Antagonistic Interactions of Kleisins and DNA with Bacterial Condensin MukB*

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MukBEF is a bacterial SMC (structural maintenance of chromosome) complex required for faithful chromosome segregation in Escherichia coli. The SMC subunit of the complex, MukB, promotes DNA condensation in vitro and in vivo; however, all three subunits are required for the function of MukBEF. We report here that MukEF disrupts MukB-DNA complex. Pre-assembled MukBEF was inert in DNA binding or reshaping. Similarly, the association of MukEF with DNA-bound MukB served to displace MukB from DNA. When purified from cells, MukBEF existed as a mixture of MukEF-saturated and unsaturated complexes. The holoenzyme was unstable and could only bind DNA upon dissociation of MukEF. The DNA reshaping properties of unsaturated MukBEF were identical to those of MukB. Furthermore, the unsaturated MukBEF was stable and proficient in DNA binding. These results support the view that kleisins are not directly involved in DNA binding but rather bridge distant DNA-bound MukBs.

SMCs are ubiquitous highly conserved proteins that have been implicated in virtually every aspect of higher order chromatin dynamics. Eukaryotic cells contain at least six different SMC complexes with functions in chromosome condensation and segregation, recombination, and repair (1–4). Bacteria carry two SMC complexes. In Escherichia coli, faithful chromosome segregation requires the action of MukBEF (5, 6). The second SMC complex, SbcCD nuclease, was implicated in the metabolism of double-strand DNA breaks (7).

The defining feature of SMCs is their structure. They consist of two globular domains connected by a long coiled-hinge-coil motif. In solution, SMCs dimerize to form an idiosyncratic V-shaped structure with two globular head domains connected at the hinge via two long coiled coils (8–10). The Walker A and B motifs, which are found in the N- and C-terminal domains of SMCs, are located at the surface of the SMC heads. This enables further association of SMCs via nucleotide-sandwiched dimerization of the head domains, leading to the formation of protein rings (11–13) or macromolecular assemblies (14–16).

SMCs act inside the cell as a part of multisubunit complexes. Among the non-SMC subunits, a conserved family of kleisins was identified (17, 18). Kleisins bind head domains in the vicinity of the ATP binding site and apparently stabilize the dimeric form of the SMC heads (11, 19, 20). In several cases, a functional interaction between kleisins and ATP has been reported (11, 14, 21).

Biophysical properties of SMCs benefit their intracellular functions. In a reaction coupled to type-2 DNA topoisomerases, condensins promote formation of DNA knots of specific topology (22–24). This property is highly conserved among condensins and indicates intramolecular DNA condensation. In contrast, cohesins promote DNA catenation, indicating predominantly intermolecular DNA interactions (25). How the structurally similar condensins and cohesins distinguish between their substrates remains unclear.

The mechanism of SMCs is under debate. Divergent models were proposed to explain the mechanism of DNA reshaping (1, 23, 24). The ring model proposed for cohesins postulates that the proteins hold DNA segments via a purely topological link (1, 10). At least for condensins, this model is at odds with the efficient binding of the proteins to linear DNA and the chirality of generated knots (22–24).

A key unanswered question in the mechanistic enzymology of SMCs is about the role of kleisins in DNA reshaping. The original experiments with 13 S condensin from frogs established that the non-SMC subunits and ATP are required for DNA compaction but not for DNA binding (26). This agrees well with genetic data, which indicate similar phenotypes for mutations in SMC and non-SMC components of the complex, and the finding that the proteolytic cleavage of kleisins leads to dissolution of cohesion between sister chromosomes (20). In contrast, the yeast SMC2/4 complex and the E. coli MukB protein promote efficient DNA knotting in the absence of ATP and the cognate accessory subunits (23, 24). Reconstitution studies with the Bacillus subtilis SMC complex indicated that the accessory subunits modulate the association of the protein with DNA (14). It remains unclear, however, if the assembly of the holoenzyme alters the DNA reshaping properties of the protein.

Here, we assessed the role of the non-SMC subunits in DNA reshaping by bacterial condensin MukB (27). In complex with MukEF, MukB plays the central role in organizing the chromosome of E. coli (5). Mutational inactivation of MukBEF results in the loss of cell viability above 30 °C, chromosome decondensation and cutting, and a high frequency of anucleate cells (28). The overproduction of MukBEF leads to the opposite effect; that is, a marked decrease in the size of the nucleoid (29). Thus, the primary function of MukBEF appears to be chromosome condensation. Accordingly, mukB phenotype is suppressed by

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mutations that increase DNA supercoiling (30, 31), presumably due to the more compact structure of highly supercoiled chromosomes (32, 33).

Within MukBEF, MukF serves as a linker between MukB and MukE (34). MukF readily dimerizes via its N-terminal domain and can also associate with MukB or MukE (34, 35). A structural analysis indicated that MukF is a kleisin (35). Accordingly, MukBEF, but not MukB, was found to form multimolecular rosette-like and fiber-like structures in the absence of ATP or DNA (36).

Purified MukB binds linear and circular DNA with similar affinities, about 30 nM MukB (24), and can bind ATP and GTP in the presence of Zn$^{2+}$ (27). The ATPase rate of MukB is the slowest reported so far for the SMC proteins and is not stimulated by single- or double-stranded DNA (24). The protein, however, supports efficient DNA supercoiling even in the absence of ATP or magnesium and promotes formation of right-handed three-noded knots, the signature activity of condensins (24). Remarkably, the overproduced MukB efficiently condensed chromosomes but failed to restore the viability of MukEF-deficient cells (29). Thus, the accessory subunits of MukBEF appear to be dispensable for DNA compaction per se but are required for other aspects of chromosome condensation.

We report here that MukF has only negative effects on MukB-catalyzed DNA reshaping. Preassembled MukBEF was unable to bind or reshape DNA; conversely, DNA binding interfered with the assembly of the holoenzyme. The interaction of MukEF with DNA-bound MukB served to displace MukB from DNA. Thus, MukEF and DNA compete for binding to MukB. We found, however, that only 50% of reconstituted MukBEF were stable; the remaining complexes were short-lived even in the presence of stabilizing magnesium. Thus, MukBEF is likely to act inside the cell as an asymmetric, half-saturated unit. We indeed found that the half-saturated MukBEF efficiently supported DNA reshaping. Furthermore, a stable MukEF-MukB-DNA complex could be formed at substoichiometric levels of MukEF. We found similar properties for MukBEF that was purified from overproducing cells. Purified MukBEF existed as a mixture of two complexes, one of which was unsaturated with MukEF. The MukEF-saturated MukBEF was unstable and acquired the DNA binding activity after dissociation of MukEF. The MukEF-unsaturated MukBEF was indistinguishable from MukB in its DNA binding and reshaping. These data support the view that DNA binding and reshaping is accomplished by the SMC subunit of the complex, whereas kleisins are responsible for other aspects of chromosome rearrangement.

**MATERIALS AND METHODS**

Proteins—DNA topoisomerases were previously described (24). MukB<sup>10</sup>, MukE<sup>F</sup>, and MukB<sup>EF</sup>, which include C-terminal His-tagged MukB or MukE, were purified from DH5α cells harboring plasmids pBB10, pBB08, or pBB03, respectively (29). The functionality of all Muk proteins on these plasmids has been previously verified (29). MukB<sup>10</sup> and MukB<sup>EF</sup> were purified using nickel-chelate chromatography followed by separation on a HiTrap heparin column (GE Healthcare) as described previously for MukB (24). Only the active, high salt fraction of MukB was analyzed here. MukB<sup>EF</sup> was purified by nickel-chelate chromatography followed by gel filtration through Sephacryl S300. No crystallizing protein was found in purified MukEF or BEF-HS. BEF-LS, the inactive low salt fraction of MukBEF, contained about 1% of acetyl carrier protein. Similar to MukB (24), acetyl carrier protein did not co-elute with DNA reshaping activities during chromatography on the Heparin II column. To obtain MukE and MukF, purified MukEF was denatured in 6 M guanidine hydrochloride, 200 mM NaCl, 20 mM HEPES, pH 7.7 and applied to the nickel column. MukF was eluted with 6 M guanidine hydrochloride, 200 mM NaCl, 20 mM HEPES, pH 7.7, 20 mM imidazole. MukE was then eluted with 20–400 mM imidazole gradient in the same buffer, and the proteins were refolded by dialysis against 20 mM HEPES, 200 mM NaCl, 50% glycerol, 2 mM EDTA, 1 mM dithiothreitol.

MukBEF was reconstituted by mixing MukEF and MukB on ice in reconstitution buffer (20 mM HEPES, 200 mM NaCl, 20% glycerol, 2 mM EDTA, 1 mM dithiothreitol) followed by 20 min of incubation. Protein concentrations were measured using Bradford assay using bovine serum albumin as a standard.

**DNA Reactions**—DNA knotting and supercoiling assays were done in reaction buffer (20 mM HEPES, pH 7.7, 40 mM NaCl, 7% glycerol, 1 mM dithiothreitol) supplemented with 2 mM MgCl$\text{$_2$}$, 1 mM MgATP, and 1 mg/ml bovine serum albumin as previously described (24). DNA binding was carried out in reaction buffer containing either 2 mM MgCl$\text{$_2$}$ or 2 mM EDTA as indicated under “Results.” The reactions were chilled on ice and analyzed by electrophoresis (4.5 h at 4 V/cm at 4°C) through 0.7% agarose in TB buffer (90 mM Tris borate (TB), pH 8.3) or EB buffer plus 2 mM MgCl$\text{$_2$}$ (see figure legends for details). DNA was visualized by staining with SYBR Gold (Molecular Probes).

**Sucrose Density Gradient Centrifugation**—30 µg of MukBEF was mixed with 30 µg each of thyroglobulin (19.2 S, 8.6 nm), catalase (11.2 S, 5.3 nm), apoferritin (17.6 S, 6.2 nm), alcohol dehydrogenase (7.3 S, 4.6 nm), bovine serum albumin (4.3 S, 3.6 nm), and carbonic anhydrase (2.8 S, 2.0 nm) in reconstitution buffer. The protein mixture was layered on top of 10–40% sucrose gradient in the same buffer (except that glycerol concentration was 10%) and centrifuged at 55,000 rpm for 12 h at 4°C in TLS 55 rotor (Beckman). The proteins in collected fractions were resolved by SDS-PAGE, visualized by Coomassie staining, and quantified using densitometry.

**Light Scattering (LS)/Size Exclusion Chromatography**—MukEF was resolved by gel filtration through a YMC-Pack Diol-300 column, and the light scattering and refractive index (RI) of eluted proteins was measured using PD2010 light scattering detector (Precision Detectors, MA) and Waters 2414 detector, respectively. The molecular mass of the proteins was calculated from LS and RI data using Discovery software (Precision Detectors, MA). The method makes use of the fact that for a given weight concentration of a protein, RI is inversely proportional and LS is directly proportional to the molecular weight of the solute (37).

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2 The abbreviations used are: LS, light scattering; RI, refractive index.
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RESULTS

MukEF Is an Elongated Complex with the Stoichiometry $E_4F_2$—MukEF was purified from cells that overproduced MukE and MukF using the C-terminal nine-histidine tag on MukE. MukEF migrated as a single peak during sucrose gradient centrifugation and gel filtration (see below). In agreement with previous data (34), the sedimentation coefficient of MukEF was found to be $7.5 \pm 0.5$ S. The Stokes radius of MukEF, as determined by gel filtration chromatography, varied depending on temperature: $5.7 \mathrm{nm}$ at $4 \degree \mathrm{C}$ and $6.5 \mathrm{nm}$ at $23 \degree \mathrm{C}$. This variation indicates conformational flexibility in MukEF and precludes the unambiguous determination of its molecular weight. It is clear, however, that MukEF is an asymmetric, most likely elongated protein. Even using the lower value for the Stokes radius, the Form factor of MukEF can be estimated as 1.4, which translates into the axial ratio of 7 (38).

When analyzed using LS/size exclusion chromatography (37), the molecular mass of MukEF was found to be $230 \pm 10$ kDa (Fig. 1A). This value is consistent with two possible complexes, $E_4F_2$ and $E_3F_3$. To distinguish between these possibilities, we measured the stoichiometry of MukEF using two approaches. First, MukEF was electrophoresed next to the calibration mixture composed of individually purified MukB, MukF, and MukE. The amount of MukF and MukE was then measured by comparing the intensities of appropriate bands on Coomassie-stained gels (Fig. 1B). Averaged from five independent experiments, the ratio of MukE to MukF was found to be $2.1 \pm 0.25$. Secondly, the individually purified MukE and MukF were mixed in various proportions and resolved by non-denaturing gel electrophoresis. Only at a MukE-to-MukF ratio of 2.0 did we find a single band on the gel (Fig. 1C). We conclude that the composition of MukEF is $E_4F_2$. The dimeric organization of MukEF indicates that the protein can indeed interact with two separate head domains of MukB, which is consistent with its putative role of a kleisin.

Bimodal Association of MukB and MukEF—Reconstitution of MukBEF was carried out by incubating purified MukB and MukEF in the buffer containing $200 \mathrm{mM}$ NaCl, $2 \mathrm{mM}$ EDTA. To evaluate the success of reconstitution, mixtures of MukB and MukEF were subjected to gel filtration through Sephacryl S300. MukB and MukEF migrate close to the excluded volume on this column, well separated from MukEF (Fig. 2A). Unexpectedly, we found that only 50% of MukEF comigrated with MukB when the equimolar mixture of MukB and MukEF was resolved by gel filtration in the presence of $200 \mathrm{mM}$ NaCl. This was not because of a poor reconstitution protocol, since all MukEF formed a complex with MukB when the proteins were mixed at the $E_2F$-to-B ratio of 0.5 (Fig. 2A). Furthermore, the assembled MukBEF was sufficiently stable to survive sucrose gradient centrifugation (Fig. 2B). Finally, we found equimolar retention of MukEF by MukB when protein mixtures were subjected to gel filtration in the presence of $40 \mathrm{mM}$ NaCl, $2 \mathrm{mM}$ MgCl$_2$, our standard reaction buffer (Fig. 2A). Only stoichiometric amounts of MukEF comigrated with MukB when higher levels of MukEF were used in reconstitution (Fig. 2A), indicating that the comigration of the proteins is due to complex formation rather than protein aggregation.

The combination of magnesium and the low salt conditions was required for complete binding of MukB to MukEF. Only about 70% of MukB bound MukEF at $40 \mathrm{mM}$ NaCl, $1 \mathrm{mM}$ EDTA (Fig. 2A, bottom panel), and magnesium had no effect on stability of MukBEF at $200 \mathrm{mM}$ NaCl (data not shown). Thus, two types of complexes are formed after reconstitution; half of the resulting MukBEF was stable, whereas the other half was stabilized by magnesium and low salt.

Saturated MukBEF Is a Multimer—In general, kleins could link together the head domains from the same or different V-shaped dimers of MukB. Either separate, ring-like molecules or multimeric assemblies would be produced in these two cases.
To distinguish between these two possibilities, we analyzed reconstituted MukBEF by gel filtration through the resin with larger pores, Sephacryl S400. Both MukB and MukBEF eluted as distinct peaks when the column was equilibrated in the EDTA-containing buffer (Fig. 2C). The Stokes radii of the proteins (8.6 nm for MukB and 9.5 nm for MukBEF) are consistent with the previous data (24, 34) and indicate the dimeric MukB at the core of MukBEF.

The situation was dramatically different in the presence of magnesium. In this case, MukB alone eluted in or close to the void volume of the column, indicating the oligomeric state of the protein (Fig. 2D, top panel). In contrast, MukBEF migrated in the included volume of the column (Fig. 2D, middle panels). Thus, the interaction with MukEF served to disrupt MukB oligomers. Unexpectedly, MukB and MukEF did not co-migrate in the presence of the equimolar level of the kleisin. This result is in apparent contradiction with our finding of the one-to-one association between MukB and MukEF (Fig. 2A, third panel).

The two experiments differ only in the resin used for gel filtration. The smaller pores of Sephacryl S300, which was used in Fig. 2A, could conceivably stabilize MukBEF via cage effects (39). In agreement with this interpretation, the unliganded MukEF in reconstitution mixture migrated faster through the column than the free MukEF (Fig. 2D). This suggests that MukBEF could indeed be falling apart during chromatography through Sephacryl S400. And indeed, both MukB and MukEF

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**FIGURE 2. Bimodal association of MukB and MukEF.**

A, Sephacryl S300 analysis of reconstituted MukBEF. MukB and MukEF were mixed in indicated proportions and, after reconstitution in 30 μl of reconstitution buffer, resolved by gel filtration through a 1 ml of Sephacryl S300 column equilibrated with the buffer containing 40 mM NaCl, 2 mM MgCl₂ (40M), 40 mM NaCl, 2 mM EDTA (40E) or 200 mM NaCl, 2 mM EDTA (200M). MukB and MukBEF migrated with similar mobilities close to the void volume of the column. Eluted proteins were resolved by SDS-PAGE and visualized by silver staining. Positions of MukB (B), MukF (F), and the His-tagged MukE (E) are indicated on the right. Asterisks mark dimeric MukB that was cross-linked during purification (24). B, sedimentation analysis of reconstituted MukBEF; reconstituted MukBEF was mixed with molecular weight standards and resolved by centrifugation through 10–40% sucrose gradient in the buffer containing 200 mM NaCl, 2 mM EDTA. The amount of each protein in collected fractions was determined using densitometric analysis of Coomassie-stained SDS-PAGE gels. Protein concentrations were further normalized to the highest level found for a given protein across the gradient. Normalized concentrations of MukB, MukF, and MukE are plotted against the values of sedimentation coefficient corresponding to each fraction. The E,F-to-B ratio during reconstitution is indicated in the top left corner of each plot. The top panel (0x) shows sedimentation profiles for MukEF and MukB that were analyzed separately from each other. C, gel filtration of reconstituted MukBEF through Sephacryl S400 in the presence of 200 mM NaCl, 2 mM EDTA. 15 μg of MukB was reconstituted with MukEF in the indicated proportions in 60 μl and resolved on a 2-ml Sephacryl S400 column. The amount of MukB, MukF, and MukE (quantified as for panel B) is plotted against Stokes radii calculated for each fraction from the mobilities of standard proteins. D, gel filtration of reconstituted MukBEF through Sephacryl S400 in the presence of 40 mM NaCl, 2 mM MgCl₂. For the bottom panel the proteins were eluted at flow rate 0.15 ml/min, which is 5-fold faster than in all other experiments.
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**FIGURE 3. Preassembled MukBEF does not bind or reshape DNA.** After reconstitution, the indicated amounts of MukB and MukEF were assayed for DNA binding and reshaping. Positions of linear (L), nicked circular (NC), supercoiled (SC), relaxed (Rlx) DNA as well as 3-, 4-, 5- and 6-noded knots are indicated on the left of the gels. A, gel shift analysis of DNA binding by reconstituted MukBEF. 10 ng of pBR322 DNA was incubated with MukBEF and resolved by agarose gel electrophoresis in the presence or absence of 2 mM MgCl₂ as indicated. B, inhibition of DNA supercoiling and knotting by MukEF. DNA knotting (top panel) and supercoiling (bottom panel) reactions were done as described previously (24). M, marker knots generated as previously described (43). C, inhibition of DNA relaxation by MukB. 10 ng (3.5 fmol) pBR322 DNA was incubated for 10 min with 2.8 pmol of MukB, reconstituted MukBEF, or mock reaction mixture. The reaction mixtures were then treated for 30 min with the indicated amounts of wheat germ topoisomerase I, deproteinized, and resolved by agarose gel electrophoresis in the presence or absence of 2 mM MgCl₂. D, inhibition of relaxation by various amounts of MukB. Reactions were done as for panel C.

eluted closer to the void volume of the column when we performed chromatography at a 5-fold greater flow rate (Fig. 2D).

To summarize, our reconstitution studies revealed an unexpectedly rich pattern of interactions between MukB and MukEF. The ratio of MukE to MukF did not change during association with MukB and remained constant throughout elution profiles (Fig. 2, B–D). Thus, the composition of MukEF-saturated MukBEF is B₂(E₂F)₂. This notation acknowledges the previous conclusion that MukB interacts with MukEF via MukF (34, 35). At this stoichiometry, however, reconstituted MukBEF exists as a multimer rather than a holoenzyme. The multimeric MukBEF appears to be a true complex rather than an aggregate since it includes equimolar amounts of MukB and MukEF even when MukB is used in excess during reconstitution (Fig. 2A). Furthermore, the multimeric MukBEF is short-lived even under stabilizing conditions, in the presence of magnesium and low salt concentration. At higher salt, only the half-saturated, stable MukBEF can be detected. The half-saturated MukBEF migrates as a single peak during gel filtration and sucrose gradient centrifugation and does not appear to contain free MukB (Fig. 2, B and C). Based on these data, the composition of the half-saturated MukBEF is B₂(E₂F)₁. Apparently, the dimeric MukEF, (E₂F)₂, dissociates into two E₂F units upon formation of the half-saturated MukBEF.

**Preassembled MukBEF Does Not Bind or Reshape DNA—** MukB was previously shown to form a stable complex with DNA and promote DNA knotting and supercoiling in the ATP- and magnesium-independent manner (24, 27). We examined here how these activities change after the association of MukB and MukEF.

MukB was reconstituted with increasing amounts of MukEF, and the resultant mixture was tested in DNA gel shift, knotting, and supercoiling assays, as described before for MukB (24). We found no new activities in MukBEF compared with MukB. Instead, the assembly of MukBEF abolished all interactions of MukB with DNA. DNA gel shift was completely inhibited at a 1:1 EF-to-B molar ratio (Fig. 3A). Greater levels of MukEF were needed to inhibit gel shift if magnesium was omitted from the gel buffer (Fig. 3A). This is consistent with our earlier finding that the saturated B₂(E₂F)₂ complex is short-lived (Fig. 2), whereas the Mg²⁺-independent B₂(E₂F)₁ complex contains moieties of MukB that are able to bind DNA. The residual DNA binding at EF-to-B ratios of greater than 1 apparently reflects the low affinity binding of MukEF to B₂(E₂F)₁ (see also Fig. 2A, bottom panel).

We found a similar disappearance of the knotting and supercoiling activities upon formation of the holoenzyme. At low levels of condensin, the addition of MukEF resulted in a gradual decline in both DNA knotting and supercoiling (Fig. 3B). At high levels of MukB, small amounts of MukEF actually stimulated supercoiling and especially knotting. This result is in accord with the previous conclusion that condensins inhibit topoisomerases and thereby limit supercoiling and knotting at high protein-to-DNA ratios (23, 24). We indeed found that high concentrations of MukEF inhibit DNA relaxation by wheat germ topoisomerase I (Fig. 3D). Thus, the initial increase in knotting further supports the view that the activity of MukB declines upon its binding to MukEF. No knotting or supercoiling could be detected when the concentration of MukEF exceeded that of MukB (Fig. 3B). Similarly, the association with MukEF completely reversed the inhibitory effect of MukB on DNA topoisomerases (Fig. 3C). We conclude that the assembly of MukBEF precludes MukB from DNA binding.

**Competition between MukEF and DNA for Binding to MukB—** We next examined if MukEF can associate with DNA-bound MukB. We found, however, only adverse effects of MukEF on the MukB-DNA complex. With or without ATP, the electrophoretic mobility of MukB-bound DNA was altered after only 5 min of incubation with MukEF, and no gel-shift was observed 60 min after the addition of kleinsins (Fig. 4A). In contrast, the complex of MukB with DNA was not affected by long incubations.

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We obtained similar results when MukBEF-DNA complex was assembled in the absence of magnesium. Without magnesium, only the half-saturated B2(E2F) complex is formed (Fig. 2A), and this complex is able to bind DNA (Fig. 2B, left panel). The addition of magnesium, which stabilizes B2(E2F)2, resulted in the rapid displacement of DNA from the complex (Fig. 2B, middle panel).

Noteworthy, elevated levels of MukEF were more efficient in removing MukB from DNA (Fig. 2C). Because we only observed stoichiometric interactions between MukB and MukEF (Fig. 2), this result is a strong indication of the competition between MukEF and DNA for binding to MukB. And indeed, direct tests confirmed that DNA interferes with the assembly of MukBEF. About 50% of the equimolar MukEF was unable to bind MukB in the presence of the excess of DNA (Fig. 2C). Thus, MukEF and DNA bind MukB on a mutually exclusive basis.

Half-saturated MukBEF Forms a Stable Complex with DNA—Finding the competition between DNA and MukEF was unexpected given the identical phenotypes of mutations in MukB, MukF, and MukE (28). We reasoned, however, that MukB, being a dimer, might act as an asymmetric unit with one monomer bound to DNA and the other to MukEF. The possibility of such arrangement has been suggested by the finding that only 50% of reconstituted MukBEFs are stable (Fig. 2).

To test this premise, we prepared the half-saturated MukBEF, B2(E2F), by reconstitution of appropriately diluted MukB and MukEF and, after incubation with DNA, resolved the mix-
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ture by gel filtration through Sephacryl S400 (Fig. 4D). To mini-

mize dissociation of MukB from DNA, gel filtration was con-
ducted at low salt in our standard magnesium-free reaction buffer. Although MukB alone forms oligomers under these conditions (top panel), MukBEF migrates as a distinct peak with the apparent Stokes radius of 12 nm (middle panel). After incu-
bation with DNA, about half of MukEF was displaced from the complex, as was found earlier (Fig. 4C). Importantly, however, MukBEF was now found with DNA, in the excluded volume of the column. We conclude that the partially saturated MukBEF can bind DNA.

Purified MukBEF Is Unstable and Can Reshape DNA after Dissociation of MukEF—To confirm that the inhibitory effect of MukEF on DNA binding was not an artifact of the in vitro reconstitution procedure, we next examined MukBEF that was purified from cells that overproduced all three subunits of the complex (“Materials and Methods”). The complex was first purified by nickel-chelate chromatography, making use of the C-terminal 10-histidine tag on MukB. Thereafter, MukBEF was fractionated on a heparin column. MukBEF eluted as two distinct peaks from this column (Fig. 5A). The fractions compris-
ing the high salt (BEF-HS) and low salt (BEF-LS) peaks were pooled (as marked in Fig. 5A) and analyzed further.

The initial survey did not reveal significant differences between the two pools of MukBEF. Both proteins migrated as single peaks during sucrose gradient centrifugation and gel filtration with no admixture of free MukB or MukEF (data not shown). The sedimentation coefficient and the Stokes radius for BEF-HS and BEF-LS were estimated as 12.5 S and 11.6 nm and as 12.1 S and 10.9 nm, respectively. These values agree well with the previous data (34) and are consistent with the B2(E2F)_2 composition determined for the reconstituted complex. Quan-
tification of the Coomassie-stained gels revealed, however, that BEF-HS is depleted in MukEF, whereas MukB and MukEF are stoichiometric in BEF-LS. The MukEF-to-MukB ratio found for BEF-HS and BEF-LS was, respectively, 1.6 ± 0.2 and 1.9 ± 0.2 (data not shown). A direct test confirmed that BEF-HS, but not BEF-LS, is able to bind extra MukEF during reconstitution (Fig. 5E). In agreement with the earlier reconstitution data (Fig. 2A), the extra MukEF remained associated with BEF-HS only in the presence of MgCl2 (Fig. 5E).

The two fractions of MukBEF differed dramatically in their DNA binding properties. BEF-LS barely supported DNA gel shift and only in the absence but not in the presence of magne-
sium (Fig. 5B) and was completely inert in DNA supercoiling and knotting (Fig. 5C). In contrast, BEF-HS was very efficient in DNA binding and reshaping. The DNA reshaping properties of BEF-HS were highly similar to those of MukB (24). The major-
ity of generated knots were trefoils, with a small admixture of the four- and five-noded knots. Of 30 trefoils that we examined by electron microscopy according to Zechiedrich and Crisoma (40), 29 were right-handed. The optimal knotting and super-
coiling required between 450 and 900 condensins per DNA, which is about 5-fold greater than for MukB (24). This is con-
sistent with the lower content of unliganded MukB moieties in the preparation of BEF-HS. Finally, high concentrations of MukBEF inhibited DNA reshaping (Fig. 5C), as was reported earlier for MukB and the yeast SMC2/4 complex (23). We con-
clude that the partially dissociated MukBEFs are responsible for the DNA reshaping activities of BEF-HS.

As stated earlier, BEF-HS migrated as a single peak through sucrose gradient and Sephacryl S400, indicating that this protein preparation does not contain free MukB. Accordingly, the complex between BEF-HS and MukEF was only stable in the presence of magnesium (Fig. 5E), as we found earlier for the half-saturated complex B2(E2F) but not for MukB (Fig. 2). We conclude, therefore, that DNA reshap-
ing is accomplished by B2(E2F) rather than by MukB.

The observed separation of MukBEF into two fractions could be due to unbalanced overproduction of the subunits of the complex. Alternatively, the purified MukBEF could be inher-
ently unstable. To distinguish between these possibilities, we subjected BEF-LS to another round of heparin chromatogra-
phy. The majority of MukBEF eluted at the higher salt during second chromatography (Fig. 5A). The DNA supercoiling activ-
ity was only found in the high salt peak (Fig. 5D). Curiously, the peak of activity did not strictly coincide with the peak of the protein, indicating that the eluted MukBEF is not homoge-
nous. To verify this conclusion, we tested MukBEF from several fractions for the ability to bind MukEF. We indeed found that the high salt fractions of MukBEF had a greater capacity for MukEF (Fig. 5F). Thus, the purified MukBEF is intrinsically unstable and regains the ability to bind and reshape DNA after dissociation of MukEF.

MukEF Is Less Abundant in E. coli than MukB—So far we de-
tected only the inhibition of DNA binding by MukEF. All DNA binding activities were found in MukB. The half-satu-
ration complex however was stable and proficient in DNA bind-
ing and reshaping. To determine whether MukBEF indeed exists in cells as a partially saturated complex, we measured the amount of all three proteins in E. coli using quantitative Western blot approach (Fig. 6). The copy number of MukB averaged 400 ± 100 per exponentially growing cell, which agrees well with an earlier estimate, 150 per cell, obtained using a different technique (41). The copy number increased to 1000 ± 220 per cell during the onset of the stationary phase. The copy number of MukE and MukF remained virtually constant throughout the growth curve, averaging 340 ± 100 and 170 ± 50 per cell, respectively (Fig. 6B). Thus, MukEF is indeed less abundant inside the cell than MukB.

DISCUSSION

MukBEF plays a central role in organizing the chromosome of E. coli. Genetic studies indicated that all three subunits of the complex are essential for MukBEF function in vivo (28). There-
fore, it came as a surprise when we found that MukB can con-
dense DNA in vitro and in vivo without any help from its kleisin, MukEF (29). We show here that MukEF in fact disrupts the interaction of MukB with DNA. Preassembled MukBEF was completely inert in DNA binding and reshaping, whereas treat-
ment of DNA-bound MukB with MukEF resulted in eventual displacement of MukB from DNA. DNA, in turn, interfered with the assembly of MukBEF, indicating that MukEF and DNA compete for binding to MukB. ATP had no obvious effect on the assembly of MukBEF or the competition between MukEF and DNA, which is consistent with the very low rate of ATP
FIGURE 5. DNA reshaping properties of purified MukBEF. Various topological (topo) forms of DNA are the same as described in Fig. 3. A, MukBEF elution profiles after heparin chromatography. Protein concentrations were determined using the Bradford assay. MukBEF was purified by nickel-chelate chromatography and further fractionated on a heparin column (Heparin I). The pools of BEF-LS and BEF-HS are marked with the gray and black bars, respectively. BEF-LS was further fractionated by another round of heparin chromatography (Heparin II; the right y axis). The fraction numbers for the Heparin II column are indicated beneath the plot. For comparison, the dashed line shows elution of MukB from a Heparin I column. B, gel shift analysis of DNA binding by BEF-HS and BEF-LS. Reactions were done as described for Fig. 3A. Gel electrophoresis was carried out in TB buffer in the absence or presence (Mg2+/H11001) of 2 mM MgCl2. NC, nicked circular; SC, supercoiled. C, DNA reshaping activities of BEF-HS and BEF-LS. DNA knotting (top panels) and supercoiling (bottom panels) were analyzed as described in Fig. 3B. cats, DNA catenanes and oligomers. L, linear. D, DNA supercoiling activity in Heparin II fractions. BEF-LS was resolved by chromatography through the Heparin II column. The eluted fractions were dialyzed against 20 mM HEPES, pH 7.7, 40 mM NaCl, 8% glycerol, 2 mM EDTA, 1 mM dithiothreitol to remove excessive salt, and 4-μl aliquots were tested for supercoiling activity. The fraction numbers are the same as in panel A. S, DNA substrate; L, load; FT, flow-through. Rlx, relaxed. E, reconstitution of BEF-HS and BEF-LS with MukEF. 40 pmol of BEF-HS or BEF-LS, as indicated, was subjected to reconstitution procedure with 20 pmol of MukEF and resolved by gel filtration through Sephacryl S300 (see Fig. 2A for details). Positions of bands from the molecular weight marker are shown on the left of the top panel. Positions of MukB (B), MukF (F), MukE (E) and the His-tagged MukE (E9) are shown on the right. Note that BEF-HS and BEF-LS contain only the endogenous MukE before reconstitution, whereas MukEF contains only the nine-histidine-tagged MukE. Thus, the association of MukEF with MukBEF can be followed by evaluating the co-migration of the His-tagged MukE with MukBEF. F, MukBEF fractions eluted from Heparin II differ in their MukEF content. 3, 6, 8.5, and 4 pmol of MukB, MukF, MukE, and the His-tagged MukE (E9) from fractions 17, 21, 23, and 26 was reconstituted with 1, 2, 2.8, and 1.3 pmol of MukE, respectively, and analyzed by gel filtration as described in panel E.
hydrolysis by MukB (24). The properties of the purified MukBEF were the same as for the reconstituted holoenzyme. The purified MukBEF was inherently unstable and readily bound DNA after dissociation of MukEF. Thus, MukEF and DNA bind MukB on a mutually exclusive basis.

This conclusion is clearly at odds with genetic experiments as well as the observation that overproduced MukBEF is a better condensin in vivo than MukB (29). The opposite would be expected if the sole function of MukEF were to displace MukB from DNA. A possible solution to this paradox lies in our finding of two modes of association between MukB and MukEF. Only the half-saturated MukBEF, B2(E2F), was stable; the binding of MukEF to B2(E2F) was short-lived and could only be detected at low salt in the presence of magnesium. It is conceivable then that MukBEF operates inside the cell in its half-saturated form. We indeed observed the ternary, DNA-MukB-MukEF complex only at subsaturating levels of MukEF (Fig. 4D). Furthermore, the MukEF-depleted fraction of purified MukBEF, BEF-HS, was active in DNA binding and reshaping, demonstrating that the partially liganded MukBEF can bind and reconfigure DNA. Finally, quantitative immunoblot analysis confirmed that MukE and MukF are present in E. coli in fewer numbers than MukB (Fig. 6). Thus, MukBEF is likely to act as an asymmetric unit.

In this view kleisins and the SMC subunit of MukBEF are responsible for different aspects of chromosome condensation. DNA binding, presumably at the base of the right-handed loops (24), is accomplished by the SMC component of the complex. Indeed, the DNA reshaping properties of the partially liganded MukBEFs were virtually identical to those of free MukB whether the complexes were reconstituted in vitro (Fig. 3) or purified from the cell (Fig. 5). An increase in the intracellular level of condensins should increase the number of chromosomal loops resulting in more compact chromosomes. The increase in the copy number of MukB (Fig. 6) may contribute to the condensation of bacterial nucleoid during the onset of the stationary phase.

The role of kleisins is different. Kleisins are postulated to bind only the DNA-free leg of the V-shaped MukB dimer (Fig. 7). By bringing distant SMCs together, kleisins provide an additional level of chromatin condensation. Experimental evidence does indicate that MukEF is prone to bridge separate molecules of MukB. Indeed, reconstituted MukBEF was always oligomeric whenever salt conditions favored more than 50% association between MukB and MukEF (Figs. 2 and 4D). Similarly, an ele-
electron microscopy study revealed that MukB EF forms multimeric rosette-like structures in the absence of DNA (36).

Inside the cell MukEF could act by directly bridging distant MukBs (Fig. 7A) or by linking MukB to cellular matrix (Fig. 7B) as was suggested based on microscopic observation of overproduced MukBEF (29) and the finding of dynamic MukEF-dependent foci of MukB-green fluorescent protein (42). In either case the extent of chromatin condensation can be controlled by modulating the interaction of kleisins with SMCs without necessarily destroying the looped chromosome organization. Both the excess and the shortage of kleisins or perhaps a proteolytic cleavage of kleisins would result in the loss of chromatin compaction (Fig. 7C).

The Role of ATP—The role of ATP in the mechanism of MukBEF remains enigmatic. We did not find any effect of ATP on the assembly of MukBEF (data not shown) or the competition between DNA and MukEF (Fig. 4). Previously, ATP was shown to be expendable for DNA reconfiguration by MukB (24) and the yeast SMC2/4 protein (23). These results are consistent with the notion that ATP plays a role of a conformational switch rather than the motor fuel (19). Apparently, MukB and the yeast SMC2/4 complex are stable in their active conformations (1671–1676).

Switch rather than the motor fuel (19). Apparently, MukB and the yeast SMC2/4 complex are stable in their active conformations (1671–1676). Recently, microarray study revealed that MukBEF forms multimeric rosette-like structures in the absence of DNA (36).

The interaction between ATP and MukB may provide the functional basis to this interaction. MukB- to MukEF-mediated multimers occurred irreversibly in MukBEF disrupts MukB-MukB interface. The conversion from such dimerization (Fig. 2A) agrees well with the finding that MukB is oligomeric under reaction conditions (Fig. 2A). This interpretation agrees well with the finding that MukB is oligomeric under reaction conditions (Fig. 2D, top panel). The primary function of ATP is to promote dimerization of the head domains of SMCs (11, 12, 19, 20); clearly, MukB does not need ATP for such dimerization (Fig. 2D).

Noteworthy, protein multimers formed in the presence of kleisins differ from those formed by MukB alone. For example, MukBEF dissociated into protomers quicker than MukB during gel filtration at low salt (Fig. 2D). Thus, the assembly of MukBEF disrupts MukB-MukB interface. The conversion from MukB- to MukEF-mediated multimers occurred irreversibly in our in vitro system. It is conceivable that ATP regulates this interconversion in vivo. The interaction between ATP and MukB has been demonstrated both structurally and functionally (12, 14, 19). Our finding that kleisins displace DNA from MukB may provide the functional basis to this interaction.

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