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

Norunn K.Torheim and Kirsten Skarstad¹

Department of Cell Biology, Institute for Cancer Research, Montebello, 0310 Oslo, Norway

¹Corresponding author
e-mail: kirsten.skarstad@labmed.uio.no

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 pBS*oriC* (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

Fig. 1. The *E. coli* *oriC* region. AT-rich regions and protein-binding sites relevant to this study are shown. The minimal fragment required for binding of SeqA protein when the DNA is fully methylated is indicated (Slater *et al.*, 1995). The annealing site for the primer, PA1, used in primer extension of the lower strand is marked with a horizontal arrow.

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.

SeqA inhibits open complex formation

The effect of SeqA on open complex formation was examined by including 1350 nM SeqA (200 SeqA molecules/plasmid) in the KMnO₄ 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 pBSoriC (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 pBSoriC 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

Fig. 3. Binding of DnaA to *oriC* plasmids in the presence and absence of SeqA. Parallel samples of pBSoriC (7 nM), DnaA (220 nM) and IHF (75 nM) were incubated with SeqA (1350 nM, 200 molecules/plasmid; right-hand panels) or SeqA storage buffer (left-hand panels) for 5 min at 37°C and gel filtered at 37°C. Fractions were collected and analyzed for DNA on a 0.8% agarose gel stained with SybrGold (A), and for the presence of DnaA and SeqA by Western blotting (B).

chloroquine (40 or 2 $\mu\text{g/ml}$; 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 pBSoriC, to which SeqA does not bind specifically (Slater *et al.*, 1995). The SeqA protein had the same effect on the topology of unmethylated pBSoriC 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

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; and 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.

plasmids. This result suggests that specific binding of SeqA to *oriC* was not required in order to change plasmid topology.

Fig. 5. Comparison of the effect of SeqA and HU on DNA topology. pBSoriC (7 nM) was incubated with SeqA (0.7–4.3 μM, corresponding to 100–600 monomers/plasmid) or HU (0.5–2.8 μM, corresponding to 70–420 dimers/plasmid), as indicated below each lane, at 37°C for 5 min (see Materials and methods) and then treated with Topo I. Samples were electrophoresed (45 V for 14 h) in 15-cm 0.8% agarose gels with chloroquine. The DNA in the gels in (A) and (B) was taken from the same samples, but electrophoresed in the presence of 40 and 2 μg/ml chloroquine, respectively. The description of plasmid species (arrows and brackets) refers to the state of the plasmids before chloroquine intercalation. Lane 1, untreated plasmid; lane 2, plasmid treated with Topo I; lanes 3–6, plasmid incubated with HU before and during Topo I treatment; and lanes 7–10, plasmid incubated with SeqA before and during Topo I treatment. Note that in (A) lane 1, the topoisomers migrate with positive supercoils due to the high concentration of chloroquine, whereas in (B) lane 1, the topoisomers migrate with negative supercoils. The extra band seen below the nicked band in (A) lanes 8–10 presumably represents plasmids that have become linearized due to prolonged storage of the samples.

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), also called pTB101 (Baker and Kornberg, 1988), consists of a 678 bp *HincII*–*PstI* fragment spanning *oriC* (–189 to +489) cloned into the pBluescript vector (Stratagene). The plasmid was purified twice on a CsCl₂–ethidium bromide density gradient as described previously (Sambrook *et al.*, 1989), followed by desalting by passage through a Sephadex G-50 Nick column (Pharmacia). Fully methylated plasmid was purified from strains W3110 or DH5, *dam*⁺ and unmethylated plasmid were purified from strain DS1310, *dam*[–].

SeqA was purified as described previously (Wold *et al.*, 1998) based on a protocol by Therese Brendler.

Probing of open complex formation with potassium permanganate

The potassium permanganate (KMnO₄) probing procedure was modified from Sasse-Dwight and Gralla (1991) (Gille and Messer, 1991; 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 ng/µl bovine serum albumin (BSA). Varying amounts of DnaA [up to 1100 ng (420 nM), incubated in 1 mM ATP for 1 min at 30°C] and 1400 ng SeqA (1350 nM), or a corresponding amount of SeqA storage buffer was added and incubation continued for 4 min. Freshly made KMnO₄ was added from an 80 mM solution to a final concentration of 7 mM. After 2 min the reaction was quenched by addition of 4 µl β-mercaptoethanol and EDTA was added to a final concentration of 20 mM. Samples were extracted with an equal volume of phenol–chloroform (Fluka) and spun through Sephadex G-50 Nick Spin columns (Pharmacia). The sites of KMnO₄ modification were visualized by primer extension reactions as described below.

Labeling of primers

The PA1 primer (5′-GAAAATGGATTGAAGCCCGGGCCGT-3′; Hwang and Kornberg, 1992) was labeled with [γ-³²P]ATP (3000 Ci/mmol) (Amersham) using the RTS T4 kinase labeling system (Gibco-BRL).

Primer extension reactions

Aliquots (4 µl) of the KMnO₄-treated samples (~20 ng) were subjected to primer extension reactions in a volume of 6 µl with 0.4 U Vent (exo-) DNA polymerase (New England Biolabs), 25 fmol γ-³²P-labeled PA1 primer and 50 fmol of each dNTP in a buffer with 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris–HCl pH 8.8, 5 mM MgSO₄ and 0.1% Triton X-100. The samples were overlaid with 15 µl mineral oil (Sigma) and incubated in a Perkin–Elmer DNA Thermal Cycler 480 for 20 cycles [1 min denaturation at 94°C, 1 min annealing at 55°C, 1 min extension at 72°C (5 min in the last cycle)]. Reactions were stopped by addition of 4 µl formamide buffer (deionized formamide with 0.37% EDTA, bromophenol blue and xylene cyanol FF). Samples were electrophoresed in 8% polyacrylamide (Bio-Rad), 8 M urea gels. The gels were exposed in a storage phosphor screen and scanned on a PhosphorImager (Molecular Dynamics STORM840).

Quantification of open complex

The gels were analyzed using ImageQuant software (Molecular Dynamics). The amount of open complex in a sample was found by volume integration of bands corresponding to the R, M and L 13mers (Figure 2). All values were normalized to correct for DNA yield and loading by dividing by values from volume integration of bands outside the AT-rich regions (marked as 'reference' in Figure 2A). These reference bands are modified both in the presence and absence of DnaA.

Gel filtration of *oriC* plasmid and proteins

Samples of 720 ng pBSoriC (7 nM), 70 ng IHF (75 nM) and 525 ng DnaA (220 nM) were incubated in reaction buffer (25 mM HEPES–KOH pH 7.6, 10 mM magnesium acetate, 5 mM ATP and 65 ng/µl BSA) in a volume of 45 µl at 37°C for 5 min in the presence or absence of 1300 ng SeqA (1350 nM). 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 for DNA by electrophoresis in 0.8% agarose, subsequently stained with SybrGold (Molecular Probes) and detected with UV light; and (ii) electrophoresed in 12% SDS–polyacrylamide gels and analyzed by Western blotting with rabbit antisera against SeqA or DnaA. 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).

Supercoiling assays

Samples (20 µl) of 320 ng pBSoriC (7 nM) in a buffer of 25 mM HEPES–KOH pH 7.6, 10 mM magnesium acetate and 5 mM ATP were incubated with SeqA (0.4–4.3 µM) or HU (0.5–2.8 µM) at 37°C. After 5 min, 14 U (1 µl) calf thymus DNA topoisomerase I (Gibco-BRL) and 4 µl 0.3 M KCl were added and incubation continued for 30 min at 37°C. The reaction was stopped by addition of 5 µl stop buffer (6% SDS, 30% glycerol, 10 mM EDTA, bromophenol blue and xylene cyanol FF). The samples were electrophoresed in 0.8% agarose with 2 or 40 µg/ml chloroquine (Sigma) for 6–7 h or 14 h at 45 V. The gel was washed three times for 20 min in 0.1 M Tris–HCl pH 7.5 or 0.5× TBE and then stained in 0.5× TBE with SybrGold. The DNA was detected with UV light.

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References

- Baker, T.A. and Kornberg, A. (1988) Transcriptional activation of initiation of replication from the *E. coli* chromosomal origin: an RNA–DNA hybrid near *oriC*. *Cell*, **55**, 113–123.
- Baker, T.A., Sekimizu, K., Funnell, B.E. and Kornberg, A. (1986) Extensive unwinding of the plasmid template during staged enzymatic initiation of DNA replication from the origin of the *Escherichia coli* chromosome. *Cell*, **45**, 53–64.
- Boye, E., Stokke, T., Kleckner, N. and Skarstad, K. (1996) Coordinating DNA replication initiation with cell growth: differential roles for DnaA and SeqA proteins. *Proc. Natl Acad. Sci. USA*, **93**, 12206–12211.
- Bramhill, D. and Kornberg, A. (1988) Duplex opening by dnaA protein at novel sequences in initiation of replication at the origin of the *E. coli* chromosome. *Cell*, **52**, 743–755.
- Broyles, S.S. and Pettijohn, D.E. (1986) Interaction of the *Escherichia coli* HU protein with DNA. Evidence for formation of nucleosome-like structures with altered DNA helical pitch. *J. Mol. Biol.*, **187**, 47–60.
- Dixon, N. and Kornberg, A. (1984) Protein HU in the enzymatic replication of the chromosomal origin of *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **81**, 424–428.
- Fuller, R.S., Funnell, B.E. and Kornberg, A. (1984) The dnaA protein complex with the *E. coli* chromosomal replication origin (*oriC*) and other DNA sites. *Cell*, **38**, 889–900.
- Funnell, B.E., Baker, T.A. and Kornberg, A. (1987) *In vitro* assembly of a prepriming complex at the origin of the *Escherichia coli* chromosome. *J. Biol. Chem.*, **262**, 10327–10334.
- Gille, H. and Messer, W. (1991) Localized DNA melting and structural perturbations in the origin of replication, *oriC*, of *Escherichia coli* *in vitro* and *in vivo*. *EMBO J.*, **10**, 1579–1584.
- Hiraga, S., Ichinose, C., Niki, H. and Yamazoe, M. (1998) Cell cycle-dependent duplication and bidirectional migration of SeqA-associated DNA–protein complexes in *E. coli*. *Mol. Cell*, **1**, 381–387.
- Hwang, D.S. and Kornberg, A. (1992) Opening of the replication origin of *Escherichia coli* by DnaA protein with protein HU or IHF. *J. Biol. Chem.*, **267**, 23083–23086.
- Kaguni, J.M. (1997) *Escherichia coli* DnaA protein: the replication initiator. *Mol. Cell*, **7**, 145–157.
- Kornberg, A. and Baker, T.A. (1992) *DNA Replication*. 2nd edn. Freeman, New York, NY.
- Lu, M., Campbell, J.L., Boye, E. and Kleckner, N. (1994) SeqA: a negative modulator of replication initiation in *E. coli*. *Cell*, **77**, 413–426.

- Margulies,C. and Kaguni,J.M. (1996) Ordered and sequential binding of DnaA protein to *oriC*, the chromosomal origin of *Escherichia coli*. *J. Biol. Chem.*, **271**, 17035–17040.
- Margulies,C. and Kaguni,J.M. (1998) The FIS protein fails to block the binding of DnaA protein to *oriC*, the *Escherichia coli* chromosomal origin. *Nucleic Acids Res.*, **26**, 5170–5175.
- Marszalek,J. and Kaguni,J.M. (1994) DnaA protein directs the binding of DnaB protein in initiation of DNA replication in *Escherichia coli*. *J. Biol. Chem.*, **269**, 4883–4890.
- Matsui,M., Oka,A., Takanami,M., Yasuda,S. and Hirota,Y. (1985) Sites of dnaA protein-binding in the replication origin of the *Escherichia coli* K-12 chromosome. *J. Mol. Biol.*, **184**, 529–533.
- Messer,W. and Weigel,C. (1996) Initiation of chromosome replication. In Neidhardt,F.C. *et al.* (eds), *Escherichia coli and Salmonella, Cellular and Molecular Biology*. 2nd edn. ASM Press, Washington, DC, pp. 1579–1601.
- Russell,D.W. and Zinder,N.D. (1987) Hemimethylation prevents DNA replication in *E.coli*. *Cell*, **50**, 1071–1079.
- Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sasse-Dwight,S. and Gralla,J.D. (1991) Footprinting protein–DNA complexes *in vivo*. *Methods Enzymol.*, **208**, 146–168.
- Schaper,S. and Messer,W. (1995) Interaction of the initiator protein DnaA of *Escherichia coli* with its DNA target. *J. Biol. Chem.*, **270**, 17622–17626.
- Sekimizu,K., Bramhill,D. and Kornberg,A. (1987) ATP activates dnaA protein in initiating replication of plasmids bearing the origin of the *E.coli* chromosome. *Cell*, **50**, 259–265.
- Sekimizu,K., Bramhill,D. and Kornberg,A. (1988) Sequential early stages in the *in vitro* initiation of replication at the origin of the *Escherichia coli* chromosome. *J. Biol. Chem.*, **263**, 7124–7130.
- Skarstad,K. and Boye,E. (1994) The initiator protein DnaA: evolution, properties and function. *Biochim. Biophys. Acta*, **1217**, 111–130.
- Skarstad,K., Baker,T.A. and Kornberg,A. (1990) Strand separation required for initiation of replication at the chromosomal origin of *E.coli* is facilitated by a distant RNA–DNA hybrid. *EMBO J.*, **9**, 2341–2348.
- Slater,S., Wold,S., Lu,M., Boye,E., Skarstad,K. and Kleckner,N. (1995) *E.coli* SeqA protein binds *oriC* in two different methyl-modulated reactions appropriate to its roles in DNA replication initiation and origin sequestration. *Cell*, **82**, 927–936.
- von Freiesleben,U., Rasmussen,K.V. and Schaechter,M. (1994) SeqA limits DnaA activity in replication from *oriC* in *Escherichia coli*. *Mol. Microbiol.*, **14**, 763–772.
- Wang,J.C. (1996) DNA topoisomerases. *Annu. Rev. Biochem.*, **65**, 635–692.
- Weigel,C., Schmidt,A., Ruckert,B., Lurz,R. and Messer,W. (1997) DnaA protein binding to individual DnaA boxes in the *Escherichia coli* replication origin, *oriC*. *EMBO J.*, **16**, 6574–6583.
- Woelker,B. and Messer,W. (1993) The structure of the initiation complex at the replication origin, *oriC*, of *Escherichia coli*. *Nucleic Acids Res.*, **21**, 5025–5033.
- Wold,S., Boye,E., Slater,S., Kleckner,N. and Skarstad,K. (1998) Effects of purified SeqA protein on *oriC*-dependent DNA replication *in vitro*. *EMBO J.*, **17**, 4158–4165.

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