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EPITOPE MAPPING AND FUNCTIONAL ANALYSIS OF MONOCLONAL ANTIBODIES TO DnaA PROTEIN

By

Wenge Zhang

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ABSTRACT

EPITOPE MAPPING AND FUNCTIONAL ANALYSIS OF MONOCLONAL ANTIBODIES TO DnaA PROTEIN

BY

Wenge Zhang

DnaA protein of *E. coli* is required for the initiation of DNA replication from the chromosomal replication origin, *oriC*. In order to correlate the structure of DnaA protein to its functions, monoclonal antibodies to DnaA protein were generated, and their inhibitory effects on the activities of DnaA protein were characterized. The epitopes of these monoclonal antibodies were precisely mapped. Monoclonal antibodies M1, M10, M12, M36, M43, M48, M60, M85, M100 and A3 recognize continuous epitopes located within amino acid residues 86-148 of DnaA protein. M7 and A22 recognize conformational epitopes. The failure of these antibodies to inhibit activities of DNA binding and ATP binding indicates that amino acid residues 86-148 of DnaA protein are not involved in these activities. The epitope bound by monoclonal antibody M7 may be involved in interaction with DnaB protein. The inhibitory effect of M1 and M60 suggests a possible interaction between DnaA protein and subunits of DNA polymerase III holoenzyme.

To

my loving daughter, Julie Ann Ju

my hushand, Nengjiu Ju

and my parents

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Chapter 1

Literature Review

The *Escherichia coli* (*E. coli*) chromosome is circular and is composed of 4,720 kilo-base-pairs (kb). *E. coli* chromosomal DNA replication begins at a unique site, *oriC*, proceeds bidirectionally, and terminates at *terC*. The leading strand is synthesized continuously and the lagging strand is synthesized discontinuously. Both strands are synthesized in the 5′ to 3′ direction. Replication of the chromosome is regulated not only to ensure the precise timing of replication in the cell cycle, but also to ensure that replication occurs only once per cell cycle. As in most macromolecular processes, the replication of the chromosome is regulated at initiation of DNA replication. The *dnaA* gene product is the initiator of chromosomal replication. Physiological and genetic studies indicate that DnaA protein plays a positive role in regulating the initiation of chromosomal replication.

1.1 The initiation of *E. coli* chromosomal replication

1.1.1 The replication origin, *oriC*

Genetic studies first indicated that *E. coli* chromosomal replication initiates at a unique site, termed as *oriC*, in the vicinity of *dnaA-ilv* [16]. The *oriC* locus was then cloned as a 9 kb *EcoR1* fragment based on its ability to confer autonomous replication to a DNA fragment which is nonreplicating by itself and contain the gene for β lactamase [1]. The recombinant DNA containing the 9 kb *EcoR1* fragment not only confers ampicillin resistance to bacteria harboring it but also was readily integrated into the *E. coli* chromosome by homologous recombination. Through mapping the integration site, the location of the origin on the chromosome was determined to be near 83 min of the *E. coli* genetic map [1]. Subcloning of this fragment located *oriC* to a 422 bp DNA fragment [2, 3]. The minimal sequence of the *E. coli* replication origin is composed of 245 base pairs. This was determined by selectively deleting flanking regions of a cloned DNA fragment containing *oriC* and measuring replication function by plasmid maintenance in *E. coli* [4]. An AT-rich 12-base pair segment near the left boundary of *oriC* is required for the maintenance of a pUC-*oriC* plasmid, but can be replaced by AT-rich vector sequences [5]. Therefore, the minimal DNA sequence for *E. coli* chromosomal replication should extend 12 bp further to the left.

Sequence comparison of the replication origin of *E. coli* with those from five

other *Enterobacteriaceae* species (Fig. 1.1) [15] revealed regions of conservation among *oriC* homolog. Highly conserved sequences are separated by regions of variable sequence but of constant length. Analyses of mutations that affect the function of *oriC* led to the proposal that *oriC* consists of two essential sequence elements: sites for initiation protein binding, and spacer elements for the precise arrangement of the protein binding sites [21].

There are several prominent features within the conserved regions (Fig. 1.1). First, the four highly conserved 9-base pair repeats TTAT(A/C)CA(A/C)A serve as binding sites for DnaA protein, and are referred to as DnaA boxes (R1-R4) [6]. The orientation of the DnaA boxes are inverted with respect to each other. DNasel footprinting analysis defined a possible fifth DnaA box with the sequence TCATTCACA [7]. This DnaA box is between an IHF binding site and DnaA box R2. When certain bases in the DnaA boxes are changed, reduced DNA binding is observed [24]. Any base pair insertion between R1 and R2 impairs *oriC* function [25]. Insertion of 10 bp between R2 and R3 [43] or insertion of 2 kb between R3 and R4 in *oriC* is tolerated [17].

Second, three highly conserved AT-rich 13-mers are present near the left boundary of *oriC*, each starting with GATC. The DNA helix at the 13-mer repeats is thermodynamically unstable in the absence of Mg^{2+}, exhibiting hypersensitivity to the single-stranded DNA specific P1 nuclease in a negatively supercoiled plasmid [10]. In the presence of Mg^{2+}, this region remains base paired. However binding of DnaA protein to *oriC* in the presence of Mg^{2+} leads to the duplex opening at the AT-rich 13-mer region [8]. By contrast, IciA pro-

tein binds to the 13-mer repeats to inhibit unwinding by DnaA protein and replication initiation [9].

The left 13-mer can be replaced by different DNA sequences with similar AT content [5, 10], suggesting that AT-richness in this region is sufficient for *oriC* to function. The precise sequence of the right 13-mer is required for *oriC* to function [5, 8]. For the middle 13-mer, there are controversial results regarding whether the precise sequence or mere AT-richness is important for *oriC* to function [5, 11].

The Dam methylation site, GATC, in which the adenine residue is methylated by DNA adenine methylase, is present 11 times within *oriC*. While only two are expected in the 245 bp origin region based on random distribution statistics, eight out of the eleven methylation sites are conserved. The first four nucleotides in the AT-rich 13-mers are also Dam methylase recognition sequences. *In vivo* and *in vitro* experiments showed that Dam methylation of *oriC* is involved in regulating the timing of initiation.

The replicated chromosomal DNA is in a transiently hemi-methylated state by virtue of pairing of the methylated template strands with the newly synthesized DNA that is not. The hemi-methylated *oriC* appears to be inactive in DNA replication *in vivo* [12]. Hemi-methylated *oriC* plasmids cannot transform *dam* mutant (defective in DNA adenine methylase), but unmethylated and fully methylated plasmids transform at high efficiency [12]. While no significant difference in the binding of unmethylated, hemi-methylated and fully-methylated *oriC* to DnaA protein was observed [18], only hemi-methylated *oriC* has an

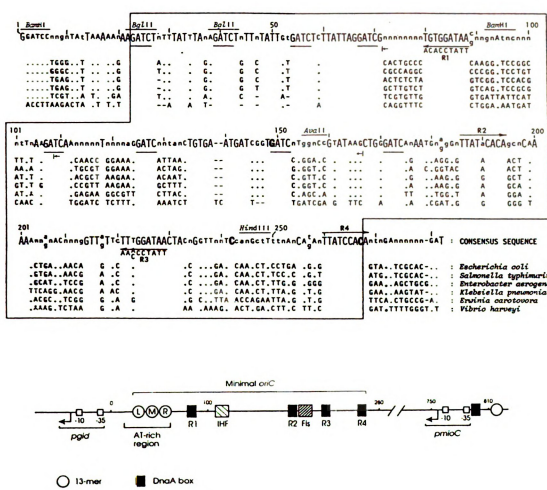

Figure 1.1: Structural features of the *E. coli* chromosomal replication origin, *oriC*

Upper part: Replication origin sequences from six bacterial species are aligned to show sequence conservation. GATC methylation sites are underlined. AT-rich 13-mers are indicated. DnaA boxes are marked as R1, R2, R3, R4. The fifth is not shown. A large capital letter indicates a consensus base in all six species. A small capital letter indicates that the base is conserved in five out of the six sequences. A lower case letter indicates that the base is present in four or three origins. A bold capital letter indicates a base substitution that inactivates the origin [35].

Lower part: A schematic representation of the *oriC* region and nearby promoter regions. Filled boxes are DnaA boxes. IHF and FIS protein binding sites are shown. The open circles represent 13-mers [47].

increased affinity for the outer membrane [46]. The outer membrane fraction inhibits the replication of hemi-methylated *oriC* plasmid specifically. Therefore, a membrane sequestration model has been proposed: Dam methylation regulates the timing of initiation by affecting the formation of a complex between hemi-methylated *oriC* and the outer membrane [13]. This complex prevents DnaA protein from interacting with *oriC*. Initiation can occur again only after remethylation of the new strand, resulting in the dissociation of *oriC* from the outer membrane to allow DnaA protein to bind and form an initiation competent DnaA-*oriC* complex. The isolation of mutants which can transform *dam*$^-$ cell with fully methylated *oriC* plasmid led to the identification of the *seqA* gene that is required for sequestration of *oriC* [22]. SeqA protein has also been shown to be responsible for the binding of the membrane fraction to hemi-methylated *oriC* [23].

Binding sites for IHF (integration host factor) and FIS (factor for inversion stimulation) are present in *oriC*. *In vivo* and *in vitro* studies showed that IHF protein facilitates the duplex opening induced by DnaA protein whereas FIS is inhibitory [19, 14, 20].

Mutations in the individual DnaA boxes, 13-mers and Dam methylation sites of *oriC* abolish its function as an origin for replication [21]. These observations and the conservation of nucleotide sequence of *oriC* among *Enterobacteriaceae* suggest the existence of a conserved mechanism for the initiation of chromosomal DNA replication.

1.1.2 The initiation of *E. coli* chromosomal replication at *oriC*

Genes required for *E. coli* chromosomal DNA replication were originally identified by genetic studies through the isolation and genetic mapping of conditional lethal mutants to the chromosome. These studies supported subsequent work on the cloning of these genes and overproduction of respective gene products by recombinant DNA methods. Two achievements made it possible to study the replication initiation *in vitro*, that then led to significant findings on the biochemistry of the initiation process. The first is the cloning of *oriC* into a plasmid for use as a substrate for *in vitro* assays. The second is the development of a reconstituted system for *in vitro* replication with purified enzymes [48]. Required proteins for replication were identified biochemically by omitting individual enzyme component from the system, and were divided into three functional groups. Initiation factors including DnaA, HU, DnaB, DnaC, DNA gyrase, and RNA polymerase recognize the *oriC* sequence to form an initiation intermediate. Elongation factors (DnaB, primase, SSB, DNA gyrase and DNA polymerase III holoenzyme) serve in DNA chain elongation. Specificity factors (topoisomerase I and RNase H) maintain dependence on *oriC*-containing plasmids by suppressing initiation of DNA synthesis at other sites [48].

Utilizing the *in vitro* reconstituted system, the initiation process was studied in detail to identify several successive stages. A speculative model for the initiation of replication from *oriC* has been proposed (Fig. 1.2) [30] that includes initial complex, open complex, prepriming complex, priming complex, followed

by bidirectional replication.

First, DnaA protein binds cooperatively to the DnaA boxes in *oriC*, forming the initial complex. Electron microscopy studies suggest that this complex consists of 20-40 DnaA protein monomers wrapped around by the origin DNA [25]. Both ADP-bound and ATP-bound forms of DnaA can bind to linear or supercoiled DNA [8]. However, footprinting analysis revealed that the ATP form of DnaA protein binds more specifically to the DnaA boxes, whereas the nucleotide-free form binds to other regions as well [31]. ATP complexed with DnaA and a supercoiled *oriC* plasmid are required for subsequent stages [8, 24].

Once *oriC* is bound by DnaA protein, the AT-rich 13-mer repeats in *oriC* are partially unwound. This duplex unwinding was detected by sensitivity to cleavage in this region by single-strand-specific P1 nuclease [8]. DnaA protein may interact with the top strand of the 13-mers to induce duplex opening. Primer extension from two primers complementary to the top and bottom strand sequence outside the 13-mer region was used to measure cleavage of each individual strand by P1 nuclease. The cleavage of the top strand is limited to only part of the middle 13-mer, while the cleavage of the bottom strand spans the three 13-mers and the adjacent AT-rich region [31]. The uncleaved region of the top strand may be due to DnaA protein protection. Mutations that only alter the specific sequence but preserve the AT-richness in the middle and the right 13-mers result in pronounced reduction of *oriC* function [57]. This suggests a sequence-specific interaction of DnaA protein with the middle and right 13-mer. This open duplex DNA with bound DnaA protein is referred to as the

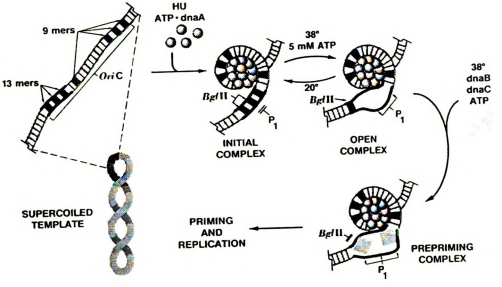

Figure 1.2: A model for replication initiation at *oriC*

The DnaA protein binds to the four 9-mers, organizing *oriC* around the protein core to form the initial complex. DnaA protein then induces duplex opening at the three AT-rich 13-mers forming the open complex. The binding of DnaB-DnaC to *oriC* forms the prepriming complex, which is followed by the priming complex and DNA replication (from [30]).

open complex. The unwinding of the 13-mers requires the ATP-bound form of DnaA protein and levels of ATP from 1 to 5 mM. Formation of the open complex requires a temperature of at least 38 °C [24] in *in vitro* assay. The histone-like protein HU or IHF is required for the unwinding of 13-mers by DnaA protein [31].

After the opening of the 13-mers, DnaB protein complexed with DnaC protein is loaded onto the opened duplex. It has been suggested that DnaA protein guides DnaB protein into the complex [30]. Cross-linking ELISA demonstrated a physical interaction between DnaA and DnaB. This interaction can be inhibited with a monoclonal antibody to DnaA protein [27]. The binding of DnaB protein produces a prepriming complex that retains a P1-sensitive configuration at lower temperature (16°C) [8]. Mapping of the P1 cleavage site showed an extended cleavage pattern compared to that of the open complex. The formation of the prepriming complex requires ATP. dATP and CTP cannot replace ATP [8].

Upon addition of SSB and DNA gyrase and in the presence of ATP, DnaB helicase further unwinds the duplex DNA. The single stranded DNA produced by DnaB helicase is coated and stabilized by SSB protein. The topological strain generated by strand unwinding by DnaB is relieved by DNA gyrase to remove positive supercoils. The extensively unwound structure that is formed, called Form I*, can be separated from other topological forms by agarose gel electrophoresis [29].

Following strand separation and loading of DnaB helicase, primase binds to

the single stranded DNA to synthesize RNA primers, thus forming the priming complex. The replication fork contains DnaB helicase, primase and DNA polymerase III holoenzyme. The moving of the replication fork ultimately results in two daughter chromosomes.

1.2 DnaA protein

1.2.1 *dnaA* mutants

The first temperature sensitive *dnaA* mutant affecting the initiation of DNA replication CRT46, was isolated and characterized by Hirota *et al.* [33]. The wild type *dnaA* gene was then cloned by its ability to complement the temperature sensitive phenotype of a *dnaA* mutant. Its location on the *E. coli* K-12 linkage map is at 83.5 min, 42 kb counterclockwise from *oriC* [34]. The coding region of 1401 nucleotides expresses a basic polypeptide with a deduced molecular weight of 52,633 daltons [36].

Other *dnaA* alleles have been isolated subsequently [39, 40]. Their respective mutations have been finely mapped by P1 transduction, then at the nucleotide level by DNA sequence analysis [41]. An identical mutation, resulting in substitution of alanine to valine at residue 184 very close to the ATP binding site, is found in *dnaA5*, *dnaA46*, *dnaA601/602*, and *dnaA604/606*. These mutants also carry a second mutation of Gly426Ser, His252Tyr, Pro296Gln, and Ala347Val respectively. The phenotypes of the above mutants are reversible upon temperature downshift from nonpermissive to permissive temperature. These mutants

reinitiate chromosomal replication in the absence of protein synthesis. All are defective in the proper timing of replication initiation relative to the bacterial cell cycle [41]. Other alleles (*dnaA205*, *dnaA203/204* and *dnaA211*) harbor different single mutations that encode substitution in the C-terminal part of the protein corresponding to Val383Met, Ile389Asn, Met411Thr respectively. The mutations of *dnaA508* affect Pro28Leu and Thr80Ile near the N-terminus.

Intragenic suppressors of the temperature sensitive *dnaA46* and *dnaA508* alleles have been obtained. These suppressor mutations not only revert the heat sensitivity of the *dnaA* alleles, but also renders them cold sensitive. The cold sensitive phenotype has been shown to be the consequence of over-initiation that results in lethality. At higher temperature, the mutant protein is partially active, resulting in normal cell growth. 2D protein gel and transcription assay showed that *dnaA* expression is enhanced in *dnaA508cos* [37]. The intragenic suppressor of the *dnaA508* allele is due to a change of GTG to ATG of the initiation codon that results in elevated expression and apparently accounts for the phenotype of this allele.

The *dnaA46-cos* allele encodes two substitutions (Gln156Leu and Tyr271His) in addition to the two mutations present in *dnaA46* [38]. Recent biochemical studies demonstrated that over-initiation by the *dnaA46cos* allele is not caused by protein over-production [53]. Instead, dnaA46cos protein possesses increased replication activity at 30 °C, and retains initiation activity for a longer period in the cell cycle compared to wild type DnaA protein [54]. The systematic study of these suppressor mutations may be of value in understanding the structural

and functional relationship of DnaA protein.

1.2.2 Biochemical properties of DnaA protein

DNA binding

Specific binding of DnaA protein to the *E. coli* replication origin (*oriC*) is essential for its function as a replication initiator. Supercoiled M13*oriC*26 DNA containing *oriC* was preferentially retained by DnaA protein on a nitrocellulose filter compared to M13*oriC*Δ221 that lacks the minimal *oriC* sequence [58]. If the plasmid was linearized, reduced binding was observed [58], suggesting that DnaA protein binds preferentially to supercoiled DNA. The blockage of the *HindIII* site located within *oriC* by preincubating M13*oriC* with DnaA protein further demonstrated that DnaA protein binds specifically to *oriC*. By comparison, the *EcoR1* site outside the *oriC* sequence was unprotected [58]. Besides binding to *oriC*, DnaA protein binds specifically to other DNA fragments including the replication origins of plasmid pSC101, pBR322, and ColE1; the promoter region of the *dnaA* gene, the promoter and N-terminal coding region of the *mioC* gene and a 971 bp *Taq1* fragment of M13 [6]. All these fragments share the common 9 bp DnaA box sequence.

DNase1 footprinting of DnaA protein at sites containing a single DnaA box showed that the region of protection centers on the 9 bp sequence and extends 40-50 bp to either side [6]. In footprinting of DnaA protein at *oriC* containing four DnaA boxes, virtually the entire sequence 5'-TTATCCACA-3' on one

strand is protected, whereas cleavage occurs at the center of the complementary strand [6]. The 9 bp consensus sequence (DnaA box) forms the core for DnaA protein binding. The four DnaA boxes in *oriC* designated R1, R2, R3, and R4 presumably act to make *oriC* the primary binding target for DnaA protein.

In a more recent study on the interaction of DnaA protein with its target recognition sequence, oligonucleotides with sequence variants of the DnaA boxes and with different flanking sequences were examined. DnaA boxes R1 and R4 of *oriC* are identical but have different flanking sequences. R2 and R3 differ from R1 and R2 at the fifth and eighth positions, respectively. The equilibrium dissociation constants and kinetic rate constants were determined. Oligo nucleotides with the sequence TT(A/T)TNCACA are bound by DnaA protein specifically [32]. DnaA boxes R1/R4(TTATCCACA) and R2 (TTATACACA) exhibit specific binding with K_D values in the range from 1 to 50 nM. R1/R4 with its natural *oriC* flanking sequence was bound with 50-fold higher affinity than R1/R4 with an averaged flanking sequence. This suggests that flanking sequences affects binding affinity. R3 (TTATCCAAA), R5 (TCATTCACA), and the DnaA box (TTTTCCACA) in the promoter region of *mioC* gene bind DnaA nonspecifically with K_D values greater than 200 nM [32]. The lack of specific binding of R3 in this assay is in agreement with an *in vivo* methylation protection study which showed that DnaA boxes R1, R2, and R4, but not R3 were protected throughout most of the cell cycle [60].

DnaA protein seems to make contact with both the major groove and minor groove [32]. Methyl groups of thymidines are only exposed in the major groove.

An oligo nucleotide of DnaA box R4 with all thymidine residues replaced by deoxyuridine in both strands or only one strand was bound with greatly reduced affinity by DnaA. Pre-treatment of the R4 oligo with drugs that bind to the minor groove impaired the binding of DnaA protein in gel retardation assays [32].

Nucleotide binding

DnaA protein binds to ATP and ADP with high affinity. The K_D values of ATP and ADP binding are 0.03 μM and 0.1 μM respectively [24]. The exchange rate of bound nucleotide with free nucleotide is very slow. Both the stability of DnaA protein and its activities are profoundly affected by nucleotide binding. The binding of ATP/ADP plays an important role in regulating the the initiation of replication through modifying the activity of DnaA protein.

Both the ATP-bound form, ADP-bound form, as well as the nucleotide-free form can bind to *oriC*. After binding, strand opening of the 13-mers requires 5 mM level of ATP. Strand separation can be detected by sensitivity to P1 endonuclease. The ADP-form fails to induce a P1 sensitive structure in *oriC*. The formation of a prepriming complex, composed of *oriC*, HU protein, DnaB, and DnaC (as measured by the retention of DnaB in the *oriC* complex and replication), is supported by the ATP-form but not the ADP-form of DnaA [24].

Extensive hydrolysis of bound ATP is not essential for the formation of the

prepriming complex. Hydrolysis of bound ATP is quite slow ($T_{1/2}$=15 min) compared to replication initiation. The non-hydrolyzable analog ATP-γS can replace ATP in forming the prepriming complex [24]. Thus the tightly bound nucleotide seems to serve as an allosteric effector. The tryptic digestion pattern of the ATP- or ADP-form of DnaA protein is distinct compared to that obtained with the nucleotide free form [28], suggesting a conformational difference among these forms. The ATP- and ADP-forms of DnaA behave as monomers in solution, while the nucleotide-free form tends to aggregate. This aggregated complex is distinct from the active complexes containing the nucleotide bound form of DnaA [25].

In addition to a domain for high affinity ATP binding, DnaA protein may have an additional low affinity ATP binding site. This speculation is based on the requirement of 1-5 mM of ATP for opening the AT-rich region in *oriC* by DnaA protein. The subsequent loading of DnaB protein at *oriC* to form the prepriming complex requires ATP at 30 μM [24]. DnaA protein (with its high affinity binding site occupied by ATP) is inactivated by mM levels of ADP in this assay [28]. Presumably, the occupancy of the low affinity binding site by ADP is responsible for the inactivation. The two levels of ATP required for DnaA function may be important for precise regulation of initiation.

Interaction with phospholipids

It is well documented that DNA replication in *E. coli* is a membrane- associated event. Interestingly, disphosphatidylglycerol (cardiolipin) was found to be strik-

ingly effective in promoting the rapid release of tightly bound ATP and ADP from DnaA protein [49]. The release of bound ADP by cardiolipin results in the reactivation of the previously inert DnaA protein for replication. The monoacidic phosphatidylglycerol is only about one tenth as effective as cardiolipin. The neutral phosphotidylethanolamine–the principal *E. coli* phospholipid, is inactive. Phosphatidylinositol, not present in *E. coli*, but with the same acidic head group as phosphatidylglycerol, possesses the same activity level. This suggests that the acidic head group is important for membrane interaction with DnaA protein.

The interaction of DnaA protein with the head group of acidic phospholipids also requires a fluid phase bilayer context. Phospholipids lacking unsaturated fatty acids were relatively inactive compared to those containing unsaturated fatty acids in effecting the release of ADP from DnaA protein [50]. Consistent with these biochemical observations, cells possessing only saturated fatty acids in their membrane due to the inhibition of oleic acid biosynthesis cannot initiate replication, and fail to grow [42].

Cardiolipin inactivates DnaA protein in the absence of bound nucleotide or *oriC* [49]. When DnaA protein was purified, half was found in an aggregated form containing phospholipids. The aggregated form was inactive in the reconstituted replication system. Phospholipase A2 treatment activates aggregated DnaA protein [56].

In vivo evidence also suggests that anionic phospholipids are involved in DnaA-dependent replication from *oriC*. Mutations in *pgsA*, which encodes phos-

phatidyl-glycerophosphate synthase, lead to arrest of cell growth due to defective biosynthesis of phosphatidylglycerol and cardiolipin. Mutations in the *rnhA*, which encodes RNaseH, promote chromosomal replication from sites other than *oriC* thereby bypassing the requirement for the *dnaA* gene. Mutations in *rnhA* gene also suppress the growth phenotype of *pgsA* mutant, suggesting that the block of the *pgsA* mutation affects initiation at *oriC* [55]. The interaction of membrane lipids with DnaA protein suggests the involvement of the cell membrane in the regulation of chromosomal replication.

1.2.3 Structural and functional domains of DnaA protein

Since the discovery of the *E. coli dnaA* gene, other bacterial homologs have been identified. In most cases, the gene order *dnaA-dnaN-recF-gyrB* is conserved. The *dnaA* gene exhibits a high degree of similarity at the nucleotide sequence level.

Amino acid sequence alignment of 14 bacterial homologs reveals conserved residues in a short N-terminal segment and a long C-terminal segment. The amino acid sequence connecting the two conserved segments is not conserved [47]. Thus three structural domains are suggested (Fig. 1.3).

Within one of the well conserved regions, a sequence motif G-X-X-G-X-G-K-T is present at residues 172 to 179. This consensus sequence is found in many proteins that bind ATP or GTP [44]. Alanine-to-valine at residue 184 is encoded by the *dnaA5* and *dnaA46* alleles. Biochemical characterization of the

purified protein demonstrated that both DnaA5 and DnaA46 are defective in ATP binding *in vitro* [45, 52].

Controlled tryptic digestion of DnaA provides some insight into one functional domain. Bound to ATP or ADP, a trypsin-resistant 30 kilodalton (kDa) fragment is obtained ([59], Carr and Kaguni, unpublished results). This fragment is inactive in *oriC* binding but retains the ability of responding to phospholipids in releasing tightly bound ATP or ADP [59], suggesting that the functions of high-affinity nucleotide binding and interaction with phospholipids may be in the 30 kDa peptide.

In a separate study to identify the DNA binding domain of DnaA protein, different regions of the *dnaA* gene were amplified and fused to *lacZ* that encodes β galactosidase. The DnaA-β galatosidase fusion was affinity purified by anti-β galactosidase antibody. The fusion containing amino acid residues 374-467 of DnaA protein was sufficient for specific *oriC* binding in a solid phase DNA binding assay. Fusion to other parts of the *dnaA* gene, including the N-terminal domain, or the C-terminal domain up to residue 374 did not bind to *oriC*. Deletion of the residues near the boundaries of this 93-amino acid domain (residues 374-467) abolished *oriC* binding activity. In addition, fusions containing the 93-amino acid domain from *dnaA204* (Ile389Asn), *dnaA205* (Val383Met), or *dnaA211* (Met411Thr) did not bind to *oriC* [51]. Therefore the C-terminal 93-amino acids are considered to be the DNA binding domain.

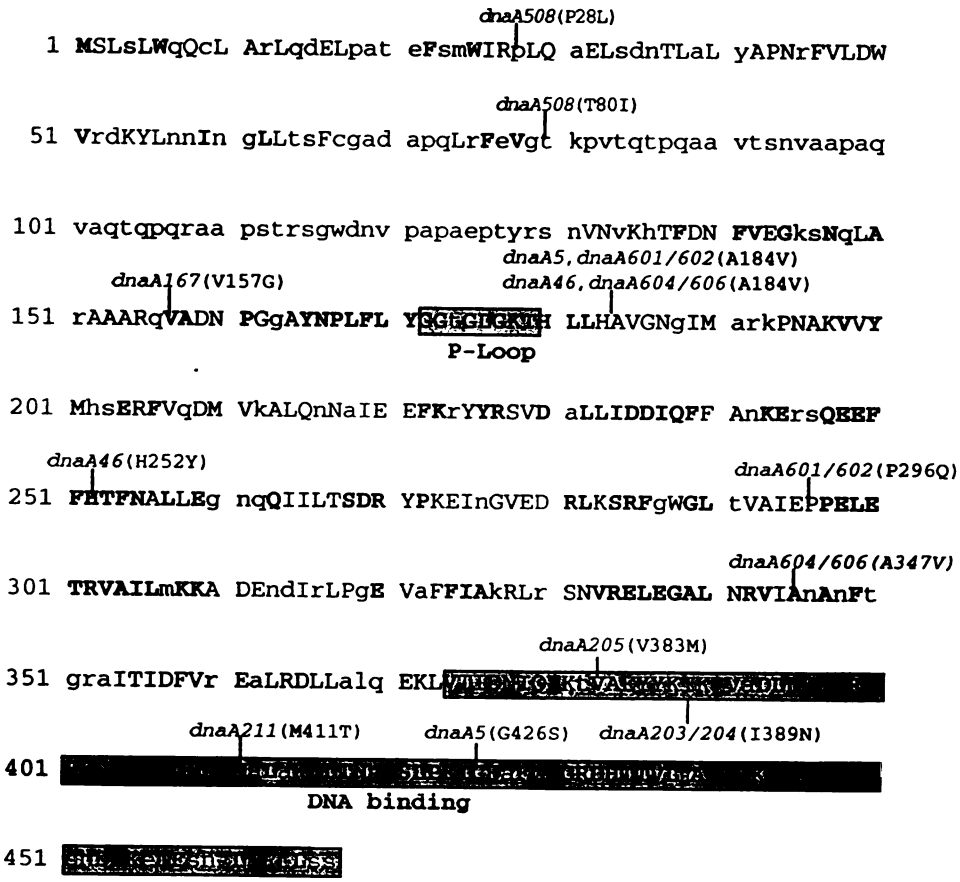

Figure 1.3: Structural and functional domains of DnaA protein
The amino acid sequence is of *E. coli* DnaA protein. Comparison of its amino acid sequence to those of 14 different bacteria revealed the conservation of the amino acid residues [47] (*dnaA* sequences from other bacteria species are not shown here). Upper case letters indicate that the amino acid is identical or shows a conservative change among 9 bacterial species, bold upper case letters indicate amino acids are identical or show a conservative change among the DnaA protein of 12 out of 14 bacterial species. The region marked "P-loop" denotes the ATP binding site. The boxed region marked "DNA binding" indicates the DNA binding domain. The mutations of the *dnaA* (TS) alleles are indicated.

1.3 Objective of the thesis

E. coli DnaA protein consists of a single polypeptide of 467 amino acids with a predicted molecular weight of 52.5 kDa. In its role in initiation of chromosomal replication, this protein possesses a number of functions, including ATP binding, sequence-specific DNA binding, interaction with DnaB protein and interaction with phospholipids. The objective of this thesis project is to correlate its structural domains to specific functions by using a monoclonal antibody approach. This study complements other ongoing projects on DnaA protein in the lab, and provides important reagents that will yield structural information that is currently lacking for DnaA protein.

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Chapter 2

Epitope mapping and functional analysis of monoclonal antibodies to DnaA protein

2.1 Introduction

DnaA protein of *E. coli* consists of a single polypeptide chain of 467 amino acids with a predicted molecular weight of 52.6 kDa [5]. DnaA protein is required for the initiation of chromosomal replication from *oriC* [23, 24, 4]. At the step of initiation of replication, DnaA protein recognizes and binds to the four 9-bp consensus sequences (DnaA boxes) in *oriC*. In the presence of 1-5mM ATP, binding induces a localized duplex opening at the AT-rich 13-mer region [46]. Subsequently, DnaA protein is proposed to guide DnaB helicase (complexed with DnaC) to bind to *oriC* through a physical interaction between DnaA protein and DnaB protein [22]. DnaB helicase further unwinds the duplex and guides primase to synthesize primers on the DNA template [27]. The elongation of primers by DNA polymerase III holoenzyme eventually results in two

daughter chromosomes.

Biochemically, DnaA protein possesses several activities important for its physiological role as a replication initiator and regulator. First, DnaA protein binds DNA specifically. It recognizes and binds to DNA containing the sequence TTAT(A/C)CA(A/C)A [7]. Second, it binds to ATP and ADP with high affinity. The ATP-bound form is active and the ADP-bound form is only partially active in forming replication complexes [29]. Third, it interacts with phospholipids. This interaction promotes the release of the tightly bound nucleotide [6]. The fast release of ADP rejuvenates inert DnaA protein by allowing it to bind ATP. Fourth, it interacts directly with DnaB protein [22]. Its structural complexity is suggested by the multiple functions of the protein. Sequence comparison of *dnaA* homologs among fourteen different bacterial species [1] reveals that DnaA protein at the amino acid level is highly conserved within a short N-terminal domain and a longer C-terminal domain. The phenotype of temperature sensitive *dnaA* mutants exhibits some correlation with the locations of mutations, suggesting the existence of specific domains that relate to specific functions.

At present, little information is known concerning functional domains of DnaA protein. It has been proposed that the consensus sequence motif GX_4GKT (P-loop) at residues 172-179 (reviewed in [2]) is involved in high affinity ATP binding [29]. Proteins encoded by the *dnaA46* and *dnaA5* alleles, both of which contain identical amino acid substitutions of alanine 184 to valine (A184V), as well as other unique substitutions, are unable to bind ATP *in vitro* [2, 3].

In a recent study, the mutant protein containing only the A184V substitution was studied biochemically and shown to be defective in ATP binding (K. M. Carr and J. M. Kaguni in press). The DNA binding activity has recently been localized to a 93-amino acid C-terminal domain [32]. Other domains of DnaA protein involved in low affinity ATP binding, interaction with phospholipids and interaction with DnaB protein are unidentified.

Monoclonal antibodies can be a useful tool to correlate the structure of a protein to its various functions. Utilizing this approach, monoclonal antibodies against DnaA protein were generated and characterized for their inhibitory effects on several activities of DnaA protein, including replication initiation, DNA binding, ATP binding, F1* formation and the interaction with DnaB protein. In the studies reported here, epitopes for these monoclonal antibodies were precisely mapped by constructing and screening a peptide library derived from the *dnaA* gene. Reported below, 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 [44]. Priming of DNA replication *in vitro* on the latter template, termed as ABC priming, involves assembly of an intermediate formed on the single-strand (ss) 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 [22] has been identified. Presumably, a domain in this region interacts directly with DnaB protein. Re-

sults 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 polumerase III holoenzyme.

2.2 Experimental Procedures

Reagents, Proteins, and DNAs: Commercial enzymes and proteins were from the following sources: bovine serum albumin, DNase I, Sigma; T4 DNA polymerase, *Tth* DNA polymerase, T4 DNA ligase, linearized pTOPE-1bp, and competent HMS174 (*recA1 hsdR rif*) lysogenized by λDE3, Novagen; Sequenase, USB.

Highly purified replication proteins were: DnaA protein (fraction IV, 2×10^5 units/mg) [4]; DnaB protein (fraction V, 6×10^5 units/mg) [8]; DnaC protein (fraction VI, 3×10^6 units/mg) [9]; primase (fraction V, 2×10^6 units/mg) [10]; single-stranded DNA binding protein (SSB) (fraction IV, 4×10^4 units/mg) [11, 12]; DNA polymerase III holoenzyme (fraction V, 2×10^5 units/mg) [13]; DNA gyrase A subunit (fraction III, 2×10^5 units/mg), and DNA gyrase B subunit (fraction V, 1×10^5 units/mg) [14]; RNA polymerase (fraction V, 250 milliunits/mg) [15, 16]; topoisomerase I (fraction III, 5×10^4 units/mg) [17]; RNase H (fraction IV, 8×10^5 units/mg) [18], and HU protein (fraction IV, 5×10^4 units/mg) [19]. Units for the above proteins are described in the corresponding references.

Monoclonal antibodies to DnaA protein were produced [17] and purified from

tissue culture supernatants with protein A or Mono-Q Superose (Pharmacia). Unless otherwise indicated, the monoclonal antibodies were in 40 mM Hepes-KOH pH 8.0, 50 mM KCl, 15% glycerol, and 2 mM dithiothreitol (DTT) by dialysis. Monoclonal antibody 2B to rat brain hexokinase, and goat anti-mouse antibodies that are specific for each immunoglobulin subclass were gifts from Professor John Wilson, Michigan State University. Antisera specific for DnaB protein was obtained from rabbits [17].

M13-A site ssDNA [12] contains a DnaA protein recognition sequence (DnaA box) in the stem of a hairpin structure formed by base pairing of an inverted repeat from the R6K γ-origin region but is not contained in the minimal DNA fragment identified as the γ-origin. M13*oriC*2LB5 contains *E. coli oriC* [16].

Epitope Mapping: Libraries of recombinant plasmids, each of which expressed a small peptide derived from DnaA protein as a fusion protein joined to T7 gene 10 protein, were constructed essentially as described by the Novagen. The *dnaA* gene was amplified by PCR, treated with DNaseI in the presence of Mn^{2+}, and fragments averaging 50-150 base pairs in size were purified from an agarose gel. The DNA fragments were then treated successively with T4 DNA polymerase and *Tth* DNA polymerase to end-fill and then add a single dA residue to the 3′ end of each fragment, followed by ligation to linearized pTOPE-1b with a single dT over hang at each 3′ end, and transformation into HMS174(λDE3), both obtained from the manufacturer. 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 pH7.9, 6 M

urea, and 0.5 M NaCl. Positive clones were identified by immunoblot analysis with the monoclonal antibodies 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 with the original plate, the 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 of 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 mutants The *dnaA*$^+$ gene and an ocher mutant at codon 184 in pACYC184 were from this laboratory (M. D. Sutton and J. M. Kaguni, manuscript in preparation). Whole cell lysates were prepared by resuspending of 10^8 cells of the plasmid bearing strain (*Sup*$^-$) collected at mid-log phase in 0.1% sodium dodecyl sulfate (SDS) and electrophoresed on 10% SDS-polyacrylamide gels. The protein was then transferred to PVDF membranes (Schleicher & Schuell). A22, M7 and M48 were used as primary antibodies and goat anti-mouse IgG HRP conjugate was used as the secondary antibody. ECL chemiluminescence kit (Amersham) was used to detect antibody-antigen complexes.

DNA Binding Assays: Fragment retention assays (25 μl) contain 6 ng of a 459 bp *Sal*1-*Xho*1 fragment containing *oriC* from pTSO182 DNA and 100 ng of *Hinf*1-digested pBR322 DNA as a nonradioactive competitor in buffer containing 40 mM HEPES-KOH (pH 7.8), 5 mM $MgCl_2$, 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 [$\alpha-^{32}$P] dATP. Monoclonal antibodies (160-250ng) were added to DnaA protein (80ng) and incubated on ice for 15 min to assay for inhibition on DNA binding activity. DNA was then added and reactions were incubated 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 above buffer equilibrated at room temperature. Radioactivity retained on the filters was determined by liquid scintillation counting.

Gel mobility shift assays [20] were performed by addition of the indicated amounts of DnaA protein and 1.48 μg of *Hae*III-digested M13*oriC*2LB5 DNA in 25 μl containing 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10% (v/v) glycerol, and 2 mM DTT. Monoclonal antibodies were added to DnaA protein and incubated for 15 min on ice to assay their inhibitory effects. After incubation at 30 °C for 10 min, the samples were electrophoresed on 1% agarose gel in 90 mM Tris-borate, and 1mM EDTA. DNA bands are visualized by staining with ethidium bromide.

ATP Binding Assays: Reactions (25μl) were performed essentially as described [29] and contained equal amounts (2 pmol) of DnaA protein and monoclonal antibodies, and 5 mM DTT, 15% glycerol, 0.01% Triton X100, and 50 mM Tris-HCl pH 8.0. Incubation was for 15 min on ice. 0.1 μM [$\alpha-^{32}$P ATP] (0.5 μCi) were then added and incubation was continued at 0 °C for 15 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. Radioactive ATP retained on the filters was quantified by liquid scintillation counting.

Replication Assays: Reaction mixtures for ABC priming (25 μl) were assembled essentially as described in [18] and contained 40 mM HEPES-KOH pH 8.0 (measured at 1 M and 20 °C), 40 mM potassium glutamate, 10 mM magnesium acetate, 4 mM DTT, 0.1 mg/ml bovine serum albumin (BSA), 4% (w/v) sucrose, 2 mM ATP, 0.25 mM each of CTP, GTP, and UTP, 100 μM each of dATP, dCTP, dGTP, and [methyl-^{3}H]dTTP (25-30 cpm/pmol), 0.1 μg M13-A site ssDNA, 1 μg SSB, 28 ng DnaA protein, 50 ng DnaB protein, 24 ng DnaC protein, 10 ng primase, and 80 ng DNA polymerase III holoenzyme. Reaction mixtures were assembled at 0 °C, then incubated for 15 min at 30 °C for DNA synthesis. To measure the inhibitory effect, DnaA protein was pre-incubated with monoclonal antibodies, then added to the reaction. Total nucleotide incorporation (in pmol) was measured by liquid scintillation counting after trichloroacetic acid precipitation (TCA) onto glass fiber filters (Whatman GF/C).

Reactions of *oriC* plasmid replication (25 μl) were similar to those described in [2] and were comparable to those for ABC priming except for the following: CTP, GTP, and UTP were at 0.5 mM each; phosphocreatine, 6mM; creatine kinase, 100μg/ml; M13*oriC*2LB5 supercoiled DNA instead of M13-A site ssDNA, 200 ng; SSB, 160 ng; HU, 25 ng; gyrase A subunit, 470 ng; gyrase B subunit, 600 ng; DnaA protein, 56 ng; DnaB protein, 55 ng; DnaC protein, 24 ng; primase, 10 ng; and DNA polymerase III holoenzyme, 80 ng. Reactions were assembled

at 0 °C and incubated for 30 min at 30 °C to measure DNA synthesis.

Assay of FI* formation: Single stage reactions (25 μl) contain 40 mM HEPES-KOH (pH 7.6), 20 mM Tris-HCl pH 7.5, 4% (w/v) sucrose, 2 mM ATP, 4 mM DTT, 11 mM magnesium acetate, 200 ng M13*oriC*2LB5 supercoiled DNA, 21 ng HU protein, 160 ng SSB, 100 ng DnaB protein, 24 ng DnaC protein, 500 ng gyrase A subunit, 1 μg gyrase B subunit, and 60 ng DnaA protein. Monoclonal antibodies were added to DnaA protein and followed by incubating on ice for 15 min to assay their inhibitory effects. Incubation was at 30 °C for 25 min. 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 90 mM Tris-borate pH 8.3, and 1 mM EDTA. DNA was detected by ethidium bromide staining.

Two stage reactions (25 μl) were performed essentially as described in [21] and contained 30 mM HEPES-KOH pH 8.0, 0.4 mg/ml BSA, 20% glycerol, 5 mM EDTA, 6 mM $CaCl_2$, 0.4 mM ATP, 80 ng M13*oriC*2LB5 DNA, and the above proteins. Monoclonal antibodies were added to DnaA protein and incubated for 15 min on ice. Reactions were incubated for 30 min at 30 °C, then placed on ice to add 500 ng gyrase A subunit, 1 μg gyrase B subunit, magnesium acetate to 10 mM, and ATP to 2mM. Incubation followed for 10 min at 24 °C. Na_2EDTA to 50 mM and SDS to 2.5% were added and electrophoresed as above.

Enzyme-linked Immunosorbent Assay (ELISA): DnaA protein (0.5μg /well) or BSA (10μg /well) was added to 96 well microtiter trays (Nunc-Immuno

Plate, Maxisorp, Inter-Med) in 50 μl of buffer containing 0.137 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4 pH 7.3 (PBS). Incubation was for 1h at room temperature. Unbound proteins were removed with four successive washes (200 μl each) of PBS containing 0.2% (w/v) BSA. The last wash was incubated for 1 h before removal. The indicated monoclonal antibodies diluted in PBS containing 0.2% BSA was added, incubated for 1 hour, and followed by three washes to remove unbound antibodies. DnaB protein (30 ng in 50 μl of PBS containing 0.2% BSA) was then added and incubated for 15 min. Glutaraldehyde (2 μl of a 2.5% solution in water) was added to 0.1%, followed by incubation for 30 min. The wells were then washed with 200 μl of PBS containing 25 mM Tris-HCl pH 7.5, and 0.2% BSA once, then three times with 200 μl of PBS containing 0.2% BSA. Rabbit antisera to DnaB protein at 10^4 fold dilution in 100 μl PBS containing 0.2% BSA was added, incubated at °C overnight, and followed by three washes to remove unbound antibodies. Antibody-antigen complexes were detected after incubation for 1 h with goat anti-rabbit IgG horseradish peroxidase conjugate. Colorimetric detection was with O-phenylenediamine (0.4 mg/ml) and hydrogen peroxide (0.3% v/v) in 100 μl of 50 mM sodium citrate, pH 4.0. After addition of 4 N sulfuric acid, absorbance was measured at 490 nm with a BioTek EL 310 plate reader.

2.3 Results

2.3.1 Identification of continuous epitopes

To identify the epitopes recognized by monoclonal antibodies to DnaA protein, a peptide library was constructed by fusing DNA fragments produced by DNase I treatment of the *dnaA* gene to part of T7 gene 10 encoding the N-terminal 260 amino acids. Expression of the fusion protein depends on the T7 RNA polymerase gene under *lacUV5* promoter control. Transformants ($2\text{-}5\times10^3$ per antibody) were replicated onto nitrocellulose filters, lysed with chloroform vapor, then screened with each antibody to identify immunoreactive clones. In addition, transformants recognized by one antibody were tested for reactivity with other appropriate antibodies. For example, transformants bound by A3 were also found to react with M100 (Table 1). Similarly, M12 and M36 bound to the same set of transformants. Clone 7 was obtained by its immunoreactivity to a monoclonal antibody not described here and was characterized early in this work. Transformants of clone 7 were bound by antibodies M85, M48, M43, M100 and M1. Table 1 listed the immunoreactivity of antibodies to various recombinants that were useful in defining the minimal epitopes. This approach is designed to identify continuous epitopes. It also assumes that amino acids from T7 gene 10 protein do not contribute to the epitopes.

Immunoreactive clones were obtained with most antibodies except for A22, and M7. The portion of DnaA protein in the fusion was deduced by DNA sequence analysis of inserts in recombinant plasmids. M1, M10, M12, M36,

M43, M48, M85 recognize linear epitopes located between residues 125-146. A3 and M100 recognize residues 104-114 and 110-114, respectively. The epitope for M60 resides in residues 86-98.

Table 2.1: Deduced epitopes of monoclonal antibodies to DnaA protein

Monoclonal Antibodies	Immunoreactive Clones[a]	Amino Acid Residues	Deduced Epitopes[b]
M36	M36-3	111-148	
	M48-4	111-146	
	M48-5	125-148	125-146
M12	M48-4	111-146	
	M48-5	125-148	125-146
	M36-3	111-148	
M85	M85-1	120-154	
	M85-3	101-144	
	M85-12	118-138	133-138
	Clone-7	133-147	
M48	M48-4	111-146	
	M48-5	125-148	
	Clone-7	133-147	133-146
M43	M43-1	131-146	
	M43-3	131-146	
	M43-4	111-141	133-141
	Clone-7	133-147	
M10	M10-6	113-146	
	M10-7	120-142	133-142
	Clone-7	133-147	
M1	M48-4	111-146	
	M48-5	125-148	133-146
	Clone-7	133-147	
A3	A3-2	94-117	
	A3-1	84-114	104-114
	M100-4	104-119	
M100	M100-4	104-119	
	M100-6	110-130	
	M100-9	101-126	110-114
	M100-11	110-127	
	A3-1	84-114	
	A3-2	94-117	
M60	M60-2	86-98	
	M60-5	70-100	86-98
	M60-6	76-112	
M7	M36-3	111-148	111-148

Continuation of Table 2.1

[a]The nomenclature of an immunoreactive transformant correlates the antibody that originally identified it.

[b]Deduced epitopes are indicated relative to the amino acid sequence of DnaA protein.

[c]Amino acids from residues 125-146 are EPTYRSNVNVKHTFDNFVEGKS, from 104-114 are TQPQRAAPSTR, from 86-98 are TPQAAVTSNVAAP.

2.3.2 A22 and M7 appear to recognize conformational epitopes

Immunoblot experiments indicated that A22, M7, and M48 were comparably immunoreactive to full length DnaA protein. However, A22 was not reactive to transformants of Table 1 that expressed fusion proteins of T7 gene 10 and portions of *dnaA* gene (data not shown). M7 was only reactive to the transformant 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 [45]. Based on this reasoning, about 40 positive clones were expected if A22 recognizes a continuous epitope. As recombinant plasmids were constructed to contain 50-150 bp of the *dnaA* gene, these results suggest that the epitope recognized by A22 resides in a longer region and may be conformational.

A collection of nonsense mutants of the *dnaA* gene have been obtained (M. Sutton and J. Kaguni, manuscript in preparation). In a non-suppressing strain, one encodes a truncated polypeptide of 147 amino acids, established as an ochre mutant by DNA sequence analysis and confirmed by size 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 as well as other mutants with nonsense codons located more distantly. As a control, M48, whose epitope resides within residues 133-146, recognizes the 147

residue long ochre peptide.

M7 and A22 bind to the 147 amino acid long ocher peptide (Fig. 2.1) and longer nonsense peptides (data not shown). However, the response of M7 to the 147 amino acid peptide was much weaker than to the full length DnaA protein (Fig. 2.1). This result suggests that the epitope recognized by M7 is not entirely in the first 147 amino acids, or that its conformation after transfer results in less binding.

Whereas these results suggest that the epitopes recognized by A22 and M7 are conformational and in the N-terminal region, they are not identical. First, the response of A22 to the ochre peptide was stronger than that of M7 (Fig. 2.1). Second, by immunoblotting M7 bound to the fusion protein encoded by plasmid M36-3 (encoding residues 111-148 of DnaA protein) whereas A22 did not. M7 and A22 also bound to a polypeptide (data not shown) which is encoded by an in-frame deletion mutant that lacks of amino acids 220-294 (J. Lipar and J. M. Kaguni, unpublished results). By comparison, neither antibody was immunoreactive with transformants containing plasmid M85-12 (encoding a fusion protein containing residues 118-138 of DnaA protein), M48-5 (residues 125-148), clone 7 (residues 133-147), M10-5 (residues 237-245), M10-6 (residues 113-146), M10-7 (residues 120-142), M62 (residues 86-98), or M60-6 (residues 76-112). The lack of immunoreactivity with these recombinants suggests that the epitope recognized by M7 and contained in plasmid M36-3 is within residues 111-148. The epitope recognized by A22 appears to be within the first 147 residues.

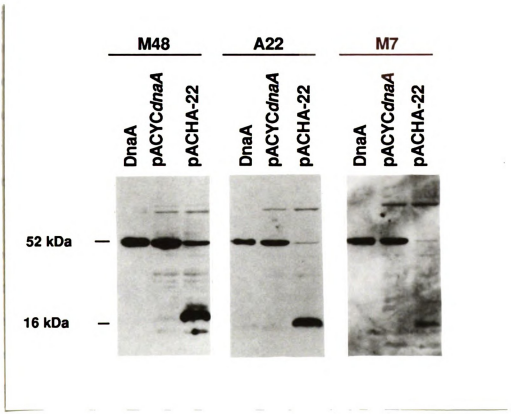

Figure 2.1: Monoclonal antibody M7 and A22 recognize conformational epitopes Whole cell lysates were prepared from *E. coli* HMS174 harboring the $dnaA^+$ gene (pACYC*dnaA*) or an ochre mutant at codon 148 (pACHA-22) in pACYC184 (M. Sutton, and J. M. Kaguni, manuscript in preparation). Purified DnaA 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 of Fig. 2.1 may be due to dissimilar electrophoretic conditions, despite precautions to treat each gel identically. (This experiment was performed by C. Margulies who was a coworker on this project)

2.3.3 DNA binding is not inhibited by monoclonal antibodies

Filter binding assays with a labeled restriction fragment containing the *oriC* sequence reveal that none of the antibodies substantially reduced DNA binding (data not shown, summarized in Table 2.3). At a saturating level of DnaA protein and in the absence of antibody, about 5.2 ng of the 6 ng *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 was regardless of the order of addition of DnaA protein, and antibodies.

Fragment mobility shift assays were also performed with a restriction digestion of a *oriC*-containing plasmid. Addition of M100, M85, and A3 as well as the remaining antibodies did not affect the DNA binding activity of DnaA protein measured by this assay (data not shown). 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.

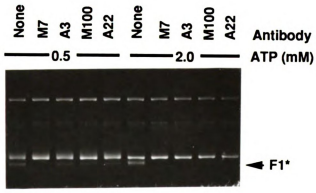

Figure 2.2: DNA binding activity is not inhibited by monoclonal antibodies Reactions (see "Experimental Procedures") contained the indicated amount of antibodies and DnaA protein. Antibodies were incubated with DnaA protein to measure their effects on DNA binding. (This experiment was performed by J. Marszalek who was a coworker on this project.)

2.3.4 ATP binding is not inhibited by monoclonal antibodies

DnaA protein binds to ATP with high affinity (K_D of 0.03 μM). The ATP-bound form is active in replication. Comparative sequence analysis revealed a conserved P-loop motif (GX_4GKT) found in many nucleotide binding proteins. The effect of monoclonal antibodies on ATP binding was examined to determine if any were inhibitory. M85 did not inhibit ATP binding (Fig. 2.1). None of other antibodies were found to reduce ATP binding activity by greater than 15% under conditions in which 0.3 ATP were bound per monomer of DnaA protein (summarized in Table 2.3)

2.3.5 Several monoclonal antibodies inhibit *oriC* replication and ABC priming

Six monoclonal antibodies inhibited DNA replication of an *oriC* containing plasmid (Fig. 2.4). M7 and A22 inhibited to near background levels at a ratio of two DnaA protein molecules per antibody molecule. By comparison, inhibition by M1, A3, M100, and M60 required higher antibody 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, inhibition was for another cause.

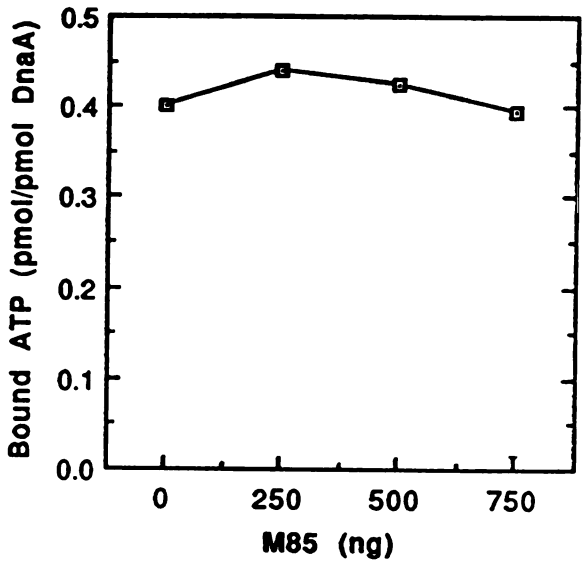

Figure 2.3: M85 do not inhibit the ATP binding activity of DnaA protein DnaA protein (2 pmol) was incubated with increasing amount of M85 at 0 °C for 15 minutes, then 0.2 μM ($\alpha-^{32}$P) ATP was added. After 10 min incubation on ice, the reaction products were filtered through nitrocellulose filters and radioactivity retained on the filters were counted.

The monoclonal antibodies were also tested for inhibition of ABC priming. This assay measures the replication activity of DnaA protein with a single-stranded M13 derivative harboring a DnaA box in a proposed hairpin structure. DNA synthesis also requires single strand DNA binding protein, DnaB, and DnaC, primase and DNA polymerase III holoenzyme. In contrast to the antibodies that inhibit *oriC* replication, only M7 and A22 inhibited ABC priming substantially (Table 2.2). Other antibodies were only slightly inhibitory (data not shown). Previous studies showed that M7 interfered with the interaction between DnaA protein and DnaB protein in DnaB-DnaC complex only if M7 was added to DnaA first [22]. A22 may inhibit ABC priming by a similar mechanism as inhibition was dependent on the order of addition (Table 2.2). Other antibodies that inhibit *oriC* replication do not inhibit ABC priming appreciably whether added before or after the BC complex (data not shown).

Table 2.2: The influence of monoclonal antibodies on ABC priming[a,b]

Incubation A	Incubation B	DNA Synthesis(pmol)	Relative Activity
None	DnaB+DnaC	243	100%
M7	DnaB+DnaC	21	8
DnaB+DnaC	M7	181	74
None	DnaB+DnaC	231	100
A22	DnaB+DnaC	90	40
DnaB+DnaC	A22	175	77

Incubation A	Incubation B	DNA synthesis(pmol)	Relative Activity
None	DnaB+DnaC	230	100%
M1	DnaB+DnaC	207	90
DnaB+DnaC	M1	185	80
A3	DnaB+DnaC	193	84
DnaB+DnaC	A3	221	96
M60	DnaB+DnaC	260	113
DnaB+DnaC	M60	240	104

a Reactions assembled on ice contained M13 A-site ss DNA, ribo- and deoxy-ribonucleotides, magnesium acetate and DnaA protein at amounts used for ABC priming (see "Experimental Procedures"). The indicated monoclonal antibodies (100 ng each), DnaB, and DnaC protein (as indicated, 50 and 24 ng each, respectively) were added in incubations (A or B), each for 10 min at 30 °C. DNA synthesis was then measured after addition of primase (10 ng) and DNA polymerase III holoenzyme (80 ng) and incubation at 30 °C for 30 min.
bThese experiments were performed by Jarek Marszalek who was a coworker on this project.

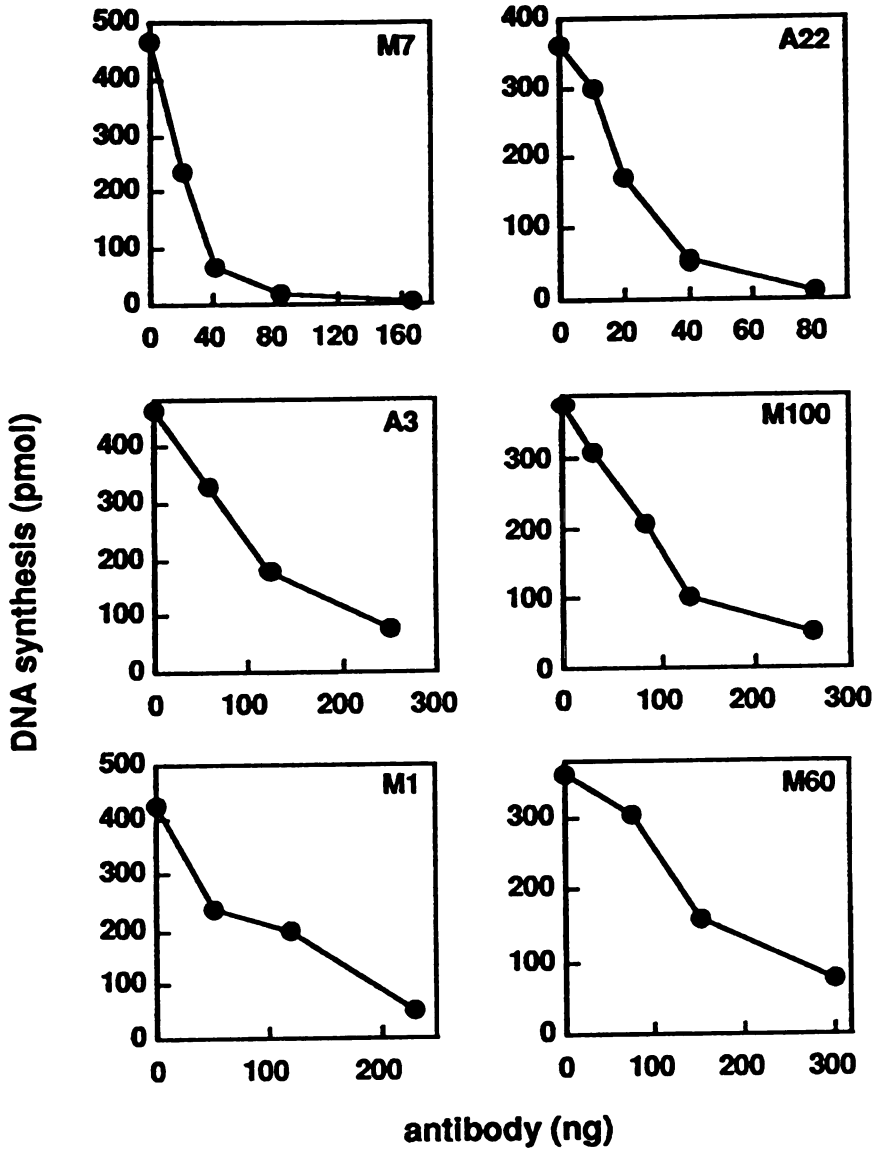

Figure 2.4: Inhibition of *oriC* replication by monoclonal antibodies. Reactions were assembled as described in "Experimental Procedures" and contained the indicated amounts of monoclonal antibodies. (This experiment was performed by J. Marszalek who was a coworker on this project.)

2.3.6 Antibody inhibition of DNA unwinding by DnaA protein

It is possible that the antibodies which specifically inhibit *oriC* replication block one or more functions of DnaA protein not essential for ABC priming. Bound to *oriC*, DnaA protein induces a localized unwinding at the three AT rich 13-mers [46], followed by binding of DnaB helicase to unwind further the parental duplex. Unwinding is detected by its sensitivity to a ssDNA specific nuclease [46, 26]. Alternatively, addition of DNA gyrase to remove the positive superhelicity in the DNA generated by DnaB helicase results in a highly negatively supercoiled DNA [21]. This topological form, termed F1*, migrates more rapidly on an agarose gel than the supercoiled plasmid DNA isolated from *E. coli*. 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 (data not shown). The remaining antibodies were not.

This assay was simplified to involve one incubation instead of two. ATP was sufficient at 0.5 mM or greater with a minimal incubation of 10 min (data not shown). Under these conditions, M7 was inhibitory at 0.5 or 2 mM ATP, whereas inhibition of by other antibodies (A22, M100, and A3) was more effective at the higher concentration (Fig. 2.5). This finding suggests that incubation of DnaA protein with 2 mM ATP may induce a conformation more favorable for antibody

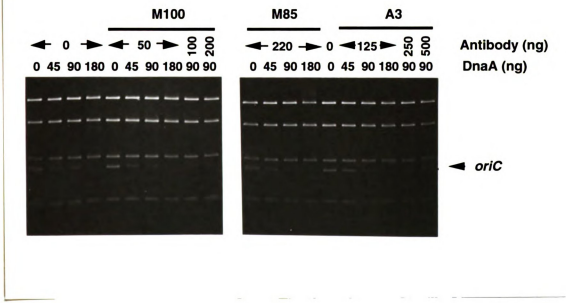

Figure 2.5: M7, A3, M100 and A22 inhibit F1* formation at 2 mM ATP. Reactions contained M7, 250 ng; A3, 250 ng; M100, 200ng; or A22, 160 ng; and ATP as indicated in a one stage incubation. (This experiment was performed by J. Marszalek who was a coworker on this project.)

binding. It also supports the suggestion that a low affinity ATP binding site exists [46] in addition to the site that confers high affinity ATP binding. The greater inhibition observed at 2 mM ATP may also explain the modest inhibition observed in the two stage incubation in which ATP was at 0.4 mM during this stage. Inhibition by A22 and M100, as well as M7 and A3 was proportional to the amount added (data not shown). The remaining antibodies were not inhibitory even at 5-fold higher levels than those used here. That M1 and M60 inhibited *oriC* replication, but ABC priming only poorly, and failed to inhibit F1* formation, suggests that they affect an unknown activity of DnaA, perhaps subsequent to unwinding.

2.3.7 Antibodies inhibit the interaction between DnaA and DnaB

DnaA protein has been shown to interact physically with DnaB protein by use of an ELISA assay [22]. In this method, immobilized DnaA protein is incubated with either DnaB alone, or as a complex with DnaC protein. The complex of DnaB bound to DnaA protein is stabilized by glutaraldehyde cross-linking. Crosslinking of DnaC protein to DnaA and BSA was not observed .

M7 inhibited the interaction between DnaA and DnaB in this assay (Fig. 2.8), this result is consistent with previous results [22]. Examination of other monoclonal antibodies indicated that they are also inhibitory (Figs. 2.7, 2.8). As a control, a monoclonal antibody to rat brain hexokinse had little effect on the binding of DnaB protein to immobilized DnaA protein (Fig. 2.6). Whereas

the ELISA method demonstrated a specific physical interaction between the two proteins, it failed to correlate the inhibition of replication with the inhibition of DnaB binding. Inhibition of the interaction between DnaA and DnaB may be due to steric hindrance. For example, binding of a divalent antibody to immobilized DnaA protein may produce a network that occludes binding of DnaB protein. Fab fragments may correlate inhibition of replication to inhibition of DnaB binding, but this has not yet been tested.

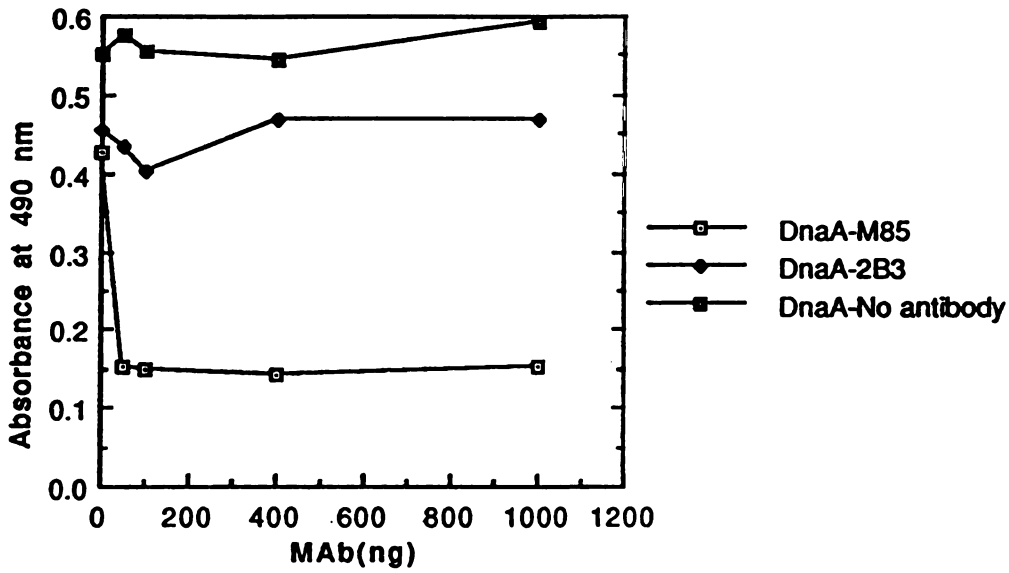

Figure 2.6: Monoclonal antibody 2B (against hexokinase) does not inhibit the interaction between DnaA and DnaB.
DnaA protein (0.5 μg/well) or BSA (10 μg/well) was added to microtiter plates. Monoclonal antibody 2B or M85 was then added at the indicated amounts, followed by incubation for 1 h to allow binding. Unbound antibodies were removed by three successive washes with PBS containing 0.2% BSA. 50 ng of DnaB protein was added and allowed to bind for 15 min. After cross-linking with glutaraldehyde, the unbound protein was removed and DnaB protein was detected by ELISA with rabbit anti-DnaB antisera.

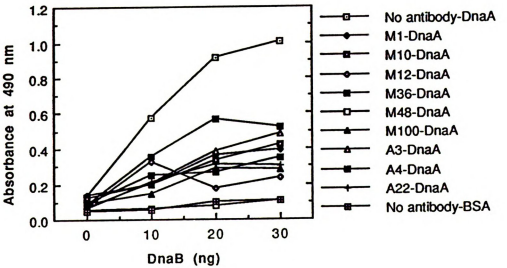

Figure 2.7: Monoclonal antibodies M1, M10, M12, M36, M48, M100 and A3 inhibit the interaction between DnaA and DnaB protein.
DnaA protein (0.5 μg/well) or BSA (10 μg/well) was added to microtiter plates. Monoclonal antibodies at the indicated amounts were then added, followed by incubation for 1 h to allow binding. Unbound antibodies were washed away by three successive washes with PBS containing 0.2% BSA. The indicated amounts of DnaB protein were added and allowed to bind for 15 min. After cross-linking with glutaraldehyde, the unbound protein was removed and DnaB protein was detected by ELISA with rabbit anti-DnaB antisera.

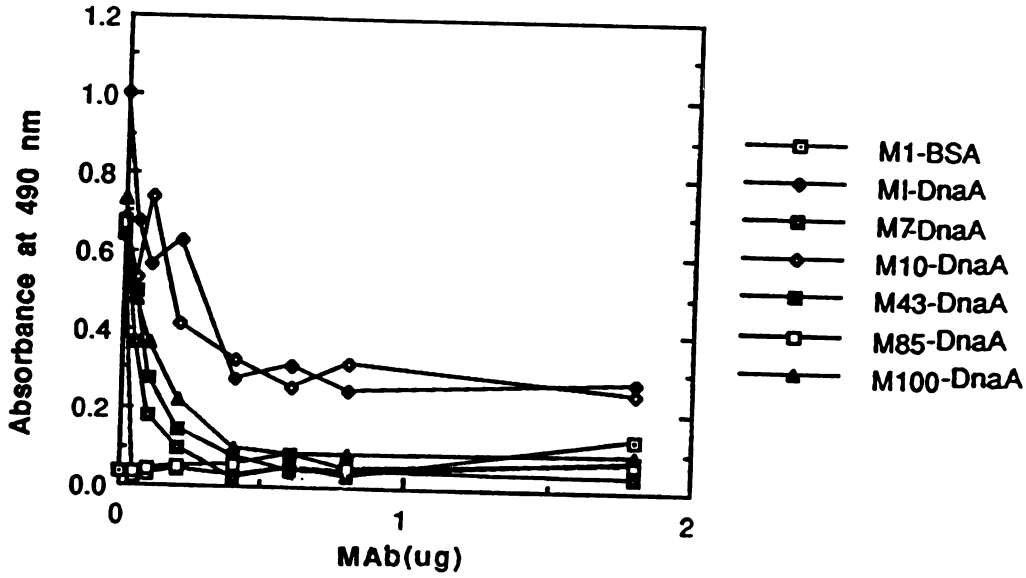

Figure 2.8: Monoclonal antibodies M1, M7, M10, M43 and M85 interfere with the interaction between DnaA and DnaB proteins.
DnaA protein (0.5 μg/well) or BSA (10 μg/well) was added to microtiter plates. Monoclonal antibodies were then added at the indicated amounts, followed by incubation for 1 h to allow binding. Unbound antibodies were removed by three successive washes with PBS containing 0.2% BSA. 50 ng of DnaB protein was added and allowed to bind for 15 min. After cross-linking with glutaraldehyde, the unbound protein was removed and DnaB protein was detected by ELISA with rabbit anti-DnaB antisera.

Table 2.3: Summary of the linear epitopes and the inhibitory effects of monoclonal antibodies to DnaA protein

Monoclonal antibodies[b]	Epitope (a.a.)	*oriC* replication	ABC priming replication	F1* formation	DNA binding	ATP binding
M36, IgG_{2B}	125-146	-	-	-	-	-
M12, IgG_1	125-146	-	-	-	-	-
M85, IgG_{2B}	133-138	-	-	-	-	-
M48, IgG_1	133-146	-	-	-	-	-
M43, IgG_1	133-141	-	-	-	-	-
M10, IgG_3	133-142	-	-	-	-	-
A3, IgG_1	104-114	+	-	+	-	-
M100, IgG_1	110-114	+	-	+	-	-
M1, IgG_3	133-146	+	-	-	-	-
M60, IgG_3	86-98	+	-	-	-	-
M7, IgG_{2A}	confro.	+++	+++	+++	-	-
A22, IgG_1	confro.	++	++	++	-	-

[a]*oriC* replication and F1* formation was performed by a Jarek Marszalek who was a coworker on this project

[b]The monoclonal antibodies were classified by an ELISA method with preparations of goat anti-mouse antibodies that are specific for each immunoglobulin subclass.

[c]+++ very strong inhibitory
++ strong inhibitory
- not inhibitory

2.4 Discussion

In order to correlate the structure of DnaA protein to its various functions, the inhibitory effect of monoclonal antibodies to DnaA protein was characterized. The epitopes for these monoclonal antibodies were precisely mapped by constructing and screening a peptide library derived from portions of the *dnaA* gene. The epitopes were deduced from the DNA sequence of the inserted fragment of recombinant plasmids isolated from positive clones. Most antibodies appear to recognize linear epitopes located within a small region near the N-terminus. The clustering of epitopes suggests that this region is highly antigenic, and surface exposed in the native conformation of DnaA protein. The epitopes of M7 and A22 are conformational and reside in longer amino acid sequences.

None of the antibodies inhibit DNA binding, which suggests that the region containing various epitopes (residues 86-146) is not involved in DNA binding. This finding is consistent with the observation that the C-terminal 93 amino acid residues are responsible for DNA binding activity. First, fusion protein containing the C-terminal region of DnaA protein from residue 379 to the end are sufficient for *oriC* DNA fragment binding [32]. Second, nonsense and missense mutants that affect the C-terminal region of DnaA protein are defective in DNA binding (M. D. Sutton and J. M. Kaguni, manuscript in preparation)

In DnaA protein, the P-loop motif, GX_4GKT located at residues 172-179, is found in many ATP binding proteins (reviewed in [28]). The structure of the P-loop has been determined by X-ray crystallographic analysis of adenylate

kinase [34, 35], elongation factor Tu [36], RecA [30] and *ras* 21 protein [31]. In *ras* 21, the conserved residues interact with the γ-phosphate of the bound nucleotide and with a magnesium ion that chelates the β- and γ-phosphates [31]. Mutagenic analysis of RecA protein [37, 38] supports the conclusion that the corresponding residues interact with the α- and β-phosphates of ATP. None of the antibodies described here inhibit high affinity ATP binding. This result is consistent with the location of respective epitopes that do not overlap the P-loop motif. In addition, the residues bound by respective antibodies are not apparently involved in interacting with adenine of ATP nor does antibody binding appear to interfere sterically or to alter the conformation of the ATP binding domain of DnaA protein.

Inhibition by M7 and A22 Antibodies M7 and A22 inhibit DnaA protein in *oriC* replication, ABC priming, and F1* 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. M7 had previously been shown to inhibit the binding of DnaB protein (from DnaB-DnaC complex) to a replication intermediate of DnaA protein bound to *oriC* [22]. By a modified ELISA procedure, M7 was shown to interfere with the binding of DnaB protein to immobilized DnaA protein [22]. The epitope recognized by M7 is 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 may be involved in the interaction between DnaA protein and DnaB protein.

Antibody binding can also induce a conformational change [39, 40]. An

alternative possibility that we can not exclude is that the binding of M7 induces a conformational change that interferes with the interaction between these two proteins.

Inhibition by A3 and M100 High affinity binding of ATP to DnaA protein is essential for its activity in inducing duplex opening at the AT-rich 13-mers in *oriC* [29, 41]. By comparison, the ADP-bound form of DnaA protein is relatively inert. Because unwinding is not required in ABC priming, both nucleotide bound- forms are active in ABC priming [44]. A3 and M100 inhibit *oriC* replication and F1* formation, but ABC priming only poorly. These observations suggest that A3 and M100 inhibit *oriC* replication by affecting the unwinding activity of DnaA protein. If so, residues bound by A3 and M100 are involved in unwinding.

Inhibition by M1 and M60 The initiation process at *oriC* involves three consecutive steps. First, DnaA protein in the ATP-bound form binds to the DnaA boxes. Second, DnaA protein induces the localized duplex opening at the AT-rich 13-mers. Third, DnaB protein binds to the *oriC* through a direct interaction with DnaA protein. The least understood result in this study is the inhibition pattern of M1 and M60. The two antibodies inhibit *oriC* replication, yet do not inhibit ABC priming and F1* formation. The ABC priming assay reflects DnaA protein's activity of binding to DNA and DnaB protein. F1* formation additionally measures the unwinding activity of DnaA protein.

The lack of effect of these antibodies in these two assays suggests inhibition of an activity subsequent to unwinding. No known biochemical activity of DnaA

protein has been identified at a step after unwinding and interaction with DnaB protein. However, genetic studies of extragenic suppressors of *dnaX* that map to the *dnaA* gene [42, 43] suggest an interaction between DnaA protein and the *dnaX* gene products, τ and γ, which are components of DNA polymerase III holoenzyme. However, the extragenic suppressors of *dnaX* have been characterized as missense and nonsense mutations at residues 213, 432, and 435 of DnaA protein [43]. This result is inconsistent with the epitopes bound by M1 and M60. Another inconsistency is that other antibodies bind to the same epitope as M1 are not inhibitory. This may be explained by a specific conformational change induced by M1 binding to result in inhibition. It is also possible that the actual epitope for M1 is a few residues different from that of the other monoclonal antibodies although the epitopes for all of them have been mapped in the same region. 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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Chapter 3

Summary and Perspective

The replication of the *E. coli* chromosome begins at a unique site, *oriC*, and is a precisely regulated event in the bacterial cell cycle. Regulation occurs at the time of initiation of chromosomal replication. Of over 20 genes whose products participate in this process, the *dnaA* gene that encodes DnaA protein is unique because of its central role in initiation. Because of this and because DnaA protein may regulate the process, it has been the focus of study in a number of laboratories.

Biochemical approaches have revealed that DnaA protein is multi-functional. First, it is a sequence-specific DNA binding protein, recognizing and binding to 9-mer sequences, termed as the DnaA boxes. DnaA protein binds to the four DnaA boxes in *oriC* in an ordered manner. Upon binding, it induces a localized duplex opening at the AT-rich 13-mers near the left boundary of *oriC*. It is proposed to guide the binding of DnaB helicase to *oriC* through a direct physical interaction with DnaB protein. DnaA protein also binds ATP and ADP with high affinity. The ATP-bound form is active in inducing duplex

unwinding whereas the ADP-bound form is relatively inert. Acidic membrane phospholipids interact with DnaA protein to promote the rapid release of tightly bound ATP or ADP.

DnaA protein is ubiquitous among bacterial species. Sequence comparison of *dnaA* homologs revealed that conserved sequences are mainly in a short N-terminal domain and a long C-terminal domain. The multiple functions of DnaA protein suggest that it may be structurally composed of several domains, each participating in a specific biochemical function required for its activity in DNA replication.

In order to correlate the structure of DnaA protein to specific functions, monoclonal antibodies to DnaA protein were produced, and the inhibitory effects on activities of DNA binding, ATP binding, unwinding of *oriC*, and replication were characterized. None of the antibodies inhibited DNA binding and ATP binding. Monoclonal antibodies M7 and A22 inhibited replication of an *oriC* plasmid, ABC priming, and the formation of a highly unwound *oriC* plasmid (Form 1*) that is dependent on the initial unwinding of the 13-mers at the left boundary of *oriC* by DnaA protein. Monoclonal antibodies A3 and M100 inhibited *oriC* replication and F1* formation. M1 and M60 inhibited only *oriC* replication. The epitopes for these monoclonal antibodies have been determined by constructing and screening a DnaA peptide library. Monoclonal antibodies M1, M10, M12, M36, M43, M48, M60, M85, M100, and A3 recognize continuous epitopes clustered at the same region (from residues 86 to 148) of DnaA protein. M7 and A22 recognize conformational epitopes within the N-terminal

147 amino acids.

These studies suggest that amino acids 86-148 are not involved in DNA binding and ATP binding, in agreement with other studies that localize the DNA binding domain to the C-terminal 93 amino acid residues, and the ATP binding domain to residues 172-179, containing a P-loop motif found in many nucleotide binding proteins. Theses studies also suggest that regions of DnaA protein are involved in interaction with DnaB protein, and in either unwinding of *oriC*, or low-affinity binding of ATP. DnaA protein may also interact with subunits of DNA polymerase III holoenzyme.

Most of the monoclonal antibodies bind to linear epitopes located in a region encompassing amino acid residues 86 to 148. This feature that limits the correlation of structure to functional activities of DnaA protein can be overcome by generating other monoclonal antibodies that bind to different regions of DnaA protein. The region of DnaA protein involved in the interaction of acidic phospholipids has not been determined. Characterization of these antibodies as well as others (not yet prepared) that bind elsewhere may be useful in identifying the domain involved in this function. Findings from this study may relate to the role of the cell membrane in DNA replication. That *E. coli* DNA replication is membrane-associated has been well documented but the molecular basis is not understood.

An ELISA assay was used to measure the interaction between DnaA and DnaB protein. With this assay, all antibodies inhibited the binding of DnaB to immobilized DnaA protein, whereas only a few inhibited the replication activity

of DnaA protein. Of the latter class, M7 was shown to inhibit binding of the DnaB-DnaC complex to DnaA protein bound to an *oriC* plasmid, or M13 containing a DnaA box motif in a hairpin structure. Apparently, the monoclonal antibodies that did not inhibit replication may block the binding of DnaA to DnaB for steric reasons by ELISA. Because the molecular weight of an IgG molecule is about 3 times larger than that of DnaA protein, the use of smaller Fab fragments in the assay might allow us to correlate directly the inhibition of replication activity with the inhibition of DnaB binding.

The inhibition patterns of M1 and M60 suggest that DnaA protein is probably involved in replication at a step after unwinding and interacting with DnaB protein. Genetic studies suggest that DnaA protein interacts with the subunits of DNA polymerase III holoenzyme. Immunoprecipitation and cross-linking ELISA can be used to investigate further the interaction of DnaA protein with other replication proteins including subunits of polymerase III holoenzyme, SSB, Primase, DNA gyrase, RNA polymerase and others.