Sequential Binding of SeqA to Paired Hemi-methylated GATC Sequences Mediates Formation of Higher Order Complexes*

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Preferential binding of the SeqA protein to hemi-methylated GATC sequences functions as a negative regulator for *Escherichia coli* initiation of chromosomal replication at *oriC* and is implicated in segregating replicated chromosomes for cell division. We demonstrate that sequential binding of one SeqA tetramer to a set of two hemi-methylated sites mediates formation of higher-order complexes. The absence of cross-binding to separate DNAs suggests that two monomers of a SeqA tetramer bind to two hemi-methylated sites on DNA. The interaction among SeqA proteins bound to at least six adjacent hemi-methylated sites induces aggregation of free proteins to bound proteins. Aggregation might be indicative of SeqA foci, which appear to track replication forks *in vivo*. Studies of the properties of SeqA binding will contribute to our understanding of the function of SeqA.

The adenine residues of GATC sequences on both strands of *Escherichia coli* chromosomal DNA are methylated at the 6-amino group (1). Upon replication, GATC sequences exist transiently in a hemi-methylated state; the newly synthesized strand remains unmethylated until it becomes methylated by Dam methylase, whereas the parental strand retains its methylation (2). For one-third of the cell cycle, replicated *oriC*, the origin of chromosomal replication containing 11 repeats of GATC sequences within 245 bp, exists in a hemi-methylated state, even though other regions are immediately methylated within 1−2 min (2, 3). This delayed methylation is caused by the binding of the SeqA sequestration protein to hemi-methylated *oriC* (3–5). In *seqA* mutants that lack SeqA function, the sequestration of methylation of *oriC* is absent. In these mutants, the timing of chromosomal initiation is disturbed, and the frequency of initiation increases (3, 6). The asynchronous and overinitiation of chromosomal replication indicates that SeqA functions as a negative modulator for chromosomal initiation at *oriC*. Negative modulation appears to be achieved by the binding of SeqA to *oriC*, which blocks initiation (7, 8).

Stable binding of SeqA to DNA requires more than one hemi-methylated site (5, 9, 10). The minimal requirement is two hemi-methylated sites that are up to 31 bases apart on the same face of duplex DNA. Cooperative interaction between SeqA proteins bound on each hemi-methylated site is a proposed requirement for stable complex formation. The *E. coli* chromosome sequence predicts that 1750 pairs of GATC sequences exist for the optimal binding of SeqA (10). The SeqA protein is a homotetramer of 21-kDa polypeptides (11). The N-terminal region of SeqA (SeqA-N; amino acid residues 1−50) donates its tetrameric property, and the C-terminal region (SeqA-C; 51−181) contains the binding domain for a hemi-methylated GATC sequence (12). Co-crystal structure analysis of SeqA-C with 12 bp of DNA containing one hemi-methylated site revealed that SeqA-C interacts with the major groove of DNA through direct hydrogen bonds and van der Waals contacts with the hemi-methylated A-T base pair and interacts with the surrounding bases and DNA backbone (12). Although the co-crystal structure suggests that a monomeric SeqA-C recognizes a hemi-methylated GATC site, whether stable binding of a SeqA tetramer requires two hemi-methylated sites remains to be elucidated (9).

SeqA forms discrete foci, which have been visualized by immunofluorescence microscopy and by the expression of a SeqA-GFP[1] fusion (10, 13−16). Foci formation is dependent upon Dam function and DNA replication, but not upon the presence of *oriC*. The number of foci is proportional to growth rate (10). SeqA proteins binding to the replicated, hemi-methylated chromosomal DNA and appearing to track the replication fork might be visualized as SeqA foci. Even though the *oriC* region contains more highly repeated GATC sequences than any other region of the chromosome and exists in a hemi-methylated state for one-third of the cell cycle, SeqA foci rarely overlap with *oriC* (13, 15). SeqA mutants possess aberrant nucleoid formation, increased frequency of anucleoid cells, and increased negative superhelicity of chromosomal and plasmid DNA (3, 13, 17−19). Overexpression of SeqA interferes with the segregation of nucleoids and causes a delay in cell division (16). These phenotypes of the *seqA* mutant, irrelevant to the role of SeqA as a negative regulator for initiation of chromosomal replication (3, 6), suggest that SeqA also participates in chromosome segregation.

The function of SeqA as a regulator for chromosomal initiation and chromosome segregation appears to be exerted by the binding of SeqA to hemi-methylated GATC sequences (4, 5, 10, 13). Recent analysis of the co-crystal structure of truncated SeqA-C and 12 bp of DNA containing a hemi-methylated GATC sequence shows that SeqA-C binds to a hemi-methylated site and its flanking region (12). However, the behavior of truncated, monomeric SeqA-C differs from the behavior of intact

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[1] The abbreviations used are: GFP, green fluorescent protein; Op-Cu(II), 1,10-Phenanthroline-copper (II).

SeqA tetramers. Together with the co-crystal structure, our findings concerning the binding of one SeqA protein to paired hemi-methylated sites, aggregation, and absence of cross-binding of the bound SeqA proteins provide insights into the function of SeqA *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—Sources were as follows: restriction enzymes and cloning enzymes, Promega; [γ-^{32}P]ATP (5000 Ci/mmol) and poly(dI-dC), Amersham Biosciences; and non- and methylated synthetic oligonucleotides, Genotech. Unless otherwise indicated, reagents were purchased from Sigma.

Bacterial Strains and Plasmid DNAs—The *E. coli* strain Top10F'(Invitrogen) was used for cloning and preparing fully methylated plasmid DNA, and the GM3819(*dam*16:km^r) strain (25) was used for preparing unmethylated plasmid DNA. *E. coli* BL21 (26) was used for overproducing the wild-type SeqA protein. *E. coli* W3SQT(*seqA*::Tet^r) was obtained by transducing W3110 (27) with P1 phage prepared from NK9050(*seqA*::Tet^r) (3) and was then used for overproducing mutant SeqA proteins. The pBMA1 (28) and pFToriC (29) plasmids were previously described. For constructing pBMA2, pBMA1 was digested with *Bgl*II, filled in with the Klenow fragment, and religated.

Proteins—The *seqA* mutant genes expressing corresponding mutant proteins were constructed by site-directed mutagenesis as previously described (11). Wild-type SeqA and mutant proteins were expressed and purified from BL21(pLys, pSS1) (5) and W3SQT [pBAD18-SeqA(K66E-R70E)], respectively, as previously described (11).

DNAs Containing Hemi-methylated Sites—Hemi-methylated DNAs containing 1−6 and 17 GATC sites are described in Fig. 1*A*. The 75-bp DNA fragment containing hemi-methylated sites was obtained by annealing synthetic oligomers, top-strand (5'-ctgggtattaaaaagaag<u>atc</u>tatt-tattta gag<u>atc</u>tgttctattgt<u>gatc</u>tcttattagg<u>atc</u>gcactgccct-3') and bottom-strand (5'-agggcagtgc<u>gatc</u>ctaataagag<u>at</u>cacaatagaacag<u>atc</u>tcaaataaatag<u>at</u><u>c</u>ttcttttaatacccag-3'). The N^6-methyladenine bases within GATC sequences of the B-strand are indicated in the corresponding figures. In Fig. 3, the 39-bp DNA fragment containing two hemi-methylated sites was obtained by annealing the oligomer 5'-ctgttctattgt<u>gatc</u>tcttattag-<u>gatc</u>gcactgccct-3' and oligomer 5'-agggcagtgc**gatc**cctaataagag**atc**a-caatagaacag-3'. N^6-methyladenine bases are indicated by boldface.

Gel Shift Assay—Unless otherwise indicated, the 20-μl reaction mixture 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, and 2 fmol of 75-bp DNA possessing four hemi-methylated sites and the specified amount of SeqA protein. The reaction mixture was incubated for 15 min at 32 °C. The subsequent steps have been previously described (5).

1,10-Phenanthroline-Copper Ion Nuclease in Situ Footprinting—1,10-Phenanthroline-copper (II) (Op-Cu(II)) footprinting was performed as previously described (9, 30) with minor modifications. The top or bottom strand of synthetic hemi-methylated 75-mer DNAs was ^{32}P-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-mer-captopropionic 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 described previously (31).

RESULTS

SeqA Binds Two Hemi-methylated GATC Sequences—Stable binding of SeqA to hemi-methylated DNA requires at least two hemi-methylated GATC sequences (9, 10). We analyzed SeqA binding to DNA by gel shift assays of DNA containing 1−6 and 17 hemi-methylated sites in the presence of competitor DNA poly(dI-dC) (Fig. 1). For DNAs containing up to six hemi-methylated sites, SeqA forms as many as three discrete, consecutive complexes (Fig. 1*B*). A new complex with the most retarded migration appeared for all samples with an odd number of hemi-methylated sites; the formation of this complex was more efficient when one additional site was added. As the number of hemi-methylated sites and the level of SeqA increased, slower migration of newly appearing complexes indicated that more SeqA proteins were bound step by step to form higher-order

complexes. With DNAs containing six hemi-methylated sites, large complexes located at the upper region and the wells of polyacrylamide gels began to appear. The DNAs containing 6 and 17 hemi-methylated sites produced three, and at least six, more discrete complexes, respectively, followed by formation of larger complexes retained near the wells. These large complexes were previously observed with DNAs containing a number of GATC sequences (3, 20). At elevated levels of SeqA, the absence of large complexes for the DNAs containing less than six hemi-methylated sites excluded the possibility that previously aggregated SeqA proteins, formed before DNA binding, were bound to the DNA.

Because the DNAs used in the preceding experiments contained different primary sequences, lengths, and numbers of GATC sequences, we used synthetic oligonucleotides containing identical sequences but having one to four N^6-methyladenine residues within GATC sequences on a strand (Fig. 2*A*). SeqA binding to the DNAs containing one or two hemi-methylated sites produced a fast-mobility complex, but formation of this complex was enhanced with two hemi-methylated sites. A new slow mobility complex appeared with three hemi-methylated sites, and this complex formation was enhanced with four hemi-methylated sites. The appearance of new complexes with an odd number of hemi-methylated sites and enhanced formation of these complexes upon addition of one site agree with the results in Fig. 1*B*. Identical mobilities in a polyacrylamide gel imply that the complexes contain the same number of SeqA molecules. The binding patterns of SeqA in such slow mobility complexes were compared by using OP-Cu(II) chemical footprinting analysis (Fig. 2*B*). For DNA containing four hemi-methylated sites, SeqA protected all four sites of DNA containing four hemi-methylated sites from chemical cleavage. On DNA containing three hemi-methylated sites and one unmethylated site, SeqA protected all three hemi-methylated sites but hardly protected the unmethylated site, suggesting that the inefficient formation of new complexes (complexes with slowest mobility) with an odd number of GATC sites (Figs. 1*B* and 2*A*) was caused by the inefficient binding of one SeqA to one hemi-methylated site. These results indicate that efficient binding of SeqA requires two hemi-methylated sites and that additional binding of SeqA to a set of two more sites mediates formation of higher order complexes.

One SeqA Tetramer Binds to Each Set of Two Hemi-methylated GATC Sequences—The requirement of two hemi-methylated sites for efficient SeqA binding (Figs. 1 and 2) and the tetrameric nature of SeqA (11, 12) raise the question as to whether the two sites are bound by one or more SeqA tetramers. To address this question, we isolated a mutant SeqA protein, SeqA(K66E-R70E), in which Lys-66 and Arg-70 residues of SeqA were substituted with glutamates. The complexes formed by SeqA(K66E-R70E) migrated more quickly than complexes formed by SeqA (Fig. 3).

Whereas a mixture of independently purified SeqA and SeqA(K66E-R70E) proteins generated two different fast mobility complexes formed by SeqA and SeqA(K66E-R70E), the mixture exhibited one additional slow mobility complex that possessed an intermediate mobility between the mobility of slow-mobility complexes formed by SeqA and SeqA(K66E-R70E) separately (Fig. 3*A*).

The *seqA* and *seqA(K66E-R70E)* genes were co-expressed in *E. coli*, and the resulting SeqA/SeqA(K66E-R70E) protein was purified. Because the expression levels of both proteins were different and SeqA is tetrameric, the four subunits of SeqA/SeqA(K66E-R70E) were heterogeneous mixtures of SeqA and SeqA(K66E-R70E) polypeptides. Therefore, SeqA/SeqA(K66E-
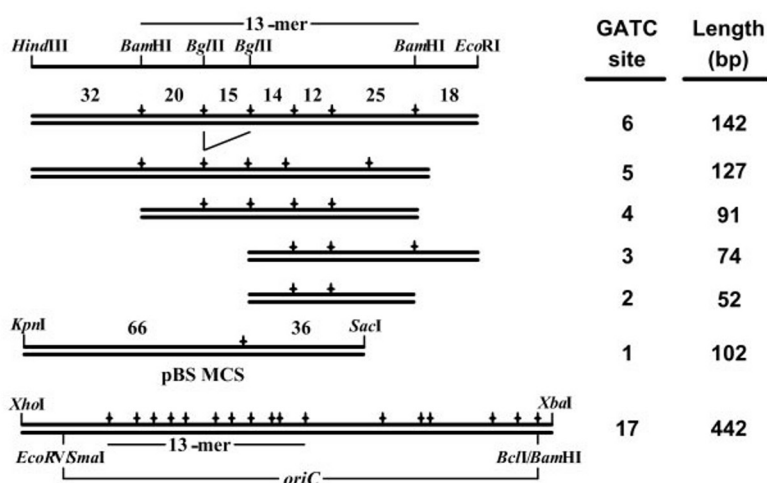
(A)

(B)

FIG. 1. **Every two hemi-methylated GATC sequences are bound by SeqA.** *A*, restriction fragments containing five and 17 hemi-methylated sites were isolated from pBMA2 and pFToriC, respectively (see "Experimental Procedures"). Other DNA fragments were obtained from pBMA1. From fully methylated plasmid DNA, the 5′-end of the top strand of the corresponding isolated restriction fragment was labeled with $[\gamma\text{-}^{32}P]ATP$ by using polynucleotide kinase and mixed with a 2-fold excess of the same restriction fragment isolated from unmethylated plasmid DNA, then heat-denatured, and renatured. 13-mer, 13-mers of *oriC* (32); crosses, GATC sequences; pBS MCS, multicloning site of pBluescript II(KS). *B*, various amounts of SeqA (0, 2, 4, 8, 16, 32, and 64 ng) were added to the gel-shift reaction mixture containing the indicated number of hemi-methylated GATC sequences. The complexes formed with an odd number of sites are indicated (★).
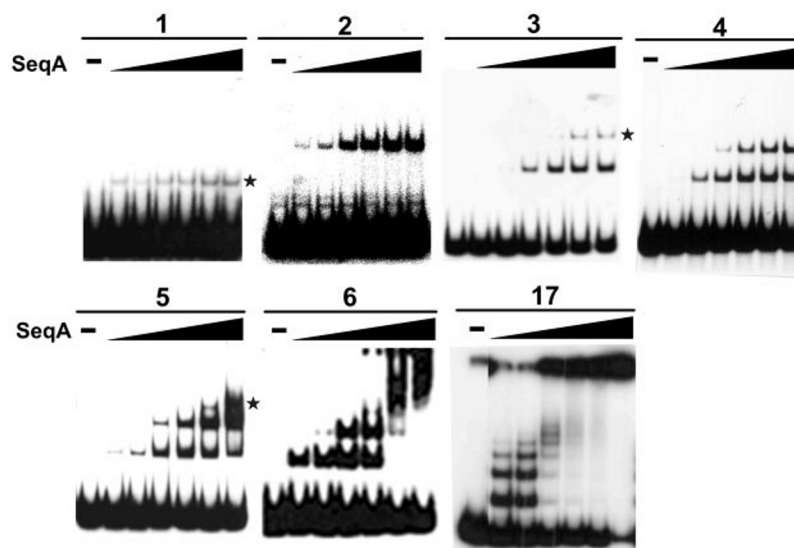
R70E) would produce complexes possessing distinctive mobilities that differed from the mobilities of complexes formed by the mixture of independently purified SeqA and SeqA(K66E-R70E) (Fig. 3*A*, *lanes 5*, *10*, and *15*; Fig. 3*B*, *lane 11*).

The ratio of amounts of SeqA and SeqA(K66E-R70E) varied with the DNA containing four hemi-methylated sites (Fig. 3*B*). As the ratio of SeqA(K66E-R70E) increased, the amount of complexes formed by SeqA decreased, and complexes formed by SeqA(K66E-R70E) appeared. Even though a fast-mobility complex with intermediate mobility was not formed, one slow-mobility complex possessing an intermediate mobility appeared and disappeared as the ratio of SeqA(K66E-R70E) increased. The formation of one slow-mobility complex and the absence of a fast-mobility complex with intermediate mobility agrees with the results shown in Fig. 3*A*. The absence of a fast-mobility complex possessing intermediate mobility indi-

cates that SeqA or SeqA(K66E-R70E) bind exclusively to each set of two hemi-methylated sites and that exchange of subunits does not occur between the purified proteins in the DNA binding reaction.

It has been proposed that to form a stable complex, one SeqA unit bound on a hemi-methylated site requires cooperative interaction with another SeqA bound on a different site (9). In this case, with DNA containing four hemi-methylated sites, one fast-mobility complex, formed by one unit each of SeqA and SeqA(K66E-R70E), and three different slow-mobility complexes, formed by three and one, two and two, and one and three units of SeqA and SeqA(K66E-R70E), respectively, with intermediate mobility must be formed in addition to the complexes formed by SeqA and SeqA(K66E-R70E) alone. Whereas co-expressed SeqA/SeqA(K66E-R70E) generated some of these intermediates, the mixing or challenging of independently pu-
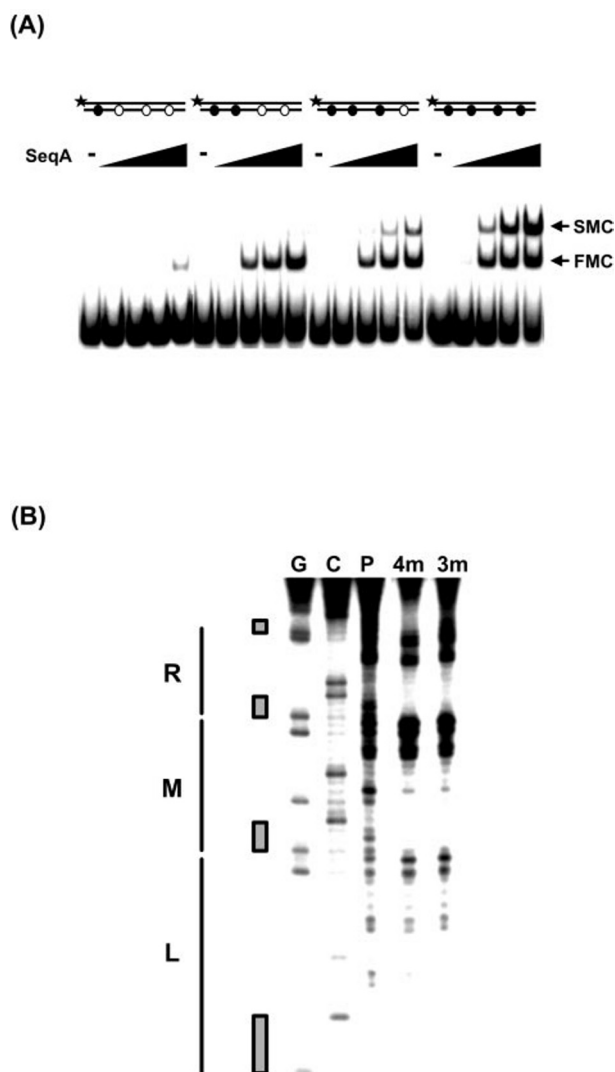
(A)

(B)

FIG. 2. **SeqA binds to a pair of hemi-methylated GATC sequences.** *A*, synthetic 75-bp DNAs containing identical sequences but having one to four N^6-methyladenine bases within GATC sequences of the bottom strand were used for gel shift assays. SeqA (0, 2.5, 5, 10, and 20 ng) was added to the reaction mixture containing the indicated DNA. Un- and hemi-methylated GATC sequences are described as *open* and *closed circles*, respectively. SMC and FMC indicate slow and fast mobility complex, respectively. *B*, the complexes treated with OP-Cu(II) were separated by polyacrylamide gel electrophoresis, then equal amounts of isolated radioactive slow mobility complexes isolated from the gel were analyzed in a 5% sequencing gel. The hemi-methylated 75-bp DNAs were ^{32}P-labeled at the 5'-end of the unmethylated top strand, indicated by (★). Free DNA (*P*); four hemi-methylated sites (*4m*); three hemi-methylated sites (*3m*). GATC sequences are denoted as *gray boxes*. *L*, *M*, and *R* indicate *oriC* 13-mer L, M, and R, respectively (32). *G* and *C* indicate Maxam and Gilbert chemical sequencing reactions of G and C, respectively.

rified SeqA and SeqA(K66E-R70E) produced no such fast-mobility complex and only one slow-mobility complex that possessed intermediate mobility (Fig. 3). The exclusive binding of SeqA or SeqA(K66E-R70E) to two hemi-methylated sites to form a fast-mobility complex and the existence of one slow-mobility complex with intermediate mobility indicate that one SeqA tetramer binds to every two hemi-methylated sites. Therefore, the slow mobility complex with intermediate mobility is consistent with the binding of one SeqA to one pair of hemi-methylated sites and the binding of one SeqA(K66E-R70E) to the pair of hemi-methylated sites.

SeqA Does Not Cross-link to the Hemi-methylated Sites on Separate DNAs—The binding of a SeqA tetramer to each of two

hemi-methylated sites (Fig. 3) raises the possibility that every two monomers of the tetramer might bind to the hemi-methylated sites on different molecules of DNA. Because this inter-DNA binding can not be discriminated in the DNA binding assays by using homogeneous DNA, for SeqA binding assays, we used a fixed amount of 130-bp DNA with six hemi-methylated sites and variable amounts of the 75-bp DNA with four hemi-methylated sites, or *vice versa* (Fig. 4). The 130- and 75-bp hemi-methylated DNA produced three and two discernible complexes, respectively. No reduction of the complexes formed with a fixed amount of DNA occurred with the increased formation of complexes with the other DNA. Each complex was formed with either 130 or 75 bp of DNA. No new complex appeared that exhibited a distinctive mobility that differed from the complexes formed by either 130 or 75 bp of DNA. These results imply that the cross-linking of a SeqA tetramer to separate DNAs does not occur under our assay conditions. However, the cross-linking of two hemi-methylated *oriC* by SeqA was proposed from the crystal packing structure of the SeqA-C-DNA complex (12). This discrepancy could be caused by differences in the molecular behaviors of an intact SeqA tetramer and truncated monomeric SeqA-C or by the presence of highly concentrated SeqA-C in the crystal.

Aggregation of SeqA to the SeqA Proteins Bound on Hemi-methylated DNA—In comparison to DNAs possessing at least five hemi-methylated sites, SeqA binding to DNAs containing 6 and 17 hemi-methylated sites produced large complexes that were retarded near the wells of the gel (Fig. 1*B*). The formation of these large, aggregated complexes was analyzed (Fig. 5). In SeqA, Val-88 interacts with the phosphate backbone for SeqA binding to a hemi-methylated GATC sequence (12); the substitution of Val-88 to Gly abolished binding to hemi-methylated DNAs (data not shown). In the absence of SeqA, SeqA(V88G)-CH, which contains a hexa-histidine-tag at the C terminus of the SeqA(V88G) protein (Fig. 5, *A* and *B*, *lane 5*), was also unable to bind to DNAs possessing four or six hemi-methylated sites, whereas SeqA-CH, which contains a His$_6$ tag at the C terminus of wild-type SeqA, was able to bind to these DNAs (Fig. 5, *A* and *B*, *lane 9*). SeqA(V88G)-CH had no effect on the formation of fast mobility (complex-I) and slow mobility (complex-II) complexes, which are produced by the binding of SeqA to two and four hemi-methylated sites, respectively (Fig. 5, *A* and *B*). The inability of SeqA(V88G)-CH to convert the complexes to larger complexes implies that SeqA(V88G)-CH can not bind to hemi-methylated sites, even cooperatively with SeqA. However, increasing the amount of SeqA(V88G)-CH shifted the most retarded complex, complex-III, which is presumably formed by the binding of three molecules of the SeqA tetramer to the six hemi-methylated sites of DNA, to the wells of polyacrylamide gels.

Cleavage of the SeqA protein with trypsin yields two fragments, SeqA-N (amino acid residues 1−50) and SeqA-C-(51−181) (12). In the absence of poly(dI-dC), although SeqA-C binds 12- and 14-bp DNAs containing one hemi-methylated site (12, 22), in our gel-shift assays containing poly(dI-dC), SeqA-C containing the DNA binding domain, did not form a stable complex with the hemi-methylated DNAs (Fig. 5*C*, *lane 10*). In addition, SeqA-C did not affect formation of SeqA-DNA complexes (I, II, and III) (*lanes 7−9*, *15*, and *16*). Further, SeqA-N did not influence formation of complex-I and -II, which were produced by SeqA binding to two and four hemi-methylated sites, respectively (*lanes 2−4*, *12*, and *13*). In contrast, SeqA-N shifted the migration of complex-III (*lanes 3* and *4*), implying that the N-terminal fragment, SeqA-N, associates with complex-III as SeqA(V88G)-CH.

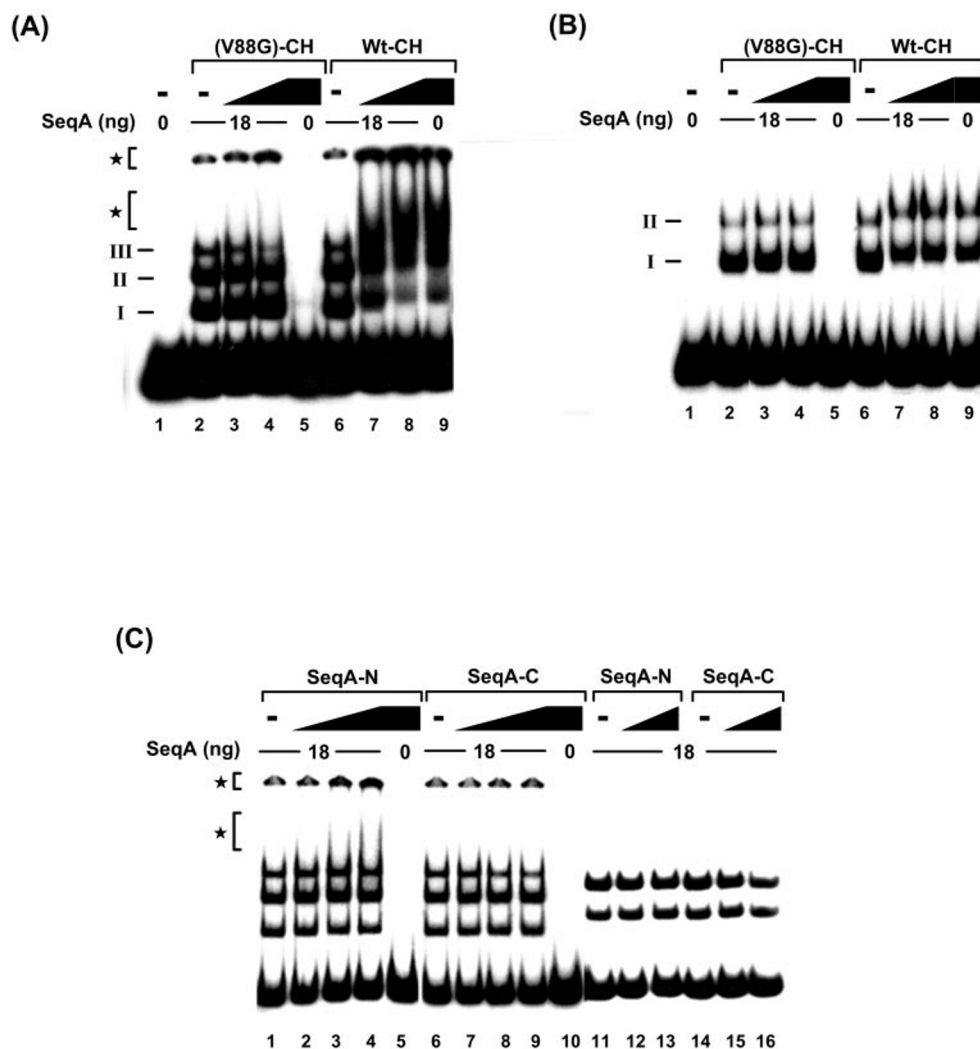Although SeqA(V88G)-CH and SeqA-N do not possess bind-

FIG. 3. **One SeqA tetramer binds to every two hemi-methylated GATC sequences.** *A*, the following DNA fragments were used for gel shift assays: 39-bp DNA containing two hemi-methylated sites (*lanes 1−5*); 75-bp DNA containing two un- and two hemi-methylated sites (*lanes 6−10*); 75-bp DNA containing four hemi-methylated GATC sequences (*lanes 11−15*). Reactions included no protein (*lanes 1, 6,* and *11*); 40 ng of SeqA (*lanes 2, 7,* and *12*); 10 ng of SeqA(K66E-R70E) (*lanes 3, 8,* and *13*); 28 ng of SeqA and 12 ng of SeqA(K66E-R70E) (*lanes 4, 9,* and *14*); 20 ng of co-expressed SeqA/SeqA(K66E-R70E) (*lanes 5, 10,* and *15*). *B*, the 75-bp DNA fragment containing four hemi-methylated GATC sequences was used with the indicated amount of protein: SeqA, 0, 40, 70, 52, 40, 28, 17, 10, 5, and 0 ng (*lanes 1−10*); SeqA(K66E-R70E), 0, 0, 3.6, 6, 9, 12, 14.5, 16, 18, and 10 ng (*lanes 1−10*); 20 ng of co-expressed SeqA/SeqA(K66E-R70E) (*lane 11*). For co-expression, wild-type *seqA* was cloned into the *Eco*RI/*Hin*dIII sites of pBAD18, and *seqA(K66E-R70E)* was cloned into the *Eco*RI/*Hin*dIII sites of pBAD33 (21).

FIG. 4. **SeqA does not cross-link to the hemi-methylated sites on separate DNAs.** The indicated amounts of DNA containing six or four hemi-methylated sites were added to gel-shift reaction mixtures containing the indicated amounts of SeqA. DNAs containing four and six hemi-methylated sites (*4* and *6 Hemi*, respectively) are indicated.

ing activity to hemi-methylated DNAs, these proteins associated with the SeqA-DNA complex formed by the binding of SeqA proteins to six hemi-methylated sites. However, this as-

sociation did not occur with complexes formed by the binding of SeqA proteins to two or four sites. These results indicate that interaction among the SeqA proteins bound on six hemi-meth-

FIG. 5. **Aggregation of SeqA to SeqA proteins bound on hemi-methylated DNA.** Purified SeqA(V88G)-CH or SeqA(Wt)-CH proteins containing a His$_6$ tag at their C termini were added to the preincubated reaction mixture containing the indicated amount of SeqA and DNA possessing six (*A*) or four (*B*) hemi-methylated sites for 3 min, then further incubated for 7 min. The following amounts of SeqA(V88G)-CH or SeqA(Wt)-CH proteins were added to the reaction mixtures: 0 (*lanes 1*, *2*, and *6*); 30 (*lanes 3* and *7*) and 60 ng (*lanes 4*, *5*, *8*, and *9*). *C,* SeqA-N-(1–50) and SeqA-C-(51–181) proteins, produced by proteolysis of the SeqA protein with trypsin, were separated as previously described (Guarné *et al.*, Ref. 12). Isolated SeqA-N-(1–50) or SeqA-C-(51–181) was added to the preincubated reaction mixture containing the indicated amount of SeqA and the DNA possessing six (*lanes 1–10*) or four (*lanes 11–16*) hemi-methylated sites for 3 min, then further incubated for 7 min. The following amounts of SeqA-N or SeqA-C were added to the reaction mixture: 0 (*lanes 1*, *6*, *11*, and *14*), 10 (*lanes 2*, *7*, *12*, and *15*), 20 (*lanes 3* and *8*) and 30 ng (*lanes 4*, *5*, *9*, *10*, *13*, and *16*). Aggregated SeqA-DNA complexes are indicated (★). DNA-protein complexes containing one, two, and three SeqA tetramers are indicated by **I**, **II**, and **III**, respectively.

ylated sites induces aggregation of the N terminus of free SeqA to the SeqA proteins bound on the DNA, not to the DNA itself.

DISCUSSION

We have demonstrated that one SeqA unit as a tetramer binds to a set of two hemi-methylated GATC sequences (Figs. 1–3). Even though the binding of monomeric SeqA-C and another C-terminal fragment (amino acid residues 71–181) of SeqA was previously detected to bind to 12- or 14-bp DNAs possessing one hemi-methylated site (12, 22), in gel-shift assays with poly(dI-dC) competitor DNA, the intact SeqA protein did not bind significantly to DNAs containing one hemi-methylated site (Figs. 1*B* and 2*A*; Ref. 9). In the presence of poly(dI-dC), SeqA-C did not bind to hemi-methylated DNA nor did it compete with SeqA for DNA binding (Fig. 5*C*). These discrepancies indicate that the molecular behavior of SeqA differs from the behavior of truncated SeqA.

A SeqA tetramer binds to two hemi-methylated sites on a DNA fragment (Figs. 1–3). SeqA was unable to cross-bind to hemi-methylated sites on separate DNAs (Fig. 4). The binding

of the truncated, monomeric C-terminal fragment of SeqA to a hemi-methylated site indicates that a SeqA monomer recognizes a hemi-methylated site (12, 22). These results suggest that two monomers in a tetramer participate in the binding of two hemi-methylated sites to form a stable complex. Dimerization of SeqA monomers by chemical cross-linking (11, 12), crystal structure of truncated SeqA-C (12), and footprint analysis of SeqA binding (9) suggest a dimeric property of the SeqA protein. A SeqA tetramer might be composed of two dimers. SeqA efficiently binds to two hemi-methylated sites in various positions and spaced up to 31-bp apart, if the two sites are not located at the opposite phase of the helix (10). Also, two methyl groups of the 6-methyladenine residues in two hemi-methylated GATC sequences can be either *in cis* or *in trans* without a significant loss of affinity for SeqA (9). This flexible recognition and binding of SeqA can be achieved in part by the binding of any monomer in a dimer of a SeqA tetramer on each hemi-methylated site.

For DNAs containing 6 and 17 hemi-methylated sites, as the

concentration of SeqA increased, large complexes appeared at the upper part and the wells of polyacrylamide gels (Figs. 1*B* and 5*A*). The appearance of aggregated complexes following the discrete complexes implies that preferential binding of SeqA proteins to the closed, hemi-methylated sites is followed by the formation of larger complexes. Even in the absence of DNA, SeqA itself reversibly aggregates in a concentration-dependent manner, even though this aggregation requires a much higher SeqA concentration than is typical for DNA binding assay conditions (11). The intrinsic aggregation property in the absence of DNA supports that the additional SeqA proteins associate with the SeqA proteins bound on hemi-methylated sites rather than associating with the DNA. The aggregated complexes with DNA containing 17 hemi-methylated sites formed at a lower concentration of SeqA than those DNAs containing six hemi-methylated sites (Fig. 1*B*). These results suggest that molecular crowdedness of the SeqA protein is a driving force for aggregation. The closeness of the SeqA proteins bound to the hemi-methylated sites enhances the aggregation property of SeqA protein by molecular crowdedness and functions as a nucleation core to recruit free SeqA proteins for aggregation. Association of these aggregated SeqA proteins with the bound SeqA proteins on the adjacent hemi-methylated sites can cause intervening DNA to loop out, as previously proposed (10, 23). These aggregated SeqA proteins on the hemi-methylated sites might be visualized, by using immunofluorescent microscopy and the GFP-SeqA fusion, as SeqA foci, which appear to track replication forks(9, 13–16, 24).

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