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SeqA blocking of DnaA-*oriC* interactions ensures staged assembly of the *E. coli* pre-RC

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Summary

DnaA occupies only the three highest affinity binding sites in *E. coli oriC* throughout most of the cell cycle. Immediately prior to initiation of chromosome replication, DnaA interacts with additional recognition sites, resulting in localized DNA strand separation. These two DnaA/*oriC* complexes formed during the cell cycle are functionally and temporally analogous to yeast ORC and pre-RC. Following initiation, SeqA binds to hemimethylated *oriC*, sequestering *oriC* while levels of active DnaA are reduced, preventing re-initiation. In this paper, we investigate how resetting of *oriC* to the ORC-like complex is coordinated with SeqA-mediated sequestration. We report that *oriC* reset to ORC during sequestration. This was possible because SeqA blocked DnaA binding to hemimethylated *oriC* only at low affinity recognition sites associated with GATC, but did not interfere with occupation of higher affinity sites. Thus, during the sequestration period, SeqA repressed pre-RC assembly while ensuring resetting of *E coli* ORC.

Introduction

Initiation of chromosome replication is precisely timed during the cell cycle, with each origin firing only once, so that all newly divided cells receive the correct genome content. (Baker and Bell, 1998;Boye et al., 2000;Early et al., 2004;Kornberg and Baker, 1992;Messer, 2002;Prasanth et al., 2004). In *E. coli*, regulation of replication initiation is focused on the assembly and disassembly of complexes comprising the chromosomal replication origin *oriC* and multiple copies of the initiator protein DnaA (Fuller et al., 1984;Leonard and Grimwade, 2005;Margulies and Kaguni, 1996;Weigel et al., 1997). DnaA is a AAA+ protein, with structural similarity to archeal and eukaryotic Orc1/Cdc6 proteins (Erzberger et al., 2002). DnaA binds to at least eight 9 mer recognition sites in *oriC* (R boxes and I sites), shown in Figure 1A (Fuller et al., 1984;Leonard and Grimwade, 2005;Margulies and Kaguni, 1996;Matsui et al., 1985;McGarry et al., 2004;Weigel et al., 1997). *In vivo*, two DnaA/*oriC* complexes have been observed on minichromosomes (plasmids using *oriC* as the sole origin of replication). First, DnaA occupies the three highest affinity sites (R1, R2, and R4), and this binding pattern persists throughout much of the cell cycle (Cassler et al., 1995;Samitt et al., 1989). In the second complex, seen only at the time DNA synthesis initiates, DnaA occupies all of its recognition sites in *oriC*, including the remaining lower affinity R boxes (R5M and R3) and I sites (Cassler et al., 1995;Ryan et al., 2002). Localized DNA strand separation within the A-T-rich DNA Unwinding Element (DUE) (Kowalski and Eddy, 1989) is coincident with assembly of the second complex (Leonard and Grimwade, 2005). Only the ATP form of DnaA is active in the strand separation process (Sekimizu et al., 1987), since occupation of I sites is required (McGarry et al., 2004) and I sites preferentially bind DnaA-ATP (Kawakami et al., 2005;McGarry et al., 2004). Once formed, the open (strand separated) complex is stabilized by direct DnaA-ATP interactions at several sites (S-M sites in Figure 1A) within the DUE

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(Speck and Messer, 2001). The fully loaded DnaA/*oriC* complex also recruits DNA helicase (Bramhill and Kornberg, 1988;Carr and Kaguni, 2002). After origin unwinding, replication fork components are loaded on the single-stranded template and DNA polymerization proceeds bi-directionally around the circular *E. coli* genome (Baker and Bell, 1998;Kornberg and Baker, 1992;Stillman, 1994).

In *E. coli*, after DNA synthesis initiates, regulatory mechanisms ensure once-per cycle origin activity (Boye et al., 2000;Camara et al., 2005;Katayama, 2001). Logically, origin complexes must also reset identically, so that all *oriC* copies start rebuilding the initiation complex from the same starting point. Thus, it is important that any mechanisms that inactivate *oriC* allow origin resetting by maintaining accessibility of DnaA to the high affinity sites. This requirement raises interesting questions regarding post-initiation sequestration of *oriC* (Bogan and Helmstetter, 1997;Campbell and Kleckner, 1990;Landoulsi et al., 1990;Lu et al., 1994;Ogden et al., 1988;Russell and Zinder, 1987;von Freiesleben et al., 2000). Sequestration requires interaction of SeqA with hemimethylated GATCs located at 11 positions within *oriC* (Figure 1A) (Bach and Skarstad, 2004;Brendler et al., 1995;Lu et al., 1994;Slater et al., 1995). Loss of SeqA or GATC sequences in *oriC* causes asynchronous initiations in cells containing multiple *oriC* copies, and measurable re-initiation during a single cell cycle (Bach and Skarstad, 2004;Boye et al., 1996;Camara et al., 2005). I*n vitro*, SeqA negatively regulates initiation of DNA synthesis (Lu et al., 1994;Torheim and Skarstad, 1999;von Freiesleben et al., 1994;Wold et al., 1998). However, despite convincing evidence that SeqA interacts with *oriC*, there are conflicting reports concerning the ability of SeqA to interfere with DnaA binding (Taghbalout et al., 2000;Torheim and Skarstad, 1999;von Freiesleben et al., 2000).

Interestingly, the weaker affinity DnaA binding sites R5M, I2 and I3 are associated with GATCs, and therefore should be targets for SeqA binding. In this paper we report that during the post-initiation sequestration period, SeqA prevented DnaA from occupying I2, I3 and R5M on hemimethylated *oriC*, but permitted binding to higher affinity DnaA recognition sites, so that chromosomal *oriC* reset to an R1-, R2- and R4-bound complex shortly after initiation of DNA replication. We propose that during the cell cycle, DnaA assembles into complexes on *oriC* that are functionally and temporally analogous to yeast ORC and pre-RC (Prasanth et al., 2004;Stillman, 2005). By focusing its activity to several key DnaA binding sites, SeqA represses re-initiation by preventing reformation of the pre-RC, while allowing resetting of the *E. coli* ORC. Proper resetting by SeqA ensures that all *oriC* copies begin the process of rebuilding the initiation complex from the same starting point.

Results

DMS footprinting reveals DnaA-*oriC* interactions *in vitro* and *in vivo*

We have measured DnaA-ATP interactions within the entire *E. coli oriC* region *in vitro* and *in vivo* by DMS footprinting of *oriC* on fully methylated supercoiled minichromosomes (pAL49) or on the chromosome using Ligation-Mediated PCR (LMPCR) (Angers et al., 2001) (Figure 1B). Different primer sets and gel run times were used to subdivide *oriC* into three sections (Figure 1B DUE -R1, lanes 1–3 and 10–12; R5M-I1-I2, lanes 4–6 and 13–15; R2-I3-R4, lanes 7–9 and 16–18). DMS preferentially methylates guanosines, and binding of DnaA reproducibly changes the methylation pattern within recognition sites, indicating occupancy of each site. When sites are fully occupied, the G in the fourth position of the 9 mer recognition site is 2–4 fold more sensitive to DMS than it is on naked DNA, and the G in the second position (if present) is approximately 2-fold less sensitive (marked by arrows in Figure 1A, quantified in Figure 1C). For the *in vitro* samples, two DnaA concentrations are shown; 10 nM (Figure 1B, lanes 2, 5, and 8), corresponding to approximately 3 molecules of DnaA per *oriC*, which is below the concentration required to unwind the 13 mer region, and 100 nM (Figure 1B, lanes 3, 6, and 9) (approximately 25–30 DnaA molecules per *oriC*) which exceeds

the previously determined concentration required for *oriC* unwinding (Ryan et al., 2002). *In vitro*, only the higher affinity sites R1, R2, and R4 give a strong footprint at the lower DnaA concentration. R1, R2, and R4 are also the only sites on chromosomal *oriC* observed to bind DnaA *in vivo* in exponentially growing cells (Figure 1B, lanes 11, 14, and 17), which is consistent with our previous *in vivo* footprints of minichromosomes (Cassler et al., 1995;Ryan et al., 2002). DnaA binding to R5M, I1, I2, and I3 can be observed *in vitro* at the higher DnaA concentration (Figure 1B, lanes 3, 6, and 9), and *in vivo* on chromosomal *oriC* in temperature-sensitive DnaC mutants (strain PC2), held at non-permissive temperature until all origins were aligned at the stage of initiation just prior to helicase loading (Figure 1B, lanes 12, 15, and 18). Because of the very low affinity of R3, binding to this site is difficult to detect (*in vitro*, a slight enhancement can be seen in a G adjacent to R3, noted by a star in Figure 1B, lanes 7–9), and so we have not included R3 in these studies. We also have noted a DnaA-induced change in methylation pattern at a site between I1 and R5M (marked by a closed circle in Figure 1B, seen in lanes 6 and 15), but it is not known if this putative site is required for *oriC* function. DnaA is also reported to bind to sites in the 13 mer region (Speck and Messer, 2001). These were not detected by *in vitro* DMS footprinting, although *in vivo* in cells aligned at initiation, each of the 13 mer regions contains a nucleotide with enhanced sensitivity to DMS (figure 1B, compare lane 10 with lane 12). Studies to determine the cause of the enhancements are currently being done.

DnaA binding at *oriC* recognition sites R1, R2, and R4 is detected *in vivo* during sequestration

Following the passage of replication forks immediately after initiation, chromosomal *oriC* becomes hemimethylated and sequestered by SeqA for 1/3 of the cell cycle (approximately 12 minutes) (Campbell and Kleckner, 1990;Lu et al., 1994). In exponentially growing cells and synchronized cells approaching initiation of DNA replication, DnaA binding to *oriC* was observed only at R1, R2, and R4 (Cassler et al., 1995;Ryan et al., 2002;Samitt et al., 1989;Figure 1B). However, these studies do not exclude the possibility that high affinity sites are unoccupied for a period of time during sequestration. In order to examine this, the interaction of DnaA with chromosomal *oriC* in *dnaC*(ts) cells during the sequestration period was analyzed. Cells were aligned at the stage of initiation by incubation at non-permissive temperature. After shifting back to permissive temperature, all *oriC* copies initiated DNA replication synchronously. Six minutes after the downshift, when *oriC* is hemimethylated (Campbell and Kleckner, 1990), DMS was added to cells to obtain *in vivo* modification patterns during sequestration (Figure 2A, lanes 1 and 3). The footprints revealed that on chromosomal *oriC*, DnaA was bound to R1, R2, and R4, but not to R5M, I1, I2, or I3 (compare with patterns obtained *in vitro*,Figure 1B). In order to study the reason why DnaA did not bind to the lower affinity sites during sequestration, it was necessary to isolate hemimethylated minichromosomal *oriC* to use as a template for *in vitro* footprinting assays. To do this, *dnaC* mutants harboring pAL49 were aligned at initiation at non-permissive temperature. After shifting back to permissive temperature, supercoiled pAL49 was isolated either after DMS treatment at 6 minutes (Figure 2A, lanes 2 and 4) or at intervals following the downshift (Figure 2B). Plasmid hemimethylation was assayed by measuring sensitivity to Hph1 cleavage (Figure 2B) at a site that overlaps a GATC, as previously described (Campbell and Kleckner, 1990). When DNA is methylated, this site is not cut. After replication, the adenine in the recognition site is methylated on one daughter molecule, and resistant to cutting, while on the other molecule it is unmethylated and sensitive. Thus, when all of the *oriC* copies are hemimethylated, 50% of them are cleaved by Hph1. The peak of hemimethylation (50% cut DNA) was obtained at 6–8 min after the shift (Figure 2B), similar to previous measurements of hemimethylation of chromosomal *oriC* in this strain (Campbell and Kleckner, 1990). At this time, the DnaA binding pattern on minichromosomal *oriC* is the same as that of chromosomal *oriC*, e. g. bound to only R1, R2, and R4 (Figure 2A, lanes 2 and 4).

It is possible that DnaA does not bind well to lower affinity sites on hemimethylated DNA. To test this, supercoiled hemimethylated pAL49 DNA isolated from aligned cells 6 minutes after the downshift was incubated with DnaA, and treated with DMS (Figure 2C, lanes 2, 3, 8, and 9). Additionally, to determine if the topology of the template affected the DMS footprint, DnaA binding was also examined using hemimethylated *oriC* PCR fragments generated using single cycle PCR (Figure 2B, Figure 2C lanes 4–6, 10–12). We found that hemimethylation did not alter DMS modification patterns obtained for purified DnaA at any R box or I site when compared to fully methylated *oriC* (compare Figures 1C and 2C), and that binding was equivalent on supercoiled and linear templates, consistent with previous reports (Ryan et al., 2002;Taghbalout et al., 2000).

SeqA specifically blocks DnaA binding at lower affinity sites on hemimethylated *oriC* DNA

R1, R2, and R4 lack GATC within the recognition site. In contrast, R5M, I2, and I3 contain GATC (overlapping for R5M and internal for I2 and I3; Figure 1A). The correlation of GATC with sites unoccupied by DnaA during sequestration raises the question of whether DnaA was absent from these sites because binding was specifically blocked by SeqA. We have tested this possibility both *in vivo* and *in vitro. In vivo*, if SeqA blocks R5M, I2 and I3, then these sites should rapidly rebind DnaA after initiation is induced in *dnaC*(ts) cells lacking SeqA. To examine this, PC2 (*dnaC*(ts) *seqA*) cells were either treated with DMS during exponential growth (Figure 3A, lanes 2 and 4), or held at non-permissive temperature to align the cells at initiation, shifted back to permissive temperature for 6 minutes, and then treated with DMS (Figure 3A, lanes 3 and 6). Interestingly, in cells lacking SeqA, chromosomal *oriC* bound DnaA at all sites 6 minutes after the downshift, similar to the pattern seen only at the time of initiation in normal cells (compare Figure 3A, lanes 3 and 6 with Figure 1B, lanes 12, 15, and 18). Similar results were seen when *oriC* on minichromosomes in SeqA mutants was examined (data not shown). Thus, shortly after initiation, cells with functional SeqA reset to an R1, R2, R4-bound state, while SeqA mutants rebound DnaA at all sites (compare Figure 3A, lanes 3 and 6 with Figure 2A, lanes 1 and 3). In exponentially growing SeqA mutants, DnaA binding was observed only at R1, R2, and R4, similar to that of normal cells (compare Figure 3A, lanes 2 and 5 with Figure 1B, lanes 11, 14, and 17), indicating that DnaA does not bind lower affinity sites continuously during the cell cycle.

The absence of SeqA appears to be most detrimental to rapidly growing cells, when *E. coli* initiates new rounds of chromosome replication before the previous round is complete (Lu et al., 1994), resulting in multi-fork replication. To determine if SeqA plays a role in resetting *oriC* to the R1, R2, and R4-bound state at slower growth rates, when there is no multi-fork replication, normal or SeqA mutant PC2 cells were grown using a less efficient carbon source (glycerol instead of glucose) to reduce growth rate, and treated with DMS in aligned cells 6 minutes after initiation was induced. As was seen for cells with multi-fork replication, *oriC* in cells containing functional SeqA reset to the R1, R2, R4-bound state (compare Figure 3B, lanes 1 and 3 with Figure 2A, lanes 1 and 3), while *oriC* in SeqA mutants rebound DnaA at all sites, including the lower affinity R5M, I2, and I3 sites (Figure 3B, lanes 2 and 4).

To determine that it was SeqA protein that was responsible for blocking the lower affinity sites, we compared the *in vitro* DMS footprints generated by incubating linear hemimethylated *oriC* with DnaA alone (50 nM, approximately 20–25 molecules per origin, Figure 4A, lanes 3,11), SeqA alone (50 nM, Figure 4A, lanes 2,10) or SeqA (25 nM and 50 nM) followed by DnaA (50 nM) (Figure 4A, lanes 4, 5, 12,13). When SeqA alone was added to hemimethylated template, only minimal changes in the DMS pattern were observed when compared to naked DNA (Figure 4A, compare lanes 2,10 with 1, 9). In the absence of SeqA, DnaA binding was detected at all recognition sites (Figure 4A, lanes 3,11). However, addition of equimolar levels of SeqA prior to addition of DnaA blocked DnaA occupancy at R5M, I2 and I3, but had little

effect on DnaA binding to R1, R2, and R4 (compare Figure 4 lane 3 with lane 5, and lane 11 with lane 13). Binding to I1 also appeared blocked by SeqA, but the footprint at this site is less pronounced than at the other sites and may be dependent on I2 binding (unpublished observations). Linear templates were used to minimize topological effects; similar results were seen on supercoiled hemimethylated templates (data not shown).

SeqA has been reported to have a 3–5 fold- higher affinity for hemimethylated DNA compared with fully methylated DNA (Slater et al., 1995). We did not observe DnaA blocking by SeqA (50 nM) at any site on fully methylated templates (Figure 4 compare lanes 7,8 with 15,16), although pre-incubation of methylated DNA with 5-fold higher concentrations of SeqA did prevent binding to R5M, I1, I2, and I3 (data not shown).

SeqA inhibits unwinding of supercoiled hemimethylated *oriC*

Previously, we found binding to both strong and weak DnaA recognition sites is required for DNA strand separation *in vitro* (Grimwade et al., 2000;McGarry et al., 2004). Thus, since SeqA inhibited DnaA binding to hemimethylated R5M, I2, and I3, it should also block open complex formation on hemimethylated *oriC*. Similarly, since SeqA did not block DnaA from binding to fully methylated templates, SeqA should not affect DnaA's ability to separate methylated DNA strands. The effect of SeqA on DnaA-induced strand separation was examined using P1 nuclease, which converts supercoiled *oriC* DNA to linear by cutting predominantly in the DUE region (Bramhill and Kornberg, 1988). DnaA (approximately 20–25 molecules per origin) is sufficient to cause both methylated and hemimethylated *oriC* supercoiled plasmids to be linearized by P1 (Figure 5A, lanes 2 and 7) consistent with previous reports (Bramhill and Kornberg, 1988). Increasing levels of SeqA (0–50 nM), reduced DnaA-catalyzed linearization by P1 on hemimethylated templates approximately 5-fold (Figure 5A lanes 3–5, and Figure 5B). However, at these levels, SeqA had little effect on DnaA-induced P1 sensitivity of fully methylated template (Figure 5A, lanes 8–10, Figure 5B).

SeqA blocking of DnaA recognition sites requires the presence of GATC

The DnaA binding sites that were blocked by SeqA were low affinity sites that were associated with a GATC sequence. In order to carefully examine whether SeqA blocking of DnaA sites requires an interior or overlapping GATC sequence, we created two mutant versions of *oriC*. First, we replaced the GATC in I2 with a GATT. Hemimethylated PCR fragments of *oriC* carrying this mutation bound DnaA as well as wild type I2 (compare Figure 6A, lanes 3 and 5). However, although SeqA blocked DnaA binding to wild-type I2, SeqA had little effect on DnaA's access to the mutated I2 (Figure 6A, compare lanes 4 and 6). This change was site specific, since SeqA did inhibit binding of DnaA to R5M on *oriC* with a mutated I2 (Figure 6A, lanes 3, 4). To verify that SeqA would inhibit DnaA binding to any site containing a GATC, we changed the R1 sequence from 5′-TGTGGATAA to 5′-TGTGGATCA. Wild-type R1 was accessible to DnaA in the presence of SeqA (Figure 6B, lanes 5, 6), but the mutated R1 was blocked (Figure 6B, lanes 3, 4). No other changes in DnaA binding to other sites in this mutant version of *oriC* were observed (data not shown).

Interestingly, the GATC to GATT mutations in I2 and I3 were very difficult to clone. Normally over 80% of the plasmids obtained using the site-directed mutagenesis procedure harbor the desired mutation, including conversions of GATC to GATT at other, non-I site *oriC* positions, and successful mutation of several non-I site GATC sequences in *oriC* has been reported (Bach and Skarstad, 2004). However, when used to mutate GATC in either I2 or I3, our procedure yielded approximately 10 fold-fewer colonies than we routinely obtain, and of those colonies, less than 5% contained the desired mutation, even though the plasmids contained a functional pBR322 origin in addition to *oriC*. Repeated attempts to clone *oriC* with GATT in both I2 and I3 gave no colonies with the desired mutation, even when *oriC* segments containing individual

mutations were ligated together. The reason for the difficulty in mutating the GATC in I2 and I3 is currently being investigated. Possibly, the mutations caused the mutated *oriC* to function in such a way that competed with the host chromosomal *oriC*, which could result in loss of viability.

Discussion

Staged assembly of *oriC*/DnaA complexes in *E. coli* is functionally and temporally analogous to yeast ORC progressing to a pre-RC

In eukaryotes, a group of conserved proteins binds to replication origins, forming the origin recognition complex (ORC). In yeast, ORC is found associated with origins throughout the cell cycle (Prasanth et al., 2004;Stillman, 2005). As cells progress toward initiation of DNA replication, additional proteins, such as Cdc6p and Cdt1 associate with ORC and recruit helicase, forming the pre-replication complex (pre-RC). The bacterial initiator protein, DnaA, is structurally homologous to CDC6/ORC1 proteins (Erzberger et al., 2002) and, like some ORC proteins, has the ability to bind and hydrolyze ATP. The data presented in this paper and in previous studies (Cassler et al., 1995;Samitt et al., 1989;Ryan et al, 2002) show that in addition to having structural similarities to ORC proteins, DnaA forms an ORC-like complex on E. coli *oriC*, that is bound to recognition sites R1, R2, and R4 throughout the cell cycle, including the time immediately after initiation during the sequestration period. At the time of initiation, a second stage of DnaA binding results in occupation of weaker affinity sites, causing DNA strand separation and recruitment of helicase (Bramhill and Kornberg, 1988;Cassler et al, 1995;Ryan et al., 2002), thus resulting in formation of a pre-RC.

SeqA regulates DnaA accessibility to *oriC* binding sites so that *oriC* ORC is reset during the sequestration period

After initiation, yeast origins reset to the ORC stage rapidly (Diffley, 2004;Prasanth et al., 2004;Stillman, 2005). In this paper, we report that in *E. coli*, after initiation of DNA replication, the ORC-like DnaA complex is also reformed rapidly, within six minutes after origin firing. At this time, *oriC* is hemimethylated, refractory to reinitiation, and presumably interacting with SeqA (Bach and Skarstad, 2004;Boye et al., 1996;Camara et al., 2005;Campbell and Kleckner, 1990;Lu et al., 1994;von Freiesleben et al., 1994). It is well established that SeqA directly contacts DNA at GATCs (Brendler and Austin, 1999;Han et al., 2004;Kang et al., 2005), and the sites that remained unoccupied during the sequestration period (R5M, I2, and I3) are the only GATC-associated 9 mer DnaA recognition sites in *E. coli oriC*, an association that is conserved among Enterobacterial chromosomes (Zyskind et al., 1983). Placement of GATC in R5M, I2, and I3 was necessary and sufficient for SeqA to block DnaA binding *in vitro*, since loss of GATC within a single site eliminated blocking of that site. Conversely, adding GATC to R1 caused SeqA to block this site, while the remaining R boxes lacking GATC remained unaffected. Our findings are compatible with the idea that one molecule of SeqA blocks one GATC-containing DnaA binding site, but our observations cannot rule out the formation of SeqA filaments in *oriC* (Guarne et al., 2005), as long as the high affinity R boxes remain accessible during sequestration.

In yeast, re-replication of an origin is prevented by inhibiting re-assembly of the pre-RC (Diffley, 2004), and the same type of negative regulation appears to ensure once per cycle regulation in *E. coli. In vitro,* blockage of DnaA binding by SeqA prevented open complex (pre-RC) formation, as detected by P1 nuclease sensitivity. In cells lacking SeqA, we observed that *oriC* did not reset to ORC shortly after initiation, but rather reassembled the fully DnaA-bound pre-RC seen in cells aligned at initiation. As would be expected in cells that reform a pre-RC after initiation, SeqA deficient strains have been reported to initiate more than once per cell cycle (Bach and Skarstad, 2004;Boye et al., 1996;Camara et al., 2005;Campbell and

Kleckner, 1990;Lu et al., 1994;von Freiesleben et al., 1994). Although the growth defects associated with SeqA mutants are more pronounced when cells are growing fast enough to have multi-fork replication (Lu et al., 1994), the regulatory role of SeqA is not restricted to fast-growing cells, since both reformation of the pre-RC and over-initiation (Boye et al., 1996) are seen in slower growing SeqA mutants. We propose that formation of the bacterial pre-RC, like the eukaryotic pre-RC, is regulated by post-initiation mechanisms (Diffley, 2004) and may be an ideal target for other regulators of initiation (see below).

SeqA is known to associate with the replication forks as they proceed bidirectionally from *oriC* (Molina and Skarstad, 2004;Yamazoe et al., 2005), and it is reasonable to suggest that I site and R5M blocking is likely to be the most efficient way for SeqA to inhibit reformation of the pre-RC since only a few SeqA molecules would need to be retained at *oriC* during the remaining sequestration period. When DnaA-*oriC* complexes were assembled prior to addition of SeqA, concentrations of SeqA that were sufficient to block GATC-containing sites were unable to displace pre-bound DnaA, nor was SeqA able to cause pre-formed open complexes to become resistant to P1 nuclease (data not shown). For this reason, we suggest that SeqA is unlikely to play a role in removing DnaA from *oriC*. Further, SeqA was approximately 5-fold less efficient at blocking DnaA from sites containing GATC on fully methylated templates, which is consistent with the reported lower affinity of SeqA for fully methylated DNA (Slater et al., 1995). Although it is possible that SeqA could bind to *oriC* before initiation if sufficient SeqA were available, it is more likely that SeqA functions after initiation when *oriC* is hemimethylated.

Previous studies show SeqA binding to *oriC* predominantly at 13 mer GATCs and the R1-IHF region, with lesser interactions at other *oriC* sites containing GATC (Skarstad et al., 2000;Slater et al., 1995;Taghbalout et al., 2000). SeqA binding in the 13 mer region could be an additional target for preventing pre-RC formation by blocking DnaA binding to the DnaA-ATP sites, which contain GATC (Speck and Messer, 2001). It has also been reported that SeqA alters the topology of supercoiled DNA (Klungsoyr and Skarstad, 2004;Skarstad et al., 2001;Torheim and Skarstad, 1999). The SeqA effects we observe are independent of DNA supercoiling, but it is likely that SeqA titration of supercoils would augment any inhibition of open complex formation caused by blocking of DnaA.

There are conflicting reports regarding SeqA's ability to inhibit DnaA binding at *oriC* (Skarstad et al., 2000;Taghbalout et al., 2000;von Freiesleben et al., 2000). Some of the disparity could be due to inconsistencies regarding the template (methylated or hemimethylated), the concentrations of SeqA, and order of addition of proteins. Our choice of SeqA and DnaA levels for the *in vitro* experiments reported here were based on the relative levels of the two proteins in cells (Messer, 2002;Slater et al., 1995) as well as our previous experience studying effects of accessory proteins on formation of DnaA-*oriC* complexes (Ryan et al., 2002). Our findings suggest that some of the disparity may also be due to the fact that only the weaker affinity DnaA recognition sites are preferentially blocked, while strong sites are not blocked or may only be blocked at much higher SeqA concentrations than those reported here. Differential blocking of DnaA would be difficult to detect by gel filtration or reporter methods used previously (Skarstad et al., 2000;Torheim and Skarstad, 1999;von Freiesleben et al., 2000).

Multiple regulators of *oriC* target pre-RC assembly

SeqA, due to its ability to block I site and R5M interaction with DnaA, must be considered a regulator of pre-RC assembly during the cell cycle. However, sequestration lasts for only one third of the cell cycle (Campbell and Kleckner, 1990), and we observed that, even in cells lacking SeqA, *oriC* is in the ORC-like state in an exponentially growing culture. Thus, after sequestration ends, other factors that modulate pre-RC assembly must come into play. The *oriC*-binding and DNA bending proteins Fis and IHF specifically target weak affinity sites, by

suppressing or promoting DnaA binding, respectively (Leonard and Grimwade, 2005). Additionally, since DnaA-ATP binds preferentially to I sites (Kawakami et al., 2005;McGarry et al., 2004), any regulators of DnaA-ATP levels, including replication dependent Hda-catalyzed conversion of DnaA-ATP to inactive DnaA-ADP (Katayama et al., 1998;Katayama and Sekimizu, 1999) or acidic phospholipid-mediated reactivation of DnaA (Crooke et al., 1992) will therefore affect pre-RC assembly. Interestingly, Hda mutants reset to ORC during the sequestration period (unpublished results), indicating that SeqA-assisted resetting of *oriC* is not sufficient to prevent the extra initiation events seen in Hda mutants (Camara et al., 2005). Since eukaryotes also utilize multiple mechanisms to control pre-RC assembly, (Diffley, 2004), it appears that this regulatory strategy may be common to all domains of life.

Materials and methods

Chemicals, proteins and enzymes

Reagent grade chemicals were purchased from Fisher Scientific, Midwest Scientific, or Sigma Chemicals. Media components were from Difco. All restriction endonucleases, T4 DNA ligase, Klenow polymerase, and *dam* methylase were purchased from New England Biolabs. Taq polymerase and polynucleotide kinase were purchased from Bioline. Pfu polymerase was purchased from Stratagene. P1 nuclease was from Roche Diagnostics. Amino-terminal His_{10}-tagged DnaA was purified as described (Li and Crooke, 1999). SeqA was a generous gift from Therese Brendler and Stuart Austin.

Plasmids and cells

pAL49 carries *oriC* as its sole origin of replication (Leonard and Helmstetter, 1986). pOC170 carries replication origins from pBR322 and *oriC* (Weigel et al., 1997). Mutant versions of pOC170 were obtained as previously described (McGarry et al., 2004) using the QuikChange site-directed mutagenesis kit (Stratagene) with 20–28 bp oligonucleotides carrying the mutations in the center.

PC2 (*dnaC*(ts), *leu-6 thyA47 deoC3 rpsL*) or PC2 *seqA* was grown in miminal salts media (Bogan and Helmstetter, 1997). For growth conditions resulting in multifork replication, the media was supplemented with 0.1%glucose, 0.2% Casamino acids, and 10 μg/ml thymine. For slow growth, the media was supplemented with 0.1% glycerol, 50 μg/ml leucine, and 10 μg/ml thymine. To align cells at initiation, exponentially growing cultures at 27°C were shifted to 39°C for one hour. A synchronized burst of initiation was induced by rapidly returning the culture to 27°C.

Generation of hemimethylated DNA

Hemimethylated supercoiled templates were isolated from *E. coli* PC2 *dnaC2*(ts) harboring minichromosome pAL49 after a temperature shift to align replication from *oriC*. After shifting back to non-permissive temperature, samples were taken at intervals and plasmid purified using Qiagen columns. Methylation was assayed by digesting an aliquot of the plasmid with *Hph*I, followed by agarose gel electrophoresis and Southern blotting. Blots were hybridized with a radiolabeled *oriC* probe (248 bp *Hind*III and *Pst*I fragment obtained from pOC170), scanned with a BioRad Personal Molecular Imager FX and images analyzed using Quantity One (BioRad) software.

Hemimethylated linear templates were generated from fully methylated PCR products by performing one additional PCR cycle. *oriC* was first amplified with 22 bp PCR primers annealing at position −184 and 343 (numbered as in Zyskind, et al., 1983), to generated a 527 bp fragment. PCR products (2μg) purified using a QIAquick PCR purification kit were treated with 8 units of *dam* methylase and 800μM S-adenosylmethionine (New England Biolabs) at

37°C overnight. The methylated DNA was amplified for one additional cycle and hemimethylation was analyzed by Hph1 digestion as described above except a 199 bp derived from an *Hph*I digestion of *oriC* DNA was used as probe.

DNA modification, Ligation-Mediated PCR, and alkaline primer extension

DMS modification of plasmid DNA *in vitro* and *in vivo* and primer extension was performed as previously described (Cassler et al., 1995;Grimwade et al., 2000). For *in vitro* reactions, DNA and protein levels are given in the figure legends. Three primers were used, two primers hybridizing at bases 272–290 and at bases 135–154 to analyze top strand modifications, and a right primer hybridizing at bases 124–142 to analyze bottom strand modifications. Ligation-Mediated PCR (LMPCR) was used to footprint chromosomal *oriC*, using the method described by Angers et al. (Angers et al. 2001). Cells were treated with DMS as previously described (Cassler et al., 1995) and chromosomal DNA was isolated using BioRad's AquaPure genomic DNA isolation kit. 5 ng of denatured DMS treated, piperidine-cleaved DNA was extended by Pfu DNA polymerase using an oligonucleotide primer (primer 1) hybridizing to regions flanking *oriC* (5′GCAAAACAGACAGGCGAAACACTG to footprint the top strand of *oriC*, 5′-CGCGGTATGAAAATGGATTGAAGCC to footprint the bottom strand). The resulting DNA fragments were ligated to a double-stranded linker (5′ CCGGAAGATCCGGCAGAAGAATGGC for top strand analysis, 5′-CCGGGCCGTGGATTCTACTCAACTTTGCT for bottom strand) to provide a primer annealing site for PCR amplification. The amplified fragments were then denatured, and extended with Pfu, primed by end-lableled oligonucleotides hybridizing to the linker sequence. Extension products were resolved on 6% polyacrylamide gels as described (Grimwade et al., 2000) and scanned on a BioRad Personal Molecular Imager FX PhosphorImager. Images were quantified using Quantity One (BioRad) software. Ratios of intensities of bands in binding sites to internal standard bands were calculated as described (McGarry et al., 2004). Deviations in band intensities among experiments were generally less than 10%.

P1 endonuclease digestions were performed as described (Grimwade et al., 2000). After digestion, supercoiled, relaxed circular, or linear forms of the plasmid were resolved by agarose gel electrophoresis. Ethidium bromide-stained gels were visualized using a BioRad Chemidoc system, and band intensities were quantified using Quantity One software.

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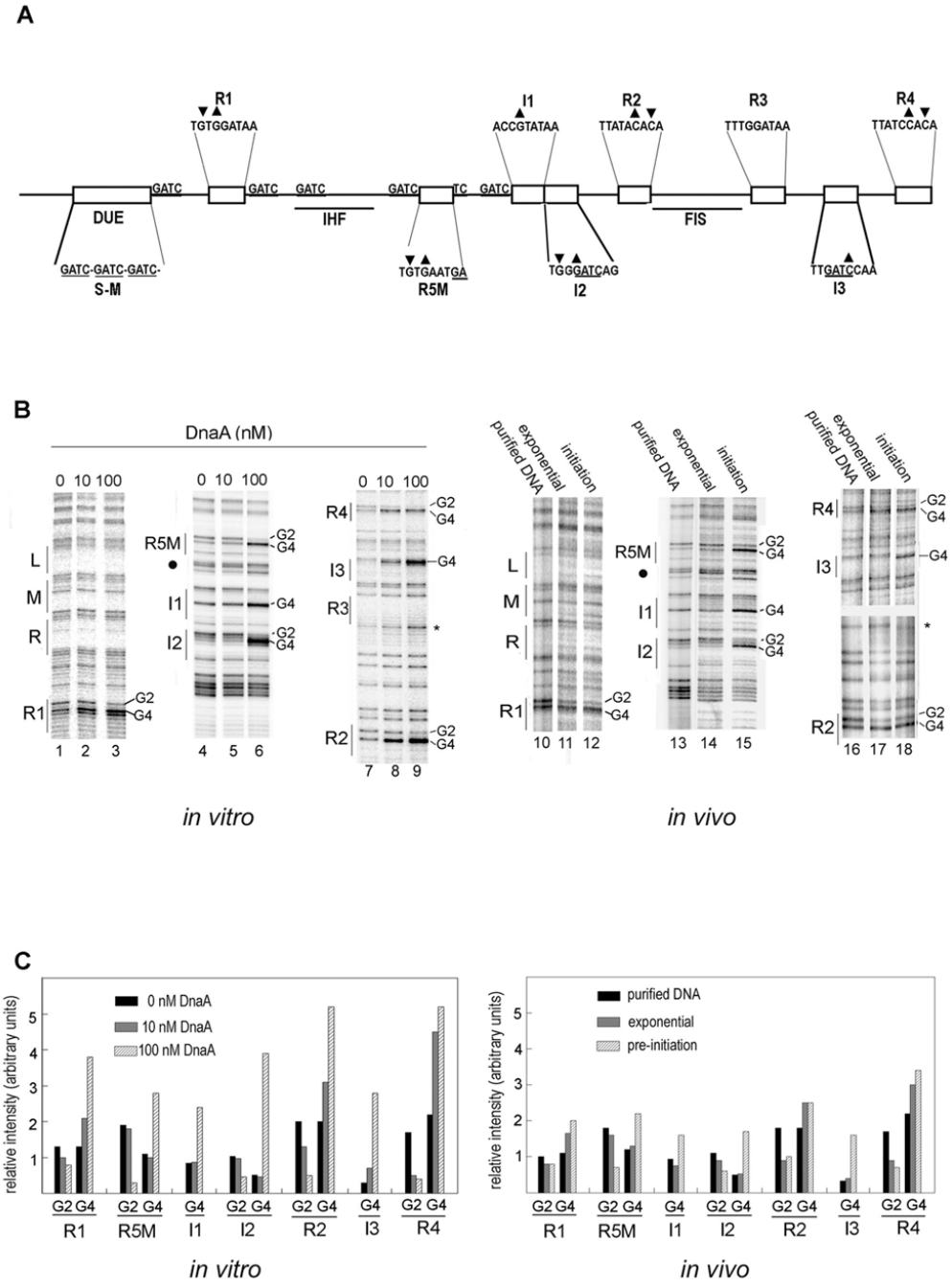

Figure 1. DnaA binding to *oriC*

A. Map of *oriC* with position and sequences of DnaA binding sites and GATC sequences indicated. GATCs associated with DnaA binding sites are underlined. Up and down arrows in the DnaA R- box and I-site recognition sequences indicate enhanced or reduced methylation by DMS. GATCs in three DnaA-ATP boxes (S-M sites) in the DNA Unwinding Region (DUE) are also marked. **B**. DMS modification patterns of *oriC in vitro* (lanes 1–9), after incubating supercoiled pAL40 minichromosme (200 fmole) with indicated concentrations of DnaA, or *in vivo* (lanes 10–18), on chromosomal *oriC* in exponentially growing cells or cells aligned at the time of initiation just prior to helicase loading, measured by LMPCR. Different primers and gel run times were used to resolve DnaA binding sites in three regions of *oriC*. DnaA binding

sites and guanines at position 2 and 4 in the recognition site are marked. The Left, Middle, and Right 13 mer AT-rich sequences in the DUE are also labeled (L,M,R). The closed circle in the center panel indicates a putative, uncharacterized DnaA binding site, and the star in the third panel marks a G whose intensity changes slightly upon R3 binding. **C.** Relative intensities of DMS modification at G2 and G4 within 7 DnaA-binding sites. I1 and I3 sites lack guanine in position 2.

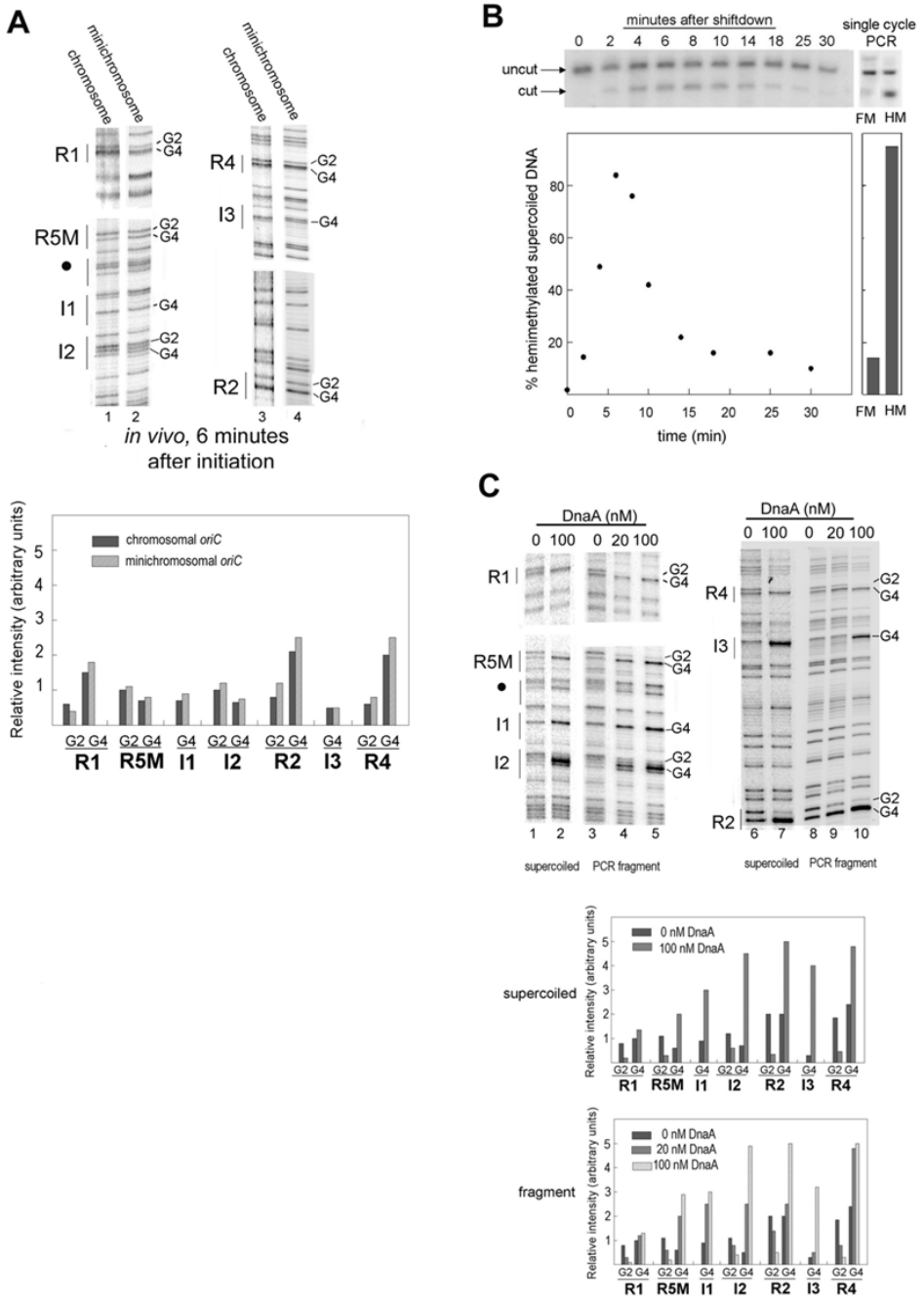

Figure 2. Binding of DnaA to *oriC in vivo* during the sequestration period and to hemimthylated supercoiled and linear *oriC in vitro*

A. Modification patterns following DMS treatment of chromosomal *oriC* (lanes 1,3) or *oriC* on pAL49 in *dnaC*(ts) cells 6 minutes after initiation (lanes 2, 4). Relative intensities of DMS-modified guanines in position 2 or 4 in the DnaA binding sites were quantified from scans of footprinting gels. **B.** pAL49 DNA was isolated from aligned *dnaC*(ts) cells at 2 minute intervals after a burst in synchronous initiation induced by return to permissive temperature, digested with Hph1, and analyzed using a Southern blot hybridized to an *oriC* probe. The percentage of cut and uncut pAL49 was quantified; 50% cutting indicates that 100% of the DNA was hemimethylated. The right panel shows the cutting pattern of fully methylated and

hemimethylated *oriC* fragments generated by PCR as described in *Experimental Procedures.* **C.** Modification patterns following DMS treatment of purified supercoiled hemimethylated pAL49 (200 fmoles) incubated with 0 or 100 nM DnaA (lanes 1, 2, 6, 7), or hemimethylated PCR *oriC* fragment (200 fmoles) incubated with 0, 20, or 100 nM DnaA (lanes 3–5, 8–10). Positions of DnaA binding sites are shown. Relative intensities of DMS-modified guanines in position 2 or 4 in the DnaA binding sites in the presence and absence of DnaA were quantified from scans of footprinting gels.

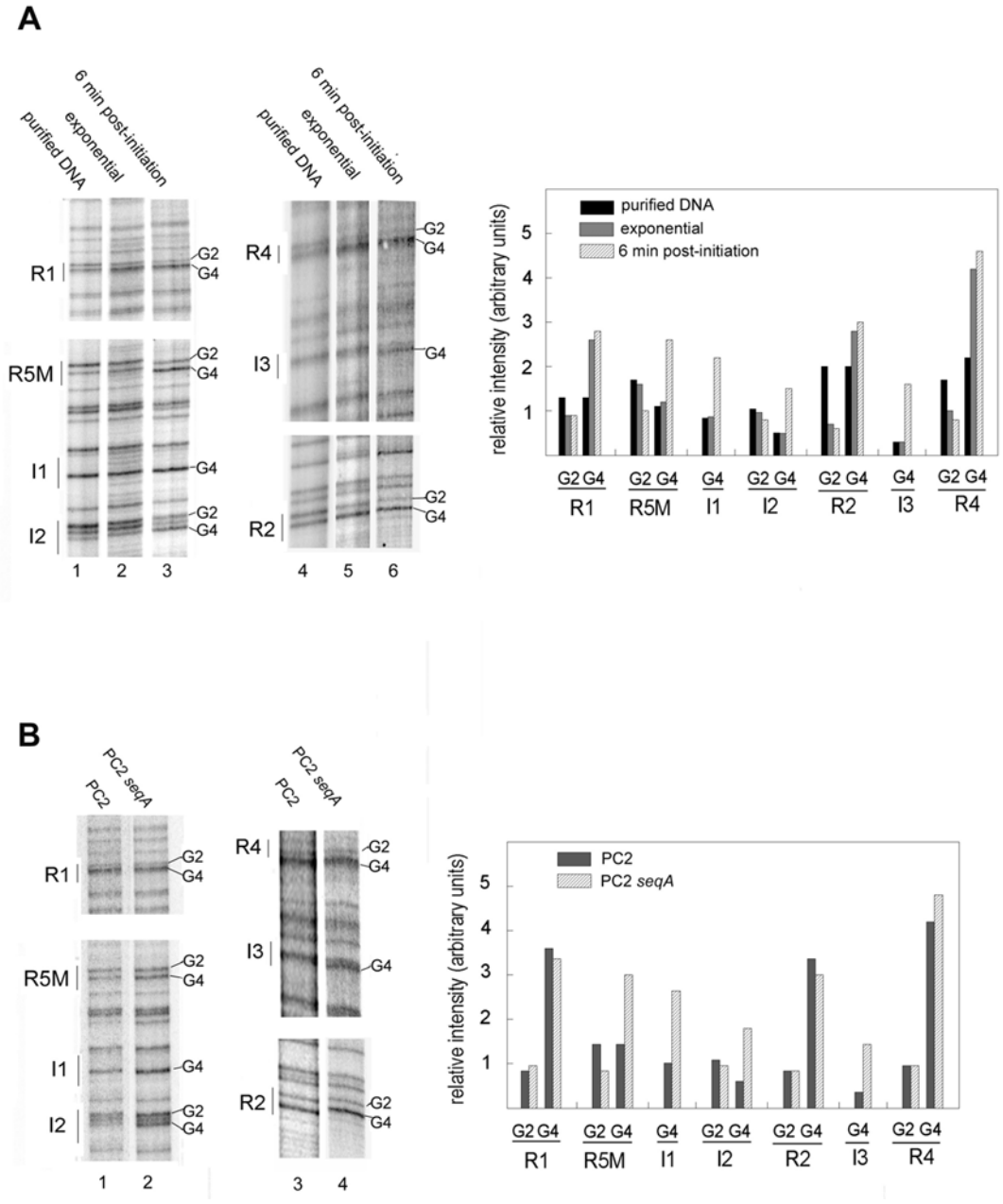

Figure 3. DnaA binds to lower affinity recognition sites shortly after initiation in cells lacking functional SeqA

A. Modification patterns following DMS treatment of purified chromosomal *oriC* (lanes 1,4) or *oriC* in *dnaC*(ts) *seqA* cells growing with multifork replication in rich media (minimal salts supplemented with glucose plus casamino acids), exponentially (lanes 2, 5) or 6 minutes after initiation was induced (lanes 3, 6). Relative intensities of DMS-modified guanines in position 2 or 4 in the DnaA binding sites were quantified from scans of footprinting gels. **B.** Modification patterns following DMS treatment of *oriC* in *dnaC*(ts) cells (lanes 1,3) or *dnaC*(ts) *seqA* cells (lanes 2,4) growing slowly in minimal salts supplemented with glycerol 6 minutes after initiation was induced. Relative intensities of DMS-modified guanines in position 2 or 4 in the DnaA binding sites were quantified from scans of footprinting gels.

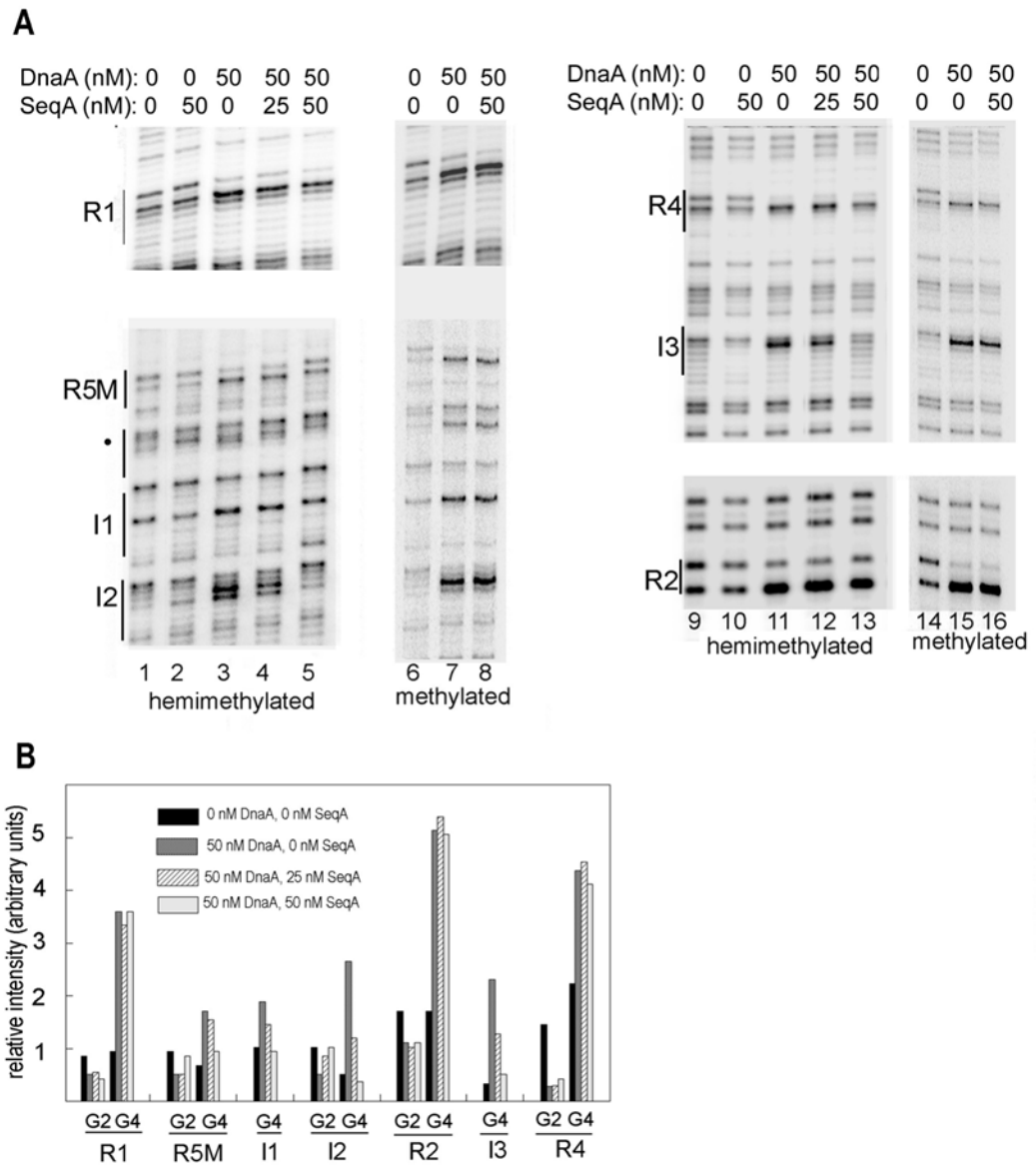

Figure 4. Purified SeqA blocks DnaA from binding to lower affinity recognition sites on hemimethylated *oriC*

A. Modification patterns following DMS treatment of hemimethylated or fully methylated PCR *oriC* fragment (125 fmoles) incubated with indicated concentrations of SeqA and DnaA. When both proteins were present, SeqA was added first. Positions of DnaA binding sites are shown. (B) Relative intensities of DMS-modified guanines in position 2 or 4 in the DnaA binding sites after incubation with SeqA and DnaA were quantified from scans of footprinting gels.

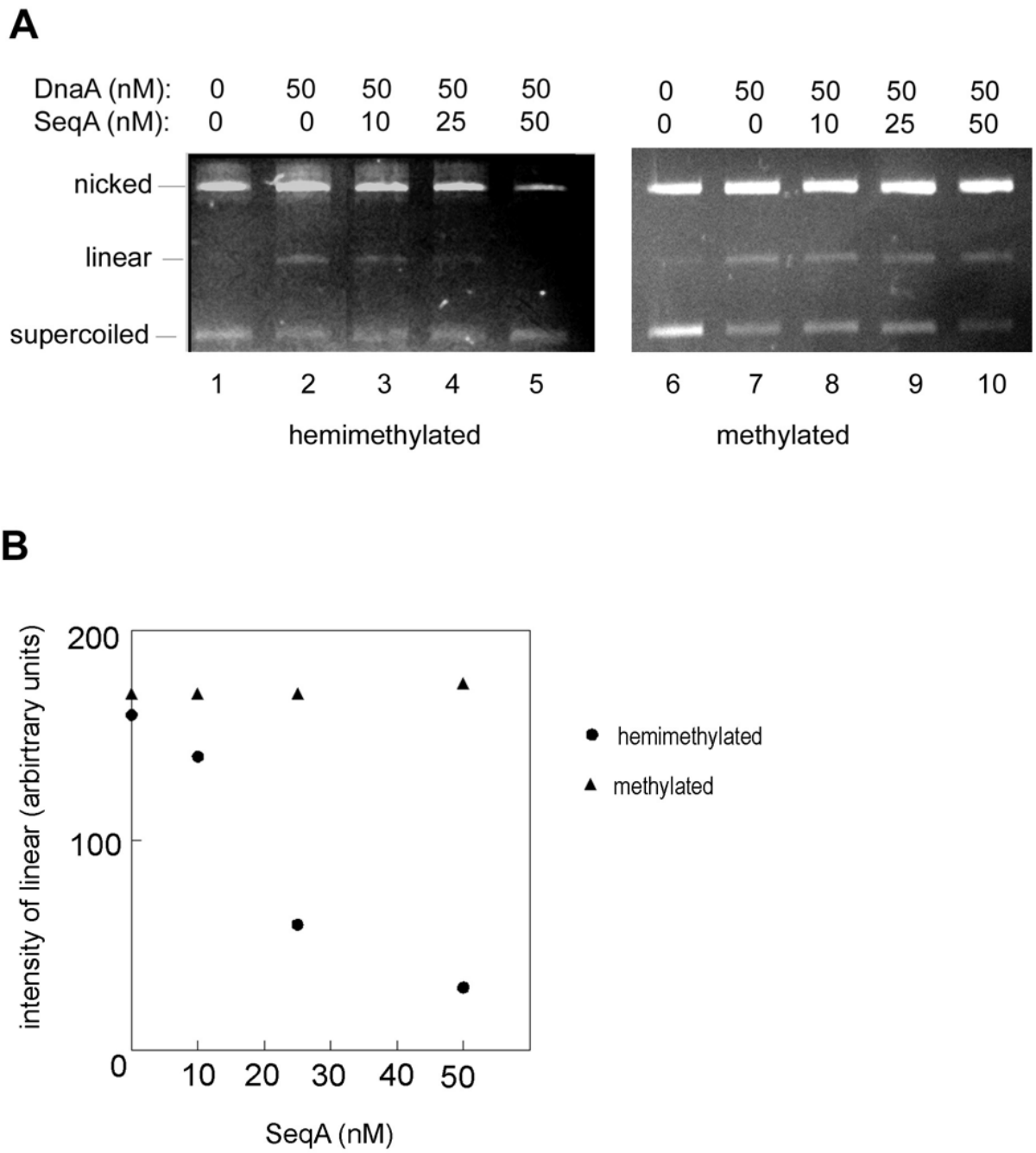

Figure 5. SeqA inhibits DnaA-induced DNA strand separation of hemimethylated supercoiled DNA

A. Purified supercoiled hemimethylated pAL49 DNA (50 ng) was incubated with the indicated concentrations of SeqA and DnaA, and treated with P1 nuclease. 10 ng aliquots of the reaction were electrophoresed, and gels were stained with ethidium bromide. The positions of uncut supercoiled, nicked and linearized forms are shown. When both proteins are present, SeqA was added first. **B.** The amount of DNA in the linear band was quantified from the gels shown in (A).

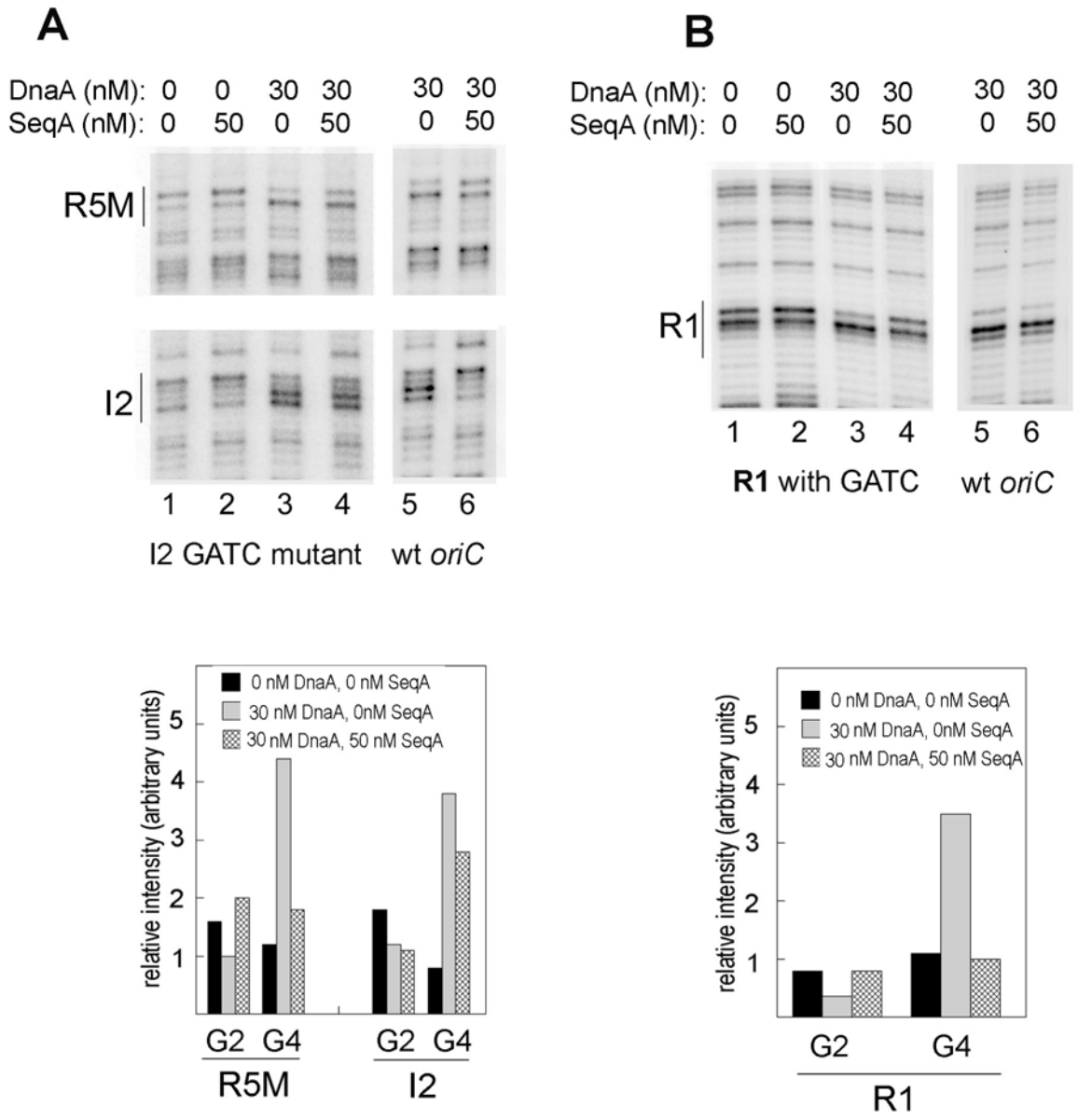

Figure 6. SeqA blocks DnaA binding only from sites associated with a GATC

A. Modification patterns following DMS treatment of hemimethylated wild type *oriC* PCR fragment (right panel) or mutated *oriC* in which I2 no longer contained a GATC sequence (left). **B.** Modification patterns after DMS treatment of hemimethylated wild type (right panel) or mutated *oriC* in which a GATC sequence was put into R1 (left). Samples were incubated with indicated concentrations of SeqA and DnaA prior to DMS treatment. When both proteins were present, SeqA was added first. Positions of DnaA binding sites are shown. The lower panels show graphs of relative intensities of DMS-modified guanines in position 2 or 4 in the DnaA binding sites after incubation with SeqA and DnaA.