Domains of DnaA Protein Involved in Interaction with DnaB Protein, and in Unwinding the Escherichia coli Chromosomal Origin*

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DnaA protein of Escherichia coli is a sequence-specific DNA-binding protein required for the initiation of DNA replication from the chromosomal origin, oriC. It is also required for replication of several plasmids including pSC101, F, P-1, and R6K. A collection of monoclonal antibodies to DnaA protein has been produced and the primary epitopes recognized by them have been determined. These antibodies have also been examined for the ability to inhibit activities of DNA binding, ATP binding, unwinding of oriC, and replication of both an oriC plasmid, and an M13 single-stranded DNA with a proposed hairpin structure containing a DnaA protein-binding site. Replication of the latter DNA is dependent on DnaA protein by a mechanism termed ABC priming. These studies suggest regions of DnaA protein involved in interaction with DnaB protein, and in unwinding of oriC, or low-affinity binding of ATP.

DnaA protein of Escherichia coli is required for the initiation of chromosomal replication from oriC, the chromosomal origin (1-3). As a DNA-binding protein, it recognizes a 9-base pair sequence, the DnaA box, present 4 times in oriC (3). On binding, localized melting of the AT-rich region by DnaA protein and 1-5 mM ATP (4) is assisted by either HU or IHF that presumably act by DNA bending (5-7). Subsequently, the binding of DnaB helicase from the DnaB-DnaC complex through direct contact with DnaA protein suggests that it orients the binding of the replicative helicase for its subsequent action to promote bidirectional replication fork movement (8). Priming of DNA replication by primase for both leading and lagging strand synthesis is proposed to occur through a physical interaction between primase and DnaB protein (9), thus establishing the elongation phase of DNA replication.

The correlation of domains of DnaA protein to its various functions is rudimentary. By comparative analysis of homologs of the dnaA gene from phylogenetically diverse microbes, sequence conservation of a P-loop motif, GXGK located at residues 172-179 (reviewed in Ref. 10), correlates with the activity of DnaA protein to bind ATP with high affinity (Kd ~ 0.03 μM) (11). In p21<sup>ras</sup> and RecA protein, corresponding residues interact with the phosphates of the bound nucleotide (12, 13). Missense mutations of the dnaA5 and dnaA6 alleles, as well as others (14), substitute a conserved alanine for valine at amino acid residue 184 near the P-loop. The mutant proteins were defective in ATP binding, suggesting that the substitution was responsible (15, 16). Together, these results implicate this region of DnaA protein in binding to ATP.

The DNA-binding domain of DnaA protein has been localized to a region from residue 379 to the carboxyl terminus (17). Other domains of DnaA protein involved in purine binding, unwinding, interaction with DnaB protein (8), or interaction with phospholipids that displace the bound nucleotide (18) are unknown. To correlate domains of DnaA protein to its various functions, a collection of monoclonal antibodies were produced and characterized biochemically. Reported here, some antibodies inhibit replication of plasmids containing oriC but not of an M13 derivative containing a DnaA protein-binding site in a proposed hairpin structure (19). Priming of DNA replication in vitro on the latter template, termed ABC priming, involves assembly of an intermediate formed on the single-stranded DNA that is dependent on DnaA protein. These results suggest that a subset of DnaA protein functions are required for ABC priming compared to those involved in replication of oriC plasmids. The region of DnaA protein bound by M7 antibody that interferes with the interaction between DnaA and DnaB protein (8) has been identified. Presumably, a domain in this region interacts directly with DnaB protein. Results with a third class of antibodies suggest that DnaA protein may act at a later step in the initiation process, perhaps through interaction with subunits of DNA polymerase III holoenzyme.

EXPERIMENTAL PROCEDURES

Proteins, DNAs, and Reagents—Reagents, commercial enzymes, and highly purified replication proteins were as described (8). Other proteins were from the following sources: bovine serum albumin (BSA)<sup>2</sup> and DNase I, Sigma; T4 polynucleotide kinase, New England Biolabs; T4 DNA polymerase, Tth DNA polymerase, T4 DNA ligase, linearized pTOPE-1b, and competent HMS174 recA1 hisd Rif^<sup>−</sup> lysogenized by λ DE3 [λ cts857 ind1Sam7 nin5 lacUV5-T7 gene1] Novagen. Monoclonal antibodies to DnaA protein were produced (20) and affinity-purified from tissue culture supernatants with Protein A-Superose (Pharmacia). Sterile buffers were used in purification of the antibodies to minimize nuclide contamination of antibody preparations. Monoclonal antibody 2B (21) to rat brain hexokinase, and preparations of

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1 Carr, K. M., and Kaguni, J. M. (1996) Mol. Microbiol. 20, in press.

2 The abbreviations used are: BSA, bovine serum albumin; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay.
goat anti-mouse antibody that were specific for each immunoglobulin subclass were gifts from Dr. J ohn Wilson, Michigan State University. 

Epitope Mapping—Libraries of recombinant plasmids, each of which expressed a fusion protein of T7 gene 10 protein joined to a small peptide derived from DnaA protein, were constructed essentially as recommended by Novagen. To construct a library, the dnaA gene was amplified by PCR using chain reaction primers with DnaA in the presence of Mn²⁺, and fragments of 50–150 base pairs in size were purified from an agarose gel. The DNA fragments were then successively with T4 DNA polymerase and Tth DNA polymerase to repair and then add a single dA residue to the 3'-end of each fragment. This was followed by ligation to linearized pTOPE-1b with a single dG residue at each end, and transformation into HMS174 (λ DE3). Both the linearized vector and the competent cells were obtained from Novagen. Colonies transferred to nitrocellulose filters were lysed with chloroform vapor, and the filters were placed on Whatman 3MM paper saturated with 20 mM Tris-HCl, pH 7.9, 6 mM urea, and 0.5 mM NaCl. Positive clones were then identified by immunoblot analysis with the monoclonal antibody of interest. Detection of antibody-antigen complexes was with horseradish peroxidase conjugated to goat anti-mouse IgG (Bio-Rad). After color development, the filters were aligned to corresponding original plates, and positive clones were colony purified and verified as immunoreactive by the above method. Plasmid DNA, isolated by an alkaline lysis procedure, was sequenced by the enzymatic method with primers that flank the site of insertion in the vector. The epitope was deduced by comparative analysis of the DNA sequences of inserts. 

The Cross-reactivity of A22 and M7 WITH dnaA Nonsense Mutations—The dnaA gene and an ochre mutant at codon 148 contained in pACYC184 were from this laboratory. Whole cell lysates were prepared by resuspension of 10⁸ cells of the plasmid-bearing strains (Sup) collected at mid-log phase in 0.1% sodium dodecyl sulfate (SDS), and electrophoresed in 10% SDS-polyacrylamide gels. DnaA protein, transferred to polyvinylidene difluoride membranes (Schleicher & Schuell), was detected by immunoblot analysis with A22, M7, and M48 as primary antibodies and goat anti-mouse IgG conjugated to horseradish peroxidase as the secondary antibody. An ECL chemiluminescence kit (Amersham) was used to detect antibody-antigen complexes.

DNA Binding Assays—Fragment retention assays (25 µl) contained 6 ng of a 459-base pair Sall-Xhol fragment containing oriC from pTS0182 DNA (22) and 100 ng of Hinfl-digested plB322 DNA as a nonradioactive competitor in buffer containing 40 mM HEPEs-KOH, pH 7.8, 5 mM MgCl₂, 2 mM DTT, and 50 mM KCl. 3' end-labeling of the oriC fragment was performed with the large fragment of DNA polymerase I and [α-³²P]ATP. Monoclonal antibodies were added, as indicated (160–250 ng), to reactions prior to DnaA protein (80 ng) and DNA to measure their effect on DNA binding activity. Following incubation at 30 °C for 10 min, the reactions were filtered through nitrocellulose filters (Millipore HAWP, 0.22 µm, 24 mm) and washed with 250 µl of the same buffer at room temperature essentially as described (25). Radioactivity retained on the filters was determined by liquid scintillation spectrometry. 

Gel mobility shift assays (23, 24) were performed by addition of the indicated amounts of DnaA protein and 1.48 µg of HaelII-digested M13oriC2LB5 DNA in 25 µl containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 0.1% (v/v) glycerol, and 2 µM DTT. Monoclonal antibodies were added before DnaA protein and DNA. After incubation at 30 °C for 10 min, the samples were electrophoresed in a 1% agarose gel in 90 mM Tris borate and 1 mM EDTA, pH 8.3, and stained with ethidium bromide. 

ATP Binding Assays—Reactions (25 µl) were performed essentially as described (11) and contained DnaA protein (2 pmol), an equimolar amount of the indicated monoclonal antibody, and 5 mM MgCl₂, 15% glycerol, 0.01% Triton X-100, and 50 mM Tris-HCl, pH 8.0. Incubation was for 15 min on ice. After addition of [α-³²P]ATP (0.5 µCi) to 0.2 µm, incubation was continued at 0 °C for 10 min followed by filtration through nitrocellulose filters (Millipore HAWP, 0.22 µm, 13 mm). The filters were then washed with 500 µl of the above buffer at room temperature. ATP retained by the filters was quantified by liquid scintillation spectrometry.

ABC Priming and DNA Replication of oriC Plasmids—Assays (25 µl) of ABC priming with M13-A site single-stranded DNA or of oriC replication (primase-primed) with M13oriC2LB5 DNA were performed as described (8). DnaA protein was preincubated with the indicated antibody in 40 mM HEPEs pH 8, 40 mM potassium glutamate, 0.1 mM MgCl₂, 4% sucrose, and 4 µM DTT for 10 min on ice. Reaction proteins for ABC priming were then added to measure DNA synthesis activity of DnaA protein as described (8). 

Assay of F* Formation—One-stage reactions (25 µl) contained 40 mM HEPEs-KOH, pH 7.8, 20 mM Tris-HCl, pH 7.5, 4% (v/v) sucrose, 2 µM ATP unless otherwise indicated, 4 mM DTT, 11 mM magnesium acetate, 200 ng of M13oriC2LB5 DNA, 21 ng of HU protein, 160 ng of single-stranded DNA-binding protein, 100 ng of DnaB protein, 24 ng of DnaC protein, 500 ng of gyrase A subunit, 1 µg of gyrase B subunit, and 60 ng of DnaA protein. Monoclonal antibodies were added before DnaA protein to measure their effect. Incubation was at 30 °C for 25 min unless noted otherwise. Reactions were stopped by addition of SDS to 2.5%, and EDTA to 10 mM. The samples were electrophoresed at 25 V for 18 h in 1% agarose gels in 0.5 µM Tris borate, pH 8.3, and 1 mM EDTA, then stained with ethidium bromide. Two-stage reactions (25 µl) were performed essentially as described (25) and contained 30 mM HEPEs-KOH, pH 8.0, 0.4 mg/ml BSA, 20% glycerol, 5 mM EDTA, 6 mM CaCl₂, 0.4 mM ATP, 80 ng of M13oriC2LB5 DNA, and the above amounts of proteins. The indicated monoclonal antibodies were added before DnaA protein. Reactions were incubated for 30 min at 30 °C for the first stage, then placed on ice followed by addition of 500 ng of gyrase A subunit, 1 µg of gyrase B subunit, magnesium acetate to 10 mM, and ATP to 2 mM. Incubation followed for 10 min at 24 °C for the second stage. Na₂EDTA to 50 mM and SDS to 2.5% were added and electrophoresis in 1% agarose gels was as described above.

Enzyme-linked Immunosorbent Assay (ELISA)—DnaA protein (0.5 µg/ml) or BSA (10 µg/ml, Fig. 7 A) were added to 96-well microtiter trays (Nunc-Immuno Plate, Maxisorp, Inter-Med) in 50 µl of buffer containing 0.137 M NaCl, 2.7 M KCl, 10 mM Na₂HPO₄, and 1.76 mM KH₂PO₄, pH 7.3 (phosphate-buffered saline). Subsequent steps were performed as described (8). After removing unbound DnaA protein, the indicated monoclonal antibody diluted in phosphate-buffered saline containing 0.2% BSA was added and incubated for 1 h. DnaB protein (except where noted, 30 ng in 50 µl of phosphate-buffered saline containing 0.2% BSA) was added and incubated for 15 min. Glutaraldehyde (2 µl of a 2.5% solution in water) was added followed by incubation for 30 min. The wells were then washed as described (8) to remove unbound protein. Retained DnaB protein was detected with rabbit antiserum specific to DnaB protein. Colorimetric detection was as described (8).

RESULTS

Identification of Continuous Epitopes—To correlate domains of DnaA protein to its various functions, a collection of monoclonal antibodies were produced. Epitopes recognized by each were then determined with a peptide library constructed by joining of random segments (50–150 base pairs) of the dnaA coding region to T7 gene 10. Expression was dependent on the T7 RNA polymerase gene under lacUV5 promoter control. Transformsants (2–5 × 10⁶ per antibody) were replica-plated onto nitrocellulose filters, lysed with chloroform vapor, then screened to identify immunoreactive clones. The portion of DnaA protein in the fusion protein was deduced by DNA sequence analysis of inserts in recombinant plasmids. In addition, transformsants recognized by one antibody were tested for reactivity with other appropriate antibodies. For example, transformsants bound by A3 were also found to react with M100 (Table I). Similarly, M12 bound to transformsants identified by M36 as well as the converse. Clone 7 was obtained by its immunoreactivity to a monoclonal antibody not described here and was characterized early in this work. Transformsants of clone 7 were bound by several antibodies (M85, M48, M43, M10, and M1). For simplicity, the immunoreactivity of antibodies to various recombinants lists those that were useful in defining the minimal epitope (Table I). This approach is designed to identify continuous or primary epitopes and not conformational epitopes. It also assumes that amino acid residues of T7 gene 10 protein do not contribute to the epitope.

Positive clones were obtained with monoclonal antibodies except for M1, M12, A22, and M7. Immunoreactive clones for M1 and M12 were not obtained presumably due to weak binding. These antibodies yielded a weaker colorimetric re-

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3 M. D. Sutton and J. M. Kaguni, manuscript in preparation.
A22 and M7 appear to recognize conformational epitopes. Immunoblot experiments indicated that A22, M7, and M48 were immunoreactive to full-length DnaA protein of 52 kDa (Fig. 1). However, A22 was not reactive to transformants of Table I that expressed fusion proteins of T7 gene 10 and portions of the dnaA gene (data not shown). M7 was only reactive to the transformant containing plasmid M36-3. No positive clones for A22 were identified after screening over 2 × 10^4 transformants. By comparison, about 2 positive clones were obtained for each 10^3 transformants screened with antibodies that appear to recognize linear epitopes, generally considered to be composed of 4–8 amino acids (26–29). Based on this reasoning, about 40 positive clones should have been detected with A22 if it recognized a linear epitope. As recombinant plasmids were constructed to contain 50–150 base pairs of the dnaA gene, this result suggests that the epitope recognized by A22 resides in a larger region and may be conformational. Alternatively, the epitope in the fusion protein may have had a negative effect on bacterial growth to result in the failure to obtain such clones.

A collection of nonsense mutants of the dnaA gene have been obtained. In a nonsuppressing strain, one encodes a truncated polypeptide of 147 amino acids, established as an ochre mutant by DNA sequence analysis and confirmed by SDS-polyacrylamide gel electrophoresis relative to molecular weight standards. To localize the portion of DnaA protein bound by A22 and M7, immunoblot analysis was performed with a nonsuppressing strain bearing this plasmid-encoded mutant (Fig. 1) as well as other mutants with nonsense codons more distally located (data not shown). As a control, M48, whose epitope is from residues 133 to 146 (Table I), recognized the 147 residue long ochre peptide (Fig. 1) and other nonsense polypeptides (data not shown).
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The epitope recognized by A22 appears to be within the first 147 residues.

**DNA Binding Activity Is Not Inhibited by Monoclonal Antibodies**—Filter binding assays (see “Experimental Procedures”) with a labeled restriction fragment containing the oriC sequence revealed that none substantially reduced DNA binding (data not shown, summarized in Table III). At a saturating level of DnaA protein (80 ng) and in the absence of antibody, about 5.2 ng of the 6 ng of oriC fragment was retained. Reactions containing antibodies resulted in retention of 4.8–5.9 ng of DNA. The failure to detect inhibition of DNA binding activity was regardless of the order of addition of DnaA protein, and antibody.

Fragment mobility shift assays (23, 24) were also performed with a restriction digest of an oriC-containing plasmid. Addition of M100, M85, and A3 (as well as the remaining antibodies, not shown) did not affect the DNA binding activity of DnaA protein (Fig. 2). Under these experimental conditions, the oriC fragment did not migrate as a discrete complex when bound by DnaA protein. Binding was inferred by the reduction in the level of unbound oriC fragment.

**ATP Binding Activity Is Not Inhibited by Monoclonal Antibodies**—DnaA protein binds ATP with high affinity (K<sub>D</sub> ~ 0.03 μM) (11). Bound to ATP, DnaA protein is active in DNA replication. Comparative sequence analysis revealed at residues 172–179 a conserved P-loop motif (G<sub>4</sub>X<sub>5</sub>GKT) that is found in many nucleotide-binding proteins (30). The effect of monoclonal antibodies on ATP binding was examined to determine if any were inhibitory. However, none were found to reduce ATP binding activity by greater than 15% under conditions in which 0.3 ATP was bound per monomer of DnaA protein (summarized in Table III).

Several Monoclonal Antibodies Inhibit oriC DNA Replication and ABC Priming—Six monoclonal antibodies inhibited DNA replication of an oriC-containing plasmid (Fig. 3). M7 and A22 inhibited to near background levels at a ratio of 2 DnaA protein molecules per antibody molecule (Fig. 3). By comparison, inhibition by M1, A3, M100, and M60 antibodies required higher levels. The remaining antibodies were not inhibitory (data not shown).

As the inhibitory antibodies did not appear to interfere with binding to DNA or ATP (summarized in Table III), inhibition of replication activity was for another cause.

Monoclonal antibodies were also tested for inhibition of ABC priming (Fig. 4). This assay measures the replication activity of DnaA protein with a single-stranded M13 derivative harboring a DnaA box in a proposed hairpin structure (19). DNA synthesis also requires single-stranded DNA binding protein, DnaB, and DnaC proteins, primase, and DNA polymerase III holoenzyme. By contrast to the antibodies that inhibited oriC replication (Fig. 3), only M7 and A22 inhibited ABC priming.

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*Fig. 1. Monoclonal antibodies M7 and A22 recognize conformational epitopes.* Whole cell lysates (see “Experimental Procedures”) were prepared from E. coli HMS174 harboring the dnaA gene or an ochre mutant at codon 148 in pACYC184. Purified DnaA protein (5 ng) served as a control for immunoblot analysis with the indicated monoclonal antibodies. In lanes containing the ochre peptide, the immunoreactive species at the position of full-length DnaA protein is chromosomally encoded. We do not know the identity of other reactive species. In this experiment, the difference in mobility of the ochre peptide in the immunoblots may be due to dissimilar electrophoretic conditions, despite precautions to treat each gel identically.

*Fig. 2. DNA binding activity is not inhibited by monoclonal antibodies.* Reactions (see “Experimental Procedures”) contained the indicated amounts of antibodies and DnaA protein. Antibodies were added prior to addition of DnaA protein to measure their effect on DNA binding.

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4 J. Lipar and J. Kaguni, unpublished results.
ingsubstantially (Fig. 4). Other antibodies were not inhibitory or only slightly inhibitory.

Previous studies showed that M7 interfered with the interaction between DnaA protein and DnaB protein from the DnaB-DnaC complex only if M7 was added first (8). A22 may inhibit ABC priming by a similar mechanism as inhibition was dependent on the order of addition (Table II). Other antibodies (M1, A3, and M60) that inhibited oriC replication did not inhibit ABC priming appreciably whether added before or after the DnaB-DnaC complex (Table II).

Antibody Inhibition of DNA Unwinding by DnaA Protein—It is possible that the antibodies that specifically inhibit oriC replication block one or more functions of DnaA protein not essential for ABC priming. Bound to oriC, DnaA protein induces a local unwinding of an AT-rich region containing 3 13-mer sequence motifs (4), followed by binding of DnaB helicase to unwind further the parental duplex. Unwinding can be detected by its sensitivity to a single-stranded DNA-specific nuclease (4, 5). Alternatively, addition of DNA gyrase to remove the positive superhelicity in the DNA generated by DnaB helicase results in a highly negatively supercoiled DNA (25). This topological form, termed F1*, migrates more rapidly than the naturally supercoiled plasmid DNA on an agarose gel. The inhibition of oriC replication activity by M1, A3, M100, and M60 may be due to inhibition of unwinding activity that is not expected to be required for ABC priming. To test this possibility, these and other antibodies were examined for their effects on the unwinding activity of DnaA protein. In an assay involving addition of DNA gyrase in a second stage of incubation, M7 and A22 were marginally inhibitory (Fig. 5). The remaining antibodies including those shown were not.

This assay was simplified to involve one incubation instead of two. ATP was sufficient at 0.5 mM or greater (Fig. 6A) with a minimal incubation of 10 min (Fig. 6B). Under these conditions, M7 was inhibitory at 0.5 or 2 mM ATP, whereas inhibition by the other antibodies (A3, M100, and A22) was more effective at the higher concentration (Fig. 7A). This finding suggests that incubation of DnaA protein with 2 mM ATP may induce a conformation more favorable for antibody binding. It also supports the suggestion that a low affinity ATP binding site exists in addition to the site that confers high-affinity ATP binding (4). The greater inhibition observed at 2 mM ATP may also explain the modest inhibition observed in the two-stage incubation. In the latter, ATP was at 0.4 mM during the first stage. Inhibition by A22 and M100 (Fig. 7B) as well as M7 and A3, not shown) was proportional to the amount added. The
remaining antibodies were not inhibitory even at 5-fold higher levels than those used here (data not shown). That M1 and M60 inhibited oriC replication (Fig. 3), but ABC priming only poorly, and failed to inhibit F1* formation (data not shown) suggests their effect on an unknown activity that may act subsequent to unwinding.

Antibody Inhibition of DnaB Binding—DnaA protein was shown to interact physically with DnaB protein by use of an ELISA assay (8). In this method, immobilized DnaA protein was incubated with either DnaB protein alone, or as the DnaB-DnaC complex. Stabilization of DnaB bound to DnaA protein required glutaraldehyde cross-linking and was specific. Cross-linking of DnaC protein was not observed to DnaA protein or to BSA. M7 antibody inhibited the interaction between DnaA and DnaB in this assay. Examination of other monoclonal antibodies including M85 that did not inhibit the replication activity of DnaA protein indicated that they also interfered with DnaB binding (Fig. 8, data not shown). M43 was described previously to have little effect (8), whereas it interfered with DnaB binding by the ELISA method. This difference was apparently due to addition of an insufficient amount in the former study where the concentration of the M43 antibody preparation was inaccurate.

As a control, a monoclonal antibody to rat brain hexokinase (2B) had little effect on the binding of DnaB protein to immobilized DnaA protein (Fig. 8). Whereas the ELISA method demonstrated a specific physical interaction between proteins, it failed to correlate the antibody inhibition of replication activity with inhibition of DnaB protein binding. Inhibition of binding to DnaB protein may be due to steric reasons. For example, binding of a divalent antibody to immobilized DnaA protein may produce a network that occludes binding of DnaB protein. Fab fragments instead may correlate inhibition of replication activity to inhibition of DnaB protein binding.

DISCUSSION

Characterization of a collection of monoclonal antibodies to DnaA protein is described here in an immunological approach to correlate the structure of DnaA protein to its various functions. As linear epitopes are 4–8 residues long (26–29), most, appear to recognize linear epitopes in a region near the amino terminus (Table III). M12 and M36 may recognize conformational epitopes; only transformants encoding residues 125 to 146 of DnaA protein were reactive. Antibodies M7 and A22 appear to recognize similar but not identical conformational epitopes. None of the antibodies inhibited DNA binding (Table III). This finding is consistent with observations indicating that the carboxyl-terminal region of DnaA protein is responsible for DNA binding activity. First, fusion proteins containing the carboxyl-terminal region of DnaA protein from residue 379 to the end are active in DNA binding (17). Second, nonsense and missense mutants that affect the carboxyl-terminal region of DnaA protein are defective in DNA binding activity.3

In DnaA protein, the P-loop motif, G

G

K

T, presumed to be involved in ATP binding, is located at residues 172 to 179 (reviewed in Ref. 10). The structure of the P-loop has been determined by x-ray crystallographic analysis of adenylyl ki-
nase (31, 32), elongation factor Tu (33), RecA (12), and p21<sup>ras</sup> protein (13). In p21<sup>ras</sup>, the conserved residues interact with the γ phosphate of the bound nucleotide and with a magnesium ion that chelates the β,γ phosphates (12). Mutagenesis analysis of RecA protein (34, 35) supports the conclusion that corresponding residues interact with the α and β phosphates of ATP (13). None of the antibodies described here inhibit high-affinity ATP binding activity (Table III). This finding is consistent with the location of respective epitopes that do not overlap the P-loop motif. In addition, the bound residues are not apparently involved in interacting with adenine of ATP nor does antibody binding appear to interfere sterically or by altering the conformation of this domain of DnaA protein.

Inhibition by M7 and A22—Antibodies M7 and A22 inhibited DnaA protein in oriC replication, ABC priming, and F1<sup>+</sup> formation involving an unwinding of the AT-rich region by DnaA protein. A critical event in each of these reactions involves a direct interaction between DnaA and DnaB protein (8). In prepriming complex formation and by ELISA, M7 was shown to interfere with the binding of DnaA protein to DnaB protein from the DnaB-DnaC complex (8). The epitope recognized by M7 appears to be within residues 111–148, whereas the epitope bound by A22 is within the first 147 amino acids. These findings suggest that residues within the M7 epitope interact with DnaB.

Antibody binding sometimes alters protein conformation (36, 37). An alternate possibility that we have not excluded is that the binding of M7 induces a conformational change that interferes with the interaction between DnaA and DnaB protein, but not with DNA binding or ATP binding.

Inhibition by A3 and M100—High-affinity binding of ATP to DnaA protein (K<sub>D</sub> = 0.03 μM) correlates with its activity in unwinding of oriC (11, 38). By comparison, ADP-bound DnaA protein is relatively inert. Both nucleotide-bound forms are active in ABC priming ((19), consistent with the lack of requirement for unwinding in this reaction. A3 and M100 inhibit activities of oriC replication and F1<sup>+</sup> formation but ABC priming only poorly. In this light, their inability to inhibit ABC priming suggests that the specific inhibition of oriC replication and F1<sup>+</sup> formation is by impeding the unwinding activity of DnaA protein. If so, residues bound by A3 and M100 may be involved in unwinding.

Inhibition by M1 and M60—The initial steps of the initiation process at oriC involve (i) binding to ATP (11), (ii) binding to DnaA boxes in oriC (#, (iii) localized unwinding of the AT-rich region containing 3 13-mer sequences (4), and (iv) interaction with DnaB protein from the DnaB-DnaC complex (8). Perhaps the least understood finding in this report was the inhibition of oriC replication activity by M1 and M60, yet lack of effect on ABC priming and F1<sup>+</sup> formation. The assay of ABC priming reflects activities of binding to DNA and DnaB protein (8). F1<sup>+</sup> formation additionally measures unwinding activity of DnaA protein (25).

The lack of effect of these antibodies in these two assays suggests inhibition of an activity required subsequent to unwinding. No known biochemical activity of DnaA protein has been identified a step after unwinding and interaction with DnaB protein. Genetic evidence of extragenic suppressors of dnaX that map to the dnaA gene (39, 40) suggests an interaction between DnaA protein and the dnaX gene products, α or γ, that are components of DNA polymerase III holoenzyme (41-43). However, of the extragenic suppressors of dnaX that have been characterized at the DNA sequence level (40), these encode missense mutations for residues 213, 432, and 435 of DnaA protein. This result is inconsistent with the epitopes bound by M1 and M60. Another inconsistency is that other antibodies bind to the M1 epitope yet are not inhibitory. This may be explained by a specific conformational alteration induced by the binding of M1 to result in inhibition (36, 37) or by them binding to distinct epitopes within residues 133–146. Further investigation is required to substantiate whether DnaA protein possesses other novel activities that are revealed by M1 and M60 antibodies.

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