Escherichia coli cell cycle control genes affect chromosome superhelicity

Tao Weitao, Kurt Nordström* and Santanu Dasgupta

Department of Cell and Molecular Biology, Uppsala University, Biomedical Center, Box 596, S-751 24 Uppsala, Sweden

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We have used ethidium bromide titration for direct measurement of the changes in the negative supercoiling of Escherichia coli chromosome caused by mutations inactivating the cell cycle functions mukB and seqA. The amounts of the intercalative agent required to relax the supercoiled chromosome in mukB and seqA mutants were lower and higher, respectively, than for the wild-type parent, confirming that these cell cycle genes modulate the topology of the E. coli chromosome. Plasmid superhelicity measured in these mutant strains showed similar effects albeit of reduced magnitude. As the effects of mukB and seqA mutations were not restricted to the chromosome alone, MukB and SeqA proteins possibly interact with factors involved in the maintenance of intracellular DNA topology. To our knowledge, this is the first direct demonstration of the influence of mukB and seqA genes on the superhelicity of the E. coli chromosome.

INTRODUCTION

The SeqA and MukB proteins in Escherichia coli play major roles in cell cycle control. SeqA is involved in sequestration, a process that regulates the frequency and timing of replication initiation in coordination with the bacterial growth rate (Crooke, 1995). SeqA is also a negative regulator of initiation of replication with inhibitory effects on DnaA and on the formation of the open complex at the replication origin (von Freiesleben et al., 1994; Torheim and Skarstad, 1999). Aberrant cell division and filament formation in SeqA-deficient strains suggest that SeqA might have targets other than oriC and might be involved in cell cycle processes other than transient inhibition of replication initiation. Intracellular locations of the SeqA foci during the cell cycle progression show a pattern of positional dynamics distinct from that of oriC (Hiraga et al., 1998; Onogi et al., 1999; Niki et al., 2000).

MukB is a very large protein with DNA- and ATP/GTP-binding domains at its C- and N-termini, respectively. Its extended coiled-coil structure gave rise to the hypothesis that MukB might function as the bacterial spindle analogue responsible for correct positioning of the replicated and decatenated chromosomes for equi-partition into the daughter cells. Inactivation of mukB results in production of anucleate cells at increasing frequency with rising temperature, attributing temperature-sensitive phenotype to the mutant strain (Hiraga et al., 1989; Niki et al., 1991; Hiraga, 1992). Recent genetic studies indicate that MukB might function as a condensation factor for the E. coli chromosome, similarly to SMC (stable maintenance of chromosome) proteins in eukaryotes and Gram-positive bacteria (Britton et al., 1998; Moriya et al., 1998; Weitao et al., 1999; Holmes and Cozzarelli, 2000; Sawitzke and Austin, 2000).

The molecular mechanisms underlying the action of SeqA and/or MukB remain obscure. We have recently shown that MukB and SeqA have opposite effects on the compactness of the bacterial nucleoids (Weitao et al., 1999). Since condensation factors and the superhelical state of the chromosomal DNA might both contribute to the compactness of the nucleoid, we undertook a direct measurement of the superhelicity of the membrane-free nucleoids from seqA and mukB mutant strains by titration with the intercalating drug ethidium bromide.

RESULTS

Titration of chromosome superhelicity in E. coli strains deficient in MukB or SeqA

Escherichia coli chromosomes are folded into nucleoids, which are nucleoprotein complexes packaged into 50–100 negatively supercoiled domains. Binding of an intercalating drug such as ethidium bromide leads to reduced negative superhelicity. With increasing concentration of the intercalator, the sedimentation rate decreases until the negative supercoiling is neutralized,
resulting in open coils (less compact, therefore slower sedimentation); further addition of the drug introduces positive supercoils to the DNA, increasing the sedimentation rate. The minimum s-value thus represents the titration end point of negative superhelicity; the drug concentration at this point provides a measure of the negative superhelicity of the nucleoids in vivo (Worcel and Burgi, 1972; Pruss et al., 1982).

Figure 1 and Table I show the results of velocity sedimentation analyses of membrane-free nucleoids (isolated by high-salt extraction, see Methods) from CM735 and its mukB and seqA derivatives. The sedimentation profiles in Figure 1, column A show nucleoids isolated from the wild type and the mukB mutant grown at 25°C (permissive temperature for growth for the mukB null mutant), then shifted to and maintained at 37°C for 2 h before harvesting. (B) Sedimentation profiles of nucleoids from wild-type, MukB+ and SeqA+ cells grown and harvested at 25°C. The top panel also includes the sedimentation profile of T4 phage particles (closed diamonds) as internal control. (C) Effect of increasing ethidium bromide concentration on the sedimentation rate of the nucleoids from the wild-type cells (0, 1.5 and 3.0 μg/ml, respectively, from top to bottom). (D) Variation in the sedimentation rates with increasing concentration of ethidium bromide for nucleoids from the wild-type, mukB and seqA strains, respectively, from top to bottom (s-value in S plotted against the dye concentration of the gradient).

Table I. Sedimentation coefficients of membrane-free nucleoids from E. coli CM735 and its mukB and seqA mutant derivatives, and the concentrations of ethidium bromide required to titrate their superhelicity.

<table>
<thead>
<tr>
<th>Strain</th>
<th>s-value in S</th>
<th>Dye concentration at the smin (μg/ml)</th>
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<tr>
<td>CM735</td>
<td>1517 ± 30</td>
<td>1.0 ± 0</td>
</tr>
<tr>
<td>mukB</td>
<td>1469 ± 30</td>
<td>0.7 ± 0.07</td>
</tr>
<tr>
<td>seqA</td>
<td>1742 ± 70</td>
<td>3.25 ± 0.35</td>
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Each value is the average from three independent experiments with the standard deviation shown except for the SeqA+ nucleoids, which were from two experiments.

The s-value was calculated by using the sedimentation rate for T4 phage particles as a standard; this phage has an s-value of 1025. Data were taken from Figure 1A.

Data taken from the minima in s-value versus dye concentration plots in Figure 1D.

show any change in the position of the slightly broadened sedimentation peak at the lower temperature. The nucleoids from seqA also did not show any temperature effect on sedimentation.
(data at 37°C not shown). For comparative sedimentation analyses of the nucleoids from the three strains, all sedimentation runs, including the titration runs, were performed with nucleoids from cultures grown at 25°C. The s-values for the wild type and its mukB and seqA derivatives (Table I) were estimated using ¹⁴C-labelled T4 phage particles (s = 1025S) as standard (see the sedimentation peak of T4 phages used as reference in the top panel of column B in Figure 1). Each value is an average from three independent runs with cells from separately grown cultures. The differences in the s-values were small but reproducible (Table I). Membrane-associated nucleoids (isolated in low salt in the presence of spermidine) from these strains had shown similar variations in the degree of compactness (Weitao et al., 1999), showing that the sedimentation rates provide reliable indicators of nucleoid structures among these strains.

The variation in sedimentation coefficients among the nucleoids merely indicated differences in their overall compactness, which could arise from either condensation or supercoiling, or from a combination of both. Suppression of the MukB phenotype by topA mutation and hypersensitivity of the mukB strain to the gyrase-inhibiting drug novobiocin (Weitao et al., 1999; Sawitzke and Austin, 2000) suggested that MukB protein might influence the supercoiling of the E. coli chromosome. Furthermore, inactivation of seqA led to compaction of the nucleoids and suppression of the mukB phenotype in the mukBseqA double mutant, indicating an opposing influence on nucleoid supercoiling by SeqA. Therefore, we examined the contributions of these genes to chromosome supercoiling by measuring the superhelical density of nucleoids from each strain directly by titration with the intercalative drug ethidium bromide.

Typical sedimentation profiles for the nucleoids from the wild-type parent CM735 strain at three concentrations of ethidium bromide are shown in Figure 1, column C (0, 1.5 and 3.0 μg/ml, respectively, from top to bottom). Figure 1, column D shows the sedimentation coefficients plotted as a function of the ethidium bromide concentration for nucleoids from the wild-type, mukB and seqA strains (top to bottom panels, respectively). As expected, the sedimentation rates decreased with increasing drug concentration, reached a minimum and then increased upon further addition of the dye; the dye concentration at the titration end points for the three strains.

**Effect of mukB and seqA mutations on the superhelicity of the plasmid pBR322**

In order to examine whether the observed consequences of mukB and seqA mutations on E. coli nucleoids arose from direct interactions of these gene products with the chromosome, or whether they resulted from a general impact of the loss of these functions on the superheliciry control within the bacteria, we tested the supercoiling of pBR322 plasmid DNA in CM735 and its mukB and seqA derivatives by ethidium bromide titration using cylindrical agarose gel electrophoresis (see Methods). The tubes were aligned by the position of the open circle (oc)-form, whose mobility was unaffected by the dye concentration. Figure 2A shows that with increasing ethidium bromide concentration, the mobility of the covalently closed circular (ccc)-form decreased, reached that of the oc-form and then increased again as positive supercoils were introduced by excessive intercalative binding. The top panel shows the plasmids from the wild-type and mukB hosts run in gels containing 0–65 μg/ml ethidium bromide. The SeqA- host yielded predominantly catenated forms of plasmid DNA with very few monomers. Titration of the dimeric plasmid molecules produced in the seqA strain is shown separately in the lower panel. The ethidium bromide concentrations in the dye-DNA complexes that ran as oc-form were 40, 30–35 and 50 μg/ml, respectively, for the plasmids from the wild-type, MukB- and SeqA- hosts. This is consistent with the relative titration pattern observed for the bacterial nucleoids. The drug concentrations corresponding to the titration end points for nucleoids and plasmids do not bear comparison due to the difference between the two experimental approaches. However, the similarity of the relative negative superhelicities estimated by these two very different methods (nucleoids: 1.0, 0.71, 3.25 and plasmids: 1.0, 0.85, 1.25 from the wild-type, mukB and seqA strains, respectively) is reassuring.

Figure 2B shows the two-dimensional (2D) chloroquine gel electrophoresis patterns of the plasmid pBR322 DNA grown in the wild type and the mukB and seqA mutant strains under two different conditions of buffer and chloroquine concentration, appropriate for low and high superhelical densities (see Methods and legend to Figure 2). The plasmid pBR322 was grown in the wild-type host in the presence of novobiocin or in a topA mutant host as controls with low and high superhelicity, respectively. As is clear from the figure, mukB mutation and inactivation of gyrase with novobiocin had the same effect on the mobility of plasmid ccc-monomers in TBE with 25 μg/ml chloroquine, suggesting that the plasmid molecules have low negative superhelicity in the absence of MukB. In contrast, the absence of SeqA and topoisomerase I affected the plasmid superhelicity similarly, causing higher mobility in TPE buffer with lower chloroquine, indicative of high negative superhelical density. In all cases, the oc-DNA molecules remained unaffected by either the mutations or the presence of novobiocin.

Use of the seqA mutant strain as host led to multimerization of the plasmid DNA (Figure 2B). Plasmid multimerization in the absence of SeqA was attributed to the possibility of higher frequency of recombination among DNA molecules with extensive single-stranded regions (higher negative supercoiling). Such recombination-dependent multimerization could then be suppressed by inactivation of the recA or recF genes. As shown in Figure 3, the plasmid DNA was predominantly monomeric in seqAreC and seqAreCF double mutants, supporting the idea that the high negative superhelicity in the absence of SeqA might have encouraged dimerization through homologous recombination. Introduction of the mukB mutation into the seqA host also resulted in monomerization of the plasmid (data not shown), possibly by reducing the negative superhelicity.

**DISCUSSION**

We had previously reported that mukB and seqA mutations have opposing effects on the nucleoid structure as shown by altered sedimentation rates and the appearance of nucleoids in fluorescence microscopy (Weitao et al., 1999). Here we demonstrate
that these mutations individually exert opposite influences on the superhelical density of the bacterial nucleoid. It is not clear from these studies whether the altered nucleoid structures result from or cause the changes in superhelicity seen in the chromosomes of the mutant strains. MukB and SeqA themselves might have topoisomerase activities but it seems more likely that they disrupt the balance among the topoisomerases either through selective interaction with topoisomerase(s) or by preventing topoisomerases from acting on the DNA modified in their presence. The domain structure of MukB resembles that of eukaryotic and bacterial SMC proteins (Britton et al., 1998; Melby et al., 1998; Moriya et al., 1998), which has been shown to interact with topoisomerase II in fission yeast and Drosophila (Hirano et al., 1997). It is tempting to speculate that MukB might be enhancing the gyrase activity in E. coli while SeqA might prevent gyrase from acting on the nucleoid structure. Recently, Torheim and Skarstad (1999) have shown from in vitro studies that SeqA binding to oriC plasmid could render it resistant to topoisomerase activities. Thus, MukB might be responsible for folding the chromosomes into negatively supercoiled, tightly condensed nucleoid-domains, while a large number of SeqA molecules, bound cooperatively to the nascently replicated chromosome segments, could temporarily protect them from topoisomerase activities. Superhelicity of the bacterial nucleoid turns out to be crucial at different stages of the bacterial cell cycle control: initiation of replication (Filutowicz, 1980; Filutowicz and Jonczyk, 1983); progression of replication fork (Filutowicz and Jonczyk, 1983; Ullsperger et al., 1988; Drlica and Zhao, 1997; Khodursky et al., 2000); segregation of replicated chromosomes and their partition (Steck and Drlica, 1984; Adams et al., 1992; Sawitzke and Austin, 2000); and, indirectly, cell division, which requires that the preceding steps be executed in correct sequence and time. As the superhelical domains are compartmentalized in a folded nucleoid, the average superhelicity as measured above might not reveal its dynamic role in cell cycle control. However, investigations into cell cycle functions that influence nucleoid structure might lead to the identification of factors responsible for its maintenance and the kinetics of its meaningful variations.

METHODOLOGICAL

Escherichia coli strains used. Escherichia coli strains MG1655 (prototroph) and CM735 (metE46 trp-3 thi-1 galK2 lacY1 or
lacZ4 mtl-1 ara-9 tsx-3 lon-1 rpsL8 or 9 sup E44 λ, Hansen et al., 1984) and their mukB and seqA derivatives have been described previously (Weitao et al., 1999). The recA and recF derivatives of the MG1655 seqA strain were constructed by generalized transduction with P1 lysates made on N8954 recA::cmr (J.L. Rosner, NIH) and JAS34 recF::Tn3 (J. Sawitzke, NCI, FCRC, Frederick, MD).

**Growth and labelling conditions.** All cultures were grown in M9 minimal medium (Sambrook et al., 1989) supplemented with 0.2% glucose and 0.4% casamino acids and methionine, histidine and tryptophan (50 μg/ml each). Stationary phase (overnight) cultures were diluted 10^-2-10^-3-fold into fresh medium and allowed to grow for 6–8 generations at 25°C. The chromosomal DNA was labelled with [methyl-3H]thymidine (5 μCi/ml, 73 Ci/mmol) for at least two generations.

**Sucrose density gradient centrifugations.** The cultures were chilled in ice and the cells were harvested by centrifugation at 4°C. Preparation of membrane-free and membrane-associated nucleoids and their centrifugations (with and without ethidium bromide) were performed according to published procedures (Worcel and Burgi, 1972; Drlica and Snyder, 1978; Pruss et al., 1982; Murphy and Zimmerman, 1997; Weitao et al., 1999). 3H-labelled T4 phage was run in a separate tube as a standard for the s-value (1025S; Worcel and Burgi, 1972); all sedimentations of membrane-free nucleoids were performed in an SW60 rotor at 16 000 r.p.m. for 30 min at 4°C (Drlica and Snyder, 1978).

**Plasmid supercoiling assay.** Exponential cultures (400 ml) of bacterial strains carrying the plasmid pBR322 were harvested at OD650 = 0.3. The plasmid DNA was extracted using the Wizard Plus Maxiprep (Promega) and stored in 0.2 ml of TE (10 mM Tris-HCl, 1 mM EDTA pH 7.4) at –20°C.

Cylindrical agarose gel electrophoresis (DeLeyys and Jackson, 1976) was performed in an assembly that held twelve glass tubes (2.5 × 150 mm) connecting the upper and lower tanks filled with TAE buffer (40 mM Tris-acetate, 1 mM EDTA; no ethidium bromide). The tubes were filled with 0.7% agarose in TAE with 0–65 μg/ml ethidium bromide (see Figure 2A) and placed vertically in the electrophoresis tank. The plasmid DNA (10 μl containing 25% sucrose and 2.5 μg/ml ethidium bromide) was loaded onto the submerged tops of the gels. The electrophoresis was run at 100 V for 105 min at room temperature. The cylindrical gels were removed carefully from the glass tubes, stained with 2 μg/ml ethidium bromide in water for 45 min and photographed.

The first dimension run for the horizontal 2D electrophoresis was performed in 0.8% agarose in TAE buffer at 2.5 V/cm. Then the gel was turned 90°, soaked for 3 h in either TPE (90 mM Tris-phosphate, 2 mM EDTA) containing 18.5 μg/ml chloroquine (Hulton et al., 1990; higher mobility for more negative supercoiling) or TBE (0.5× 45 mM Tris–borate, 1 mM EDTA) containing 25 μg/ml chloroquine (Hinton et al., 1992; higher mobility for less negative supercoiling) and run at 1 V/cm for 20 h. The plasmid DNA was detected by Southern blotting with a 32P-labelled probe specific for the plasmid origin and the tet' gene on pBR322.

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**REFERENCES**


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