Complex formation of MukB, MukE and MukF proteins involved in chromosome partitioning in *Escherichia coli*

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**mukF, mukE and mukB genes** are essential for the process of chromosome partitioning in *Escherichia coli*. We have studied protein–protein interactions among MukB, MukE and MukF proteins by co-immunoprecipitation and sucrose gradient sedimentation experiments, using *mukFEB* null cells harboring plasmids carrying the wild-type or mutant-type *mukFEB* operon. MukB forms a complex with MukF and MukE. Analysis of mutant MukB proteins suggested that MukF and MukE bind the C-terminal globular domain of MukB. MukF is indispensable for an interaction between MukB and MukE; however, MukF itself is able to associate with MukB even in the absence of MukE. We have also found that MukF has a Ca²⁺-binding activity. Although purified MukF was able to make a complex either with MukE or MukB, a complex consisting of the three Muk proteins was barely detected *in vitro*. However, increasing the Ca²⁺ or Mg²⁺ concentration in the reaction partially restored complex formation. This suggests that Ca²⁺ or Mg²⁺ may be required for the formation of a complex consisting of the three Muk proteins, and thus may participate in a particular step during chromosome partitioning.

**Keywords:** Ca²⁺-binding protein/chromosome partitioning/motor protein/MukB–MukE–MukF

**Introduction**

In eukaryotic cells, replicated chromosomes are inherited by two daughter cells using a specialized mitotic apparatus containing a spindle pole body and spindle microtubules. However, it has been a mystery how prokaryotic cells such as *Escherichia coli* transmit two duplicated chromosomes with high accuracy after chromosomal replication. Until very recently, in contrast to eukaryotes, bacterial cells were regarded as lacking anything like a mitotic apparatus. Nevertheless, on the assumption that bacteria also possess their own partitioning mechanism for the chromosome, even if it might be primitive, we performed a genetic screening to search for genes whose products are essential in an active way for chromosome partitioning (Hiraga *et al.*, 1989). First, we identified a novel gene *mukB* (Niki *et al.*, 1991). Missense and null *mukB* mutants produce anucleate cells with much higher frequency than wild-type cells (5–15% versus 0.03%). The *mukB* mutants also show temperature-sensitive growth: the mutant cells cannot grow at temperatures >30°C. We also found recently that MukB is involved in cell-cycle-dependent segregation of SeqA–DNA complexes (Hiraga *et al.*, 1998). SeqA itself is involved in controlling initiation of chromosome replication (Lu *et al.*, 1994).

The *mukB* gene encodes a 170 kDa protein (Yamanaka *et al.*, 1994a), one of the largest proteins in *E. coli*. MukB has globular domains at both ends and forms a homodimer by means of an α-helical coiled-coil rod structure in the center of the protein (Niki *et al.*, 1991, 1992). Such a structure reminds us of the motor proteins, such as eukaryotic kinesin and myosin. Biochemical studies further revealed that the N-terminal globular domain has ATP/GTP-binding, ATPase and GTPase activities, while the C-terminal globular domain has a DNA-binding activity (Niki *et al.*, 1992; Saleh *et al.*, 1996; Lockhart and Kendrick-Jones, 1998).

In order to determine how MukB plays a role in the chromosome partitioning process, we have searched specifically for MukB-associated proteins using genetic screening. A linkage group, *smbB*, of suppressors of a *mukB106* mutant was located in the *rneE* gene. All the *smbB* mutations encoded truncated RNaseE proteins lacking the C-terminal half. This was accompanied by an increased level of MukB, probably via an effect on mRNA degradation (Kido *et al.*, 1996). In addition, when the wild-type *cspC* and *cspE* genes were present in elevated copy numbers, the products of which are homologs of a major cold shock protein CspA in *E. coli*, the effects of the *mukB106* point mutation were suppressed (Yamanaka *et al.*, 1994b). However, it is still obscure how overexpression of the genes is involved in chromosome partitioning. All the above suppressors of *mukB* mutations thus may suppress *mukB* mutations indirectly.

Through the process of sequencing the region upstream of the *mukB* gene, we found two other novel genes, *mukF* and *mukE*, which are also essential for chromosome partitioning. These two genes compose an operon with *mukB* and *smtA* in the order *smtA–mukF–mukE–mukB* (Yamanaka *et al.*, 1996). The *mukF* gene encodes a 51 kDa protein containing a leucine zipper motif in the middle and an acidic amino acid cluster domain near the C-terminus. The *mukE* gene encodes a 27 kDa protein with as yet no identifiable features from database searches. Overexpression of any one of these three genes does not suppress the phenotypes caused by a defect in any of the other genes, indicating that the three gene products may...
constitute an active complex or, alternatively, they may independently play an indispensable role at different stages of chromosome partitioning.

Here we first report the existence of a complex consisting of MukF, MukE and MukB in a wild-type cell lysate, and then describe its conceivable structure. From the results of reconstitution experiments using the purified Muk proteins, we found that formation of the complex is stimulated by Ca$^{2+}$ or Mg$^{2+}$.

**Results**

**Phenotypes of a mukFEB deletion mutant**

We have established mukFEB deletion strains OT5 and OT7 as described in Materials and methods. These strains carry the disrupted smtA-mukF-mukE-mukB genes on the chromosome (see Figure 1B). However, it was previously reported that the smtA gene, encoding an S-adenosylmethionine-dependent methyltransferase, is not essential for cell growth and is not involved in chromosome partitioning (Yamanaka et al., 1995). Cells of both OT5 and OT7 form colonies only at a temperature <25°C, thus showing temperature-sensitive growth, as observed before for null mutants of individual mukF, E and B genes. Temperature-sensitive growth was complemented with plasmid pAX850 carrying the mukFEB genes, but not with plasmids carrying only one or two of these individual muk genes. Even at the permissive temperature, 22°C, these mukFEB null mutant strains still produced a significant number of anucleate cells, as also reported previously for mukF, mukE or mukB null mutants (Yamanaka et al., 1996). The doubling time of the mukFEB null mutants at 22°C in L medium was similar to that of individual muk null mutants.

**Physical interactions among Muk proteins**

As the first test of any physical interactions among MukB, MukF and MukE proteins, the Muk proteins in a cell lysate were immunoprecipitated using polyclonal antibodies raised against each Muk protein. The specificity of the antibodies was confirmed by Western blotting using a lysate of cells overexpressing three Muk proteins and affinity-purified antibodies against each Muk protein (Figure 2A). Each purified antibody recognized only the specific Muk protein but not the other Muk proteins, even in a cell lysate from the labeled cells was subjected to immunoprecipitation. Even in the absence of MukB, anti-MukF and anti-MukE antibodies (but not anti-MukB antibodies) co-precipitated MukF and MukE simultaneously (Figure 2B, top right), indicating that those two proteins could form a complex by themselves. In the absence of MukE, anti-MukF antibodies could precipitate MukF and MukB simultaneously. However, anti-MukB antibodies co-precipitated MukF poorly (Figure 2B, bottom left). This could be attributed to the insensitivity of anti-MukB, the decreased expression of MukF and MukB that is observed when MukE is missing (data not shown), and also the inefficient formation of a MukFEB complex (see Figure 3H and below). On the other hand, when MukF was absent, MukB was completely unable to form a detectable complex with MukE, indicating that MukF was indispensable for the interaction between MukB and MukE (Figure 2B, bottom middle). It should be noted that in the absence of any particular Muk protein, specific antibodies against this protein precipitated little, if any, of the other Muk proteins, demonstrating again the specificity of the antibodies.

MukF possesses a leucine zipper motif in the center of the protein (Figure 7C). Similarly, there is also a putative leucine zipper motif (1212–1233) in the C-terminal globular domain of MukB (Figure 7B). Such motifs are known to be involved in protein–protein associations. It is likely that this leucine zipper motif plays a critical role in MukF activity because the mukF233 missense mutation (Leu233 to Pro) located adjacent to this motif (see Figure 7C), which may disrupt the $\alpha$-helical repeat sequence, fails to suppress temperature-sensitive growth in mukF null mutant cells (Yamanaka et al., 1996). We examined a role for the leucine zipper motif of MukF in complex formation using the cell lysate from mukFEB null mutant cells, harboring plasmid pMY1010. This plasmid carries the mukFEB operon with the mukF233 mutation. The altered MukF233 protein was still able to form a complex with MukE and MukB (Figure 2B, bottom right), indicating that the leucine zipper motif in MukF may not be required for formation of the MukFEB complex. On the other hand, this motif of MukF could presumably be involved in association with another MukF molecule in the MukFEB complex or other unknown proteins.

**Mutational analysis of the MukB protein**

The MukB protein has two globular domains at each end that are linked by $\alpha$-helical coiled-coil structures in the middle (Niki et al., 1991, 1992). It is thus very important to know which globular domain is responsible for interaction with MukF or MukE in order to elucidate a molecular mechanism for how the MukFEB protein complex is involved in the process of active chromosome partitioning. We introduced mutations, including those isolated previously (Saleh et al., 1996), in either the N- or C-terminus of the mukB gene in the mukFEB operon on pAX850. These mutated derivatives of plasmid pAX850 were introduced into the mukFEB null mutant cells of strain OT5.
Fig. 1. Plasmids used in this study. The top bar represents arrangement of genes, restriction enzyme sites and putative promoters (P1, P2 and P3) in the mukFEB operon in the E.coli chromosome. (A) Structures of plasmids. Shaded bars show open reading frames. Solid lines show vector DNA. Restriction enzyme sites with double lines indicate disruption of the corresponding sites, causing a frameshift mutation. Solid bars show positions of non-coding regions downstream of the frameshift mutations. Asterisks represent the position of point mutations. The name of point mutations and the amino acid substitution are in parentheses on the right. Arrowheads in vector DNA show controllable promoters of T5-lac and T7-lac. (B) Chromosome of bacterial mukFEB null mutant strains, OT5 and OT7. The hatched bar represents the inserted *kan* segment in place of the Xhol deletion. B, BamHI; Bg, BglII; C, ClaI; E, EcoRI; K, KpnI; N, NcoI; Ns, NsiI; P, PstI; X, XhoI.
Table I summarizes the results of co-immunoprecipitation experiments studying interactions between the mutant MukB proteins and MukF–MukE. The mutant MukB1–1371 protein, lacking 115 amino acids from the C-terminal region, abolished any detectable interaction with both MukF and MukE, in addition to the DNA-binding activity of MukB. In contrast, the mutant MukB255–1486 protein, lacking the N-terminal 254 amino acids, retained the ability to interact with MukF and MukE. We next introduced point mutations into the N- or C-terminal globular domain of MukB and examined complex formation of these mutants with MukF and MukE. Two mutant proteins, MukB1008 (Gln1429 to Arg) and MukB1009 (Leu1403 to Pro), which have a point mutation in the C-terminal globular domain, could not associate with either MukF or MukE. However, these mutants retained the DNA-binding activity of MukB. On the other hand, another point mutation in the C-terminal domain, MukB1013 (Val1381 to Leu), did not affect complex formation but impaired DNA-binding activity. From these results, we conclude that MukF and MukE proteins associate with the C-terminal globular domain of MukB. Interestingly, a mutation in the nucleotide-binding site of the Walker motif A (MukB1004, Lys40 to Ile) (Walker et al., 1982) retained the ability to form a complex, indicating that nucleotide binding or nucleotide hydrolysis of MukB is not necessary either for interactions with MukF and MukE or for the DNA-binding activity of MukB.

Sucrose gradient sedimentation of Muk complexes

To confirm a physical interaction among the three Muk proteins and to examine the Muk complex in a semi-quantitative way, cell lysates were subjected to sedimentation through a 10–60% sucrose gradient. The presence of the three Muk proteins in each fraction was examined by Western blotting using specific antibodies.

All three Muk proteins showed the same sedimentation coefficient of ~11.5S in wild-type cells (Figure 3A) as well as in cells overexpressing the three Muk proteins from plasmid pAX850 (Figure 3B). It should be noted that free Muk proteins are hardly detected, suggesting that almost all molecules of each Muk protein in the cell are recruited into the complex. On the other hand, MukB, MukF and MukE proteins sedimented differently at 8.6S, 5.3S and 2.7S, respectively, when the other two Muk proteins were absent (Figure 3C, D and E). These results indicate that the three Muk proteins form a large complex, which can be detected in cell lysates. To measure the Stokes’ radii of each Muk protein and the MukFEB complex, gel filtration was performed using the same lysate. The Stokes’ radii of MukB, MukF, MukE and MukFEB were measured as 9.2, 4.7, 3.1 and 12.3 nm, respectively (Table II). Each purified Muk protein showed similar values of the sedimentation coefficient and Stokes’ radius as in the cell lysates (data not shown). After combining the two parameters (Siegel and Monty, 1966), the native molecular weight was estimated as 330 kDa for MukB, 110 kDa for MukF, 35 kDa for MukE and 600 kDa for the MukFEB complex. It is thus reasonable to suppose that MukB and MukF form a dimer, MukE is present as a monomer by itself, and the three Muk proteins make a large complex as big as 600 kDa. In addition, MukB1008 was sedimented at 8.6S, separating from a 7.3S complex consisting of MukF and MukE (Figure 3F and 3G), consistent with the co-immunoprecipitation experiment.

In the absence of MukB, the MukF and MukE proteins were apparently tightly bound together, generating a 7.3S complex (Figure 3G). In the absence of MukE, MukB showed sedimentation coefficients of 25S and 8.6S, while MukF sedimented at 25S and 5.3S (Figure 3H). Presumably, the 25S material corresponds to a MukFB complex and the smaller 8.6S and 5.3S complexes may correspond to a MukB dimer (340 kDa) and a MukF dimer (102 kDa), respectively. It is notable that the MukFB complex gave a larger sedimentation coefficient (25S) than the MukFEB complex (11.5S; see Figure 3H and B). Therefore without MukE, MukB and MukF seem to form an aberrantly large complex in which multiple MukF molecules may bind to a MukB dimer. The amount of this complex is lower than that of free MukB and MukF; therefore, this may result in the inefficient co-immunoprecipitation of MukB and MukF, shown in Figure 2B. In the absence of MukF, MukB and MukE sedimented separately (Figure 3I) at the positions of a MukB dimer (8.6S) and a MukE monomer.
Fig. 3. Sedimentation analysis of Muk proteins of a cell lysate by sucrose gradient centrifugation. A cell extract from wild-type cells (PB103) or mukFEB null mutant cells (OT5), harboring the indicated plasmids, was subjected to sedimentation through a 10–60% sucrose gradient. Proteins in each fraction were analyzed by SDS–5% PAGE for MukB and SDS–PAGE (10% acrylamide) for MukF and MukE, and were detected by Western blotting using specific antibodies. Positions of MukB, MukF and MukE are shown with arrows. Marker proteins, thyroglobulin (19.2S), ferritin (17.6S), catalase (11.3S), aldolase (7.3S), bovine serum albumin (4.3S), ovalbumin (3.5S) and chymotrypsinogen (2.4S), were also subjected separately to the same sedimentation. Note that errors of collected volume in each fraction are corrected by the actual volume in the fractions.

(2.7S), respectively. This indicated that MukB cannot bind MukE in the absence of MukF. We therefore concluded that MukF links MukB and MukE in the MukFEB complex.

Oligomerization of MukF and MukE proteins
As described above, in the absence of MukB, MukF and MukE were able to form a complex by themselves, and MukF can make a homodimer per se. We next asked how MukE molecules exist in the MukFE complex. We transformed mukFEB null mutant cells with plasmid pMY134 DNA, which can express MukE tagged with 11 amino acids (T7 tag) at the C-terminus. To the resultant transformant, we introduced a compatible plasmid encoding the authentic mukF, mukE or mukB genes in various combinations. Proteins in the transformants were labeled with 35S and a cell lysate was prepared for immunoprecipitation. When anti-T7 monoclonal antibody was used for immunoprecipitation, MukE was co-immunoprecipitated with MukE-T7 only when MukF was co-expressed (Figure 4, compare lanes 3 and 4). In contrast, MukB did not affect the association between MukE-T7 and MukE (Figure 4, lane 5). This result showed that more than two MukE molecules were able to associate only in the presence of MukF, probably through dimerization of MukF.

Ca2⁺-binding activity of the Muk proteins
MukF protein has another characteristic domain: an acidic amino acid cluster in the C-terminal region. Mutational analysis of the mukF gene revealed that this region is also essential for MukF function in vivo (Yamanaka et al.,
Table I. Complex formation and DNA-binding activity of various MukB mutant proteins

<table>
<thead>
<tr>
<th>MukB mutation</th>
<th>Substitution</th>
<th>DNA binding*</th>
<th>Complex formation with MukF &amp; MukE**</th>
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<tbody>
<tr>
<td>MukB 106</td>
<td>S33F</td>
<td>NT</td>
<td>+</td>
</tr>
<tr>
<td>MukB 1004</td>
<td>K40I</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>MukB 33</td>
<td>D1201N</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>MukB 1013</td>
<td>V1381L</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>MukB 1009</td>
<td>L1403P</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>MukB 1016</td>
<td>F1404L</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>MukB 1014</td>
<td>D1406N</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>MukB 1015</td>
<td>M1428L</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>MukB 1008</td>
<td>D1426R</td>
<td>-</td>
<td></td>
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<tr>
<td>MukB (1-1371) deletion</td>
<td>-</td>
<td></td>
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<tr>
<td>MukB (255-1488) deletion</td>
<td>NT</td>
<td></td>
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</table>

(A) Summary of the DNA-binding activity and complex-forming activity of MukB mutants. *DNA-binding activity was examined using purified mutant MukB proteins and 32P-labeled HpaII DNA fragments of pUC118 (Saleh et al., 1996; our unpublished data). **Complex formation was examined using a cell lysate containing the mutant MukB proteins (this study). NT, not tested.

(B) A schematic representation of positions of the mukB mutations used in this study. Closed bars represent N- and C-terminal globular domains. Open and hatched bars represent coiled-coil regions.

Table II. Hydrodynamic properties of Muk proteins

<table>
<thead>
<tr>
<th>Muk protein or Muk complex</th>
<th>Stokes’ radius (nm)</th>
<th>S20,w (S)</th>
<th>Calculated mol. wt (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MukF</td>
<td>4.7</td>
<td>5.3</td>
<td>110</td>
</tr>
<tr>
<td>MukE</td>
<td>3.1</td>
<td>2.7</td>
<td>35</td>
</tr>
<tr>
<td>MukB</td>
<td>9.2</td>
<td>8.6</td>
<td>330</td>
</tr>
<tr>
<td>MukFEB</td>
<td>12.3</td>
<td>11.5</td>
<td>600</td>
</tr>
</tbody>
</table>

Stokes’ radii and sedimentation coefficients were determined experimentally as described. The native molecular weight of the proteins was calculated from these values (Siegel and Monty, 1966) using a partial specific volume of 0.73 cm³/g.

1996). By analogy with eukaryotic calreticulin, in which an acidic domain is responsible for its low-affinity Ca²⁺-binding activity, we examined whether MukF also possesses a Ca²⁺-binding activity. Using purified His₆-tagged MukF, MukE and MukB proteins, we carried out [⁴⁵Ca²⁺]

overlay experiments in the presence of excess Mg²⁺, as described in Materials and Methods. As anticipated, His₆-tagged MukF showed a distinct Ca²⁺-binding activity compared with bovine serum albumin (Figure 5A) or His₆-tagged MukB (data not shown). His₆-tagged MukE also displayed a weak Ca²⁺-binding activity. Interestingly, FtsZ, as predicted by Löwe and Amos (1998) from crystallography, demonstrated a slightly higher Ca²⁺-binding activity than His₆-tagged MukF (Figure 5C; Discussion).

Reconstruction of the Muk complex using purified Muk proteins in vitro

We purified independently the three Muk proteins, each with six histidine residues (His₆ tag) or with 11 amino acid residues followed by His₆ (T7-His₆ tag), using metal-affinity chromatography. With these purified proteins, we attempted to reconstruct a complex consisting of the three proteins in vitro. After mixing MukF-His₆ with either MukB-His₆ or MukE-His₆, MukF-His₆ was immunoprecipitated using affinity-purified anti-MukF antibodies. MukE-His₆ (Figure 6A, lane 4) or MukB-His₆ (Figure 6A, lane 5) was co-immunoprecipitated with MukF-His₆, demonstrating that we had succeeded in making MukFE and MukFB complexes in vitro. In the presence of the three Muk-His₆ proteins in the reaction mixture, both MukE-His₆ and MukB-His₆ were co-immunoprecipitated with MukF-His₆ (Figure 6A, lane 6), suggesting the existence of both MukFE and MukFB complexes.

Next we performed the following experiments. MukF-His₆, MukB-His₆ and MukE-T7-His₆ were mixed and immunoprecipitated with monoclonal antibody against the T7 tag. Under the standard conditions, MukF-His₆ was co-immunoprecipitated with MukE-T7-His₆ efficiently; however, only a small amount of MukB-His₆ was co-immunoprecipitated (Figure 6B, lane 1). This indicated that the MukFE complex was dominant in the reaction,
Fig. 5. Ca\(^{2+}\)-binding activity of Muk proteins by \[^{45}\text{Ca}^{2+}\] overlay. Purified FtsZ, His\(_{6}\)-tagged MukE and MukF, or bovine serum albumin were electrophoresed in SDS–PAGE (10% acrylamide) and blotted on a nitrocellulose membrane. The membrane was incubated in a solution containing \[^{45}\text{Ca}^{2+}\], autoradiographed (A) and finally stained with 0.1% Amido-Black solution (B). Reading from the left, samples contained 2.5, 5.0 or 10.0 \(\mu\)g of each purified protein. The numbers on the left indicate the molecular size (kilodaltons) of standards used. The density of the autoradiography was measured with MacBAS (Fuji film) and plotted against the amount of the proteins applied (C).

in contrast to the studies with crude cell lysates above, and that the MukFEB complex was only a very minor constituent under these conditions. This may be due to the loss of some unknown factor(s) during purification, essential for formation of the MukFEB complex, or alternatively due to a requirement for an intermediate complex before the final complex is generated. To test the former possibility, the cell lysate from mukFEB null mutant cells was added to the mixture of the three purified Muk proteins; to test the latter possibility, two of the three proteins were pre-incubated in advance and then the third one was added to the reaction. However, neither of these strategies improved the formation of the complex of the three Muk proteins (data not shown).

Since we had demonstrated that MukF is a Ca\(^{2+}\)-binding protein, we investigated whether the addition of Ca\(^{2+}\) in the reaction mixture would promote complex formation with the three proteins effectively. In fact, increasing the concentration of Ca\(^{2+}\) did result in increasing amounts of MukB-His\(_{6}\) co-immunoprecipitated with
MukF-T7-His$_6$ and MukF-His$_6$ using the anti-T7 monoclonal antibody (Figure 6B, lanes 2–6). This effect of Ca$^{2+}$ was maximal with 10–30 mM CaCl$_2$. A further increase in the Ca$^{2+}$ concentration had a negative effect on formation of the complex and finally completely inhibited its formation at 100 mM CaCl$_2$. Importantly, at any concentration of Ca$^{2+}$, none of the Muk proteins were precipitated with control rabbit IgG (data not shown). This indicated that the Muk proteins were not precipitated in a non-specific way, e.g. resulting from aggregation by increasing the concentration of Ca$^{2+}$.

It is known that Mg$^{2+}$ can compete with Ca$^{2+}$ for the binding site of proteins in some cases. A similar experiment was therefore performed using MgCl$_2$ instead of CaCl$_2$ in the reaction mixture (Figure 6B, lanes 7–11). Increasing the concentration of Mg$^{2+}$ resulted in co-immunoprecipitation of MukB-His$_6$, but was slightly less effective than with Ca$^{2+}$. Mg$^{2+}$, however, had an additive effect on formation of the MukFEB complex in the presence of Ca$^{2+}$ (data not shown).

Interestingly, MukF-His$_6$ was detected as a doublet through SDS–PAGE (see Figure 6A and B), suggesting degradation or some modification of MukF-His$_6$ such as phosphorylation or another post-translational modification. Amino acid sequence analysis of the two bands of the doublet revealed that both of the protein bands were in fact MukF-His$_6$, with identical N-termini.

**Discussion**

We have shown here for the first time that MukF and MukE associate in vitro with MukB, all of which are essential for chromosome partitioning in *E. coli*. It is conceivable that the three Muk proteins may also act as a complex in vivo, since none of the three proteins are dispensable for proper chromosomal DNA partitioning. To facilitate the analysis of interactions among mutant Muk proteins, we introduced mutations into *mukB* or *mukF* genes in the context of the *mukFEB* operon on a plasmid. From the co-immunoprecipitation experiments using various mutant MukB proteins, mutants that have an amino acid substitution in the C-terminal domain can be classified into three groups. The first group contains MukB1013 and MukB1015, which retain the ability to associate with MukF and MukE. Overexpression of these mutant MukB proteins can suppress temperature-sensitive growth of the *mukFEB* null mutant OT5, when both the wild-type MukF and MukE proteins are also overexpressed (our unpublished data), but not when wild-type MukF and MukE are at the wild-type level (Saleh *et al*., 1996). This is one piece of *in vivo* genetic evidence suggesting an association between MukB, MukF and MukE. The second group of mutants contains MukB33 and MukB1014, which also retain the ability to associate with MukF and MukE, although in this case overexpression of these MukB mutants with the wild-type MukF and MukE proteins does not suppress the temperature-sensitive growth of OT5 cells. Thus, MukB mutant proteins in this class can associate with MukF and MukE to make a complex; however, the resultant complex is not active because of some other deficiency. This might involve the failure to associate with proteins other than MukF and MukE, or a deficiency in an unknown function residing in the C-terminal domain of MukB. The third group contains MukB1008, MukB1009 and the C-terminal truncated MukB1–1369 protein. These proteins are unable to make a detectable complex with MukF and MukE, and are thus unable to suppress the temperature-sensitive growth of OT5 cells, even with an excess of MukF and MukE. The properties of this third group of MukB proteins suggest that complex formation of the three Muk proteins is essential for the Muk proteins to exert their activities.

Mutational analysis using truncated MukB or missense MukB mutants shows that the C-terminal globular domain is responsible both for complex formation with MukF and MukE and for the DNA-binding activity. However, it should be noted that the capacities for DNA binding and for complex formation can be separated. MukB1013 is deficient in DNA binding but is capable of forming a complex, confirming that the three Muk proteins associate together in the absence of DNA. On the contrary, MukB1008 and MukB1009 retain the DNA-binding activity but are deficient in complex formation, indicating that MukF and MukE are unnecessary for the DNA-binding activity of MukB. MukB1004 and MukB1006, which have an amino acid substitution in or close to the Walker motif A, respectively, retain the ability to associate with MukF and MukE. These MukB mutants are likely to have a defect in the ATP/GTP-binding or ATPase activity, suggesting that nucleotide binding or hydrolysis is not necessary for complex formation.

It is still unclear how many molecules constitute the MukFEB complex because of the large size of MukB molecules compared with MukF and MukE. However, we have some suggestive results, as follows. From sucrose gradient sedimentation experiments, when MukB is absent (Figure 3G) or the mutant MukB1008 protein is unable to associate with MukF or MukE (Figure 3F), MukF and MukE can still form a complex, which has a sedimentation coefficient of ~7.3S. This value corresponds to ~150 kDa in the case of globular proteins. MukF can form a homodimer by itself and MukE forms oligomers dependent on the MukF dimer (Figure 4), suggesting that two MukF molecules and two MukE molecules are brought together into the MukFEB complex, resulting in a heterotetramer (155 kDa). MukB can associate with MukF in the absence of MukE, but not with MukE in the absence of MukF (Figures 2B, 3H and I). Mutational analysis of the MukB protein showed that the C-terminal globular domain of MukB is important for MukFEB complex formation. The evidence above suggests that the MukFEB complex associates with a MukB homodimer and presumably the MukF subunit, but not MukE, binds directly to the C-terminal globular domain of MukB. By combining two hydrodynamic parameters, the MukFEB complex is predicted to be ~600 kDa (Table II). After subtracting 340 kDa for a MukB dimer from 600 kDa, a mass of 260 kDa remains. Considering that the MukB molecules form a homodimer in an antiparallel arrangement (Melby *et al*., 1998), the 260 kDa may correspond to two sets of the MukFEB complex.

Previously, we predicted that regulation of MukF activity may involve Ca$^{2+}$ binding (Yamanaka *et al*., 1996), by analogy with the sequence of calreticulin in eukaryotic cells. Calreticulin is a ubiquitous major Ca$^{2+}$-binding protein localized in the endoplasmic reticulum (Michalak...
**A MukB (N-globular domain)**

![Amino acid sequence alignment of the N-terminal globular domain of MukB from six bacterial species. Possible Walker A and B-like motifs (Walker et al., 1982) are boxed. Identical amino acids to those of E.coli are shown in red. Different amino acids from those of E.coli are shown in blue. Gaps are given as dashes. Sequences not available due to incomplete sequencing are indicated by slashes. Asterisks denote positions of mutations used in this study. E.c., Escherichia coli; S.t., Salmonella typhi; Y.p., Yersinia pestis; V.c., Vibrio cholerae; H.i., Haemophilus influenzae; H.ac., Haemophilus actinomycetemcomitans.](image)

**B MukB (C-globular domain)**

![Amino acid sequence alignment of the C-terminal globular domain of MukB. Conserved amino acids in a putative leucine zipper motif are boxed.](image)

**C MukF**

![Amino acid sequence alignment of MukF. A region containing a leucine zipper motif and an acidic region are shown. Conserved leucine residues and acidic residues are boxed.](image)

Fig. 7. (A) Amino acid sequence alignment of the N-terminal globular domain of MukB from six bacterial species. Possible Walker A and B-like motifs (Walker et al., 1982) are boxed. Identical amino acids to those of E.coli are shown in red. Different amino acids from those of E.coli are shown in blue. Gaps are given as dashes. Sequences not available due to incomplete sequencing are indicated by slashes. Asterisks denote positions of mutations used in this study. E.c., Escherichia coli; S.t., Salmonella typhi; Y.p., Yersinia pestis; V.c., Vibrio cholerae; H.i., Haemophilus influenzae; H.ac., Haemophilus actinomycetemcomitans. (B) Amino acid sequence alignment of the C-terminal globular domain of MukB. Conserved amino acids in a putative leucine zipper motif are boxed. (C) Amino acid sequence alignment of MukF. A region containing a leucine zipper motif and an acidic region are shown. Conserved leucine residues and acidic residues are boxed.

et al., 1992). The C-terminal quarter of calreticulin possesses an acidic cluster, which is responsible for high-capacity Ca\(^{2+}\) binding (Baksh and Michalak, 1991). A C-terminal, truncated MukF protein lacking the acidic region failed to associate with MukB and MukE (our unpublished data). We therefore assume that the C-terminal acidic domain of MukF might be responsible both for binding Ca\(^{2+}\) and for the formation of the MukFEB complex.
Norris et al. (1988) have previously proposed that fluctuations in the intracellular level of free Ca$^{2+}$ could provide a mechanism for the initiation of DNA replication, chromosome partitioning and cell division. Although there is circumstantial evidence that Ca$^{2+}$ is implicated in cell cycle events, any regulatory role for Ca$^{2+}$ in E. coli cells in vivo is still obscure. To date, there have been some reports on the presence of prokaryotic calmodulin-like proteins (Laoudj et al., 1994; Smith, 1995). However, detailed analysis of bacterial calmodulin-like proteins and, importantly, their corresponding genes has not yet been described. In this study, we demonstrated that the gene product of mukF is a Ca$^{2+}$-binding protein. The optimum concentration of Ca$^{2+}$ necessary for MukFEB complex formation in vitro is substantially higher than the values reported for the average concentration of free Ca$^{2+}$ (0.1 μM) in E. coli cells in vivo (Gangola and Rosen, 1987). The high concentration required for the formation of the MukFEB complex in vitro may be placed in better perspective by the fact that one genome equivalent of mukF in E. coli cells in vivo is stimulated by calmodulin (Hisanaga and Pratt, 1984).

The significance of the association of MukF and MukE with MukB still remains to be assessed. Eukaryotic calmodulin binds cytoplasmic dynein from sea urchin in a calcium-dependent manner and the dynein ATPase activity is stimulated by calmodulin (Hisanaga and Pratt, 1984). In addition, Ca$^{2+}$ regulates myosin activity by binding to the regulatory light chain directly or through the activation of myosin light chain kinase by calmodulin. Since the overproduction of MukF, but not MukE or MukB, in wild-type cells results in the production of anucleate cells, mimicking the deletion of any of the muk genes (Yamanaka et al., 1996), it is feasible that Ca$^{2+}$-dependent MukF, in association with MukE, which may also bind Ca$^{2+}$ weakly, modulates the biochemical activities of MukB, such as its DNA-binding and ATPase activity.

MukF, MukE and MukB have been found so far only in E. coli, Haemophilus influenzae (Fleischmann et al., 1995), Vibrio cholerae (Melby et al., 1998), Haemophilus actinomycetemcomitans, Yersinia pestis (H.P. Erickson, personal communication and http://note.cellbio.duke.edu/ Faculty/ηEErickson/ηENucleus) and Salmonella typhi (sequence data were obtained from the Sanger Center via the website at http://www.sanger.ac.uk/Projects/S._typhi/blast_server.shtml). Among these bacterial species, amino acid sequence analyses are well conserved in MukB, particularly in N- and C-terminal globular domains (Figure 7A and B), MukF (Figure 7C) and MukE (our unpublished data). It should be noted that point mutations in MukB used in this study affect well-conserved amino acids among different bacterial species.

**Materials and methods**

**Bacterial strains and media**

All strains used are derivatives of E. coli K-12. MC1061 (araD139 Δara, leu797 ΔlacX74 galU galK hor hsr kam’ strA) (Casadaban and Cohen, 1980) and its derivative MSS (MC1061 ArnlA::cat) (Ohmori et al., 1995). XL1-blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 recA1 lacI1ΔlacProAB lacY1 araD139 Δara leu797 ΔlacX74 galU galK hor hsr kam’ strA) was used as a host for both general cloning and protein purification. PB103 (dadiR tcpE61 trpA62 tna-5) (de Boer et al., 1989), A33 (PB103 mukF::kan), A34 (PB103 mukE::kan) (laboratory strains) and GC7528 (PB103 ΔmukB::kan) (Niki et al., 1991) were used. The site-specific integration of prophage ΔADE3 into the chromosome of a host cell was performed using the ADE3 Lysogenization Kit (Biolab, Oxford). L medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract and 0.5% NaCl, pH 7.4) and M9 medium supplemented with 0.5% glucose, 1 mM MgSO$_4$ and required L-form amino acids (50 μg/ml each) were used for cell growth. When necessary, antibiotics were added to media as follows if not specified: ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; kanamycin, 30 μg/ml; chloramphenicol, 30 μg/ml; streptomycin, 120 μg/ml.

**Plasmids**

A summary of plasmids used in this study is depicted in Figure 1. Plasmid pAX850 carries the 8.5 kb BamHI fragment containing the mukFEB operon (Yamanaka et al., 1996). Plasmids pMY1001, pMY1002, pMY1004, pMY1006, pMY1003 and pMY1005 were constructed by replacing the 4.6 kb HindIII fragment in pAX850 with the corresponding fragments in pCX13, pCX9, pCX14, pCX8 (Saleh et al., 1996), pCX16 and pCX15, respectively (our unpublished data). Plasmids pKX733 and pKX732 carry mutations mukB33 and mukB106 in the mukFEB operon on pACYC184, respectively (Yamanaka et al., 1994a). Elimination of the Kan site in mukB gave rise to C-terminal truncation of MukB in the mukFEB operon (pMY1007). Introduction of a frameshift at the Cis site in mukF, the Nis site in muke and the SerEII site in the mukB gene on pAX850 resulted in elimination of each gene (pMY1008, pMY1009 and pKX746, respectively). Plasmid pMY1010 carries the mukFEB operon with the mukF233 mutation, which was transferred from plasmid pKX305 (Yamanaka et al., 1996). Plasmids pKX748, pKX766, pKX768, pKX750 and pAX840 were described previously (Yamanaka et al., 1996). mukF and muke genes were amplified by PCR and cloned into an expression vector pQE60 (Qiagen), yielding plasmids pKX789 and pMY1015, respectively. Both the N- and C-terminal regions in the mukB gene were amplified by PCR and ligated into plasmid pQE60, together with the 3.6 kb XhoI–KpnI fragment containing the middle portion of mukB from pAX840 (Niki et al., 1991). The resultant plasmid was named pMY171. Addition of isopropyl-β-D-thiogalactopyranoside (IPTG) induces production of MukF, MukE and MukB tagged with His$_6$ at their C-terminus, because expression of the mukB gene suppressed temperature-sensitive growth of the mutant at its C-termirus, encoded by pKX789, pMY105 and pMY171, respectively. Plasmids pMY115 and pMY116 are derivatives of pQE60, which can express wild-type MukB and mutant MukB1004 (Lys-40 to Ile) proteins without any tags, respectively. A pET21a DNA (Novagen) was digested with EcoRI, filled in with Klenow enzyme and recircularized with ligase, resulting in pET21aRI. PCR-amplified mukE gene was ligated with the Ncol–Ndel-digested pET28b DNA, yielding pMY119. Expression plasmid pMY119 expresses MukE protein tagged with T7-His at its C-terminus. An XbaI–BamHI fragment from pMY119 was ligated with XbaI–BamHI-digested pET21aRI DNA, resulting in pMY134. Plasmid pMY134 expresses MukE tagged with the T7 peptide at its C-terminus. A 4.2 kb Avll–BamHI fragment from pAX850 was filled in with Klenow enzyme and ligated with pRSETB DNA (Invitrogen), which was digested with ScaI and filled in. The resulting plasmid pMY121 expresses the N-terminal truncated MukB (MukB255–1486), tagged with His$_6$ at its N-terminus. All of the tagged wild-type Muk proteins function normally in vivo because expression of the plasmid-encoded muk gene suppressed temperature-sensitive growth of the corresponding null mutant cell.

**Construction of a mukFEB deletion mutant**

The kanamycin-resistance-conferring gene cassette from pACYC177 (Chang and Cohen, 1978) (1.4 kb HaelI fragment) was replaced with a 2.9 kb XhoI fragment on pAX850. The muk gene fragment with the kanamycin-resistance-conferring gene was excised and inserted into a BamHI site in plasmid pKHS002 (Ohmori et al., 1995). The resulting construct was introduced into strain MC1061 at 22°C and kanamycin-resistant (20 μg/ml) streptomycin-resistant (120 μg/ml) and ampicillin-sensitive (50 μg/ml) transformants, which carry the deleted mukFEB genes in the chromosome, were confirmed by Southern hybridization. The resulting strain was named OT5. The disrupted mukFEB genes in strain OT5 could be transduced with phage P1vir into the wild-type strain PB103 only below 25°C in the presence of kanamycin. One of the transductants, OT7, was used for further analysis.
**Purification of His<sub>6</sub>-tagged Muk proteins**

MukF-His<sub>6</sub>, MukE-His<sub>6</sub>, and MukB-His<sub>6</sub> were overexpressed in *E. coli* strain XL1-blue (DE3, pLY195) and YM944 (XL1-blue[pMY171]), respectively. MukE-T7-His<sub>6</sub> was overexpressed in YM334 (XL1-blue[DE3, pMY119]). Cells were grown at 30°C in 1 l of L medium containing tetracycline and 1% glucose and supplemented with ampicillin (YM198, YM171, YM944) or kanamycin (YM334). When the turbidity of the cells reached 50 Klett units (Klett–Summerson colorimeter with a No. 54 green filter), 1 l of 54% IPTG was added. The cells were grown at 30°C for a further 2 h before harvesting. The cell pellets were resuspended with 20 ml of sonication buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10% glycerol, pH 8.0) with 0.5 mg/ml lysozyme and incubated on ice for 30 min. The cells were lysed by three cycles of freeze–thawing and sonication. Cell lysates were cleared by centrifugation at 75 600 g at 4°C for 30 min, followed by a gentle passage through a 0.45 μm filter. Chelating-Sepharose FF (Pharmacia) was immobilized with Ni<sup>2+</sup> and equilibrated with sonication buffer. Four milliliters of a Sepharose suspension for MukF or MukE and 8 ml for MukB were used for purifying each His<sub>6</sub>-tagged protein. The supernatant was loaded onto a Chelating Sepharose FF column at a flow rate of 0.4 ml/min. The column was washed with 15 vol of the sonication buffer and then 20 vol of the sonication buffer with 20 mM imidazole. Bound proteins were eluted with a 20–500 mM linear gradient of imidazole in the sonication buffer. Fractions containing the His<sub>6</sub>-tagged proteins were pooled and precipitated with 35% ammonium sulfate. The precipitated proteins were dissolved with a small volume of buffer A [25 mM HEPEs–KOH, 0.1 mM EDTA, 1 mM diethiothreitol (DTT), 0.5 mg/ml lysozyme and 10% glycerol, pH 7.6] and dialyzed against buffer A × 50 μl slurry of 30% protein A–Sepharose 4F (Pharmacia) pre-equilibrated with buffer YA was added. The samples were mixed at 4°C with gentle mixing for 1 h and centrifuged at 8000 g for 1 min. The precipitated Sepharose was washed six times with 1 ml of ice-cold buffer YA, suspended with 25 μl of SDS-PAGE loading buffer, boiled for 5 min and centrifuged briefly at room temperature. The supernatant was analyzed by SDS–10% PAGE. Where non-labeled proteins were used, the gel was stained with Coomassie Brillant Blue R250. Where radiotiosephosphate-labeled proteins were used, dried gel was autoradiographed using a BAS2000 image analyzer (Fuji film).

**Sucrose gradient sedimentation and gel filtration**

Cells were grown in 20 ml of L medium supplemented with appropriate antibiotics where necessary. When the turbidity of the culture reached 80 Klett units, cells were harvested by centrifugation, washed with 10 ml of 25 mM HEPEs–KOH pH 7.6 and finally suspended with 200 μl of buffer B containing 0.5 mg/ml lysozyme. The cell suspension was incubated on ice for 30 min and treated with three cycles of freeze–thawing. Then 3 μl of 1 M MgCl<sub>2</sub> and 1 μl of 20 mg/ml DNase were added to the cell suspension. After incubation at 25°C for 1 h, the supernatant was recovered by centrifugation for 30 min at 4°C. A 2 ml sucrose gradient (10–60%, w/w) was prepared by diluting 0.36 ml each of 10, 17.5, 25, 32.5 and 40% sucrose onto 0.2 ml of 60% (w/w) sucrose in 25 mM HEPEs–KOH pH 7.6. 25 mM KCl, 0.1 mM EDTA, 2 mM DTT, 10% glycerol in a polycollomer centrifuge tube (Beckman; 11 × 34 mm). The tubes with the sucrose solution were incubated at 37°C for 1 h and then at 4°C for 1 h before use. The cell lysate in 70 μl was layered onto the sucrose gradient and centrifuged at 55 000 r.p.m. at 4°C for 13 h in a Beckman TLS-55 rotor. Approximately 100 μl of fractions were collected from the bottom of the tube after centrifugation.

The same lysates were subjected to gel filtration column chromatography using a Superose 6 HR10/30 column (Pharmacia; 1.0 × 30 cm). The standard proteins (Stokes’ radius) used were as follows: thyroglobulin (8.50 nm), ferritin (6.10 nm), catalase (5.22 nm), aldolase (4.81 nm), bovine serum albumin (3.55 nm), ovalbumin (3.05 nm) and chymotrypsinogen A (2.09 nm). [6th Ca<sup>2+</sup> overlay experiment]

The [6th Ca<sup>2+</sup> overlay experiment was performed according to the method of Maruyama et al. (1984). Purified proteins were subjected to SDS–PAGE and transferred to a nitrocellulose membrane (Ppritech, et al., 1987), the corresponding bands were trimmed and sent to Panpharm Laboratories Co., Ltd for raising antibodies in rabbits. Sera were affinity purified using immobilized proteins on a nitrocellulose membrane (Pringle et al., 1991). The purified antibodies did not cross-react with each other, proving the specificity of the antibodies. Anti-T7-tag monoclonal antibody directed against the polypeptide of 11 amino acids (MAMSTGQGQMG) was purchased from Novagen.

**In vivo labeling of proteins**

Cells having an indicated plasmid were grown at 22°C in M9 medium supplemented with 0.5% glucose, 1 mM MgSO<sub>4</sub> and 1% L-amino acid (50 μg/ml each), except for methionine and cysteine, and containing appropriate antibiotics. When the turbidity reached 80 Klett units, 1.2 ml of cells was removed, labeled with 4 μl of radiolabeled Pro-mix t<sup>35</sup>S [in vitro cell labeling mix (37 TBq/mmol; Amersham) at 37°C for 15 min and chased with 0.2 mg/ml unlabeled methionine and cysteine at 37°C for 2 min with agitation. The labeled cells were harvested by centrifugation and washed three times with saline. After the final wash, the cell pellet was suspended with 0.1 ml of buffer YA (25 mM HEPEs–KOH, 100 mM NaCl, 5% glycerol, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride, pH 7.6) supplemented with 1.0 mg/ml of lysozyme, incubated on ice for 30 min and subjected to three cycles of freeze–thawing. The cleared lysate was prepared by centrifugation at 15 000 g at 4°C for 20 min, followed by addition of NP-40 at 0.2%. **Immunoprecipitation**

Reaction mixture in buffer YA was mixed with 1 μg of affinity-purified polyclonal antibodies or T7 monoclonal antibody. After 1 h on ice, a 50 μl slurry of 30% protein A–Sepharose 4F (Pharmacia) pre-equilibrated with buffer YA was added. The samples were mixed at 4°C with gentle mixing for 1 h and centrifuged at 8000 g for 1 min. The precipitated Sepharose was washed six times with 1 ml of ice-cold buffer YA, suspended with 25 μl of SDS-PAGE loading buffer, boiled for 5 min and centrifuged briefly at room temperature. The supernatant was analyzed by SDS–10% PAGE. Where non-labeled proteins were used, the gel was stained with Coomassie Brilliant Blue R250. Where radiotiosephosphate-labeled proteins were used, dried gel was autoradiographed using a BAS2000 image analyzer (Fuji film).

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5883
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