Escherichia coli SeqA protein affects DNA topology and inhibits open complex formation at oriC

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Chromosome replication in Escherichia coli is initiated by the DnaA protein. Binding of DnaA to the origin, oriC, followed by formation of an open complex are the first steps in the initiation process. Based on in vivo studies the SeqA protein has been suggested to function negatively in the initiation of replication, possibly by inhibiting open complex formation. In vitro studies have shown that SeqA inhibits oriC-dependent replication. Here we show by KMnO4 probing that SeqA inhibits open complex formation. The inhibition was not caused by prevention of DnaA binding to the oriC plasmids, indicating that SeqA prevented strand separation in oriC either directly, by interacting with the AT-rich region, or indirectly, by changing the topology of the oriC plasmids. SeqA was found to restrain the negative supercoils of the oriC plasmid. In comparison with the effect of HU on plasmid topology, SeqA seemed to act more cooperatively. It is likely that the inhibition of open complex formation is caused by the effect of SeqA on the topology of the plasmids. SeqA also restrained the negative supercoils of unmethylated oriC plasmids, which do not bind SeqA specifically, suggesting that the effect on topology is not dependent on binding of SeqA to a specific sequence in oriC.

Keywords: Escherichia coli/Initiation of DNA replication/open complex formation by DnaA/SeqA/ supercoiling

Introduction

Initiation of DNA replication in Escherichia coli takes place at a unique origin, oriC, and is a precisely controlled process (Kornberg and Baker, 1992; Boye et al., 1996; Messer and Weigel, 1996). The minimal oriC (Figure 1) contains five specific binding sites for the initiator protein DnaA (Skarstad and Boye, 1994; Kaguni, 1997), termed DnaA boxes R1–R4 and M (Fuller et al., 1984; Matsui et al., 1985; Schaper and Messer, 1995). DnaA binds to these boxes in an ordered but non-cooperative fashion in vitro and forms an initial complex (Margulies and Kaguni, 1996; Weigel et al., 1997). Next, ATP-bound DnaA separates the DNA double helix at the AT-rich region in the left part of oriC (Figure 1) and forms an open complex (Sekimizu et al., 1987; Bramhill and Kornberg, 1988). This opening reaction is strongly stimulated by the presence of the architectural proteins IHF (integration host factor) or HU (Dixon and Kornberg, 1984; Skarstad et al., 1990; Hwang and Kornberg, 1992). The DNA template must also contain unrestrained negative supercoils to make strand separation energetically favorable, unless transcriptional activation is provided (Baker and Kornberg, 1988). DnaA then directs the DnaB helicase/DnaC complex to the open complex (Baker et al., 1986; Funnell et al., 1987; Bramhill and Kornberg, 1988; Marszalek and Kaguni, 1994). Binding of DnaB to oriC generates the prepriming complex (Sekimizu et al., 1988).

A candidate for a negative regulator of the initiation process is the SeqA protein, which was identified as a factor involved in sequestration of hemimethylated, newly replicated oriC (Lu et al., 1994; von Freiesleben et al., 1994), a mechanism rendering hemimethylated origins inert to initiation (Russell and Zinder, 1987). In vivo studies of seqA mutant cells showed that SeqA also affects the initiation of DNA replication from fully methylated oriC in a negative fashion (Lu et al., 1994; von Freiesleben et al., 1994; Boye et al., 1996). Open complex formation has been suggested to be the inhibited step (Lu et al., 1994); another suggestion has been that SeqA modulates the activity of the DnaA protein directly (von Freiesleben et al., 1994). In vitro, SeqA binds specifically to fully methylated oriC fragments (Figure 1; Slater et al., 1995). In vitro replication studies have shown that SeqA is capable of inhibiting replication both by preventing formation of prepriming complexes and by inhibiting replication from pre-made prepriming complexes (Wold et al., 1998). In the present study we used KMnO4 probing to detect open complex formation by DnaA, and show that SeqA inhibits strand separation in oriC. Furthermore, we find that SeqA has a profound effect on DNA structure by restraining negative supercoils.

Results

Open complex formation depends on the DnaA concentration

Strand separation at the three AT-rich 13mers in oriC can be studied using potassium permanganate (KMnO4) probing. KMnO4 modifies thymines and, to a lesser extent, cytosines in single-stranded (ss) or kinked B-DNA regions (Sasse-Dwight and Gralla, 1991). The modified bases are non-functional as templates for DNA polymerase in replication elongation reactions. We performed KMnO4 probing on pBSoriC (7 nM) in the presence of IHF (75 nM) and DnaA protein (0–420 nM) at 30 and 37°C. The samples were analyzed for modification of bases in the lower strand in oriC by performing primer extension reactions from the PA1 primer (Figure 1). The extension products were analyzed on a sequencing gel (Figure 2A).

In agreement with earlier findings, the presence of products in the AT-rich R, M and L 13mer regions, corresponding to modification of ssDNA, increased with increasing

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DnaA concentrations both at 37 and at 30°C (Figure 2A, lanes 1–5 and 11–15, and 2B). At high DnaA concentrations (>200 nM) open complex formation reached a plateau. At even higher concentrations (>450 nM), DnaA inhibited open complex formation (data not shown). The formation of open complex was more efficient at 37°C than at 30°C.

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The effect of SeqA on open complex formation was examined by including 1350 nM SeqA (200 SeqA molecules/plasmid) in the KMN04 probing reactions (Figure 2A, lanes 6–10 and 16–20). Otherwise, reaction conditions were as described above. At moderate to high DnaA concentrations (>110 nM), addition of SeqA inhibited the formation of open complexes both at 30 and at 37°C (Figure 2B). Low concentrations of SeqA (25–50 SeqA molecules/plasmid) did not inhibit open complex formation, whereas higher amounts of SeqA (400 SeqA molecules/plasmid) inhibited the reaction even further (data not shown).

**SeqA does not inhibit the binding of DnaA to oriC plasmids**

Both SeqA and DnaA bind to oriC. To address whether the presence of SeqA hindered the DnaA binding to oriC, DnaA–pBSoriC complexes were formed in the absence and presence of SeqA under conditions where SeqA inhibits open complex formation (7 nM pBSoriC, 75 nM IHF, 220 nM DnaA and 1350 nM SeqA). The samples were incubated at 37°C and gel filtered through agarose columns at 37°C to preserve open complexes. Aliquots of the collected fractions were analyzed by gel electrophoresis and stained for DNA (Figure 3A). The presence of proteins in the fractions was determined by Western blotting (Figure 3B). The coelution of DnaA and DNA is a measure of the extent of complex formation between DnaA and oriC. The amount of DnaA that bound to the oriC plasmids in the presence of SeqA, relative to its absence, was calculated for three different experiments, each based on three different Western blots. These data showed that the presence of SeqA did not inhibit binding of DnaA to oriC. In fact, it appeared that slightly more DnaA (10–20%) bound to the oriC plasmids in the presence of SeqA. Control samples without DNA were gel filtered to verify that the protein found in the DNA-containing fractions was present due to binding to the plasmids and not due to aggregation (data not shown). The experiments also showed that ~10% of the SeqA (~20 molecules/plasmid) bound strongly enough to coelute with the DNA.

**The plasmid topology changes in the presence of SeqA**

SeqA preferentially binds to the left part of the minimal oriC (Slater et al., 1995), which includes the region of initial strand separation (Figure 1). Therefore, binding of SeqA could inhibit the opening reaction directly. Alternatively, SeqA could be capable of restraining negative supercoils in the plasmid in the same way as HU (Skarstad et al., 1990) and Fis protein (Margulies and Kaguni, 1998), thus making strand separation unfavorable.

To investigate the latter possibility, calf thymus DNA topoisomerase I (Topo I) was used as a tool. This enzyme will relax negatively supercoiled DNA if the supercoils are unrestrained (Wang, 1996; Figure 4A). Plasmid pBSoriC (7 nM), which had ~20 negative supercoils (determined by band counting), was incubated with SeqA (0.4–2.7 μM) and treated with Topo I. The resulting topoisomers were separated on an agarose gel containing a high concentration of chloroquine (40 μg/ml; Figure 4). Topo I treatment in the absence of SeqA led to complete relaxation of the DNA (Figure 4B, lane 2). At high levels of SeqA (400 SeqA molecules/plasmid) the plasmids were only slightly affected by Topo I (Figure 4B, lane 7); on average five of the ~20 supercoils were relaxed (compare lane 1 with lane 7), i.e. ~15 of the supercoils remained. This result suggests that SeqA interacted with the plasmids, affecting the topology in such a way that, on average, 15 negative supercoils/plasmid were restrained and not accessible to Topo I. At lower levels of SeqA a subpopulation of plasmid molecules was completely relaxed by Topo I (Figure 4B, lanes 4–6), indicating that SeqA restrained most of the negative supercoils in some plasmids, while leaving others unaffected. A control with SeqA storage buffer corresponding to the highest amount of SeqA added, did not inhibit the enzymatic activity of Topo I (data not shown). Data from several experiments show that the ratio of SeqA molecules to plasmid molecules must be 400–600 to affect the whole population of plasmids. At 200 SeqA molecules/plasmid, which is the level used here to inhibit open complex formation, SeqA restrained the negative supercoils of about one-third of the plasmids.

**A comparison of the effect of SeqA and HU on plasmid topology**

The histone-like protein HU is known to restrain negative supercoils of DNA (Broyles and Pettijohn, 1986; Baker and Kornberg, 1988; Skarstad et al., 1990). To gain more insight into how SeqA affects DNA topology, we made a comparison with the HU protein. Plasmid pBSoriC (7 nM), was incubated with increasing amounts of SeqA.
Fig. 2. SeqA inhibits open complex formation. (A) KMnO$_4$ reactions were performed on pBS orIC (7 nM) at 30 or 37°C as described in Materials and methods. The DnaA concentration was varied as indicated at the top of the figure (lanes 1, 6, 11 and 16, no DnaA; lanes 2, 7, 12 and 17, 55 nM; lanes 3, 8, 13 and 18, 110 nM; lanes 4, 9, 14 and 19, 220 nM; and lanes 5, 10, 15 and 20, 420 nM DnaA). A concentration of 220 nM DnaA corresponds to 33 DnaA molecules/plasmid. The concentration of SeqA was 1350 nM (200 SeqA molecules/plasmid, lanes 6–10 and 16–20). Samples were subjected to primer extension reactions with $^{32}$P-labeled primers (PA1, Figure 1) and the products were separated on an 8% polyacrylamide gel with urea. Dideoxy-sequenced pBS orIC plasmids were used as sequence markers (lanes marked T, A, C and G). The regions of each lane included in the quantification of open complex (AT-rich 13mers: R, M and L) and reference bands are indicated to the right. (B) Extent of open complex formation at different DnaA concentrations with SeqA (circles) and without SeqA (squares), at 30°C (open symbols) and at 37°C (closed symbols) was found by analyzing the marked regions in (A) as described in Materials and methods. The background signal level in the absence of DnaA and SeqA at 37°C (lane 1) was set to 1.

(0.7–4.3 μM) or HU protein (0.5–2.8 μM) and treated with Topo I. The resulting DNA topology was analyzed on agarose gels with two different concentrations of chloroquine (40 or 2 μg/ml; Figure 5). The gel with high chloroquine concentration resolves the highly negatively supercoiled topoisomers, whereas the gel with low chloroquine concentration resolves the slightly supercoiled topoisomers (Figure 5). Incubation with increasing amounts of HU (lanes 3–6, both panels) led to a widened distribution of topoisomers that gradually shifted from the position of relaxed form towards more supercoiled species. Data from several experiments indicated that addition of 70 HU dimers/plasmid restrained, on average, two supercoils of each plasmid. In agreement with the results in Figure 4, SeqA at 100–200 SeqA monomers/plasmid (lanes 7 and 8, both panels) either shifted a subpopulation of the plasmids completely or had little effect. Thus, at the intermediate levels of protein used here, SeqA only affected a subpopulation of the plasmids, whereas HU affected all of them.

At higher amounts of protein (Figure 4, 400 SeqA monomers/plasmid, lane 9, or 420 HU dimers/plasmid, lane 6), both SeqA and HU restrained supercoils in all the plasmids. The number of restrained supercoils/plasmid was ~15 and ~10, in the presence of SeqA and HU, respectively. This indicates that the potential of SeqA to restrain negative supercoils is comparable to that of the classical histone-like protein HU.

**SeqA also restrains negative supercoils in unmethylated plasmids**

To investigate whether the effect of SeqA on DNA topology requires specific interaction with orIC, we performed the Topo I assay described above on unmethylated pBS orIC, to which SeqA does not bind specifically (Slater et al., 1995). The SeqA protein had the same effect on the topology of unmethylated pBS orIC as on the topology of the methylated form (data not shown). The level of SeqA required to affect the whole population of unmethylated plasmids was the same as for methylated
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**Fig. 4.** Effect of SeqA on DNA topology. (A) The principle for using Topo I as a tool to investigate the level of unrestrained negative supercoils of plasmids. The plasmids are treated with Topo I, deproteinized (by addition of SDS) and electrophoresed in the presence of chloroquine. (I) A negatively supercoiled plasmid is relaxed upon Topo I treatment, chloroquine intercalation rewinds the relaxed plasmid in the opposite direction. (II) A plasmid with restrained supercoils is not relaxed by Topo I, chloroquine intercalation unwinds the negatively supercoiled plasmid and, if present in excess, chloroquine will rewind the plasmid in the opposite direction. (B) pBSoriC (7 nM) was incubated with SeqA (0.4–2.7 μM, corresponding to 50–400 molecules/plasmid) at 37°C for 5 min (see Materials and methods). Calf thymus DNA topoisomerase I was added and incubation was continued for another 30 min. Samples were electrophoresed (45 V for 6 h) in a 10-cm 0.8% agarose gel with 40 μg/ml chloroquine. The description of plasmid species (arrows and bracket) refers to the state of the plasmids before chloroquine intercalation. Lane 1, untreated plasmid; lane 2, plasmid treated with Topo I; lanes 3–7, plasmid incubated with SeqA before and during Topo I treatment. Note that at 40 μg/ml chloroquine, negatively supercoiled plasmids will lose their negative supercoils and acquire a few positive supercoils. Plasmids relaxed by Topo I will all become highly positively supercoiled and migrate as unresolved topoisomers in front. (C) A hypothetical scheme of how SeqA by cooperative binding builds a protein complex on the plasmid and restrains most of its negative supercoils.

**Discussion**

We have given the first evidence that SeqA can inhibit initiation of DNA replication at an early stage *in vitro*, namely at the stage of open complex formation. The results agree well with earlier *in vivo* studies (Lu et al., 1994; von Freiesleben et al., 1994; Boye et al., 1996), all pointing to a negative effect of SeqA on the initiation of replication. *In vitro*, SeqA affects oriC-dependent replication negatively by inhibiting both the formation of prepriming complexes and replication from pre-made prepriming complexes (Wold et al., 1998). It is likely that at least some of the effects seen on prepriming complex formation originate from the effects of SeqA on open complex formation shown here.
Effect of SeqA on binding of DnaA to oriC

The binding of DnaA protein to oriC is non-cooperative and ordered (Margulies and Kaguni, 1996; Weigel et al., 1997), indicating that the initial complex of DnaA with oriC is a well-defined structure. We have shown by gel filtration analysis that the presence of SeqA does not reduce the amount of DnaA protein bound to the oriC plasmids. These experiments show that SeqA does not inhibit the initial binding of DnaA to oriC, but do not address whether SeqA affects the structure of the DnaA complex at oriC. The reason for the somewhat higher amount of DnaA bound to oriC plasmids in the presence of SeqA compared with in the absence of SeqA is not known, but might indicate that fewer DnaA molecules are tightly bound in the open complex compared with the initial complex. In an earlier gel-filtration study by Wold et al. (1998) it was shown that the formation of prepriming complexes was inhibited in the presence of SeqA. In that study DnaA was not recovered in initial complexes. However, magnesium was excluded from those reactions (in order to maintain prepriming complexes) and lack of magnesium possibly explains the observed destabilization of the initial complexes.

Restraint of negative supercoils by SeqA

SeqA profoundly influences the topology of the oriC plasmids. The effect seems, in part, to be different from the restraint of negative supercoils by HU. HU changes the topology of all plasmids in the population in a continuous, concentration-dependent manner. In contrast, SeqA appears to have a more cooperative effect on individual plasmids: it either changes the supercoiling to a certain level or it has little effect. This presumably reflects a different binding and/or mechanism of action for SeqA compared with HU. Whereas HU probably is distributed evenly among plasmids, affecting them all similarly and simultaneously, SeqA seems to have a preference for plasmids that are already bound by other SeqA molecules. This results in restraint of almost all supercoils in a subpopulation of plasmids, rather than restraint of a few supercoils in all the plasmids. A hypothetical scheme of how SeqA might restrain most of the negative supercoils in a plasmid is shown in Figure 4C. The cartoon is based on the following facts: (i) SeqA is capable of restraining most of the negative supercoils in a single plasmid (this study); (ii) SeqA seems to act cooperatively when affecting individual plasmids in the plasmid population (this study); and (iii) SeqA binds cooperatively to DNA (Slater et al., 1995).

The histone-like protein HU binds to DNA as a heterodimer, whereas the stoichiometric binding of SeqA to DNA is unknown. Assuming that SeqA binds as a monomer, we find that 400 SeqA monomers/plasmid is at least as efficient as an equivalent amount of HU (420 dimers/plasmid) in restraining negative supercoils. The mechanism behind this effect on topology may, however, be different in the two cases.

The amount of SeqA needed to inhibit open complex formation (200 SeqA molecules/plasmid) is sufficient to change the topology of a subpopulation of the plasmids. It is probable that the plasmids that are topologically affected by SeqA are those that are impaired in open complex formation. Since we have been unable to detect inhibition of open complex formation at low levels of SeqA, we propose that the major contribution to the inhibition comes from restraint of negative supercoils. However, it should be noted that we cannot rule out that a more specific effect of SeqA is responsible for part of the inhibition of open complex formation.

The effect of SeqA on DNA topology is not dependent on specific binding to oriC

SeqA protein binds specifically and cooperatively to a fully methylated oriC fragment (Slater et al., 1995). The binding has a certain specificity for the left half of oriC containing the AT-rich 13mers, DnaA box R1, the IHF binding site and DnaA box M (Figure 1). It is, however, not clear whether a specific sequence, the combination of GATC sites or other structural determinants are important for binding. SeqA does not bind specifically to unmethylated oriC (Slater et al., 1995). However, SeqA had essentially the same effect on the topology of unmethylated plasmids as on fully methylated plasmids. Thus, specific binding of SeqA to oriC is not required to affect the topology of the oriC plasmids, nor is there a requirement for methylation of GATC sites.

SeqA may affect replication in two ways

Wold et al. (1998) showed that replication of fully methylated oriC plasmids in vitro was inhibited by low levels of SeqA protein (25 molecules/oriC plasmid), and then further inhibited at higher levels (>200 molecules/oriC plasmid). Replication of unmethylated oriC plasmids was affected only at high levels of SeqA. These data suggested that SeqA inhibits replication in vitro in two different ways: (i) SeqA is capable of specifically inhibiting replication of fully methylated oriC plasmid; and (ii) SeqA inhibits replication of both fully methylated and unmethylated plasmid when present in high amounts. Since we have shown here that both fully methylated and unmethylated plasmids are topologically affected in the presence of high amounts of SeqA, we suggest that the mechanism of inhibition in the latter case is the restraint of negative supercoils by SeqA.

SeqA may affect chromosome topology in vivo

The effect of SeqA on plasmid topology shown here may reflect a similar effect of SeqA on chromosome topology in vivo. The fact that initiation of replication in SeqA-less cells occurs at a lower mass than in wild-type cells (Boye et al., 1996) and that these cells exhibit an ‘overinitiation’ phenotype (Lu et al., 1994; von Freiesleben et al., 1994; Boye et al., 1996), could be an indirect effect of the topological state of the origin in the absence of SeqA. Since the amount of SeqA in vivo has been estimated to be only 1000 molecules/cell (Slater et al., 1995), it is reasonable to assume that SeqA does not influence the DNA topology by binding all over the chromosome. Rather, SeqA is more likely to have a specific effect on certain structures or topological domains. Localization studies indicate that the major part of the SeqA protein in the cell is part of complexes that might function as folding centers for refolding of daughter chromosomes (Hiraga et al., 1998). Thus, our finding that SeqA can affect plasmid topology may prove to be important, not only in the timing of replication, but also in the process
of partitioning and segregation of daughter chromosomes after replication.

Materials and methods

**Purification of DNA and proteins**

Plasmid pBSoriC (3640 bp) was cloned into SmaI and HindIII sites of pUC18 (Stratagene, La Jolla, CA). The plasmid was purified twice on a preparative agarose gel. The purified plasmid was digested with BamHI and SmaI and purified on a preparative agarose gel. The purified plasmid was used as a template for in vitro transcription

**Probing of open complex formation with potassium permanganate**

The potassium permanganate (KMnO₄) probing procedure was described in Sasse-Dwright and Grolla (1991) and Woelker and Messer, 1993. Reactions (50 μl) with 800 ng pBSoriC (7 nM) and 80 ng IHF (75 nM) were incubated for 2 min at 37°C or 30°C in a buffer with 25 mM HEPEs-KOH, pH 7.6, 10 mM magnesium acetate, 5 mM ATP and 65 μg/ml bovine serum albumin (BSA). Varying amounts of DNA were incubated in 1 mM ATP for 1 min at 30°C and 1400 ng SeqA (1350 nM), or a corresponding amount of SeqA. The samples were extracted with an equal volume of 4 M KCl and 0.1 M Tris-HCl pH 7.5 or 0.5 M KCl and washed three times for 20 min in 0.1 M Tris-HCl pH 7.5 or 0.5 M KCl and then stained in 0.5 M TBE with SybrGold. The DNA was detected with UV light.

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**References**


References


**Quantification of open complex**

The gel was analyzed using ImageQuant software (Molecular Dynamics). The amount of open complex in a sample was calculated by density scanning of the bands corresponding to the R, M and L 13mers (Figure 2). The integrated density was normalized to the DNA yield and loading by dividing by values from volume integration of bands outside the AT-rich regions (marked as ‘reference’ in Figure 2A). Reference bands are modified both in the presence and absence of DnaA.

**Gel filtration of oriC plasmid and proteins**

Samples of 700 ng pBSoriC (7 nM), 70 ng IHF (75 nM) and 525 ng DNA (220 μg) were incubated in reaction buffer (25 mM HEPEs-KOH pH 7.6, 10 mM magnesium acetate, 5 mM ATP and 65 μg/ml BSA) in a volume of 45 μl at 37°C for 5 min in the presence or absence of 1300 ng SeqA (1350 μl). Aliquots (40 μl) of the mixtures were loaded onto pre-equilibrated 1 ml Bio-Gel A-15m columns and eluted with reaction buffer at 37°C. Ten-drop fractions (~170 μl) were collected. Aliquots of the fractions were: (i) analyzed by DNA electrophoresis in 0.8% agarose, subsequently stained with SybrGold (Molecular Probes) and detected with UV light; and (ii) electrophoresed in 12%–polyacrylamide gels and analyzed by Western blotting with rabbit antisera against SeqA or DNA. The antibodies were detected using the Vistra ECF Western blotting kit (Amersham) and scanned on a PhosphorImager (Molecular Dynamics STORM840). Quantification was carried out using ImageQuant software (Molecular Dynamics).


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