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E. coli SeqA Protein Binds *oriC* in Two Different Methyl-Modulated Reactions Appropriate to Its Roles in DNA Replication Initiation and Origin Sequestration

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Summary

The *seqA* gene negatively modulates replication initiation at the E. coli origin, *oriC*. *seqA* is also essential for sequestration, which acts at *oriC* and the *dnaA* promoter to ensure that replication initiation occurs exactly once per chromosome per cell cycle. Initiation is promoted by full methylation of GATC sites clustered in *oriC*; sequestration is specific to the hemimethylated forms generated by replication. SeqA protein purification and DNA binding are described. SeqA interacts with fully methylated *oriC* strongly and specifically. This reaction requires multiple molecules of SeqA and determinants throughout *oriC*, including segments involved in open complex formation. SeqA interacts more strongly with hemimethylated DNA; in this case, *oriC* and non-*oriC* sequences are bound similarly. Also, binding of hemimethylated *oriC* by membrane fractions is due to SeqA. Direct interaction of SeqA protein with the replication origin is likely to be involved in both replication initiation and sequestration.

Introduction

In Escherichia coli, DNA replication initiates at a single genetic locus, *oriC*. The timing of initiation is precisely controlled. During steady-state growth at any given growth rate, initiation always occurs at a fixed time in the cell cycle. If a cell contains several chromosomes and several copies of *oriC*, as occurs during rapid growth, all origins present are initiated in synchrony (Skarstad et al., 1986). The timing mechanism is very tightly coupled to the physiological state of the cells: the coefficient of variation of the cell mass at initiation is less than 10% (E. B., unpublished data). The physiological signals sensed by the timing mechanism and the molecular processes that confer precise timing are unknown.

Replication initiation is further regulated in a second respect: each copy of *oriC* is initiated exactly once per cell cycle (e.g., Skarstad et al., 1986; Koppes and Nordstrom, 1986). Even when multiple origin copies are present, initiation occurs at every *oriC* copy without any copy undergoing more than a single initiation. This aspect of replication control is understood in a general way. A mechanism called sequestration ensures that a newly replicated origin is temporarily refractory to initiation (Russell and Zinder, 1987; Ogden et al., 1988; Campbell and Kleckner, 1990; Hansen, et al., 1991; Landoulsi et al., 1990; Lu et al., 1994). With such a process available, initiation potential can temporarily overshoot the required level, thus ensuring that initiation occurs at all origins present. Newly initiated origins are sequestered for a certain period while initiation potential is still high; then, after initiation potential has fallen to a safe level, origins are released from sequestration and are readied for the next round of initiation, which occurs some time later.

The *dnaA* gene promoter is also sequestered, with a resulting transient block to initiation of transcription (Campbell and Kleckner, 1990). Since the *dnaA* gene is replicated only 1 min later than the origin, sequestration at *dnaA* is temporally coordinated with sequestration at *oriC* and probably contributes to a drop in initiation potential following replication initiation (Campbell and Kleckner, 1990; Theisen et al., 1993).

Several proteins act positively to promote replication initiation. The major origin-recognition protein is DnaA, while DnaC protein recruits DnaB helicase to the origin (Baker and Wickner, 1992). Two small accessory proteins, integration host factor (IHF) and factor for inversion stimulation (FIS), play auxiliary roles (Messer and Weigel, 1995).

Replication initiation and sequestration both are critically dependent upon two other factors: Dam-mediated N^6-adenine methylation at a series of GATC sites densely packed within *oriC* (e.g., Løbner-Olesen et al., 1994; Lu et al., 1994; Smith et al., 1985; Messer et al., 1985) and a recently discovered protein, SeqA (Lu et al., 1994), which negatively modulates initiation and is required for sequestration (Lu et al., 1994; von Freisleben et al., 1994).

Replication initiation and sequestration require two different versions of GATC methylation (Figure 1). When replication initiation occurs, *oriC* is fully methylated at all GATC sites on both strands. This methylation positively modulates initiation. Replication generates two symmetrically hemimethylated forms of *oriC*. Sequestration acts specifically on these hemimethylated forms, which are uniquely refractory to Dam methylation as compared with other newly replicated GATC sequences and are blocked from undergoing replication initiation. *oriC* remains in its sequestered state for approximately one third of a doubling time, at which point it is rapidly remethylated (Campbell and Kleckner, 1990). Once full methylation is restored, two thirds of a cell cycle elapse before the origin again undergoes initiation.

The identification of SeqA as a negative modulator of chromosomal replication and initiation is particularly interesting because it raises the possibility that replication initiation, like many other biological processes, is regulated by a homeostatic tension between opposing positive and negative forces. It has been proposed that the SeqA protein acts directly at *oriC* as a factor that either is required

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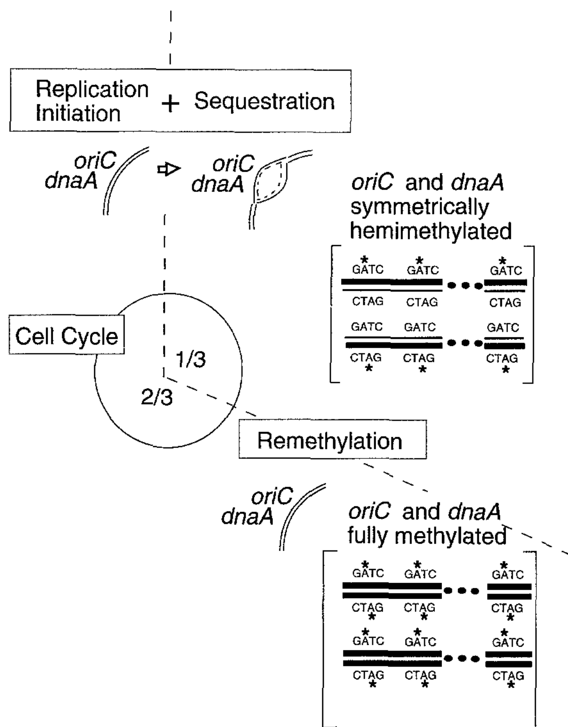

Figure 1. A Cycle of Replication Initiation and Sequestration Involves Changes in the Methylation Status of *oriC* and the *dnaA* Promoter

for cooperative interactions at the origin or determines the sensitivity of such interactions to physiological regulatory signals (or both) (Lu et al., 1994). Less direct roles have also been suggested (von Freisleben et al., 1994).

For sequestration at *oriC*, as defined by genetic assays and altered remethylation kinetics, SeqA protein is essential; SeqA is similarly essential for sequestration of the *dnaA* promoter. SeqA may be the only nonessential protein involved in this aspect of regulation control (von Freisleben et al., 1994). Genetic interactions suggest that sequestration could involve interactions between SeqA and DnaA proteins (Lu et al., 1994).

Newly replicated (hemimethylated) *oriC* can also be found in association with a subcellular fraction enriched for outer membrane proteins, perhaps as a component of the sequestration block, chromosome segregation, or both (Ogden et al, 1988). Such fractions also bind exogenously added hemimethylated DNA, as measured by a filter binding assay (Chakraborti et al., 1992; Kusano et al., 1984) or by DNase I footprinting (Herrick et. al., 1994), and specifically block initiation from hemimethylated *oriC* plasmids in vitro (Landoulsi et al., 1990).

We have now purified SeqA protein and have used a gel retardation assay to analyze the interaction of SeqA with GATC-containing DNA fragments encoding *oriC* or non-*oriC* sequences in various methylation states. The observations presented suggest that direct binding of SeqA to the replication origin plays an important role in the modulation by SeqA of both replication initiation and origin sequestration. More specifically, these results support our previous suggestion that SeqA negatively modulates replication initiation by antagonizing open complex formation. They further suggest that SeqA binding is responsible for the specificity of sequestration for hemimethylated DNA, but that additional factors are required to target sequestration to specific loci.

Results

Purification of SeqA Protein

An E. coli strain carrying the *seqA* gene in a controllable overexpression vector exhibits isopropyl-β-D-thiogalactopyranoside (IPTG)-dependent induction of a protein having about the mobility expected for SeqA, ~21 kDa (Figure 2A). SeqA protein was purified from such cells (Figure 2B; see Experimental Procedures). The protein elutes from the final column step primarily in a broad peak of minimum predicted M_r ~120 kDa (assuming a globular shape), approximately a hexamer; a small peak at the position expected for monomers is sometimes observed. The final material comprises a single visible band on a 12% SDS–polyacrylamide gel stained with Coomassie blue (Figure 2B). Scanning of several gels indicates that no other species is present at more than 0.5% the level of SeqA protein (data not shown).

Substrates for SeqA Retardation Assays

Purified SeqA protein has been analyzed for interactions with both a DNA fragment encoding the E. coli origin of

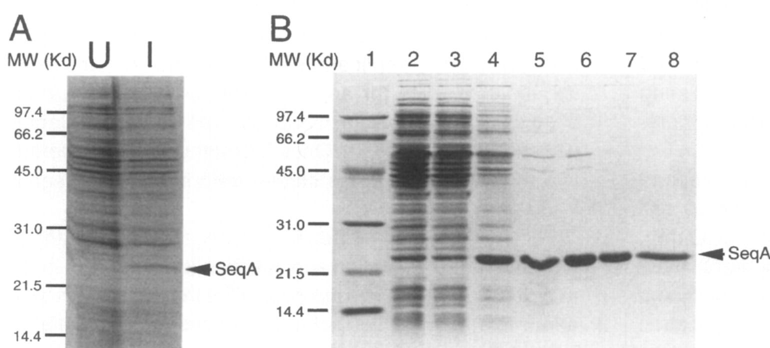

Figure 2. Overproduction and Purification of SeqA Protein

(A) SeqA protein was overproduced by IPTG induction of a T7 promoter-*seqA* fusion, and lysates were prepared from uninduced (U) and induced (I) cells.

(B) Purification of SeqA protein from the induced cells. Lane 1, Bio-Rad low M_r markers. Lane 2, cleared lysate of induced cell extract. Lane 3, fraction soluble after 25% ammonium sulfate precipitation. Lane 4, fraction precipitated by 35% ammonium sulfate. Lane 5, eluate from phenyl-Sepharose column. Lane 6, fraction precipitated by 30% ammonium sulfate. Lanes 7 and 8, eluates from first and second Superose columns. Lane 8 contains 2.7 µg of the final purified SeqA preparation.

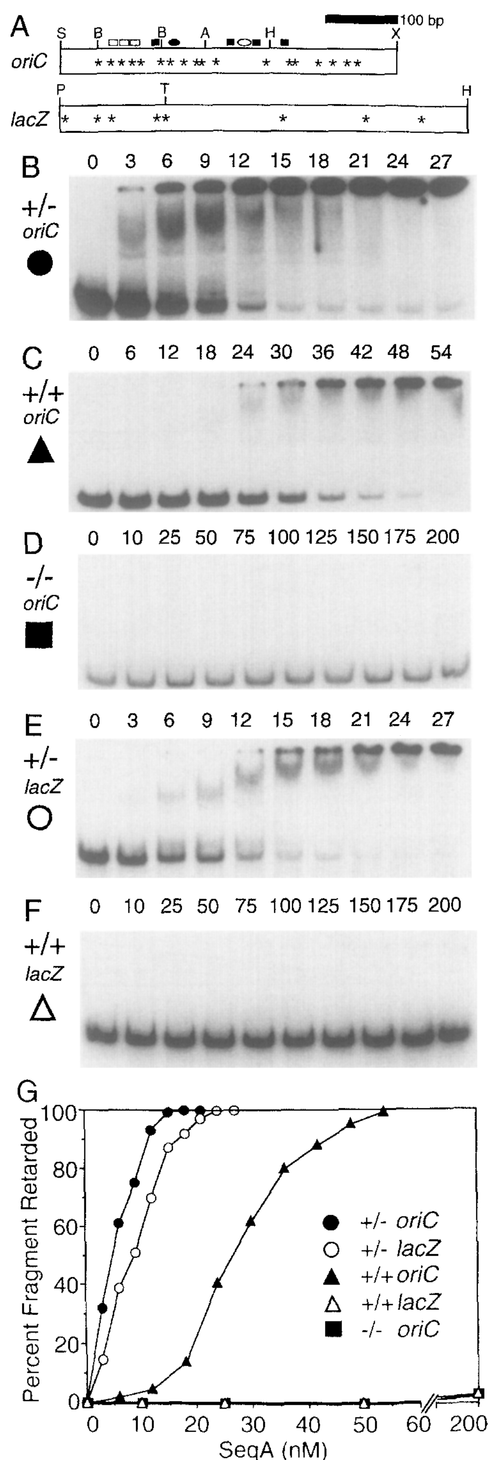

Figure 3. SeqA Binding to *oriC* and *lacZ* DNA Fragments of Varying Methylation Status

(A) 465 bp *oriC* and 565 bp *lacZ* substrate fragments. Asterisks represent GATC sites for Dam methylase modification. Within *oriC*, closed boxes indicate DnaA protein–binding sites (reviewed by Skarstad and Boye, 1994; Messer and Weigel, 1995); open boxes indicate 13-mer repeat sequences (Bramhill and Kornberg, 1988); closed and open ovals are IHF- and FIS-binding sites, respectively (Filutowicz and Roll, 1990; Polaczek, 1990; Bonnefoy and Rouviere-Yaniv, 1992; Gille et al., 1991; Filutowicz et al., 1992). Single-letter codes for restriction sites are as follows: S, SmaI; X, XhoI; A, AvaII; B, BamHI; H, HindIII; P, SpeI; T, TaqI. The TaqI site in *lacZ* overlaps a GATC site that is not sequestered in vivo (Campbell and Kleckner, 1990). The SmaI and XhoI sites at the ends of the *oriC* fragment are also AvaI sites; *oriC* fragment was prepared by cleavage with AvaI.

(B–F) Gel retardation analysis of SeqA binding to *oriC* and *lacZ* fragments of the indicated methylation status. All reactions contain purified SeqA protein at the indicated nanomolar concentration. Plus/plus indicates fully methylated DNA; minus/minus indicates unmethylated DNA; plus/minus indicates an equimolar mixture of the two symmetrically hemimethylated forms. Symbols are as in (G).

(G) Fraction of substrate fragment retarded as a function of SeqA protein concentration as obtained by quantitation of gels in (B)–(F) (see Experimental Procedures). Open and closed circles are hemimethylated *lacZ* and *oriC*, respectively. Open and closed triangles are fully methylated *lacZ* and *oriC*, respectively. The closed square is unmethylated *oriC*; unmethylated *lacZ* was not assayed.

replication and a non-*oriC* control fragment. The full-length 465 bp *oriC* substrate contains the minimal origin plus a bit of flanking sequence and encodes 18 GATC sites (Figure 3A). The control substrate is a 565 bp DNA fragment from the *lacZ* gene and encodes eight GATC sites, one of which has been assayed and found not to be sequestered in vivo (Campbell and Kleckner, 1990).

Unmethylated, hemimethylated, and fully methylated versions of these fragments were prepared using approaches specifically developed for this analysis (see Experimental Procedures). Pure unmethylated DNA was prepared using a *dam*$^-$ host. Pure fully methylated DNA was prepared using a *dam*$^+$ host, followed by in vitro treatment with E. coli Dam methylase; in the resulting preparations, at any given GATC sequence on either strand, greater than 99.95% of molecules are methylated. Hemimethylated substrate was obtained by denaturing and reannealing a mixture of unmethylated and fully methylated DNAs and then purifying a mixture equimolar for the two complementary hemimethylated forms away from other species.

SeqA Retardation of Full-Length *oriC* and *lacZ* Fragments

SeqA binding to methylated and hemimethylated substrate DNAs was examined by gel retardation (see Experimental Procedures). In brief, purified protein was incubated with the desired substrate fragment in 20 μl reactions containing PIPES buffer, 250 mM potassium glutamate, 2 mM EDTA, 1 nM labeled substrate fragment, 2 μg of calf thymus DNA, and varying concentrations of SeqA protein. After incubation for 30 min at 37°C, reaction mixtures were electrophoresed through a nondenaturing polyacrylamide gel and the percentage of label that migrated more slowly than the position of free substrate fragment (i.e., the percent retardation) was quantified.

SeqA binds the fully methylated *oriC* fragment with high affinity. At 25 nM SeqA, 50% of substrate is retarded, and 100% is retarded at ~60 nM. Retardation of the fully methylated *oriC* fragment requires more than a single molecule of SeqA protein. The sigmoidal shape of the retardation curve, with a Hill coefficient of 4.7 (data not shown), implies that the fractional retardation of DNA substrate molecules depends on approximately the fifth power of the protein concentration. This retardation is not due to a contaminating protein, as no other species is present in

the purified protein preparation in an amount sufficient to explain the level of retardation observed (see Experimental Procedures).

In sharp contrast, SeqA does not detectably retard the fully methylated *lacZ* control fragment, even at SeqA concentrations several times higher than that required for 100% retardation of the *oriC* fragment.

SeqA also interacts strongly with the two hemimethylated substrate preparations, which behave similarly to one another. 50% retardation of *oriC* and *lacZ* fragments occurs at 6 nM and 9 nM SeqA, respectively, and 100% retardation occurs at 15 nM and 20 nM, corresponding to molar ratios of SeqA monomers to substrate DNA fragment of 15:1 and 20:1, respectively (Figure 3). The number of SeqA molecules required for retardation of these substrates has not been determined except that it is less than 12.

No retardation of the unmethylated *oriC* fragment is observed. Since reaction mixtures contain a 300-fold excess, by weight, of unmethylated GATC-containing competitor DNA, the simplest possibility is that SeqA does not differentiate between unmethylated *oriC* and unmethylated random DNA sequences. SeqA binding to unmethylated *lacZ* fragment was not tested.

All reactions in which binding is observed yield complex arrays of retarded species (Figure 3): at lower levels of protein, retarded fragment is found in several discrete high mobility bands and in a broad region in the upper portion of the gel that extends up to the wells; at higher protein levels, 100% of retarded fragment is in the wells of the gel. The transition from higher to lower mobility species likely reflects meaningful aspect(s) of SeqA–DNA interactions rather than nonspecific aggregation of SeqA onto SeqA–DNA complexes at high protein concentration: this transition occurs at a lower SeqA concentration for hemimethylated substrates than for fully methylated substrates, an effect not expected for simple, nonspecific aggregation via protein–protein interactions. Mobility differences might reflect variations in the nature or conformation of individual DNA–protein complexes, association of two or more complexes, or both.

SeqA protein has been purified independently via its ability to bind the bacteriophage P1 origin and has been shown to bind strongly to hemimethylated DNA substrates (Brendler et al., 1995).

SeqA Retardation of Fully Methylated *oriC* Subfragments

The substrate determinants within *oriC* required for SeqA-mediated gel retardation of fully methylated DNA were dissected using eight fully methylated subfragments of the full-length *oriC* substrate, which differ substantially with respect to number of GATC sites and types of other determinants present, i.e., DnaA-binding sites, 13-mer "melting" sequences, and IHF- and FIS-binding sites. Six of these subfragments correspond to three complementary pairs, each containing the information from the full fragment: SmaI–HindIII (S-H) plus HindIII–XhoI (H-X), SmaI–AvaII (S-A) plus AvaII–XhoI (A-X), and SmaI–BamHI (S-B)

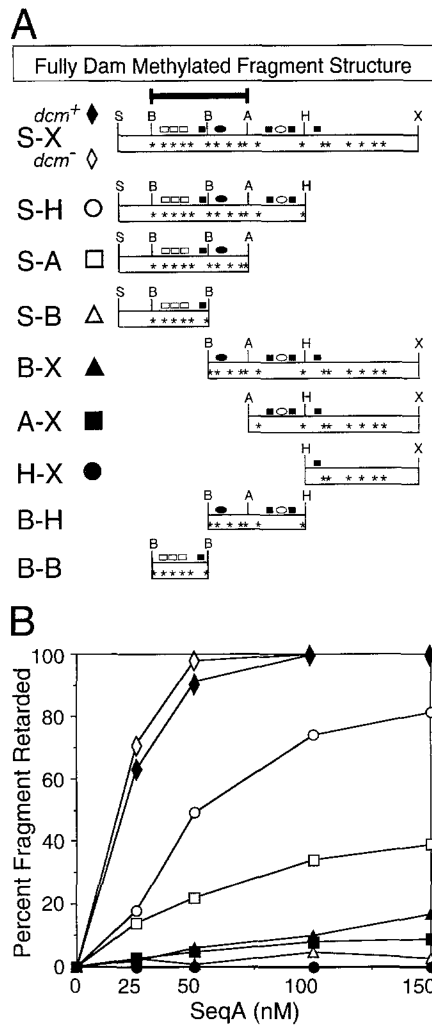

Figure 4. SeqA Binding to Fully Dam-Methylated *oriC* Subfragments

(A) Full-length *oriC* fragment and subfragments in fully methylated form were used as substrates for SeqA retardation assays. Binding reactions were identical to those described in Figure 3, except that the substrate fragment concentration was 0.1 nM instead of 1 nM. Restriction sites and genetic determinants are symbolized as in Figure 3. Positions of GATC sites (asterisks) in *oriC* with respect to the SmaI site at the left end (Messer and Weigel, 1995) are: base pairs 49, 70, 86, 101, 114, (–) 140, 154, 172, 189, 196, 218, 286, 319, 327, 360, 382, 400, and 413. The closed horizontal bar at the top represents the left half of the minimal origin (see text). Within this region, two arrays of evenly spaced GATC sites are underlined in the list above; the minus sign in the list above represents the spacer between the two arrays (see Discussion). Pairs of complementary subfragments are denoted by the closed and open forms of a particular symbol, circle, triangle, or square. Closed and open diamonds denote full-length fragments from *dcm*+ and *dcm*− strains, respectively. The number of GATC sites including GATCs at terminal BamHI sites rendered duplex during labeling reactions are as follows: SmaI–XhoI, 18; S-H, 12; S-A, 10; S-B, 6; B-X, 13; A-X, 8; H-X, 6; B-B, 6; and B-H, 8. S-A and A-X subfragments were derived from DNA grown in a *dam*+ dcm− host to permit AvaII digestion. One Dcm recognition site overlaps a GATC site near the AvaII site of *oriC*; three others occur on either side of the left-most BamHI site and 31 bp to the left of the AvaII site, respectively; Dcm methylation status has no effect on SeqA binding.

(B) Fraction of substrate fragment retarded as a function of SeqA protein concentration, as determined by quantitation of corresponding gels. Substrate fragments are denoted as in (A).

plus BamHI–XhoI (B-X). BamHI–BamHI (B-B) and BamHI–HindIII (B-H) subfragments were also analyzed. Each subfragment was prepared by digestion of full-length fully methylated fragment (Figure 4). Retardation was assayed as above, except that a lower concentration of labeled substrate fragment was used (Figure 4 and legend).

Maximally efficient retardation of fully methylated *oriC* requires determinants located along the entire length of the substrate fragment. None of the eight fragments examined is retarded as efficiently as the full-length fragment (Figure 4; see below). Only two of the eight are retarded with any reasonable efficiency, S-A and S-H; for two additional subfragments, B-X and A-X, retardation is just barely detectable. The remaining four exhibit no detectable retardation at the range of SeqA concentrations examined (Figure 4; data for B-B and B-X at 50 nM SeqA; see below).

Determinants particularly important for SeqA retardation of fully methylated *oriC* occur within a region that extends from the left-most BamHI site to the AvaII site, which comprises the left half of the minimal replication origin. The two subfragments that exhibit substantial retardation both carry this region. Most strikingly, cleavage at the second BamHI site located within the region essentially abolishes retardation: neither resulting subfragment, S-B or B-X, is retarded efficiently. The important region is also not present in its entirety in the other four nonretarded subfragments. The two subfragments that exhibit reasonable binding also contain some sequences to the left of the minimal origin (S-B), but this region contains no GATC sites or other known determinants that might be suspected to contribute to SeqA binding (Figure 4A).

Fully efficient retardation requires determinants beyond the important left-half segment, however. The S-A fragment contains this segment, but its reactivity is increased by addition of the AvaII–HindIII segment, and reactivity of the resulting S-H fragment is even further increased by addition of the remaining H-X segment (Figure 4). Furthermore, the enhancing effect of the additional sequences requires that they be present in *cis* to the primary region: the S-A and A-X subfragments exhibit the same retardation curve when both are present in the same reaction (data not shown) as when assayed individually (Figure 4).

Fully methylated GATC sites must contribute to SeqA retardation of full-length fully methylated *oriC*, since unmethylated *oriC* substrate is not detectably retarded under the conditions used here (i.e., in the presence of unmethylated competitor DNA; see Figure 3 and above). The specific contributions of fully methylated GATCs are unclear, however. Analysis of subfragments reveals that the presence of multiple GATC sites per se is insuffcent: the H-X, S-B, B-B, and B-H fragments, which are not retarded at all, contain six, six, six, and eight GATCs, respectively, and the two fragments that are retarded weakly, A-X and B-X, contain eight and 13 GATCs, respectively. Moreover, the fragments that show the most retardation both contain fewer than 13 GATCs: 12 for S-H and 10 for S-A. An economical hypothesis is that one or more particular GATC sites or a specific array of GATC sites within the left-half origin segment (or both) are especially important (see Discussion).

SeqA Retardation of Hemimethylated *oriC* Subfragments

The B-B, B-H, and H-X subfragments of *oriC* have also been examined in their hemimethylated forms. Each of the two symmetrically hemimethylated forms was examined individually in a reaction mixture in which it was the only form labeled (Figure 5). All six species interact with SeqA as strongly as does the full-length hemimethylated *oriC* substrate population, or nearly so. At 50 nM SeqA, where all of the full-length fragment is retarded, each of the six hemimethylated species also exhibits 100% retardation; at a SeqA concentration of 5 nM, where ~45% of full-length fragment is retarded (see Figure 3), all three subfragments exhibit 30%–50% retardation. The two hemimethylated forms of each subfragment give very similar retardation patterns, but minor differences can be discerned for the B-B substrate.

The behavior of hemimethylated subfragments contrasts sharply with that observed for fully methylated subfragments. In parallel analysis, the fully methylated forms of the B-B, B-H, and H-X subfragments exhibited no retardation at 50 nM SeqA (Figure 5).

Level of SeqA Protein In Vivo

In wild-type strain MG1655 growing exponentially in minimal glucose medium, SeqA protein is present at the level of about 1000 molecules per cell (see Experimental Procedures), which corresponds to about 0.4 ng of SeqA per

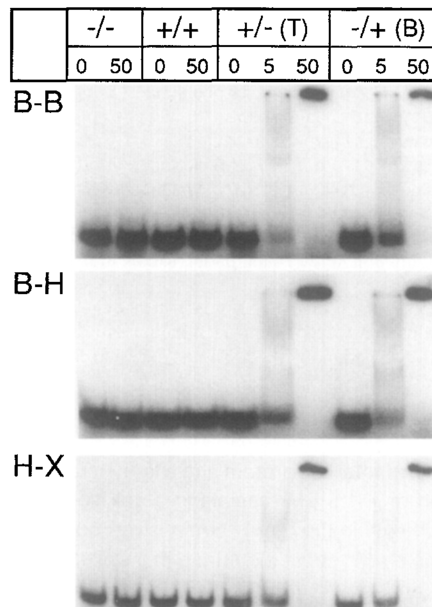

Figure 5. SeqA Retardation of Hemimethylated *oriC* Subfragments in Mixtures in which Only One Hemimethylated Form Is Labeled

Labeled form are as follows: unmethylated (minus/minus); fully methylated (plus/plus); top strand methylated (plus/minus (T)); bottom strand methylated (minus/plus (B)). Top and bottom strand designations refer to *oriC* as diagrammed in Figure 3A. Fragments are denoted as in Figures 3 and 4. Reaction conditions are identical to those in Figure 4. The nanomolar concentration of SeqA in each reaction is indicated at the top.

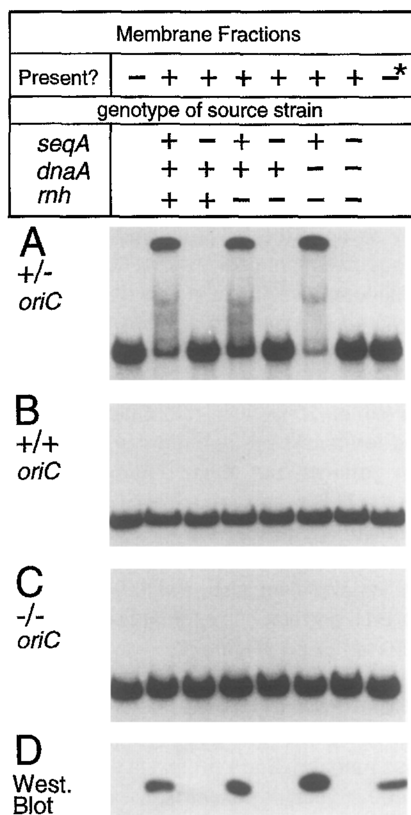

Figure 6. SeqA-Dependent Retardation of *oriC* Substrate Fragments by Crude Subcellular Outer Membrane Fractions Prepared from Exponentially Growing Cells

Crude membrane fractions were prepared from the indicated strains according to Ishidate et al. (1986); binding was assayed exactly as in Figures 3 and 4, except that 5–10 μg of membrane protein replaced purified SeqA. For each pair of isogenic $seqA^+$ and $seqA^-$ strains, reactions contained equal amounts of total added protein. SeqA protein concentration in the reaction mixture was 4 nM for $seqA^+dnaA^+rnh^+$ 3.5 nM for $seqA^+dnaA^+rnh^-$, and 4.5 nM for $seqA^+dnaA^-rnh^-$.

(A–C) Binding to hemimethylated (plus/minus), fully methylated (plus/plus), and unmethylated (minus/minus) full-length *oriC* fragments was performed exactly as in Figure 3. The asterisk indicates a reaction identical to the first lane in each panel, except that 10 μg of bovine serum albumin was also present.

(D) Immunoblot detection of SeqA protein in membrane preparations. Membrane protein (10 μg) from each membrane preparation used in panels (A)–(C) was analyzed. The asterisk indicates a standard containing 100 fmol of purified SeqA protein.

microgram of total cell protein and a total intracellular concentration of ~1.5 μM (assuming a cell volume of 1 fl). The SeqA level in the $dnaA^-$ strain is reproducibly higher than in the $dnaA^+$ strain examined in parallel (*dnaA::Tn10 rnh::Tn9* and $dnaA^+$ *rnh::Tn9*; 1.3-fold). This difference could reflect negative transcriptional regulation of *seqA* expression by DnaA protein (Lu et al., 1994) or destabilization of SeqA protein by interaction with DnaA protein.

SeqA Protein Accounts for All of the Hemimethylated *oriC*-Binding Activity in a Crude Subcellular Outer Membrane Fraction

Certain subcellular membrane fractions contain an activity that causes gel retardation of hemimethylated *oriC* DNA, but not of fully methylated or unmethylated *oriC* fragments (L. Rothfield, personal communication). Analysis of such membrane preparations in parallel with purified SeqA protein suggests that SeqA protein is responsible for all of this gel retardation activity for three reasons. First, in membrane fractions prepared from $seqA^+$ cells, efficient retardation is observed for the hemimethylated forms of both full-length *oriC* (Figure 6) and *lacZ* (data not shown) substrate fragments. In membrane fractions prepared from a *seqA* mutant strain, in contrast, no retardation is observed (Figure 6). Second, the levels of retardation observed with *oriC* and *lacZ* fragments are comparable with that expected for the concentration of SeqA protein present, ~75% binding at ~4 nM SeqA (compare with Figure 3). Third, the level of SeqA protein and the level of hemimethylated *oriC* fragment retardation observed per microgram of total membrane protein vary coordinately in a particular mutant situation: both levels are about 2-fold higher in membranes prepared from a strain carrying a *dnaA::Tn10* null mutation as compared with those prepared from an isogenic $dnaA^+$ strain (see Experimental Procedures). The difference between the SeqA levels in these two strains likely reflects differences in total cellular SeqA levels (see above).

Since preparations made from *dnaA::Tn10* cells bind hemimethylated DNA, DnaA protein is not required either directly for *oriC* binding activity in these membrane preparations or indirectly for localization of SeqA protein to these fractions.

No retardation of unmethylated or fully methylated fragment by these membrane fractions is observed (Figure 6). This result is fully explained by the lower affinity of SeqA protein for fully methylated and unmethylated substrates: at the concentration of SeqA present in these reactions, neither species is expected to exhibit significant binding (4 nM SeqA; see Figure 3). The low level of SeqA in the membrane preparations, ~10 nM, precludes assessment of retardation at higher protein to membrane concentrations. SeqA protein is not enriched in the membrane fraction as compared with total cellular protein; instead, it is present at about half the whole cell level, 0.2 versus 0.4 ng per microgram of protein, respectively.

Discussion

SeqA Retardation of Fully Methylated *oriC* and Its Role in Negative Modulation of Replication Initiation

SeqA specifically retards a fully methylated full-length *oriC* substrate fragment via a high affinity interaction, 50% retardation at a SeqA concentration of 25 nM. Retardation involves multiple SeqA protein monomers (approximately five). These observations provide strong circumstantial evidence that SeqA negatively modulates replication initiation directly, by interacting with the replication origin per se.

The smallest region required for replication initiation on a small plasmid minichromosome in vivo extends approximately from the left-most BamHI site through the right-most DnaA-binding site (Messer and Weigel, 1995). The most important interactions between SeqA and the fully

methylated form of *oriC* occur along a 150 bp segment that comprises the left half of this "minimal" origin, with cooperation in *cis* between regions on both sides of the right-most BamHI site playing a crucial role. Interactions with GATC sites are presumably an important component of SeqA binding. The left half of the minimal origin includes two arrays of four GATC sites spaced about 15 bp apart; the two arrays are separated by twice this increment, 30 bp (Figure 4A and legend). It is attractive to suppose that SeqA interacts coordinately with these arrays. Direct interaction of SeqA with GATC methylation is entirely compatible with prior genetic observations (Lu et al., 1994). Such analysis failed to detect any such interaction, but this is the expected result, because the positive requirement for methylation is epistatic to the negative role of SeqA protein.

Determinants outside of the critical left-half region are also required for maximally efficient retardation of fully methylated *oriC* by SeqA. Such determinants could comprise additional fully methylated GATC sites, other specific DNA sequences, particular DNA structural features, the simple nonspecific presence of duplex DNA, or a combination of these factors.

SeqA modulation of replication initiation may also involve activities of SeqA beyond its binding to fully methylated DNA. SeqA can exert a deleterious effect even when the origin is not fully methylated: overproduction of SeqA is lethal to a *dam::Tn9* strain, as it is to other mutants compromised for replication initiation (*dnaAts* and IHF$^-$; Lu et al., 1994). Also, strong and qualitatively specific genetic interactions between *seqA* and *dnaA* mutations have led to the proposal that SeqA might interact directly with DnaA (Lu et al., 1994).

Further Evidence That SeqA May Negatively Modulate Replication Initiation by Antagonizing Open Complex Formation

Formation of an open complex is a critical feature of replication initiation in vitro and in vivo (Bramhill and Kornberg, 1988; Gille and Messer, 1991). Previous observations have led to the suggestion that SeqA negatively modulates replication initiation by antagonizing the transition of the initiation complex from a closed form to an open form (Lu et al., 1994). The observations presented here provide additional support for this: the left half of the minimal origin, which contains the most important DNA substrate determinants for SeqA-mediated retardation of fully methylated *oriC*, is also the region of the origin that plays the most specific and crucial role in open complex formation.

More specifically, there is a good correspondence between individual determinants that contribute to open complex formation and specific regions required for SeqA retardation of fully methylated *oriC* (Figure 4A). First, the left half of the origin is bounded on its left end by the region in which the duplex opens during replication initiation, both in vitro and in vivo (Bramhill and Kornberg, 1988; Hwang and Kornberg, 1992; Gille and Messer, 1991). Since this melting region comprises a tandem array of highly conserved 13-mer sequences, each beginning with GATC, SeqA binding to these sites could influence origin opening very directly. Second, IHF protein is important for replication initiation in vivo (reviewed by Messer and Weigel, 1995; Lu et al., 1994), probably because it promotes open complex formation (Skarstad et al., 1990; Hwang and Kornberg, 1992). The unique IHF-binding site of *oriC* occurs at the right end of the left-half region, and the corresponding IHF footprint extends across the interstitial BamHI site at which cleavage destroys retardation by SeqA (Messer and Weigel, 1995). Third, DnaA protein, bound to its R1 site, another 9-mer site(s), or both within the origin, also interacts with the 13-mer region in the open complex via a second type of binding site (Bramhill and Kornberg, 1988; Yung and Kornberg, 1989). The R1 site is adjacent to the BamHI site where cleavage disrupts SeqA binding.

Conversely, the right half of the minimal origin, which is less important for SeqA retardation, is not directly involved in open complex formation. Correspondingly, this region contains the binding site for FIS protein (Messer and Weigel, 1995), and there are no genetic interactions between *seqA*$^-$ and *fis*$^-$ mutations (Lu et al., 1994).

SeqA Retardation of Hemimethylated *oriC* and Its Relation to Sequestration

SeqA also binds strongly to hemimethylated DNA (50% retardation at 6–9 nM). SeqA retards *lacZ* DNA and diverse *oriC* subsegments essentially as well as it retards hemimethylated full-length *oriC*. It is plausible that retardation requires only a single hemimethylated GATC site. Some of the subfragments analyzed contain as few as six GATC sites.

In vivo, sequestration exhibits two distinct features: it occurs only at particular genetic loci, thus far *oriC* and the *dnaA* promoter, and it occurs only when the GATC site or sites at these loci are in their hemimethylated form. The strong interaction of SeqA with hemimethylated DNA is presumably important for the second feature; SeqA does not appear to be responsible for the locus specificity, however, as SeqA retardation of hemimethylated substrates is not very sensitive to the genetic composition of the substrate beyond the presence or absence of GATC(s).

The interaction of SeqA with hemimethylated GATC sites could confer sequestration specificity for that form either because SeqA binds preferentially to the hemimethylated forms as compared with the fully methylated form and/or because SeqA binding to hemimethylated DNA is qualitatively unique and is more effective than SeqA binding to fully methylated DNA in blocking access of the DNA to Dam methylase and other proteins required for replication initiation or transcription. This distinction is important because, at least at *oriC*, SeqA might already be present on the origin before it becomes hemimethylated. The specific nature or intimacy of the SeqA interaction with hemimethylated *oriC* might therefore be as important as binding per se, or more so.

It has been suggested previously that DnaA protein determines the locus specificity of sequestration at both *oriC* and *dnaA* (Lu et al., 1994). This possibility is even more attractive now that it is clear that SeqA protein itself cannot be responsible. In addition, a sequestered GATC site in the *dnaA* promoter region is located at the beginning of a 13-mer sequence, which is in turn adjacent to a DnaA

9-mer binding site. Perhaps sequestration is accomplished by the combined effects of SeqA protein interacting with the component GATC site in its hemimethylated form and DnaA protein interacting with the 9-mer and 13-mer sequences.

Binding of Hemimethylated *oriC* by Outer Membrane Fractions

A subcellular "outer membrane" fraction contains an activity that gel retards hemimethylated *oriC* DNA; this activity is SeqA. SeqA may therefore also be the activity responsible for the ability of such fractions to interact with hemimethylated *oriC* in filter binding and replication inhibition assays (see Introduction).

Subcellular membrane fractions prepared from synchronized cells that have recently initiated DNA replication have newly replicated *oriC* sequences preferentially associated (see Introduction). SeqA could be responsible for this association. This remains to be established, however, as the membrane fractions analyzed here were prepared from exponentially growing cells.

The presence of SeqA in outer membrane fractions could reflect a normal, biologically significant interaction between SeqA protein and specific membrane-associated subcellular components. SeqA protein is, however, present in the membrane fraction at a lower level with respect to total cellular protein than in whole cells. Thus, SeqA could be present nonspecifically along with other contaminating proteins or via association with residual DNA; also, SeqA may tend to precipitate nonspecifically onto membranes during fractionation, since the protein is only soluble in vitro at high salt concentrations and membrane fractions are prepared under low salt conditions. The data presented here demonstrate that DnaA protein, at least, is not required for localization of SeqA to membranes.

Membrane fractions from synchronized cells have recently been shown to protect hemimethyated *oriC* DNA from nuclease digestion, and this protection has been attributed to the product of the *hobH* gene (Herrick et al., 1994). The relationship of these findings to those reported here remains to be established. *hobH* and *seqA* are clearly two different genes (Lu et al., 1994; Herrick et al., 1994), and in the current experiments, no binding of hemimethylated *oriC* could be detected in membrane fractions prepared from asynchronously growing *seqA*$^-$ hobH$^+$ cells. It is possible that there are two different proteins, each capable of binding hemimethylated *oriC*, but that HobH activity is not detected in the fractions and assays described here. Alternatively, however, it seems possible that the binding activity observed by Herrick et al. (1994) corresponds to SeqA and has been attributed inappropriately to HobH protein. In support of this possibility, in contrast to observations reported by Herrick et al. (1994), a *hobH::kan* disruption mutation confers no perturbation of cell growth or DNA replication initiation (E. B., unpublished data).

Experimental Procedures

Purification of SeqA Protein

SeqA protein was overexpressed in E. coli strain BL21/λDE3 harboring pLysS and pNK3506, which contains the *seqA* gene fused to the T7 promoter of pET11a (Studier et al., 1990). An overnight culture of this strain was grown at 37°C in M9ZB media (Studier et al., 1990) containing chloramphenicol (20 μg/ml), ampicillin (25 μg/ml), and timentin (16 μg/ml) with glucose omitted. Cells were diluted into fresh medium and grown to OD_{600} = 0.6, at which point *seqA* expression was induced by addition of IPTG to 0.5 mM. After 1 hr, the cells were harvested by centrifugation, weighed, and resuspended in an equal weight of buffer A (50 mM Tris [pH 7.5], 5 mM EDTA, 1 mM DTT). The cell suspension was frozen in liquid nitrogen and stored at −80°C in a graduated 50 ml tube.

The starting material for SeqA purification was 16.6 g of frozen cell suspension (8.3 g wet weight of cells). Cells were thawed; they lysed owing to T7 lysozyme produced by pLysS. In autolysed cells, nearly all SeqA is insoluble (pellets at 16,000 × g). To solublize SeqA, an equal volume of buffer A containing 4 M NaCl was added to autolysed cells, and the tube was gently rotated for 60 min at 4°C. The lysate was cleared by centrifugation at 80,000 × g for 2.5 hr (Beckman 70.1 Ti rotor). SeqA was precipitated from the supernatant (24 ml, 420 mg of protein) in a 25%–35% ammonium sulfate fraction and resuspended in buffer A containing 2 M NaCl. The resuspended material (15 ml, 63 mg of protein) was loaded on a phenyl–Sepharose CL-4B column (Pharmacia) equilibrated in buffer A plus 2 M NaCl. The column was washed with buffer A plus 2 M NaCl and then buffer A plus 1 M NaCl, and SeqA was eluted in buffer A plus 1 M NaCl and 50% ethylene glycol. The eluate was dialyzed against three changes of buffer A plus 1 M NaCl to remove ethylene glycol, and SeqA was precipitated from the solution (28 ml, 8.7 mg of protein) with 30% ammonium sulfate. The precipitate was resuspended in a small volume of buffer A plus 1 M NaCl and dialyzed against three changes of buffer A plus 1 M NaCl. The dialyzed fraction (4 ml, 6 mg of protein) was adjusted to 10 mM CHAPS by adding 1 ml of 50 mM CHAPS in buffer A plus 1 M NaCl. SeqA was subjected to two sequential FPLC runs on a Superose 12 gel filtration column (Pharmacia) equilibrated with buffer A plus 1 M NaCl and 10 mM CHAPS. CHAPS increases SeqA purity by sharpening the peak of a similarly eluting contaminant. Fractions corresponding to the broad peaks of several runs were pooled, dialyzed against buffer A plus 1 M NaCl and then against buffer A plus 1 M NaCl and 50% ethylene glycol, and stored at −20°C. SeqA (~550 μg) was recovered at 70 μg/ml. The identity of purified SeqA was confirmed by N-terminal sequence analysis.

Preparation of Substrate DNA Fragments

oriC and *lacZ* substrate fragments were prepared using two derivatives of pBluescript SK(+) (Stratagene) that lack the KpnI and SacI polylinker sites, respectively. pNK3512 and pNK3514 harbor the 465 bp *oriC* AvaI fragment (Figure 3A) cloned into SmaI and XhoI polylinker sites; pNK3516 and pNK3518 harbor the 546 bp BsrFI–Eco47III *lacZ* fragment (Figure 3A) cloned into the XmaI and EcoRV polylinker sites. Unmethylated plasmid DNA was isolated from NK6372 (*dam-13::Tn9*). Fully methylated plasmid DNA was isolated from MG1655 (*dam*$^+$) except as noted and treated in vitro with E. coli Dam methylase (New England Biolabs). Full-length fragments were obtained by digestion with AvaI (*oriC*) or SpeI plus HindIII (*lacZ*); AvaI cuts at both SmaI and XhoI sites.

Each hemimethylated fragment used in Figures 3 and 6 was prepared by combining a pair of methylated and unmethylated plasmid DNAs that also differ with respect to KpnI and SacI sites (see above). For each plasmid pair, the two plasmid DNAs were linearized with ScaI, mixed, denatured and reannealed (with NaOH treatment extended to 10 min; Horiuchi and Zinder, 1972), digested with both SacI and KpnI, and electrophoresed through 0.7% agarose. Unmethylated and fully methylated duplexes in the mixture were cleaved by SacI and KpnI, respectively. The two hemimethylated species remain uncleaved and were eluted from the gel as such and then digested as above to release the desired fragment.

Fragments used for the analysis in Figure 5 were isolated from pBluescript SK(+) *oriC* derivatives harboring the B-B fragment (pNK3508) or the B-X fragment (pNK3509). Desired fragments or subfragments were purified from methylated and unmethylated versions of each plasmid following digestion with enzymes that would permit end filling at only one end (Sambrook et al., 1989). Hemimethylated subfragments were made by hybridizing single-end labeled fully meth-

ylated fragment to a 20-fold excess of unlabeled unmethylated fragment; 5% of label remains in fully methylated DNA.

Analysis of Fully Methylated *oriC* Fragment for the Possible Absence of GATC Methylation on One or Both Strands

The fully methylated *oriC* fragment preparation used in all assays presented was analyzed to determine the probability that one or more GATC sites lack N^6-adenine methylation on one or both strands. A small amount of this preparation, radiolabeled at the 3′ end of each strand, was hybridized with a 20-fold excess of unmethylated fragment to give a mixture of the two symmetrically hemimethylated forms, each labeled on the 3′ terminus of the methylated strand. This mixture was then cleaved with MboI, which digests only unmethylated GATC sites. Any molecule in the original fully methylated fragment preparation that contained an unmethylated GATC site would yield a completely unmethylated GATC site in the new hemimethylated DNA sample. Such unmethylated sites would be cleaved by MboI, producing a corresponding end-labeled single-stranded species detectable in a DNA sequencing gel.

As one control, parallel samples were left undigested or were digested either with Sau3A, which cleaves at GATC sites irrespective of methylation status, or with DpnI, which cleaves methylated sites efficiently but also slowly digests hemimethylated DNA. Also, analogous preparations of fully methylated and unmethylated fragment were made by hybridizing a small amount of labeled fragment with an excess of unlabeled fragment of the same methylation status and were treated analogously.

In all control reactions, the specificities of the three enzymes for DNA in the three methylation states were as expected. In the critical digest of hemilabeled hemimethylated DNA with MboI, no bands corresponding to nicking or cleavage at internal GATC sites were detected. Evaluation of background and the intensities of bands detected in control digests suggests that a signal representing cleavage of 0.05% of the substrate DNA would have been detected. Thus, the fully methylated fragment preparation is ≥99.95% methylated at every GATC site. Even if every adenine residue was unmethylated at a frequency just below the level of detection, only 1.8% of the substrate DNA preparation would have a single hemimethylated GATC site (.05% × 18 sites × 2 strands).

Bacterial Strains

NK6366 is equivalent to MG1655 (Bachman, 1987); NK6367–NK6372 are derivatives of MG1655 containing additional mutation(s) as follows: NK6367, *seqAΔ10*; NK6368, *rnh::Tn9*; NK6369, *rnh::Tn9 seqAΔ10*; NK6370, *rnh::Tn9 dnaA::Tn10*; NK6371, *rnh::Tn9 dnaA::Tn10 seqAΔ10*; NK6372, *dam-13::Tn9*. *rnh::Tn9* and *dnaA::Tn10* alleles are derived from E. coli AQ3519 (from T. Kogoma). *dam-13::Tn9* allele is derived from GM2159; this strain and GM31 were gifts of M. Marinus. *seqAΔ10* allele is an in-frame deletion-insertion allele; the ApoI–EagI fragment specifying codons 35–154 of *seqA* is replaced by a short EcoRI–EagI fragment of pNB8. A *seqAΔ10* strain is phenotypically Pgm^+ (data not shown).

Gel Retardation

The gel retardation procedure was developed and generously provided by L. Rothfield (University of Connecticut). A typical reaction of 20 μl contained buffer B (250 mM potassium glutamate, 10 mM PIPES buffer, 2 mM EDTA, [pH 7.0]), 2 μg of calf thymus DNA, 2 or 20 fmol of DNA fragment, and the appropriate dilution of pure SeqA (diluted in buffer A containing 1M NaCl) or crude membrane preparation. All reaction components except substrate DNA(s) were mixed in a volume of 19 μl and incubated at room temperature for 20 min; 1 μl of substrate fragment in TE was added, and incubation was continued at 37°C for 30 min. A 4 μl aliquot of loading buffer (buffer B plus 40% sucrose) was then added, and the reaction was loaded on a 3.5% SDS–polyacrylamide gel (50 mg of bisacrylamide per gram of total polyacrylamide) and subjected to electrophoresis at 4°C in running buffer B. Radioactivity in gels was quantitated with a Fujix BAS 2000 imaging system. The fraction of DNA retarded by interaction with SeqA protein was determined by comparing, within each lane, the amount of radioactivity at the position of free fragment with the amount of radioactivty within the range of positions occupied by retarded fragment.

SeqA Antibodies and Immunoblotting

Purification of SeqA was initially followed using an antiserum raised against a synthetic polypeptide and subsequently by antiserum produced against the purified protein; in crude cell extracts, the latter antiserum revealed one prominent SeqA-specific band and a low nonspecific background.

To determine the number of SeqA protein molecules per cell, 1–2 ml of culture at OD_{450} = 0.15 was concentrated, incubated at 95°C for 2 min in SDS loading buffer, electrophoresed through a 12% SDS–polyacrylamide gel, and analyzed by immunoblot with anti-SeqA antiserum by comparison with known amounts of pure SeqA protein present on the same gel. Bound antibody was detected by ECL (Amersham, UK); relative band intensities were quantified by scanning densitometry. Concentrations of cells and total protein concentration in each culture sample were determined by Coulter counter and Bradford analysis, respectively.

SeqA levels in whole cells were determined using a single set of samples, one from each strain of interest, analyzed in four independent immunoblots. A single immunoblot in which protein was transferred to nitrocellulose using wet blotting (Sambrook et. al, 1989) yielded numbers of SeqA molecules per cell as follows: $dnaA^+rnh^+$ = 908 ± 15; $dnaA^+rnh^-$ = 1143 ± 46; $dnaA^-rnh^-$ = 1435 ± 34. Three immunoblots performed with semi-wet blotting gave similar values: 847 ± 458, 1040 ± 697, and 1380 ± 837, respectively. SeqA levels in membrane fractions were determined by duplicate analysis of the preparations analyzed in Figure 6 using the wet blotting method. SeqA protein levels (nanograms per microgram of total protein) were as follows: $dnaA^+rnh^+$ = 0.12 and 0.17; $dnaA^+rnh^-$ = 0.15 and 0.2; $dnaA^-rnh^-$ = 0.33 and 0.36. Each of these values is about one third of that observed in the same whole cell sample.

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