The SeqA protein regulates chromosome initiation and is involved in segregation in *Escherichia coli*. One SeqA protein binds to two hemi-methylated GATC sequences to form a stable SeqA-DNA complex. We found that binding induced DNA bending, which was pronounced when the two sequences were on the same face of the DNA. Two SeqA molecules bound cooperatively to each pair of hemi-methylated sites when the spacing between the sites was ≤30 bp. This cooperative binding was able to stabilize the bending of a wild type to a single hemi-methylated site, or mutant form of SeqA protein to hemi-methylated sites, although such binding did not occur without cooperative interaction. Two cooperatively bound SeqA molecules interacted with another SeqA bound up to 185 bp away from the two bound SeqA proteins, and this was followed by aggregation of free SeqA proteins onto the bound proteins. These results suggest that the stepwise interaction of SeqA proteins with hemi-methylated GATC sites enhances their interaction and leads to the formation of SeqA aggregates. Cooperative interaction followed by aggregation may be the driving force for formation of the SeqA foci that appear to be located behind replication forks.

The adenine residues of GATC sequences in *Escherichia coli* are methylated on their 6 amino group by Dam methyltransferase (1, 2). Upon replication, the GATC sequences on the newly replicated strand remain transiently unmethylated, leading to a hemi-methylated state of the DNA duplex. The new strand is subsequently methylated by Dam, and the duplex becomes methylated on both strands. The initiation of chromosome replication at the origin of chromosomal replication (oriC), which contains repeated GATC sequences, is tightly controlled (3, 4). Once initiation is fired, reinitiation from the newly formed oriC is prevented by an oriC sequestration process affected by the binding of SeqA protein to the newly replicated, hemi-methylated origin (5, 6). The hemi-methylated state of the replicated oriC is maintained for about one-third of the cell cycle, whereas it persists in other chromosomal regions for at most 2 min. Further, the asynchronous and overinitiation of chromosomal replication characteristic of seqA mutants indicates that SeqA is a negative modulator of chromosomal initiation at oriC (5, 7).

Topoisomerase IV is essential for decatenating and segregating replicated chromosomes at cell division (8–12). Together with DNA gyrase, it also removes the positive supercoils that accumulate in front of replication forks and growing mRNA transcripts. SeqA has been shown to promote the relaxation and decatenating activity of topoisomerase IV (13). This appears to result from a specific interaction between topoisomerase IV and SeqA. Besides the asynchrony and overinitiation of chromosomal replication, seqA mutants have an aberrant nucleoid structure, an increased frequency of abnormal segregation, and increased negative superhelicity of chromosomal and plasmid DNA (5, 14–16). These findings suggest that interaction with SeqA is required for proper functioning of topoisomerase IV in *in vivo*. In addition, SeqA functions as a transcriptional regulator of the bacteriophage Apr promoter (17).

The SeqA protein exists as a homotetramer of 21-kDa polypeptides (18). The N-terminal region of SeqA (SeqA-N; amino acid residues 1–50) is responsible for its tetrameric property and for aggregation of the protein (18–20), whereas the C-terminal region (SeqA-C; 51–181) binds to hemi-methylated GATC sequences (19, 21, 22). SeqA-C interacts via hydrogen bonds and van der Waals contacts with the major groove of DNA, with the hemi-methylated A-T base pair and also with the surrounding bases and DNA backbone. The NMR structure of hemi-methylated GATC revealed that it has an unusual backbone structure and a remarkably narrow major groove and suggested that this peculiar structural feature might contribute to recognition of hemi-methylated GATC sites by SeqA protein (23). To form a stable SeqA-DNA complex in the presence of competitor DNA, one SeqA tetramer binds to each of two hemi-methylated GATC sequences (20) that are up to 31 bases apart on the DNA (24). The sequential binding of SeqA tetramers to hemi-methylated sites leads to the formation of higher order complexes (20). Further, the binding of SeqA proteins to at least six adjacent hemi-methylated sites induces the aggregation of free proteins onto the bound proteins, thus implying cooperative interaction between the SeqA proteins. Such aggregation may be responsible for the SeqA foci that have been visualized by immunofluorescence microscopy and by expression of a SeqA-green fluorescent protein (GFP) fusion protein (14, 24–27). These foci appear to track the replication fork, and their number is proportional to growth rate (24). Here, we show that binding of SeqA to hemi-methylated DNA induces a conformational change of the DNA; the bound SeqA proteins interact cooperatively with each other, which increases their tendency to interact and aggregate.
The GATC sequences containing an $N^6$-methyladenine base are underlined. The spacing is indicated as the number of nucleotides from a modified adenine residue to another as previously described (24).

### Table I

**DNA containing four hemi-methylated GATC sites**

The 12-, 16-, and 19-bp spacers between the second and third GATC sequences on DNAs containing four hemi-methylated sites were constructed by inserting the annealed oligomers into the BamHI/EcoRI sites of pBluescript II(KS). Spacings of similar size, 16 bp were obtained by inserting the corresponding annealed oligomers into the Ncol/Ndel sites of the plasmid DNA containing the 19-bp spacer. Plasmid DNAs containing the inserts were isolated from *E. coli* (Top10F (dam-)) or GM3819 (dam-16::Km) (28) and then digested with the indicated restriction enzymes to yield DNAs of similar size. -130 bp. Fully methylated DNAs were dephosphorylated and labeled with [$\gamma$-32P]ATP at their 5'-end with T4 polynucleotide kinase. The unmethylated and fully methylated fragments were mixed, and hemi-methylated fragments were generated by heat denaturation and renaturation. The second and third GATC sequences are underlined. Ncol and Ndel restriction sites are in boldface.

| Spacing (bp) | DNA sequences | Restriction sites |
|--------------|---------------|------------------|
| 12           | 5'—gtccgacctgtatctgactggagcagctggag—3' | Sall/KpnI         |
|              | 5'—gtccgacctgtatctgactggagcagctggag—3' | XhAI/KpnI         |
| 16           | 5'—gtccgacctgtatctgactggagcagctggag—3' | Sall/KpnI         |
|              | 5'—gtccgacctgtatctgactggagcagctggag—3' | XhAI/KpnI         |
| 19           | 5'—gtccgacctgtatctgactggagcagctggag—3' | Sall/KpnI         |
|              | 5'—gtccgacctgtatctgactggagcagctggag—3' | XhAI/KpnI         |
| 24           | 5'—gtccgacctgtatctgactggagcagctggag—3' | Sall/ApaI         |
|              | 5'—gtccgacctgtatctgactggagcagctggag—3' | XhAI/HindIII      |

### Table II

**DNA containing six hemi-methylated GATC sites**

The 40-, 60-, 100-, and 185-bp spacers between the second and third GATC sequences on DNAs containing six hemi-methylated sites were constructed by inserting the annealed oligomers into the BglII sites of pBlM1 (31). Plasmid DNAs containing the inserts were isolated from *E. coli* (Top10F (dam-)) or GM3819 (dam-16::Km) (28) and then digested with EcoRI/HindIII. Hemi-methylated DNA probes labeled with [$\gamma$-32P]ATP at their 5'-ends were prepared as described in Table II.

| Spacing (bp) | DNA sequences |
|--------------|---------------|
| 40           | 5'—gtccgacctgtatctgactggagcagctggag—3' |
|              | 5'—gtccgacctgtatctgactggagcagctggag—3' |
| 60           | 5'—gtccgacctgtatctgactggagcagctggag—3' |
|              | 5'—gtccgacctgtatctgactggagcagctggag—3' |
| 100          | 5'—gtccgacctgtatctgactggagcagctggag—3' |
|              | 5'—gtccgacctgtatctgactggagcagctggag—3' |
| 185          | 5'—gtccgacctgtatctgactggagcagctggag—3' |
|              | 5'—gtccgacctgtatctgactggagcagctggag—3' |
EXPERIMENTAL PROCEDURES

Materials—Sources were as follows: restriction enzymes and cloning enzymes, Promega; [γ-32P]ATP (5000 Ci/mmol) and poly(dI-dC), Amersham Biosciences; T4 polynucleotide kinase, New England Biolabs; Pyrobest DNA polymerase and T4 DNA ligase, Takara; Pyrobest DNA polymerase and T4 DNA ligase, Takara; QIAEX II gel extraction kit, Qiagen; site-directed mutagenesis kit, Invitrogen; and unmethylated and methylated synthetic oligonucleotides, Genotech. Unless otherwise indicated, additional reagents were from Sigma.

Bacterial Strains and Plasmid DNA—E. coli strain Top10F (Invitrogen) was used for cloning and preparing fully methylated plasmid DNA, and the GM3819 (dam16:Km r) strain (28) was used for preparing unmethylated plasmid DNA. E. coli BL21 (29) was used for overproducing wild-type SeqA protein and E. coli W3SQT (seqA::Tetr) (20) for overproducing mutant SeqA proteins. The pBend2 plasmid (30) was a gift from Dr. Adhya. The pBMA1 (31) and pFToriC (32) plasmids have been previously described.

Proteins—The mutant forms of seqA were constructed by site-directed mutagenesis as previously described (18). Wild-type SeqA and mutant proteins were expressed and purified from BL21(pLys, pSS1) (6) and W3SQT (pBAD18-SeqAK66E-R70E), respectively, as previously described (24).

Crude fractions of W3SQT harboring plasmids overproducing the mutant proteins SeqA(E9K) or SeqA(E5,9K) were prepared according to Lee et al. (18) with minor modifications. E. coli W3SQT cells harboring the indicated seqA mutant in pBAD18 (33), or pBAD18 alone, were grown in LB medium to an optical density at 600 nm of 0.3–0.4, followed by the addition of L-α-arabinose to a concentration of 0.1%. Three hours after induction, the cells were harvested and resuspended in 25 mM HEPES (pH 7.8), 1 mM EDTA, and 1 mM dithiothreitol. Cells were lysed and the proteins partially purified as described (34).

DNAs Containing Hemi-methylated Sites—The synthetic 75-bp DNA fragments containing two hemi-methylated sites with variable spacing

![Fig. 1. DNA bending induced by the binding of SeqA protein to two-hemimethylated GATC sequences. A, plasmid pBend2 (30) was modified to contain the indicated spacings between the two GATC sites. B, 5’-end of the top strand of the corresponding restriction fragment of the fully methylated plasmid DNA described in A was labeled with [γ-32P]ATP using polynucleotide kinase and mixed with a 2-fold excess of the same restriction fragment from unmethylated plasmid DNA. The sample was then heat-denatured and renatured. Each reaction contained 20 ng of SeqA protein and 2 fmol of hemi-methylated DNA (the molar ratio of SeqA tetramer over the DNA was ~100), and the DNA-protein complexes were separated on a 4.5% polyacrylamide gel using a long gel electrophoresis system. C, SeqA (0, 2.5, 5, 10, 20, 40) was added to reaction mixtures containing the indicated DNAs (Table I). The extent of SeqA-DNA complex formation with each DNA was determined and is plotted in D. One unit of SeqA binding activity is defined as a shift of one-fourth of the input DNA by SeqA protein. The number of nucleotides between the adenine bases of the two GATC sequences on the DNA containing two hemi-methylated sites is indicated (B and C).]
are described in Table I. These were obtained by annealing synthetic oligomers, top strand (5'-ctgggttagatctgattagagatgtctttgtgtatgagttggctggct-3') and bottom strand (5'-aggagtcgcctacttaaagatagctctacttttaataatctctcatact-3'). The GATC sequences are underlined, and the N methylation adenine bases within the GATC sequences of the B-strand are indicated in the corresponding figures. The DNAs containing four hemi-methylated sites with variable spacing (Figs. 2 and 3) between the second and third GATC sequences are described in Table II and the DNAs containing six hemi-methylated sites with variable spacing (Fig. 5) between the third and fourth GATC sequences are described in Table III. The hemi-methylated DNAs were obtained by annealing synthetic oligomers.

**Gel Shift Assays**—Unless otherwise indicated, the 20-μl reaction mixtures contained 10 mM Tris-HCl, pH 7.6, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 μg of poly(dI-dC), 10% glycerol, 5 μg of bovine serum albumin, −2 fmol of hemi-methylated DNA, and the specified amount of SeqA protein. These were incubated for 15 min at 30 °C. Subsequent steps have been previously described (6).

1.10-Phenanthroline-Copper Ion Nuclease in Situ Footprinting—1,10-phenanthroline-copper (II) (Op-Cu(II)) footprinting was performed as previously described (35, 36) with minor modifications. The top or bottom strand of synthetic hemi-methylated 175-mer DNAs was 32P end-labeled and incubated with SeqA for 15 min at 30 °C, followed by treatment with 200 μM 1,10-phenanthroline-copper and 5.8 mM 3-mercaptopropionic acid for 35 s at 30 °C. The cleavage reaction was stopped by adding 0.1 volume of 28 mM 2,9-dimethyl-1,10-phenanthroline. SeqA-bound DNA and free DNA were separated by polyacrylamide gel electrophoresis (5% w/v). Subsequent steps were performed as before (37).

**RESULTS**

**Binding of SeqA Protein to Two Hemi-methylated GATC Sequences Induces DNA Bending**—A SeqA protein as tetramer can bind to two hemi-methylated GATC sequences (20) that are separated by up to 31 bp (24). We examined the possibility that the DNA is bent as a result of this event. The DNA fragments used were identical in size but varied in the relative location of a pair of hemi-methylated sites (Fig. 1, A and B). The indicated spacing refers to the number of nucleotides between the N methylation adenine nucleotides of the GATC sequences. A bend near the center of a DNA fragment maximally retards the electrophoretic mobility of a DNA-protein complex (30). With DNA in which the hemi-methylated sites were separated by 19 bp, so that they were on the same phase of DNA helix, SeqA binding produced a typical DNA-bending pattern. Although bending was not initially apparent with a 16- or 25-bp spacing, such that the two hemi-methylated sites were on opposite phases, a pattern of bent DNA did appear when electrophoresis was continued for longer periods (data not shown). These observations suggest that the extent of bending is dependent on the relative position of the two hemi-methylated sites on the DNA helix and is favored when the two hemi-methylated sites are on the same phase.

We compared the affinity of SeqA for DNA containing different spacings between the two hemi-methylated GATC sequences (Fig. 1, C and D). Its affinity for hemi-methylated DNA containing an 11-, 21-, or 31-bp spacing was higher than for a 16- and 26-bp spacing. The more efficient binding of SeqA to DNA with the two hemi-methylated sites on the same phase of the DNA helix is in agreement with a previous report (24). Therefore, the phase difference between the two hemi-methylated GATC sequences affects the affinity of SeqA for it as well as DNA bending.

**Interaction between Two SeqA Proteins Bound on Hemi-methylated DNA**—We analyzed the interaction between SeqA proteins bound to each of two hemi-methylated GATC sequences by employing DNA containing four hemi-methylated sites (Fig. 2). The spacing between the first and second and between the third and fourth sites was fixed, while the spacing between the second and third sites varied. The lengths of the DNAs were similar, with no more than 4 bp difference (Table I). SeqA formed fast and slow mobility complexes with all of these DNAs (Fig. 2, A and B). We showed previously that the fast and slow mobility complexes are formed by binding one and two molecules of SeqA, respectively, (20). The fast mobility complexes migrated similarly regardless of the spacing. The slow mobility complexes involving DNA with spacing of >30 bp also migrated at similar rates whereas the slow mobility complexes involving spacing of ≤30 bp migrated at different rates. If the bound SeqA proteins interacted, these complexes would each have a unique nucleoprotein structure, thereby migrating differently through the polyacrylamide gel. The absence of any difference in the migration of the slow mobility complexes formed with spacings of >30 bp suggests that the SeqA proteins bound to those DNAs did not interact. More slow mobility complexes were formed with DNA having a spacing of 19 or 27 bp than with DNA having a spacing of 32 bp (Fig. 2C), supporting the idea that SeqA binds cooperatively to DNA with a spacing of ≤30 bp. The altered migration and efficient formation of slow mobility complexes when the spacing between the two sites is...
Fig. 3. Cooperative binding of SeqA to hemi-methylated DNA. A, synthetic 75 bp DNAs of identical sequence but having one to four N<sup>6</sup>-methyladenine bases within the GATC sequences on the bottom strand (20) were used for gel-shift assays. SeqA (0, 0.5, 1, 2, 4, 8, 16, and 32 ng) was added to reaction mixtures containing the indicated DNAs. Unmethylated and methylated GATC sequences on the bottom strands are indicated by open and closed circles, respectively. SMC and FMC indicate slow and fast mobility complexes, respectively. The amounts of SMCs and FMCs formed were measured and are plotted in B. C, adenine bases in the fourth GATC site of the hemi-methylated DNAs used in Fig. 2A were changed to thymine. 0 (lane 1), 10 (lanes 2, 7, and 12), 20 (lanes 3, 8, and 13), 40 (lanes 4, 9, and 14), and 60 ng (lanes 5, 10, 15–18) of SeqA were added to reaction mixtures containing the indicated DNA. The number of nucleotides between the adenines of the second and third GATC sequences is indicated.

≤30 bp indicated that the SeqA proteins bound to each pair of hemi-methylated sites interact.

Interactions between SeqA Proteins Stabilize the Unstable Binding of SeqA to a Hemi-methylated Site—The binding of a SeqA tetramer to a pair of hemi-methylated GATC sequences yielded a fast mobility complex when the DNA contained two sites, but not to any significant extent when it contained only one site (20). We therefore compared the binding of SeqA to four synthetic oligonucleotides containing identical sequences but with one, two, three, or four N<sup>6</sup>-methyladenine residues within the GATC sequences on one strand (Fig. 3, A and B). Formation of fast mobility complexes increased with the number of hemi-methylated GATC sequences. Furthermore a new slow mobility complex that contained two SeqA proteins appeared with three hemi-methylated sites, and formation of this complex was enhanced with four hemi-methylated sites. Evidently the formation of a slow mobility complex by binding of SeqA to DNA containing three hemi-methylated sites is more favorable than formation of the fast mobility complex with DNA containing one hemi-methylated site, although these complexes result from the binding of one SeqA to one hemi-methylated site. The greater efficiency with which the slow mobility complex is formed in the first case suggests that the SeqA tetramers bound to two hemi-methylated sites on the DNA facilitate or stabilize the binding of an additional SeqA to an unbound hemi-methylated site.

To examine this cooperative binding, we varied the spacing between the second and third hemi-methylated site (Fig. 3C), and we changed the un-methylated GATC sequence at the fourth site to a GTTC sequence to prevent any possible contribution from it. Slow mobility complexes were formed with spacings of 12 and 19 bp, but not when the spacing was greater. This shows that the SeqA proteins bound to two hemi-methylated sites are able to facilitate the binding of an additional SeqA to the third hemi-methylated site but only when the third site was separated from the second by <32 bp. This conclusion
is of course consistent with our finding that the cooperative interaction between two SeqA proteins bound to hemi-methylated DNA requires a spacing of \( \leq 30 \) bp (Fig. 2).

**Interactions between SeqA Proteins Stabilize the Unfavorable Binding of Mutant SeqA Proteins to Hemi-methylated Sites**—SeqA(E9K) protein, in which a Glu-9 is substituted for a Lys, is not able to form fast mobility complexes, only slow mobility complexes, with DNA containing four hemi-methylated sites (18). The introduction of the additional change of Lys for Glu-5 abolished formation of the slow mobility complex (Fig. 4A). Because instability of SeqA(E5,9K) hampered its purification, a partially purified fraction obtained from a seqA-null mutant harboring a SeqA(E5,9K)-overproducing plasmid was used for binding assays. To compensate for any effect of contaminating proteins, the assays were controlled by addition of a partially purified fraction prepared from a seqA-null mutant-containing vector. Despite its inability to bind to hemi-methylated sites, SeqA(E5,9K) could convert the fast mobility complex formed by SeqA to a slow mobility complex (Fig. 4B), indicating that SeqA bound to hemi-methylated sites helps SeqA(E5,9K) bind to additional sites.

To throw further light on the binding of SeqA(E5,9K) as a result of interaction with SeqA, we used SeqA(K66E/R70E), which forms complexes that migrate more rapidly, in place of SeqA. The substitutions of Lys-66 and Arg-70 with glutamates might increase the electronegativity of SeqA and lead to the faster migration of the SeqA(E5,9K)-DNA complexes (Fig. 4C, lanes 2–5 and lanes 7–10, respectively). The mock fraction prepared from the seqA-null mutant containing vector plasmid was added to contain 1.6 and 3.2 \( \mu \)g (lanes 2–5 and lanes 7–10, respectively) of total protein. C, gel-shift assays with synthetic 75-bp DNA containing four hemi-methylated sites and 30 ng of purified SeqA and 6 ng of purified SeqA(K66E/R70E). Lanes 4–6: 0 and 3.2, 0.2 and 3.0, 0.8 and 2.4 \( \mu \)g of SeqA(E5,9K) and mock partially purified fractions, respectively. D, slow mobility complexes formed in the presence of SeqA(Wt), SeqA(K66E/R70E) or SeqA(K66E/R70E) + SeqA(E5,9K) shown in C were analyzed by OP-Cu(II) footprinting as described under “Experimental Procedures.”
Stepwise Interaction of SeqA Proteins Bound to Hemi-methylated DNA Augments Their Ability to Interact—A SeqA molecule bound to two hemi-methylated sites is able to interact cooperatively with a molecule on another site separated from it by up to 30 bp (Figs. 2 and 3). We next examined the interaction of two SeqA proteins cooperatively bound on four hemi-methylated sites with an additional SeqA using DNAs containing six hemi-methylated sites in which the spacing between the second and third sites was varied, while the spacing between all the other sites was fixed (Fig. 5). As the amount of SeqA was increased, the binding of a SeqA to pairs of hemi-methylated sites was observed to result in the sequential formation of three discrete complexes (complex I, II, and III) (Fig. 5A). After these three complexes were formed, there appeared larger complexes in both the upper region of the gel (complex IV) and the well (complex V). The formation of the large complexes is in agreement with our previous finding that SeqA proteins bound to six or more adjacent hemi-methylated sites tend to form aggregates with the free proteins (20). The absence of both the discrete and the large complexes when the GATC sequences were fully methylated demonstrates that the formation of these complexes is dependent upon hemi-methylation. Quantification of the large complexes (complex V) (Fig. 5B) showed that for a spacing of up to 60 bp between the second and third sites, the yield of the large complex was constant; thereafter, increased spacing resulted in a reduction in the yield.

We then changed one or two of the GATC sequences at the left end of the DNA that were separated by 60 bp to GTTC sequences (Fig. 5C). With six hemi-methylated sites, increasing amounts of SeqA generated complexes I, II, and III followed by the large complexes, as shown in Fig. 5A. The DNA with five hemi-methylated sites produced complexes similar to the above except for the inefficient formation of complex III. With only four hemi-methylated sites, only complexes I and II containing one and two SeqA proteins, respectively, were formed.

In summary, a SeqA tetramer bound to two hemi-methylated sites interacts cooperatively with another SeqA bound at a distance of ≤30 bp (see Fig. 6). The cooperative interaction
Cooperative Interaction of SeqA Proteins

between these two SeqA proteins enhances their capacity for interaction, thereby favoring the formation of a larger complex involving another SeqA bound up to 200 bp from the leftmost binding site. Such interaction between three bound SeqA proteins triggers the further aggregation of free proteins with the bound proteins, generating large aggregates.

DISCUSSION

The C-terminal region of SeqA (amino acid residues 71–181) contains a domain that binds to hemi-methylated GATC sequences while the N-terminal region (1–50) is responsible for tetramer formation (19, 22). The two regions are connected by a flexible linker. Although SeqA is a homotetramer (18, 19), chemical cross-linking and the footprinting pattern point to a basic dimeric configuration of the tetramer (35, 38). We speculate that the SeqA tetramer has a Y shape (Fig. 6A). In this structure the N-terminal regions of the four subunits are opposed while the C-terminal regions connected to the N termini by the flexible linkers are partially split in two with two of the subunits closer than the other two. In the presence of competitor DNAs, binding of a SeqA protein to two hemi-methylated sites is required to form a stable SeqA-DNA complex (20). The two hemi-methylated sites can be separated by up to 31 bp and located on different phases of the helix (Fig. 1: 24). The flexible binding of SeqA could be achieved if one of the pairs of closely connected subunits recognizes one of the two hemi-methylated sites so that the other pair of subunits can bind to the other site, binds the DNA between the two hemi-methylated sites (Fig. 1) and so stabilizes the binding of the tetramer to the two hemi-methylated sites (Fig. 6A).

A SeqA tetramer bound to two hemi-methylated sites can interact with further molecules. Cooperative interaction between the two SeqA proteins occurs when the pair of hemi-methylated sites are located within ≤30 bp, otherwise they bind independently (Fig. 2). This cooperative binding is able to stabilize the binding of a wild-type to a single hemi-methylated site (Figs. 3 and 5B) or a mutant SeqA molecule to additional hemi-methylated sites (Fig. 4). Cooperativity was more obvious with DNA containing a larger number of hemi-methylated sites (Fig. 5). Thus two cooperatively SeqA proteins bound to four adjacent hemi-methylated sites could interact with a SeqA protein bound to two hemi-methylated sites 185-bp apart on the same DNA or stabilize the binding of another SeqA to a single hemi-methylated site 60-bp apart (Fig. 5). Interaction between the three SeqA proteins could then induce aggregation of free SeqA proteins on them (Fig. 5 and Ref. 20). We propose that stepwise interaction of the bound SeqA proteins at hemi-methylated sites augments their capacity for interaction and aggregation; consequently, as the number of interacting SeqA protein increases, this facilitates recruitment of SeqA that is free or bound distally at hemi-methylated sites, thus expanding the complex. This stepwise interaction followed by aggregation may be the driving force for the formation of the SeqA foci that appear to track replication forks in vivo (14, 24–27).

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