-L-glutamic acid (Sigma-Aldrich), 2 μ M of a 41 nt oligonucleotide, 1 mg ml⁻¹ BSA and 0.02% Tween 20). To assess DNA bridging, 4.5 ng pBIO DNA was incubated for 1 h with 0.3 μ l of the washed beads in 3 μ l TENG, and then washed twice in Reaction buffer (20 mM HEPES-KOH, pH 7.7, 50 mM NaCl, 2 mM MgCl₂, 5% glycerol, 1 mM DTT, 0.02% Tween 20, 1 mg ml⁻¹ BSA). The beads were then resuspended in 12 μ l Reaction buffer containing 1.2 μ g MukB (3.5 pmol), 10 ng pUC40 DNA (3.8 fmol), 10 ng (3.5 fmol) pBR322 DNA or 10 ng (34 fmol) PCR-generated linear 443 bp DNA as indicated in the legend to Figure 1. After 30 min incubation, the beads were washed with 12 μ l Reaction buffer and

either supplemented with the next reactant or the Stop buffer (10 mM TrisCl, pH 7.9, 1 mM EDTA, 200 mM NaCl, 0.2% SDS, 0.5 mg ml⁻¹ proteinase K). The results were virtually identical when we included 1 mM MgATP into the Reaction buffer (data not shown). The eluted DNA was resolved by gel electrophoresis through 0.8% agarose gel alongside with the serially diluted pUC40 DNA with known concentration, stained with SYBR Gold (Molecular Probes) and quantified using densitometry. Under these conditions, up to 20% of pUC40 DNA could be bound to the bead-tethered pBIO DNA (Figure 1B).

Essentially the same procedure was used to quantify the bound MukBEF. In this case, however, each reaction contained 6 μ g beads with 30 ng of immobilized pBIO DNA, the beads were incubated with 5.3 pmol MukB or MukBEF, nonspecific binding was suppressed by including 1 mM spermidine instead of BSA into the Reaction buffer, and the bound protein was eluted by boiling the beads in the SDS-PAGE loading buffer.

Magnetic tweezers

Magnetic tweezers manipulations were performed as previously described (Cui *et al*, 2008), except that we selected beads with two attached DNAs. This system was initially developed to study DNA braiding (Strick *et al*, 1998; Charvin *et al*, 2005) and was later applied to explore DNA unlinking by topoisomerases (Charvin *et al*, 2003; Stone *et al*, 2003; Neuman *et al*, 2009).

Beads with two attached DNAs are randomly produced when biotinylated DNA is mixed with the beads and can be later identified by their characteristically symmetric hat curves (the DNA extension versus rotation curves) both at high and low stretching forces (Figure 6) (Marko, 1997; Strick *et al*, 1998; Stone *et al*, 2003; Charvin *et al*, 2005). In contrast, beads with single DNAs produce symmetric hat curves only at low forces because of the force-induced DNA melting (Strick *et al*, 1996, 1998; Marko, 1997; Bryant *et al*, 2003), whereas three or more DNAs yield asymmetric hat curves (Figure 6). In our experiments, about 2% beads were attached to two DNAs.

Kinetic models

Sequential binding. The first model envisions sequential binding of several MukBs, each of which binds DNA in a single event. This

Figure 6 DNA extension versus rotation curves (hat curves) for beads with one, two or more attached DNAs. DNA nanomanipulations were performed as previously described using the 2.8 μ m pBB10 DNA (Cui *et al*, 2008). (A) The hat curves for beads with one attached DNA. Such beads produce bell-shaped hat curves at low stretching force owing to formation of plectonemes in DNA (Strick *et al*, 1998; Charvin *et al*, 2005). At higher forces, supercoiled DNA undergoes structural transitions resulting in plateau regions that span either negative rotations only (DNA melting) or include positive rotations as well (formation of P-DNA) (Allemand *et al*, 1998). (B, C) Beads with two (B) or more (C) DNAs produce characteristic bell-shaped spiked curves even at high forces (Strick *et al*, 1998; Charvin *et al*, 2005). The spike appears due to the geometric constraints of the system, wherein the first intertwining of the DNAs leads to significant DNA contraction (Strick *et al*, 1998; Charvin *et al*, 2005; and Figure 3A). Insets show the same hat curves on an expanded scale to highlight the spike region. The data were analysed according to the model developed by Charvin *et al* (2005) and fit to equations (1 and 2) of the previous study. When three (or more) DNAs are present (C), two of the three DNAs will be closer to each other resulting in an asymmetric spike region. In this case, the equations that describe well the negative rotations (Fit left) are poorly applicable to the positive rotations (Fit right), and vice versa.

mechanism can be described using the following kinetic scheme:

where D_i denotes DNA crossings with i MukBs holding them together, and k_{fi} and k_i are microscopic rate constants for, respectively, binding and dissociation of i -th MukB to the bridge. When DNAs are crossed by rotating the bead (Figure 3), the reaction is shifted forward yielding multiprotein complexes. The average number of bound MukBs would be expected to increase with increasing time of incubation and the extent of DNA overlap.

Once the DNAs are untwisted, further protein binding becomes impossible (all k_{fi} s in equation (1) turn to zero), and the complex dissociates. The well known solution to this scheme can be expressed as a series of exponents:

$$D(t) = \sum A_i \cdot \exp(-k_i t), \quad (2)$$

where $D(t)$ is the total number of remaining bridges, t is the time, and the amplitudes A_i s can be expressed as a function of the rate constants k_i s and the abundance of each of the D_i species at the onset of dissociation, D_i^0 . Given that MukB–DNA complex is destabilized by applied force (Cui *et al*, 2008), one can predict that $k_1 > k_2 > k_3 > \dots$ because for $i > 1$, stretching force is distributed between progressively greater number of MukB bridges.

In the limiting case, when $k_i \gg k_{i+1}$, A_i equals D_i^0 . In this case, the amplitude of the fastest exponent reflects the abundance of complexes with the fewest MukBs, whereas the other exponents describe decay of the more complex structures. It follows then that an increase in the initial number of bound MukBs (which can be achieved, for example, by extending the binding phase of the reaction) would result in a decline of both the observed rate constants and the proportion of the rapidly decaying species. This conclusion holds for the more general case of $k_i > k_{i+1}$, when relationship between A_i and D_i^0 becomes more complex.

Bridge isomerization model. The second model postulates that the initial unstable bridge undergoes isomerization—perhaps in an

ATP-modulated manner—which results in a more stable structure. Dissociation of such structure can be analysed in terms of the following kinetic scheme:

where D_1^* denotes MukB bridge stabilized by the postulated conformational transition. Note that, in contrast to equation (1), k_{f2} does not necessarily turn to zero after the DNAs are untwisted. In this model, the decay of the bridges would be given by:

$$D(t) = A_1 \exp(-\lambda_1 t) + A_2 \exp(-\lambda_2 t),$$

$$\lambda_{1,2} = \frac{(k_1 + k_2 + k_{f2}) \pm \sqrt{(k_1 + k_2 + k_{f2})^2 - 4k_1 k_2}}{2} \quad (4)$$

where λ_1 and λ_2 are the observed rate constants, and A_1 and A_2 are, again, related to the abundance of the stable and unstable species at the onset of dissociation, $(D_1)^0$ and $(D_1^*)^0$. In contrast to the mechanism in equation (1), longer protein binding times might increase the proportion of the stable species among all formed complexes, but not the observed rate constants.

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Conflict of interest

The authors declare that they have no conflict of interest.

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