The EMBO Journal Vol.17 No.14 pp.4158–4165, 1998

Effects of purified SeqA protein on *oriC*-dependent DNA replication *in vitro*

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***In vivo* studies suggest that the *Escherichia coli* SeqA protein modulates replication initiation in two ways: by delaying initiation and by sequestering newly replicated origins from undergoing re-replication. As a first approach towards understanding the biochemical bases for these effects, we have examined the effects of purified SeqA protein on replication reactions performed *in vitro* on an *oriC* plasmid. Our results demonstrate that SeqA directly affects the biochemical events occurring at *oriC*. First, SeqA inhibits formation of the pre-priming complex. Secondly, SeqA can inhibit replication from an established pre-priming complex, without disrupting the complex. Thirdly, SeqA alters the dependence of the replication system on DnaA protein concentration, stimulating replication at low concentrations of DnaA. Our data suggest that SeqA participates in the assembly of initiation-competent complexes at *oriC* and, at a later stage, influences the behaviour of these complexes.**

Keywords: DnaA/*Escherichia coli*/initiation of DNA replication/*in vitro* replication/SeqA

Introduction

Replication of the *Escherichia coli* chromosome starts at a single locus, *oriC*. Initiation of replication is precisely controlled in several aspects. During steady-state growth, initiation occurs at a specific time in the cell division cycle and at a specific cell mass. Normally this coupling of initiation to cell growth is extremely tight; the coefficient of variation in individual cell mass at the time of initiation is <9% (Boye *et al.*, 1996). In rapidly growing cells containing several copies of *oriC*, all origins are initiated in synchrony (Skarstad *et al.*, 1986). Furthermore, a mechanism called sequestration acts at *oriC* to ensure that a newly replicated origin is temporarily refractory to initiation (Russell and Zinder, 1987; Ogden *et al.*, 1988; Campbell and Kleckner, 1990; Landoulsi *et al.*, 1990; Lu *et al.*, 1994).

Genetic and physiological studies suggest that SeqA plays important roles in control of replication (Lu *et al.*, 1994; von Freiesleben *et al.*, 1994; Boye *et al.*, 1996). First, SeqA is a negative regulator of the onset of DNA replication. This role was inferred originally from the effects of SeqA elimination or SeqA overproduction in mutant strains compromised for initiation. Subsequent studies specifically identified SeqA as a regulatory factor for initiation. In the absence of SeqA, initiation occurs at a lower cell mass compared with wild-type cells and origins may be initiated twice in the same cell division period (Boye *et al.*, 1996). Secondly, SeqA is essential for sequestration (Lu *et al.*, 1994). These two effects of SeqA are distinguished in part by the methylation status of GATC sites in *oriC*: initiation occurs on fully methylated DNA whereas sequestration affects *oriC* in the hemimethylated state that arises as a result of its replication.

Biochemical analysis has shown that SeqA binds *oriC* in a methylation-modulated fashion (Slater *et al.*, 1995). SeqA binds fully methylated *oriC* specifically via a highly co-operative but relatively low affinity interaction. This binding requires multiple determinants throughout *oriC* (Slater *et al.*, 1995), probably explaining why SeqA is reported not to bind to a fully methylated 70 bp *oriC* fragment (Brendler *et al.*, 1995). SeqA also binds hemimethylated *oriC* and certain other hemimethylated DNAs by a higher affinity, less co-operative interaction (Brendler *et al.*, 1995; Slater *et al.*, 1995). Unmethylated *oriC* is not bound by SeqA (Slater *et al.*, 1995).

There is no information regarding the precise biochemical stage(s) of initiation at which SeqA exerts its effect(s). Genetic studies seem to implicate open complex formation as a critical step because the mutants affected by SeqA elimination or overproduction appear to be specifically defective in that stage; furthermore, manipulation of SeqA levels has no effect on a mutant whose corresponding biochemical defect is just after open complex formation (Lu *et al.*, 1994). Physiological studies provide no additional information as to the specific point of SeqA action: the time of replication initiation is inferred from flow cytometry analysis, which essentially monitors the cell mass at which bulk synthesis of new DNA begins. Thus, an effect of SeqA on initiation timing could be executed at any point at or before escape of the replication fork from the origin region.

Here we present a biochemical investigation of the effects of purified SeqA protein on replication initiation from *oriC in vitro*. These studies provide direct biochemical evidence for a functional interaction between SeqA and DnaA proteins at *oriC* during early stages of the initiation process. We demonstrate that SeqA can act negatively both on the formation of the pre-priming complex and on replication from this complex once formed, and suggest that SeqA ensures development of a fully elaborated nucleoprotein complex at *oriC*.

Results

Effects of SeqA protein in reconstituted replication reactions

Replication of *oriC* plasmids *in vitro* can be reconstituted with purified replication proteins (Kaguni and Kornberg,

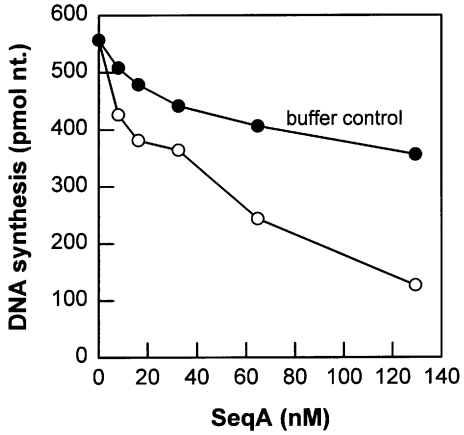

Fig. 1. SeqA protein inhibits replication from *oriC in vitro*. The indicated amounts of SeqA or SeqA buffer were added to the reaction mixture on ice before incubation at 29°C. The replication reaction was as described in Materials and methods (RNA polymerase-independent reaction). DNA replication is measured in nucleotides (nt) synthesized. SeqA at 10 nM corresponds to three SeqA molecules per *oriC*.

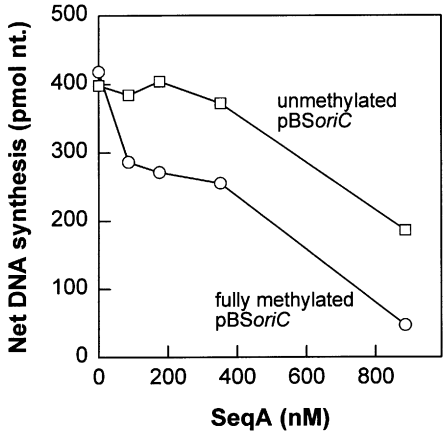

Fig. 2. SeqA-mediated inhibition of replication of fully methylated and unmethylated pBS*oriC*. Unmethylated pBS*oriC* was purified from a *dam*$^-$ strain, whereas the fully methylated pBS*oriC* was purified from a *dam*$^+$ strain and additionally methylated *in vitro*. Apart from the plasmid templates used, the replication reaction was as in Figure 1. The ordinate values are net numbers, adjusted for inhibition by the SeqA storage buffer at each concentration of SeqA (~50% inhibition by buffer at the highest SeqA concentration).

1984). We have examined the effect of purified SeqA protein in such a reaction. In the protocol employed for this work, replication proteins [DnaA, DnaB, DnaC, SSB, primase, gyrase (A and B subunits), DNA polymerase III holoenzyme and HU or IHF] were incubated with supercoiled *oriC* plasmid DNA as template along with ATP and deoxynucleotides (see Materials and methods for details). Replication is measured as the amount of radioactively labelled deoxynucleotides incorporated into newly synthesized DNA, which reflects the combined effects of initiation and elongation. Thus, an effect at either of these two steps will be detected as altered replication efficiency.

A complete reaction mixture containing DnaA protein at 50 nM was incubated on ice in the absence of SeqA protein; SeqA was then added at 0–130 nM and initiation was finally allowed to occur by transfer of the reaction to 29°C, which permits open complex formation. Total nucleotide incorporation was assayed after 20 min. Under these conditions, the presence of SeqA significantly inhibited replication, over and above a small amount of inhibition due to the presence of salt in the SeqA storage buffer (Figure 1). When added before shifting to 29°C, the order of SeqA addition relative to the other replication proteins did not affect the inhibition. Also, the same level of inhibition was observed if SeqA was allowed to bind to the DNA template [by pre-incubating SeqA and template (pBS*oriC*) at 29 or 37°C for 20–25 min] before adding the other proteins, or if SeqA was pre-incubated with the complete set of replication proteins (at 0°C) prior to addition of pBS*oriC* (data not shown).

In vivo, the initial DNA duplex opening reaction at *oriC* is dependent on transcriptional activity by RNA polymerase. In the *in vitro* replication system used above, initiation is independent of transcription. This difference arises because the level of free, unrestrained supercoils in the purified plasmid DNA template is higher than in a native bacterial chromosome (Ogawa *et al.*, 1985; van der Ende *et al.*, 1985), since supercoils are restrained *in vivo* by the binding of structure-modifying proteins (Pettijohn, 1988; Drlica, 1992). Dependence on transcriptional activation can be achieved *in vitro* by reducing the level of free supercoils in the template DNA to the *in vivo* level by adding a high level of HU protein (Baker and Kornberg, 1988; Skarstad *et al.*, 1990). SeqA inhibited both RNA polymerase-dependent and -independent replication reactions to a similar extent (data not shown).

The ability of SeqA to inhibit DNA replication was sensitive to the methylation status of the template plasmid: at low SeqA levels (≤150 nM), inhibition was observed on a fully methylated template but not on an unmethylated template (Figure 2). This is parallel to what is observed for SeqA in binding *oriC*-containing DNA fragments in gel retardation assays (Slater *et al.*, 1995). A further inhibition of the reaction was observed at higher SeqA concentrations, and this inhibition appeared to be independent of methylation status. This effect may be analogous to the *in vivo* finding that overproduction of SeqA protein is inhibitory to growth of a *dam*$^-$ strain (Lu *et al.*, 1994).

In the standard reconstituted replication reaction, the DnaA concentration used (~50 nM) is that required to give maximal DNA synthesis. In trying to make the reaction more sensitive to SeqA, we carried out reconstituted reactions at other DnaA concentrations. We found that at low DnaA concentrations (<20 nM), SeqA (at 180 nM, sufficient to give a significant inhibition at normal amounts of DnaA) actually stimulated the reaction considerably (Figure 3). This stimulatory effect has also been confirmed in the RNA polymerase-dependent replication reaction. Furthermore, stimulation was observed irrespective of the order of addition of DnaA and SeqA into the reaction (data not shown).

Effects of SeqA on formation of pre-priming complexes

When supercoiled *oriC* plasmid, ATP, DnaA, DnaB, DnaC and HU or IHF proteins are incubated together at an appropriate temperature, the pre-priming complex forms: DnaA protein, assisted by HU or IHF, opens the DNA duplex in *oriC* and the DnaB helicase becomes inserted

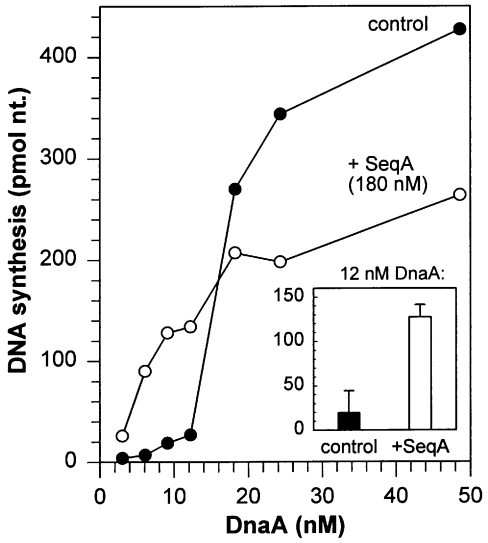

Fig. 3. SeqA stimulates *in vitro* replication at low DnaA concentrations. The indicated amounts of DnaA were added to replication reactions containing 180 nM SeqA (54 molecules per *oriC*) before incubation. The control contained the corresponding amount of SeqA buffer. The shown data points are average values obtained from two independent experiments; the range of variation between the two is <15 pmol for the four lowest values of DnaA. The inserted frame shows average stimulation by SeqA (180 nM) at 12 nM DnaA, obtained from five independent experiments. The error bars indicate the standard deviation.

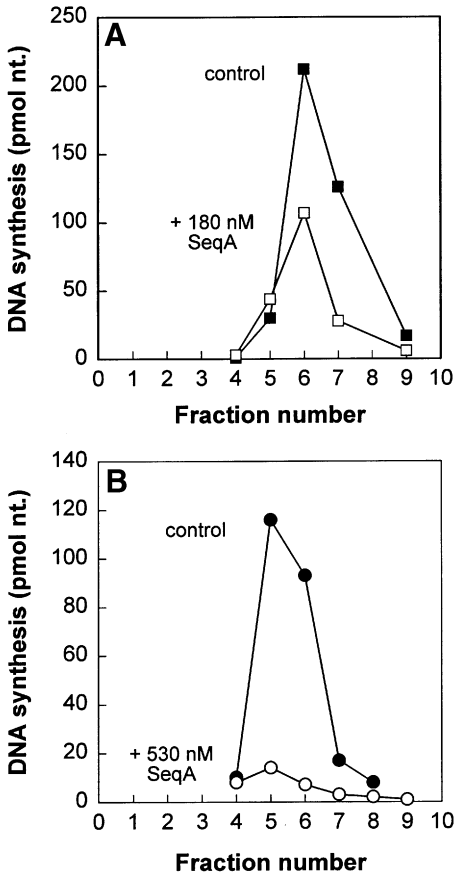

Fig. 4. Replication capacity of gel-filtered complexes assembled in the presence or absence of SeqA. Pre-priming complexes were assembled in the presence of SeqA [(**A**) 180 nM SeqA (54 molecules per *oriC*) or (**B**) 530 nM SeqA] or the corresponding volume of SeqA storage buffer (control), and isolated by gel filtration through Biogel A15-m columns. Replication was measured by adding the rest of the replication proteins to 20 μl of the filtered fractions.

into the unwound region (Baker *et al.*, 1986; Funnell *et al.*, 1987; Bramhill and Kornberg, 1988; Sekimizu *et al.*, 1988a; Marszalek and Kaguni, 1994). To determine whether SeqA affects replication before or after this stage, complexes were assembled on an *oriC* plasmid in the presence and absence of SeqA, and the resulting protein–*oriC* complexes were separated from unbound proteins by gel filtration (Materials and methods). Fractions from the gel columns were collected and assayed for replication activity by adding the rest of the replication proteins [primase, SSB, gyrase and DNA polymerase III (Pol III) with β-subunit]. Complexes assembled in the presence of SeqA exhibited a considerably reduced capacity for subsequent replication compared with complexes assembled in the absence of SeqA (Figure 4).

To investigate whether the inhibitory effect of SeqA may be due to its preventing pre-priming complex formation, the levels of DnaA, DnaB and DnaC in the gel-filtered fractions were measured by electrophoresis and immunoblotting (Figure 5). In the presence of SeqA, the DNA-containing fractions contained significantly reduced amounts of DnaA, DnaB and DnaC compared with the control without SeqA: DnaA and DnaB were reduced by 80–85% and DnaC by >95% (Figure 5B). Thus, SeqA can indeed prevent the formation of a pre-priming complex.

In the control without SeqA, the fractions active for replication (fractions 5–7; Figure 5A) contained 70–90% of the DnaA, DnaB and DnaC molecules loaded onto the column (6.0 pmol of DnaA and 7.9 pmol of DnaB and DnaC were loaded). As observed also by Marszalek and Kaguni (1994), the isolated pre-priming complexes contained DnaC; the amount was almost equimolar to DnaB (Figure 5B).

SeqA protein co-eluted with the DNA (pBS*oriC*) (Figure 5A). To verify that binding of SeqA is dependent on *oriC*, we compared the SeqA elution in parallel reactions containing either pBS*oriC* or the vector plasmid lacking *oriC*. Pre-priming complex mixtures containing pBS*oriC* or vector plasmid (both fully methylated) and SeqA, were incubated and gel filtered. In contrast to the case for pBS*oriC*, SeqA did not co-elute with non-*oriC* DNA; rather, SeqA eluted in later fractions (not shown). *oriC* nucleates binding of a large number of SeqA molecules: ~100% of the SeqA eluted in the DNA-containing fractions when present at 180 nM (54 SeqA molecules per *oriC*), and even at 560 nM (160 molecules per *oriC*) >80% of the SeqA eluted in these fractions.

Effects of SeqA on the activity of pre-formed pre-priming complexes

The interaction of SeqA with established pre-priming complexes was investigated as follows: pre-priming complexes were formed and gel filtered in the absence of SeqA, fractions were collected, and each fraction was split in two and incubated with either SeqA or control buffer. Replication activity was measured by adding the rest of the replication proteins. Again, SeqA strongly inhibited replication (Figure 6), showing that SeqA can act negatively even after the pre-priming complex is formed. To

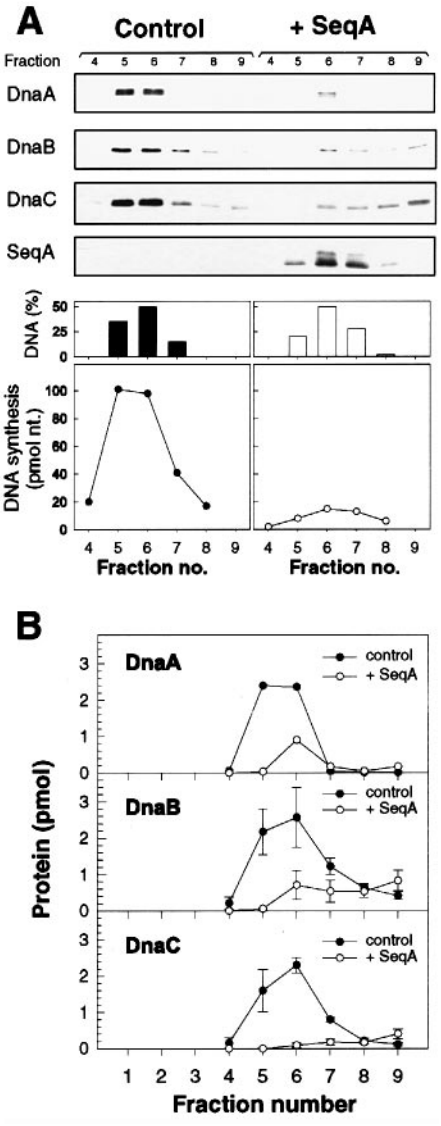

Fig. 5. A pre-priming complex is not formed in the presence of SeqA. The pre-priming complex mixture was incubated in the presence of 530 nM SeqA or the corresponding volume of SeqA buffer, and isolated by gel filtration through Biogel A15-m columns. (**A**) The upper panels show the levels of DnaA, DnaB, DnaC and SeqA proteins in the gel-filtered fractions (immunoblot analyses with antisera against DnaA, DnaB, DnaC and SeqA, respectively). The two lower panels show the amount of DNA recovered in each fraction (as a percentage of that loaded onto the column) and the amount of DNA synthesis (in pmol of nucleotides) obtained after adding the rest of the replication proteins to 20 µl of each fraction (see Materials and methods). (**B**) Quantification of the amount of DnaA, DnaB and DnaC present in the complexes assembled in the absence (●) and presence (○) of SeqA. Relative band intensities were quantified by scanning densitometry of chemiluminiscence exposed films. The values for DnaA are obtained from the immunoblot in (A), whereas the values for DnaB and DnaC are average values from three independent experiments (one of which is shown in A), each quantified from at least two differently exposed films. Standard deviations are indicated by bars.

investigate whether this inhibition is due to SeqA disrupting the already established pre-priming complexes, we analysed the integrity of pre-formed pre-priming complexes after incubation with SeqA. Gel-filtered pre-priming complexes were incubated with control buffer or with SeqA (350 nM, corresponding to ~160 molecules per *oriC*

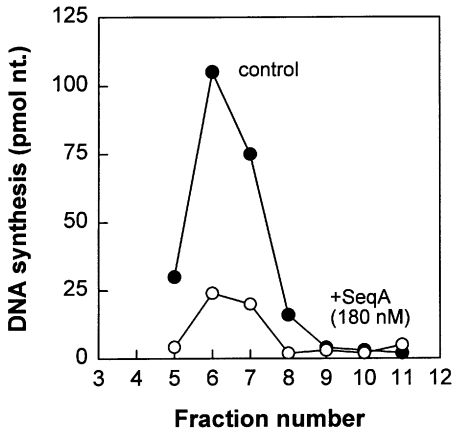

Fig. 6. SeqA can inhibit replication when added to a pre-made pre-priming complex. The experiment was as in Figure 4A, except that SeqA or the corresponding volume of SeqA storage buffer was added after the pre-priming complexes had been formed and gel filtered. SeqA or buffer was incubated with the gel-filtered DNA complexes for 5–10 min at 29°C before the rest of the replication proteins were added. SeqA was added to each fraction to a concentration of 180 nM. However, as the amount of DNA template in each fraction was not quantified in this experiment, the ratio of SeqA to *oriC* may differ somewhat from that in Figure 4A (54 molecules per *oriC*).

in this reaction), and filtered through a second gel column to remove unbound proteins. Immunoblot analyses of the fractions showed that SeqA did not significantly change the levels of complex-associated DnaA, DnaB or DnaC proteins when added at this stage (Figure 7), in contrast to the significant reduction in protein levels detected when SeqA was present at pre-priming complex formation (Figure 5). The minor differences with and without SeqA seen in Figure 7 are within the experimental uncertainty in the immunoblotting and quantification procedures (see legend to Figure 7). Thus, SeqA is not able to displace significant amounts of proteins from an established pre-priming complex, indicating that the inhibitory effect on replication at this stage is due to mechanisms other than disruption of the pre-priming complex.

Discussion

SeqA can affect DNA replication at early stages in vitro

The experiments described here demonstrate that purified SeqA protein can affect DNA replication initiated from *oriC in vitro* in reactions containing pure replication components. These findings complement and extend earlier genetic evidence that SeqA affects DNA replication *in vivo* (Lu *et al*., 1994; von Freiesleben *et al*., 1994) and biochemical evidence that SeqA binds *oriC* in a methylation-sensitive manner (Slater *et al*., 1995).

The early stages of replication initiation are an orderly process including formation of an initiation-competent complex, then an open complex and finally a pre-priming complex. More specifically, DnaA protein binds to *oriC* and induces a localized unwinding of the AT-rich region that is proposed to be the entry site for DnaB helicase (Fuller *et al*., 1984; Bramhill and Kornberg, 1988). DnaA bound to *oriC* interacts with DnaB, complexed to DnaC, to permit binding of DnaB (Marszalek and Kaguni, 1994), thereby generating the pre-priming complex (Sekimizu *et al*., 1988a).

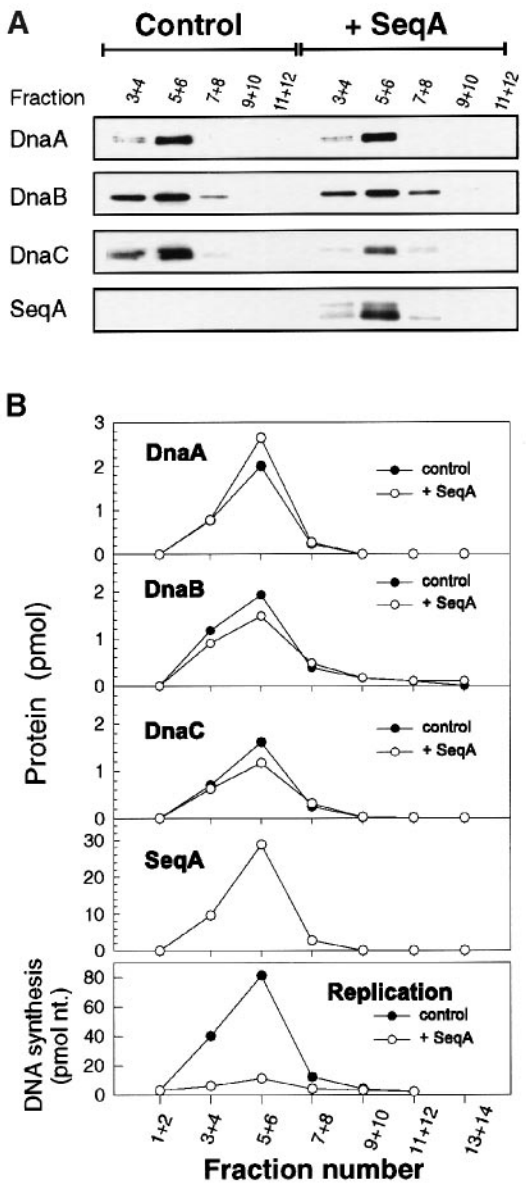

Fig. 7. Can SeqA remove proteins from pre-made pre-priming complexes? Pre-priming complexes were assembled in the absence of SeqA and gel filtered as in the previous figures. The fractions assumed to be active for replication (fractions 5–7) were pooled, and SeqA (350 nM, corresponding to ~180 molecules per *oriC* assuming this pool contained 100% of the loaded DNA) or the corresponding amount of SeqA storage buffer as a control were added. After incubation (5–10 min at 29°C), the mixture was gel filtered through a second Biogel A-15m column, and 10-drop fractions (~140 µl) collected. (**A**) Immunoblot showing levels of DnaA, DnaB, DnaC and SeqA in the fractions from the second column, in the absence and presence of SeqA. The fraction numbers correspond to those in the previous figures. (**B**) The four upper panels show quantification of the immunoblots shown in (A). The lower panel shows the replication activity of the corresponding fractions after adding the rest of the replication proteins to 20 µl of each fraction. DNA eluted in exact proportion to replication activity (not shown). The data for DnaA and SeqA are from the immunoblot shown in (A) whereas the others are average values from two experiments, one of which is shown in (A). In each experiment, quantification was by scanning densitometry of three differently exposed films. For the DnaB and DnaC values, the total range of variation between differently exposed films and between the two experiments is larger than the differences with or without SeqA (about ± 0.5 pmol at the peak values).

The current findings demonstrate that SeqA has the potential to influence one or more of these steps. In a fully reconstituted reaction, SeqA stimulates replication when DnaA protein is at limiting concentration (⩽20 nM) and inhibits replication when the DnaA concentration is higher. Furthermore, in a staged reaction, SeqA inhibits formation of a pre-priming complex. Inhibition occurs at the concentrations of DnaA where inhibition of replication is observed in the reconstituted reaction, suggesting that these two effects are the same.

SeqA can stimulate replication

We favour the view that SeqA mediates its stimulatory effect by changing the distribution of DnaA molecules amongst the *oriC* regions present. At the DnaA concentrations where SeqA stimulates replication, the DnaA:*oriC* ratio is very low (<2:1 at 6 nM DnaA). Since DnaA binding to its five binding sites on linear *oriC* fragments is not co-operative (Messer *et al*., 1997), DnaA molecules are, in the absence of SeqA, probably distributed randomly amongst the available *oriC*s. Consequently, at low DnaA concentrations, relatively few *oriC*s contain enough DnaA molecules to undergo initiation. SeqA might exert its stimulatory effect by recruiting all of the DnaA molecules into functional initiation assemblies, with relatively little DnaA protein remaining at other sites, thus increasing the number of *oriC*s that are competent for initiation.

Alternatively, rather than changing the distribution of DnaA molecules on *oriC* templates, SeqA might instead increase the activity of the nucleoprotein complexes, thus permitting some of the 'marginal' complexes to be functional for early stages, via either direct and/or indirect effects of SeqA.

SeqA can inhibit formation of a pre-priming complex

Isolated *oriC*–protein complexes assembled in the presence of SeqA are defective in replication and contain SeqA protein in large amounts (~100 molecules per *oriC*); the DnaA, DnaB and DnaC contents of these complexes were reduced by 80–95% compared with complexes assembled without SeqA present. These findings could reflect effects of SeqA protein on initial assembly of DnaA onto *oriC*; alternatively, initiation-competent complexes may form but be unstable at later steps, e.g. during open complex formation. In either case, subsequent stable binding of DnaB and DnaC would be inhibited.

SeqA can inhibit replication after formation of the pre-priming complex

SeqA inhibits the standard reconstituted replication reaction when added together with the other proteins prior to incubation, i.e. before pre-priming complexes are formed. This inhibition is affected by the methylation status of the DNA template: at low levels of SeqA, only methylated templates are inhibited. We infer that this reflects the specific binding of SeqA to fully methylated *oriC*. However, at higher concentrations of SeqA, replication of an unmethylated template is also inhibited. The fact that SeqA can act negatively, independently of the template methylation status, is in accordance with the *in vivo* observation that SeqA overproduction is lethal to methylation-deficient *dam*$^-$ cells (Lu *et al.*, 1994).

Replication inhibition by SeqA also of an unmethylated *oriC* plasmid indicates that binding to *oriC* is no prerequisite for this mode of inhibition, suggesting that SeqA can inhibit replication by a direct interaction with the replication proteins. In accordance with this, we find that SeqA can inhibit replication also after the pre-priming complex is formed. This inhibition apparently occurs without the disruption of the pre-priming complex, as measured by the presence of its normal contents of DnaA, DnaB and DnaC proteins. We cannot exclude, however, that SeqA displaces these proteins from the complex at a later stage. Whether SeqA acts before or after Pol III has started replicating is not addressed in the present experiments. Hence, it is not clear whether SeqA inhibits progression from the pre-priming complex to the start of elongation, or inhibits elongation directly by interacting with the replication fork. However, preliminary data indicate that SeqA can inhibit *in vitro* replication at the elongation stage, in addition to an inhibitory effect occurring earlier in the initiation sequence (data not shown). Also, *in vivo* observations suggest that high levels of SeqA can inhibit elongation: flow cytometry experiments show that after strong SeqA overproduction, DNA replication is inhibited and the replication forks are stopped before they reach the terminus (our unpublished results).

Implications for the role of SeqA in DNA replication in vivo

In vivo, two different effects of SeqA on DNA replication have been distinguished thus far. First, SeqA delays replication initiation without perturbing the precision with which initiation is coupled to cell physiology (Boye *et al*., 1996); the precise step of replication initiation affected by SeqA *in vivo* is not known. Secondly, SeqA is required for sequestration of *oriC* from re-methylation immediately following replication initiation and, in a presumably related effect, for blocking replication of hemimethylated *oriC* plasmids (Lu *et al*., 1994).

How might the effects of SeqA observed in the current study be relevant to its *in vivo* roles? The *in vivo* effect of SeqA involves a change in the timing of the process, not on the probability that initiation will occur at all, whereas the current study has examined the effects of SeqA on the probability of occurrence of various steps. Also, staged reactions provide opportunities for effects that would, in an unpaused reaction, be unlikely on kinetic grounds. Nonetheless, despite these limitations, the findings presented here provide strong support for the notion that SeqA plays an integral role in replication initiation. Specifically, the data imply that SeqA modulates the assembly of an initiation-competent protein–DNA complex at *oriC* and then influences the behaviour of that complex once it has formed. Initiation complexes assembled in the absence of SeqA are capable of initiation but are probably defective with regard to more sophisticated behaviour such as replication timing and co-ordination, and sequestration. In this sense, SeqA might be considered a 'stringency factor', i.e. a component that constrains the initiation process to behave in accord with the available regulatory inputs. Accordingly, the observed SeqA-induced delay of initiation *in vivo* (Boye *et al*., 1996) may be a result of SeqA preventing the initiation process until the stage is set for a highly controlled and co-ordinated initiation. The delay may occur at any of the stages where SeqA effects have been observed: initial assembly, early stages or even exit of replication from *oriC*. Once the origin region itself has been replicated, SeqA presumably becomes bound to the newly hemimethylated *oriC*, perhaps together with residual DnaA molecules, to mediate sequestration.

Materials and methods

Reagents

Ribonucleoside triphosphates, deoxyribonucleoside triphosphates, polydI–dC·polydI–dC and Sephadex G-50 Nick columns were from Pharmacia; [γ-^{32}P]ATP (>5000 Ci/mmol) and [α-^{32}P]dTTP (800 Ci/mmol) from Amersham; polyvinyl alcohol (mol. wt 30 000–70 000) and Igepal from Sigma; and *S*-adenosyl methionine (SAM) from New England Biolabs.

Plasmid DNA

Plasmid pBS*oriC* (3640 bp), also called pTB101 (Baker and Kornberg, 1988), consists of a 678 bp *Hinc*II–*Pst*I fragment spanning *oriC* (–189 to +489 bp) cloned into the pBluescript vector.

The plasmids were purified by banding twice in $CsCl_2$-ethidium bromide density gradients as described (Sambrook *et al.*, 1989), followed by desalting over Sephadex G-50 Nick columns. Unless otherwise stated, the plasmid used as template in replication reactions was fully methylated, i.e. grown in a wild-type strain (W3110). To eliminate the possibility of SeqA inhibiting replication by binding to persistent hemimethylated GATC sites on the DNA template, the experiment shown in Figure 1 was repeated with a template additionally methylated *in vitro* to ensure complete methylation at all sites. The additional *in vitro* methylation did not affect the inhibition by SeqA. The DNA template used in the experiments was, therefore, not additionally *in vitro* methylated, unless stated explicitly. *In vitro* methylation with Dam methylase and SAM was performed according to the manufacturer's instructions, and followed by a second filtration through Sephadex G-50 Nick columns. Unmethylated pBS*oriC* was obtained from a *dam*$^-$ strain (DS1310).

Enzymes

The gyrase B subunit and Pol III* were purified as described by Mizuuchi *et al.* (1984), and Maki *et al.* (1988), respectively. Primase and gyrase A subunit, provided by H.Nakai, were purified as described by Kruklitis and Nakai (1994). DnaA protein, provided by E.Crooke, was purified as described by Sekimizu *et al.* (1988b). DnaB–DnaC in equimolar complex was provided by N.Dixon. SSB, β-subunit of DNA polymerase III holoenzyme and HU [purified as described by Kaguni and Kornberg (1984); Crooke (1995)] were a gift from A.Kornberg. IHF protein was a gift from H.E.Nash. The *E.coli* RNA polymerase was purchased from Pharmacia, and Dam methylase from New England Biolabs.

Purification of SeqA

We have used two preparations of SeqA. The purification of the first of these has been described before (Slater *et al.*, 1995). The other preparation, which is functionally indistinguishable from the first, was purified by a procedure based on a protocol provided by T.Brendler (Brendler *et al.*, 1995):

SeqA was overexpressed in *E.coli* strain B834 (DE3) harbouring pLysS and pSS1, which is pET11a (Studier *et al.*, 1990) with *seqA* under pT7 control (Slater *et al.*, 1995). An inoculum of cells freshly transformed with pSS1 was grown in M9ZB medium (Studier *et al.*, 1990) containing ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml). Growth was at 25°C, to improve solubility of SeqA. However, the solubility seems to be the same when grown at 37°C, so growing at higher temperature may be preferable. As the inducible production of SeqA was found to vary considerably, the response to isopropyl-β-D-thiogalactopyranoside (IPTG) was checked by microscopy (the responding cells tend to become longer) in several parallel cultures after inducing with 0.4 mM IPTG for a few hours. A culture verified to respond to IPTG was diluted 1:500 into M9ZB (5 l) and grown to an OD_{600} of 0.45, when IPTG was added to 0.4 mM. The cells were grown with IPTG for 1–2 generations before harvesting. The cell pellets were resuspended in an equal weight of 250 mM potassium chloride, 25 mM HEPES–KOH pH 7.5, 10 mM magnesium acetate, 20 mM spermidine, 0.1 mM EDTA, 2 mM dithiothreitol (DTT) and 10% (v/v) sucrose. The cells were lysed by freezing

and thawing, and the cell lysate centrifuged (45 min, 183 000 *g*, 1°C) in a Beckman 60Ti rotor. The supernatant contained very little SeqA and was discarded. SeqA was recovered from the pellet fraction by sonication in an equal volume of buffer [1 M KCl, 25 mM HEPES pH 7.5, 10 mM magnesium acetate, 20 mM spermidine, 2 mM DTT, 0.1% Igepal (v/v), 10% (w/v) sucrose]. The sonicated pellet was centrifuged (45 min, 183 000 *g*, 1°C). The supernatant (Fraction I, 10.2 ml) was removed and the proteins precipitated with 0.35 g/ml ammonium sulfate. The precipitate was dialysed in 300 mM ammonium sulfate, 40 mM HEPES pH 7.5, 1 mM magnesium acetate, 0.1 mM EDTA, 2 mM DTT, 0.1% Igepal (v/v) and 15% (v/v) glycerol (Fraction II, 2 ml). Fraction II was diluted to a final salt concentration of 200 mM ammonium sulfate (with little loss of protein), loaded onto a heparin–agarose column [45 ml matrix, equilibrated in buffer A (25 mM HEPES pH 7.5, 1 mM magnesium acetate, 0.1 mM EDTA, 2 mM DTT and 15% glycerol) containing 200 mM ammonium sulfate], eluted with a linear gradient of ammonium sulfate (0.2–1 M ammonium sulfate in buffer A, 360 ml), and collected in 5 ml fractions. SeqA eluted as a single peak (480 mM ammonium sulfate) of high purity (>99.5% as judged by silver-stained SDS–PAGE gels). Finally, SeqA was concentrated by centrifugation through Centricon (No. 10 or 30; Amicon) which gave ~10× concentration, although with some (10–50%) loss of protein.

Reconstituted DNA replication

The RNA polymerase-independent reaction (25 µl) contained 30 mM Tricine-KOH (pH 8.2), 12 mM magnesium acetate, 2 mM ATP, 0.04% polyvinyl alcohol, 200 ng of supercoiled DNA template (fully methylated pBS*oriC* unless otherwise stated; equal to 600 pmol of nucleotides, or 84 fmol of molecules), 125 ng DnaB–DnaC in an equimolar complex, 180 ng of gyrase A subunit, 180 ng of gyrase B subunit, 450 ng of SSB, 15 ng of primase, 112 ng of Pol III*, 26 ng of β subunit of Pol III, 8 ng of HU (Figures 1 and 3) or IHF, and 64 ng of DnaA unless otherwise stated, and dATP, dTTP, dCTP and dGTP each at 0.1 mM, with [α-^{32}P]dTTP at 30–200 c.p.m. per pmol of deoxynucleotides. All data shown are obtained from RNA polymerase-independent reactions.

The RNA polymerase-dependent reaction (25 µl) additionally contained UTP, GTP and CTP each at 0.5 mM, 100 ng of HU and RNA polymerase at the indicated amounts. DnaA protein was added at the indicated amounts.

Mixtures were assembled at 0°C, incubated at 29°C for 20 min and then precipitated with 500 µl of cold 10% trichloroacetic acid containing 100 mM PP_i. Total nucleotide incorporation was measured by liquid scintillation counting after filtration onto GF/C glass-fibre filters.

Replication reaction staged at pre-priming complex formation

The first stage contained DNA template, DnaA, DnaB, DnaC and HU or IHF (in the same amounts as above) in 20 µl of pre-priming buffer [30 mM Tricine–KOH, pH 8.2, 5 mM ATP, 0.25 mg/ml bovine serum albumin, 0.01% (v/v) Brij 58, 0.3 mM EDTA, 60 mM potassium glutamate and 20% (v/v) glycerol]. Incubation was at 29°C for 10 min. The pre-priming complexes thus formed were gel filtered. The gel filtering was performed at room temperature through Biogel A15-m: 100 µl of pre-priming mixture was loaded onto 1 ml columns equilibrated with pre-priming buffer, and five-drop fractions (~70 µl) were collected. The rest of the replication proteins were added to the fractions in the amounts described above and the mixture incubated at 29°C for 20 min. A 20 µl aliquot of each fraction was used for the replication reaction. In the experiments when immunoblot analysis of the filtered fractions was also performed, the rest of the fraction was added SDS loading buffer.

Acknowledgements

We thank our collaborators Elliott Crooke, Nicholas Dixon and Hiroshi Nakai, with whom we maintain and share our supplies of *E.coli* replication proteins. We are grateful to Arthur Kornberg and Howard E.Nash for the kind gifts of pure proteins and to Therese Brendler for providing a purification scheme for SeqA. The technical assistance of Anne Wahl is gratefully acknowledged. This work was supported by grants from the Research Council of Norway and from The Norwegian Cancer Society (E.B., K.S.) and The National Institutes of Health, USA (N.K.).

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Received August 28, 1997; revised May 18, 1998; accepted May 19, 1998