Characterization of DnaAΔ96-120:
The role of domain II of DnaA in replication initiation

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ABSTRACT

A 25 amino acid deletion in domain II of the *Escherichia coli* replication initiator protein DnaA was characterized in order to further understand the mechanism by which DnaA serves as a master regulator protein of DNA replication initiation in bacteria. DnaA plays a major role in the assembly of the replisome during DNA replication and the 4 domains of DnaA has been extensively studied with the exception of domain II which was initially characterized as a non-essential flexible linker region. Mutant *dnaAΔ96-120* was found to serve as a suppressor of mutant *seqA* phenotypes that have been shown to cause excessive over-initiation of replication (Sutera and Lovett, 2006). This suggests that this 25 amino acid region of domain II is somehow involved in the mechanisms of DNA replication initiation. As DnaA is known to interact with DtaA (*DnaA*-initiator-associating factor), DNA at the origin, and DnaB and DnaC in the assembly of the replication machinery, these interactions were of particular interest in determining why the deletion of residues 96-120 of domain II mitigates such over-replication. Through colony morphology studies, DNA damage assays, and flow cytometric analysis, it was confirmed that *dnaAΔ96-120* results in under-initiation of DNA replication and causes suppression of the mutant *seqA* phenotype. Additionally, DNA damage survival assays and flow cytometry suggest that *dnaA* and *diaA* are epistatic. Through protein-protein interaction experiments it was determined that the deletion of 25 amino acids of domain II did not inhibit binding of DnaA to DtaA. Flow cytometry and DNA damage survival assays suggest that *dnaAΔ96-120* impacts DNA replication by causing under-initiation and that the non-essential domain II plays a regulatory role in initiation of replication.
INTRODUCTION

In order for a species to survive they must have successfully acquired a way to pass genetic material from one generation to the next. The fidelity and successful completion of replication of the genome are necessary in all organisms for the transmission of appropriate genetic material from mother to daughter cell and to ensure that growth of the organism proceeds. In the prokaryotic *Escherichia coli (E. coli)* replication of the single, circular chromosome occurs bi-directionally from the chromosomal origin of replication, oriC, to the terminus, ter. In nutrient rich media, replication and division of *E. coli* can occur approximately every twenty minutes. The rate at which DNA replication occurs is directly related to the frequency of initiation and thus the overall rate of DNA synthesis (Messer 1987). The timing between rounds of replication, known as the eclipse period, is central to the survival of the organism (Boye, *et al.* 2000). This timing relies on a number of positive and negative regulation mechanisms that are central to the process of replication.

*oriC*'s role as the origin of replication is due in large part to its dense regulatory signal sequence content. Approximately 245 nucleotide base pairs in length, *oriC* contains an AT-rich region consisting of three 13-mer repeats and 5 binding sites for the sole initiator protein in *E. coli* DnaA, the product of the gcnc *dnaA* (Messer 2002). These fully methylated binding sites, termed DnaA boxes and called R1, M, R2, R3 and R4 are 9 nucleotide base pair nonpalindromic sequences of the general consensus 5'-TT^A/T-eNcA-eA3'. Monomeric DnaA protein has a high affinity for the five DnaA boxes in either its ATP or ADP bound state. DnaA binds to the R1, R2, and R4 sites throughout the majority of the cell cycle (Zakrzewska-Czerwinska, *et al.* 2007; Asklund and Aflung, 2009).
2005; Yokoyama, et al. 2003). In addition to the five high affinity DnaA boxes, 3 low affinity ATP-DnaA boxes, L, M, and R, are present in the AT-rich region of oriC. ATP bound DnaA binds to these regions of the general sequence 5' - AGATCT-3' in a cooperative fashion. In addition to the DnaA and ATP-DnaA boxes located within oriC, 11 recognition sequences for Dam (DNA adenine methyltransferase) are present, playing a crucial role in the negative regulation of initiation (Messer 2002). Additional regulatory sequences are present throughout oriC.

**Replication Initiation**

Initiation of replication begins with the binding of the 52 kDa protein DnaA to oriC and the subsequent recruitment of a number of replication factors for the assembly of the replisome. The assembly of multiple, active ATP-DnaA molecules on oriC is stimulated by homotetramers of the DnaA-binding protein DiaA (Keyamura, et al. 2007). DiaA tetramers serve to stabilize and enhance the binding of multiple ATP-DnaA molecules to the low affinity DnaA boxes of oriC and therefore, DiaA serves an important role in positively regulating initiation and in coordination of the cell cycle (Katayama 2008). Though DnaA can bind to oriC in both its ADP and ATP bound states, only ATP-bound DnaA is essential and active in replication (Speck and Messer 2001; Messer 2002). As each molecule of the ATP complexed DnaA protein binds to the DnaA boxes in oriC a 40° bend is introduced at each site unwinding a 28 nucleotide base pair section in the AT-rich region. This open region is expanded to 44-46 base pairs (bp) when single stranded DNA-binding protein (SSB) is introduced (Messer 2002; Speck and Messer 2001). The remodeling of oriC to the open complex is mediated by approximately 20-30 DnaA monomers.
Following the conversion of oriC to the open complex, two double hexamers of inactive DnaB helicase bound to the DnaC helicase loader are recruited and positioned by DnaA into the unwound AT-rich DNA-unwinding element (Mott and Berger 2007). One complex is loaded for each of the replication forks that will travel bi-directionally from oriC to ter. ATP hydrolysis then activates the helicase activity of DnaB and releases DnaC from the complex allowing the two DnaB hexamers to move past each other in the 5' to 3' direction expanding the opening to 65 bp. This expansion is followed by the assembly of a DnaB and DnaG primase complex on the single stranded DNA. This complex synthesizes leading strand ribonucleic acid (RNA) primers. The sliding clamp is then assembled on the RNA-primed DNA as a homodimer of the β-subunit of DNA polymerase III (pol III) is loaded by the clamp loader, the pol III γ complex (Messer 2002; Katayama, et al. 1998). Assembly of the replisome is thus completed and DNA replication proceeds.

The structure of DnaA sheds light onto its complex function as the essential replication initiator protein. DnaA has been found to be comprised of four functional domains. Each functional domain has been characterized. Domain I, spanning residues 1-56, and part of Domain II, residues 57-129, is involved in the oligomerization of DnaA at oriC and the interaction with DnaB helicase that is required for initiation. Specifically, N-terminal amino acid residues 2-86 are involved in oligomerization of DnaA and residues 24-86 and 130-148 are required for the interaction with DnaB and form physical contacts between the two proteins (Messer, et al. 1999; Simmons, et al. 2003; Scitz, et al. 2000). Additionally, it has been shown that residues 111-148 or 130-219 of DnaA promote binding to DnaB in vitro (Scitz, et al. 2000). Though part of Domain II is
involved in the oligomerization of DnaA and the interaction with DnaB, the larger part of
the domain is classified as a flexible linker region and can tolerate deletions and
substitutions in this region (Messer et al. 1999). Domain III, residues 135-373, contains
ATP-interaction motifs of the AAA+ family and plays an important role in mediating
replication as it is responsible for ATP binding, ATP recognition in the initiation
complex, ATP hydrolysis, and also functions in DnaB loading, residues 135-148 are
particularly important for the interaction with DnaB (Katayama 2008; Messer, et al.
1999). Domain IV comprises 94 C-terminal amino acids from Val374 to Ser467 and is
required for the binding to DNA (Yoshida, et al. 2003).

**Replication Control**

For viability of the organism it is important that cell cycles are tightly regulated
and that sufficient time is allowed between replication cycles for growth. The cell has
several important mechanisms for controlling DNA replication and the eclipse period in
between rounds of replication. In *E. coli*, regulation of initiation occurs through titration
of DnaA molecules by the datA locus, the regulatory inactivation of DnaA (RIDA) by
ATP hydrolysis mediated by the sliding clamp Hda, and the sequestration of oriC by
important role at oriC for initiation they are not isolated to this region. Some 300 DnaA
boxes capable of binding DnaA are interspersed throughout the chromosome. At the datA
locus a particularly high capacity for binding DnaA is observed. This high binding
capacity serves to decrease the availability of DnaA molecules, thereby limiting the
number of DnaA molecules free to bind to oriC and regulating initiation (Messer 2002).
RIDA functions to regulate DNA replication through the hydrolysis of ATP-DnaA to the
Inactive ADP-DnaA which occurs when ATP-DnaA interacts with Hda in the presence of the pol III β-sliding clamp (Su’etsugu, et al. 2005).

In addition to regulation of initiation by way of DnaA, oriC is subject to regulation. Repeated, palindromic GATC sequences that serve as recognition sites for Dam are located in both oriC and the promoter region of dnaA (Katayama 2008; Messer 2002). Adenine residues in the 11 GATC sequences in oriC are methylated in both strands of parental DNA; full methylation of these sites is required for efficient initiation. Immediately following initiation the nascent strand of DNA is hemimethylated at these GATC sequences during the eclipse period, which can extend for one minute to one-third of the cell cycle (Messer 2002). SeqA has a high affinity for hemimethylated oriC, a lower affinity for the fully methylated form, and will not bind to unmethylated DNA (Messer 2002). Thus, following replication when the hemimethylated form of GATC sites persists, SeqA binds and sequesters oriC from action by DnaA and further rounds of replication allowing the cell to “catch up”. The duration of the eclipse period is influenced by the duration of hemimethylation and the concentration of Dam and thus dictates the time between rounds of initiation at an origin (Boye, et al. 2000). As an additional method of regulation, SeqA sequesters the dnaA promoter resulting in short term suppression of DnaA (Messer 2002). After cell division occurs the oriC and dnaA promoter are released from sequestration due to the full methylation by Dam and initiation can resume (Boye, et al. 2000). As a result of the important role SeqA plays in regulation, seqA mutant phenotypes are subject to gross over-initiation resulting in cells that grow poorly as the result of an insufficient amount of time for growth between replication events during the cell cycle. These seqA knockouts show an increased
sensitivity to DNA damaging agents such as azidothymidine (AZT), hydroxyurea, and UV light due to the convergence of multiple, uncontrolled replication forks upon sites of DNA damage (Sutera and Lovett 2006). DnaA and seqA are thus seen to play antagonistic roles in DNA replication. Mutant dnaA alleles, if not lethal, are observed to under-initiate rounds of replication and serve to alleviate the sensitivity resultant from the over initiation caused by mutant seqA phenotypes (Sutera and Lovett 2006).

Figure 1.- Replication Control by DnaA and SeqA

From Boye, et al. 2000

Figure 1.-

a) DnaA serves as the essential initiator protein of DNA replication and is active when bound to ATP though it also binds ADP. SeqA plays a role as a negative regulator of replication.
b) DnaA binds to the origin in its ATP bound form and causes unwinding of the AT-rich region of the origin. It then recruits DnaB bound to the DnaC helicase loader.
c) Binding of DnaB inactivates DnaA allowing for the rest of the replisome to assemble and replication to begin.
d) SeqA sequesters the origin by binding newly formed, hemimethylated DNA and preventing the initiation of subsequent rounds of replication.

As dnaA is an essential gene and the viability of the cell is dependent on its functionality, the fact that the deletion of 25 residues in domain II of the DnaA protein is
not only tolerated but serves as a suppressor of mutant seqA phenotypes presents an interesting question. Why does this mutant allele prove to be beneficial to the cell, displaying increased viability when exposed to DNA damaging agents and what are the specific interactions that involve this domain? The rather sweeping assessment of domain II as dispensable is inaccurate for mutations in this region have been shown to be beneficial to the cell in certain cases (Seitz, et al. 2000). For this reason the mutant allele of dnaA containing a deletion of 25 amino acids, residues 96-120, in domain II of the protein warrants further examination. To this end, dnaAΔ96-120 was isolated as a suppressor of seqA::FRT cat obgE::Tn5 which displays over-initiation of DNA replication as ObgE plays a role in replication fork stability, chromosome segregation, cell division and the timing of replication initiation (Foti, et al. 2005). Of particular interest in the characterization of domain II are the potential interactions and binding capacities of domain II DnaA mutants with DiaA, DnaB, and the DnaA binding sites of oriC.

MATERIALS AND METHODS

Media and Antibiotics

Bacteria were primarily grown in rich Luria-Bertani (LB) media containing 1% w/v tryptone, 0.5% w/v yeast extract, and 0.5% w/v sodium chloride. LB plate media was the same as above with the addition of 1.5% w/v agar. Minimal plate media contained 56/2 salts, 0.2% w/v glucose, 0.001% vitamin B1, and 2% w/v agar. LCG media used for the creation of P1 transducing lysates contained LB media with 0.1% glucose and 2mM calcium chloride. LCG top agar contained 0.7% w/v agar and LCG plate media contained
1% w/v agar. Selection of strains was achieved through the use of 100 µg/mL ampicillin (Ap), 15 µg/mL chloramphenicol (Cm), 30 µg/mL kanamycin (Kn), and 15 µg/mL tetracycline (Tc) when necessary.

**Bacterial Strains**

All bacterial strains used were *Escherichia coli* K-12 isogenic to MG1655. Strains were constructed by electroporation transformation and P1 transduction using P1 bacteriophage. Cell growth and culture density was determined by measuring the optical density at 600 nm (O.D.₆₀₀).

Growth curves were performed using a BioScreen C instrument at 25 °C and 37 °C in LB media. Multiple growth curves were averaged and then a semi-log plot was constructed for O.D.₆₀₀ versus time in hours. The doubling time was represented as 2/slope during exponential phase and converted into minutes.

The following strains and plasmids were used in the characterization of domain II of DnaA.
### Table 1. *Escherichia coli* K-12 Strains and Plasmids

<table>
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<th>Strain</th>
<th>Relevant Genotype</th>
<th>Source or Derivation</th>
</tr>
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<tbody>
<tr>
<td>STL2168</td>
<td>F- ompT hsdS(r, m) dcm' gal λ (DE3)</td>
<td>Cm' seqA gene disruption in MG1655</td>
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<td>STL7222</td>
<td>seqA::FRT cat</td>
<td>Mg1655</td>
</tr>
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<td>STL8297</td>
<td>dnaA46 tnaA::Tn10</td>
<td>Tc' transductant P1 NK9219 X Mg1655</td>
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<td>Tc' Kn' Cm' transductant P1 STL12842 X STL12697</td>
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</table>

^* oss denotes obgE::Tn5 seqA::FRT cat suppressor
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<tr>
<th>Plasmids</th>
<th>Relevant Genotype</th>
<th>Source or Derivation</th>
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<td>pDONR201</td>
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<td>Native <em>kan dnaA</em></td>
<td>Gateway™</td>
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<td>pSTL378</td>
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</table>

Isolates of the above strains were chosen from plates following construction and re-struck to single colonies using a sterile toothpick to ensure appropriate antibiotic resistances. At least 2 isolates of those re-struck and confirmed were then frozen for storage at -70°C in LB media with the addition of 15% w/v glycerol.

**PCR for DNA Sequencing**

DNA sequences, both chromosomal and plasmid, were amplified using PCR (polymerase chain reaction) and sent to the Dana Farber Cancer Institute for sequencing. PCR reactions for sequencing purposes were composed of sterile ddH$_2$O, 25mM magnesium chloride, 0.2mM dNTPs, approximately 2.5 ng/µl chromosomal DNA, 5 ng/µl primer, 1.25 units of GoTaq DNA polymerase (Promega Corporation), and 10 µl GoTaq Flexi Buffer.

Colony PCR was completed by streaking strains to single colonies on appropriate plate media. A single colony was then picked off the plate using a sterile toothpick and resuspended in 25 µl sterile ddH$_2$O and microwaved for 45 seconds at highest power setting. 5 µl of this resuspension was then used as the template DNA in the PCR reaction.
Chromosomal DNA for PCR amplification was isolated using an Epicentre Masterpure™ DNA Purification Kit. Plasmid DNA was isolated using the Sigma GenElute™ Plasmid Miniprep Kit.

Primers used for PCR amplification of dnaA derivatives were constructed by Kathryn Moore and created by the Dana Farber Cancer Institute. 61545dnaAGWN1: 5’-
GGGGACAGTTTTGTACAAAAAACAGGGCTTTCTATCGACCTTTTGTTCGAGTGGA
GT- 3’ and 61546dnaAGWN2: 5’-
GGGGACACTTTGTACAAGAAAGCAGTGTTCAATTTCATAGGTTACGATGAC
AA- 3’

PCR reactions were performed in a BIO-RAD DNA Engine Tetrad2 Peltier Thermal Cycler. The following schedule was shown to be most efficient: 1) 95° C for 2 min, 5 times at [2) 95° C for 30 s, 3) 50° C for 30 s, increase by 1° C every cycle 4) 72° C for 3 min 30 s], 20 times at [6) 95° C for 30 s, 7) 55° C for 30 s, 8) 72° C for 3 min 30 s], 9) 72° C for 5 min, 10) 4° C forever. PCR products were purified using a Qiagen Inc. QIAQuick® PCR Purification Kit and verified for approximate size using 0.8% agarose gel electrophoresis.

dnaA derivative strains were confirmed via sequence analysis using primers 31F:
5’-GGATCTCTTTCGGATA-3’, 811R: 5’-CAACGCCGTTGATCTTTCGGATA-3’,
991F: 5’-TCTAAGCTAGTGAGCTGGAAAAGGG-3’, and 1111R: 5’-
TATTGTGATGTTGACCAGTTTTC-3’ constructed by the Dana Farber Cancer Institute.
**Gateway™ Plasmid Creation**

For construction of native and fusion expression plasmids the Gateway™ Cloning Technology System provided by Invitrogen was used. The BP recombination reaction to construct native and fusion pDONR-201 entry vector plasmids was as follows: appropriate attB-PCR fragments were created using appropriate primers as outlined in the Gateway™ Cloning Technology System manual and purified using Qiagen Inc. QIAQuick® PCR Purification Kit. 7 µl attB-PCR product, 1 µl pDONR201 donor vector, and 0.5 µl BP Clonase™ II enzyme mix were added to a microcentrifuge tube. The reaction was incubated at 25° C for 4 hours and then transformed into electrocompetent DH5α cells and plated on LB Kn plates to select for recombinants. pDONR201 recombinant entry vectors were then isolated using Sigma GenElute™ Plasmid Miniprep Kit and confirmed by sequence analysis by the Dana Farber Cancer Institute using primers DCattL1S: 5’-TCGCGTTAACGCTAGCATGGATCTC-3’ and 5’-GTAACATCGAGATTTTGAGACAC-3’.

The LR reaction was then used to create expression clones as follows: 1 µl of destination vector plasmid DNA, 7 µl of entry vector DNA, and 1.5 µl LR Clonase™ II enzyme mix were added to a microcentrifuge tube. The reaction was then mixed and incubated at 25° C for 4 hours. 1-2 µl was then drop dialyzed and transformed into the appropriate strain background and the remaining reaction was allowed to incubate overnight. If the transformation failed, the remaining reaction mixture was used for a second transformation in the morning. Recombinants were selected for using appropriate antibiotic LB plate media. Expression clone plasmids were then verified via diagnostic restriction enzyme digest.
Outward PCR Plasmid Creation

Primers were constructed to create a dnaAΔ96-120 tagged with six histidine (his) residues from the Mori collection his tagged dnaA plasmid. Reverse pCA24NΔdnaA primer: 3'-GACGTTGCTCGTCACCTGGCGTTGTTGCAGTTCACC-5' and forward pCA24NΔdnaA primer 3'-AACCGTCCCCGGCCGGCAGAACCAGCTATCGTTCTAAG-5' were used to construct a his tagged dnaAΔ96-120 through colony PCR.

Electroporation Transformation

Electroporation transformation was completed in order to introduce plasmids carrying desired genotypes into specific strain backgrounds.

Competent cells of STL242 (MG1655), STL2895, STL8297, and STL2168 were made by the following protocol. A standing overnight culture was grown in 2 mL LB media at 37° C using selective media when appropriate. A larger culture was then inoculated using the overnight culture in a 1:100 dilution and grown with vigorous shaking at 37° C to an OD600 of 0.5-1.0. The culture was then centrifuged at 4,000 rpm for 10 minutes at 4° C and the supernatant decanted, cells were resuspended in 50 μl of cold 1mM Hepes, pH 7.0. Cells were centrifuged again at 4,000 rpm for 10 minutes at 4° C and the supernatant decanted, cells were resuspended in 10 ml cold 7% dimethyl sulfoxide (DMSO). A final centrifugation was done for 10 minutes at 4,000 rpm at 4° C and resuspended in 2 ml cold 7% DMSO. Cells were stored at -70° C.

Transformations were performed using a BIO-RAD MicroPulser. 1 mL LB media was aliquoted into sterile test tubes. DNA was drop dialyzed to remove salt using 0.025
μm Millepore nitrocellulose filters. 40 μL competent cells were added to a control test tube. 40 μL competent cells were mixed with 1-2 μl of drop dialyzed DNA in a sterile microcentrifuge tube and then transferred to an electroporation cuvette and pulsed. The 1 ml of LB media was then added to the cuvette to resuspend the cells using a sterile pipette tip. The resuspended cells were then poured back into the test tube and placed at 37° C for 1 hour. 0.1 ml of each culture was plated onto selective plate media and incubated overnight at 37° C. Growth was expected on test plates and not on the control plate.

**P1 Transducing Lysates and P1 Transductions**

P1 transducing lysates for P1 transduction were made according to the following protocol: A 5 ml standing overnight culture of a strain with the desired genotype was grown at 37 °C. A 1:1 dilution of the standing overnight was done into a fresh aliquot of LCG and grown shaking for 1 hour at 37 °C. LCG plates for 6 dilutions and a control were warmed to 37 °C and LCG top agar was melted at 65° C. Aliquot 0.2 ml of culture into 7 small test tubes and label 0-5 and control. Place 10 μl of 242 P1 stock into tube 0 and vortex. Perform a 1:10 serial dilution with tube 0 through tube 5 vortexing after each addition. Do not place any P1 stock into the control tube. Incubate the tubes for 30 minutes and then add 2.5 ml of molten LCG top agar to each tube, vortex, and spread onto a pre-warmed LCG plate. Incubate the plates upright for 5 hours or overnight at 37° C.

Choose a plate that has small, bumping plaques for harvest. Scrape off the top layer of agar using a sterile metal spatula and place in a 15 ml Corex glass centrifuge tube, add 2 ml of LB media and 0.5 ml chloroform, and vortex hard for 30 seconds. Let
the mixture stand at 25°C for 10 minutes and then centrifuge at 4°C for 10 minutes at 4,000 rpm. Remove the supernatant broth with a sterile pasteur pipette and place in a sterile screw-cap glass tube with 0.5 ml chloroform. Store at 4°C.

P1 transductions were performed as follows: A 5 ml standing overnight culture of the strain to be transduced was grown in LCG media at 37°C. The culture was aliquoted into 3 microcentrifuge tubes and spun at 13,000 rpm for 10 minutes. The supernatant was removed and the pellet was resuspended in a small amount of LCG media. 5 µl of P1 transducing lysate was added to one tube, 20 µl of P1 transducing lysate was added to the second, and nothing to the third as a control. The tubes were incubated at 37°C for 30 minutes and then 0.1 ml of 1M sodium citrate was added to each tube. The mixture was then plated on selective media and incubated overnight at 37°C.

Temperature Assays

Colony morphology of the given strains was observed through streaking to single colonics on appropriate LB and minimal plate media pre-warmed to the appropriate temperature and incubating overnight to 2 days on 25°C, 30°C, 37°C, and 42°C.

AZT Survival Assays

Viability of strains when subjected to DNA damaging agents was assessed through azidothymidine (AZT, Sigma) survival assays according to the following protocol. 0.2 ml standing overnight cultures were grown at 25°C, 30°C, and 37°C for assays at each of the given temperatures. Cultures were then diluted 1:20 and grown with vigorous shaking at the same temperature until an OD_600 of approximately 0.4 was reached. 0.1 ml of each culture was pipetted into a 96-well plate and 1:10 serial dilutions
through 10^-5 into 56/2 salt solution were completed in the adjacent wells, each addition was followed by adequate resuspension. Following dilution, 0.2 ml of each well was plated on two each of the following doses of AZT in both minimal and LB plate media: 0 ng/ml, 12.5 ng/ml, 50 ng/ml, and 75 ng/ml. The plates were then incubated overnight to 2 days at the temperature at which the culture was grown. Colonies were then counted for each strain assayed and fraction survival was calculated at each given AZT dose.

**Complementation Testing**

Complementation testing was completed by transformation of the hisDnaA and hisDnaAΔ96-120 plasmids into dnaA46 to verify function of the plasmid. Following transformation of the plasmid into the temperature sensitive dnaA46 background, the strain should have lost its temperature sensitivity ensuring that the plasmid was efficiently producing desired protein. Transformants were thus plated on appropriate antibiotic plate media following the serial dilution procedure described for AZT survival assays and grown at 30°C, 37°C, and 42°C using empty MG1655 and dnaA46 strains as controls.

The following day, colonies were counted and complementation rates were calculated to check for complementation between the suppressor plasmids and the wild-type MG1655 and dnaA46.

**Flow Cytometry**

DNA content and replication synchrony were assessed using flow cytometry. 2 ml standing overnight cultures of strains were grown at 25°C and 37°C in both minimal and LB media. The following day cultures were diluted 1:100 into fresh media and grown
with vigorous shaking to an OD$_{600}$ of .2 (log phase). 1 mL of the culture was fixed in 95% ethanol. The remaining culture was treated with 300 µg/ml rifampicin (Sigma) a replication initiation inhibitor, and 32 µg/ml cephalexin (Sigma) an inhibitor of cell division. The culture was then grown in the dark with vigorous shaking for 2.5-3 hours for higher temperatures and rich media and 4-5 hours for lower temperatures and minimal media to complete already initiated replication events. Samples were then spun down at 4,000 rpm for 10 min at 4° C and resuspended in 500 µl of 1x Dulbecco’s phosphate-buffered saline (PBS, Gibco) and stained with a 1:100 dilution of Invitrogen Pico Green dye in 25% DMSO in total darkness for 1-3 hours at 25° C. The samples were then diluted using 500 µl of a 1:1000 dilution of Pico Green dye in 1x PBS. Samples were analyzed using a FACSCalibur flow cytometer and BDCellQuest and FlowJo software version 6.4.1.

**Protein-Protein Interaction Pull-down Experiment**

Protein-Protein interactions of DiaA and DnaA were determined by completing pull-down experiments.

A plasmid carrying biotin-binding domain (BBD) fusion to DiaA under control of the T7 promoter, was constructed and then transformed into BL21(DE3) for protein expression. Likewise, MG1655 was transformed with a plasmid expressing His-tagged wild-type DnaA or His-tagged DnaA$\Delta_{96-120}$, expression of these proteins was controlled by the lac promoter. Strains were induced to over-express proteins by the following protocol: inoculation of a larger volume of LB media was done with a 1:100 dilution of a standing overnight culture and then grown with vigorous shaking at 37° C to an OD$_{600}$ of 0.6-0.8. 1 µM IPTG was then added to the culture and the cells were grown at 37° C with
vigorous shaking for 2 hours. The cells were then centrifuged at 4,000 rpm in a swinging bucket rotor for 10 minutes. The supernatant was then discarded and cells were resuspended in Tris-Sucrose (10% w/v sucrose and .05 M Tris pH 7.5) at 1% of the original volume for storage at -20°C until use.

Crude lysates of over-expressed cells were completed for use in pulldown experiments as follows: 100 µL of cells were thawed and incubated at 4°C for 5 minutes with the addition of 4 mM EDTA, 1 mM DTT, and 10 mg/mL lysozyme in Tris-Sucrose in a microcentrifuge tube. 200 mM of NaCl was then added and incubated at 4°C for 25 minutes. The cells were then heat shocked 3 times for 15 seconds in a 37°C water bath and placed on ice followed by centrifuging at 13,000 rpm for 15 minutes in a microcentrifuge.

Pull-down experiments were completed as follows using Novagen® Streptavidin beads: 100 µL of a 50% slurry of Novagen® Streptavidin agarose beads were equilibrated through 3 washes with 500 µL of Blank Lysis Buffer (1 mM dithiothreitol (DTT), 200 mM NaCl, 10% w/v ultrapure sucrose, 5 mM Tris pH 7.5) for each reaction. All manipulations of the streptavidin agarose beads were done at 4°C. Centrifugation was done at 4,900 rpm. After each wash beads were centrifuged for 1 minute and the supernatant was then discarded. After equilibration of the beads an equal volume of a cleared lysate of the biotinylated DiaA (BBD-DiaA) strain was added and rotated for one hour at 4°C. Beads were then gently washed using 500 µL of wash buffer (50 mM Tris pH 7.5, 150 mM NaCl) and centrifuged for 1 minute. The supernatant was pipetted off and discarded. The wash was repeated a total of 3 times. 10 µL of BBD-DiaA bound to the streptavidin beads was resuspended in 10 µL of 2x FSB (4% sodium dodecyl sulfate
(SDS), 200 mM DTT, 120 mM Tris pH 6.8, 0.002% bromophenol blue, 10% glycerol) and stored at -20° C for SDS-Page and western blotting. 90 μL of His-tagged DnaA or DnaAΔ96-120 cleared lysate was added to each 90 μL of BBD-DiaA-streptavidin beads and rotated for 1 hour at 4° C. Samples were then washed using 500 μL of wash buffer in the manner mentioned above. 10 μL of the samples were resuspended in 10 μL of 2x FSB. Additionally, 10 μL of each clear lysate used was resuspended in 10 μL of 2x FSB for analysis by SDS-Page and Western blotting. Frozen samples were thawed and boiled and equal volumes were loaded onto a 12% SDS-Page gel with a 4% stacking gel according to the procedure found in BioRad® Mini Protean II Manual.

Gels were transferred onto a PVDF membrane following the procedure described in Mini Trans-Blot® Electrophoretic Transfer Cell Instruction Manual provided by BioRad®. Membranes were blotted following the protocol described in the QIAexpress® Detection and Assay Handbook, using a primary Qiagen® Penta-His antibody and secondary mouse IgG antibody (HRP) from Genetex, Inc. Membranes were then treated using the SuperSignal® West Pico Chemiluminescent detection system manufactured by Thermo Scientific.

**DNA Binding Experiment**

DNA binding experiments to test the efficacy of DnaA domain II mutants binding to OriC were done using radioactive γ^{32}P labeled oligonucleotides. Dana Farber Cancer Institute created oligonucleotides equivalent to the entire length of OriC 5'- AGAAAGACCTGGGATCCTGGGTATTTAAAAAGAAGATCTATTTATTTAGAGATATGCTTTCTATTGTGATCTCCTTATTAGGATCGCCTGCCCTGGGATAAACAAAGGAGTC-3' and anti 5'-
GATCCTGTTTATCCACAGGGCAGTGCATCTCTAATAAGAGATCAACAATAGAA
CAGATCTCIAAAATAAAATAGATCTTCTTTTAATACCCAGGATCCCAAGGTCTTT
CT-3’. Prior to kinasing the oligos they were ethanol precipitated as follows: Oligos were
diluted in sterile ddH₂O to 1μg/ml in a microcentrifuge, 10% of the volume of 3M
sodium acetate pH 4.9 was added, 3 volumes of 100% ethanol were then added and
vortexed well. The tube was then placed in a dry ice and ethanol bath for 30 minutes. The
mixture was then centrifuged at 13,000 rpm for 15 minutes at 4°C. The supernatant was
decanted and the pellet was dried at 37°C and resuspended in 50 μl sterile ddH₂O.

The oligos were kinased as follows: 50 pmol of primer was incubated with 10% volume
T4 polynucleotide kinase buffer (New England Bio Labs), 10% volume γ^32P, 1 unit
T4 polynucleotide kinase (1 μl), and ddH2O. The mixture was then adequately mixed
and incubated in a 37°C water bath for 40 minutes. The kinased oligos were then cleaned
using Sephadex G-50 Quick Spin Columns for radiolabeled DNA purification from
Roche.

The kinased primer was then annealed to its anti-strand by the following
protocol: Equal pico molar amounts of oligo were added to a microcentrifuge tube, 10% volume
annealing buffer 10x, and sterile ddH₂O were added and mixed. The tube was
then boiled for 5 minutes and allowed to cool slowly in the water bath to room
temperature and stored at 4°C until use the same day.

The pull-down experiment was completed as above with the addition of 0.3 mM
ATP to all of the buffers used. Following washes after rotation of the streptavidin beads,
diaA, and dnaA or dnaAΔ96-128 crude lysate for 1 hour at 4°C 1 pmol annealed oligo was
added and allowed to rotate in the cold for one hour. The beads were then washed as
described two times and the samples were run on a 6% denaturing acrylamide gel at 100 V for 30-40 minutes. The gel was then dried using a BIO-RAD model 583 Gel Dryer and autoradiographic film exposed overnight.

RESULTS

Suppressors of mutant seqA phenotypes were originally isolated by phenotypic differences. seqA::FRT cat obgE::Tn5 strains were observed to have very small, slow growing colonies as well as larger, more robust ones (figure 2). It was surmised that these larger colonies were in fact suppressors that spontaneously arose during normal growth at 37° C on rich media.
Figure 2.- seqA' suppressor colony morphology

Figure 2.- *obgE::Tn5 seqA::FRT cat* and *oss2* *obgE::Tn5 seqA::FRT cat* were streaked to single colonies on LB plate medium at 25° C and 37° C. Colony morphology of *obgE::Tn5 seqA::FRT cat* at 25° C and 37° C on LB media show small colony morphology and poor growth at low temperatures with the exception of larger suppressor colonies. The suppressor colonies *oss2* *obgE::Tn5 seqA::FRT cat* show better growth on LB at low temperatures. A.) *obgE::Tn5 seqA::FRT cat 25° C*. B.) *obgE::Tn5 seqA::FRT cat 37° C*. C.) *oss2* *obgE::Tn5 seqA::FRT cat 25° C*. D.) *oss2* *obgE::Tn5 seqA::FRT cat 37° C*.

The genetic source of this suppressor phenotype was mapped to mutant *dnaA* (Moore, K. Lovett Lab, 2005). It was determined through sequence analysis of *dnaA* that a 75 base pair deletion in domain II was responsible for the suppression of the observed seqA::FRT cat mutant phenotype.
Figure 3.- Graphical representation of DnaAΔ96-120

Figure 3.- Graphical representation of DnaAΔ96-120. The bolded section and enlarged protein and DNA sequence show the deleted region of domain II. A) Domain I, amino acid residues 1-86, involved in oligomerization and in interaction with DnaB. B) Domain II, amino acid residues 87-134, constitutes a flexible loop, linker region. C) 75 base pair deletion of amino acid residues 96-120 within domain II of DnaA. D) Domain III, amino acid residues 135-373, contains the binding site for ATP/ADP, ATPase function, a second interaction site with DnaB, and is required for local DNA unwinding. E) Domain IV, amino acid residues 374-468, essential for specific binding to DNA.

The deletion occurred at short repeated DNA sequences, 5' - AACGTC - 3', that may act as hotspots for mutagenesis.

Alleviation of mutant seqA phenotypes by mutant dnaA was observed in colony morphology studies on rich and minimal media at 25° C, 30° C, 37° C, and 42° C. Data is shown at 25° C and 37° C on LB plate medium (figure 4, figure 5). At lower temperatures and on minimal media (data not shown) the morphological differences are quite obvious as the rate of replication is slowed and replication forks converge on sites of DNA damage with less frequency, thus increasing viability.
Figure 4. - 25° C colony morphology images show suppression of seqA mutant phenotype by mutant dnaAΔ96-120 and diaA. Strains streaked to single colonies on LB media at 25° C. A.) MG1655 B.) seqA::FRT cat C.) dnaA(Δx) D.) dnaAΔ96-120 E.) diaA::FRT kan F.) dnaAΔ96-120 seqA::FRT cat G.) diaA::FRT kan seqA::FRT cat H.) dnaAΔ96-120 diaA::FRT kan seqA::FRT cat I.) dnaAΔ96-120 diaA::FRT kan seqA::FRT cat.

At 25° C on both LB and minimal media (data not shown) seqA::FRT cat colonies are small and temperature sensitivity is observed in comparison to more robust wild-type MG1655 colonies (figure 4, B and A). dnaAΔ96-120 seqA::FRT cat and diaA::FRT kan seqA::FRT cat strains are larger than seqA::FRT cat colonies and slightly smaller than
wild-type indicating that dnaAΔ96-120 and diaA::FRT kan serve to suppress the seqA::FRT cat mutant phenotype (figure 4, F and G). dnaA(5x) is a low temperature sensitive mutant allele of dnaA with decreased viability at temperatures lower than that for optimal growth. In a comparison of dnaA(5x) which gives rise to a mixed population of small colonies and larger suppressors at low temperatures, dnaAΔ96-120 colonies are all of similar size and look much like wild-type (figure 4, C and D). diaA::FRT kan colonies look very similar to dnaAΔ96-120 and dnaAΔ96-120 diaA::FRT kan colonies look similar to both single mutant strains. Additionally, the dnaAΔ96-120 diaA::FRT kan seqA::FRT cat triple mutant looks no different than the double mutants listed above (figure 4, I, D, E).
Figure 5.- Colony Morphology at 37° C

Figure 5.- 37° C colony morphology figures show mild suppression of mutant seqA::FRT cat phenotypes by dnaAD96-120 and diaA::FRT kan. Strains were struck to single colonies on LB media and grown at 37° C. A.) MG1655 B.) seqA::FRT cat C.) dnaA(Sx) D.) dnaAD96-120 E.) diaA::FRT kan F.) dnaAD96-120 seqA::FRT cat G.) diaA::FRT kan seqA::FRT cat H.) dnaAD96-120 diaA::FRT kan I.) dnaAD96-120 diaA::FRT kan seqA::FRT cat

At 37° C suppression of the small colonies of seqA::FRT cat is observed by its pairing with dnaAD96-120 and diaA::FRT kan mutations (figure 5, B, F, G). dnaAD96-120 colonies were larger than those of dnaA(Sx) (figure 5, D and C). At 37° C dnaAD96-120
**seqA::FRT cat** grows as large, discrete colonies similar to wild-type. **diaA seqA::FRT cat** colonies present a larger morphology than **seqA::FRT cat** but not as large as wild-type or **dnaAΔ96-120 seqA::FRT cat** suppressed strains (Figure 5, F, G, B, and A). **diaA::FRT kan** colonies were smaller than both those of wild-type and **dnaAΔ96-120** but not as small as **seqA::FRT cat** colonies. **dnaAΔ96-120 diaA::FRT kan** colonies looked much like **diaA::FRT kan** and **diaA::FRT kan seqA::FRT cat** colonies as did **dnaAΔ96-120 diaA::FRT kan seqA::FRT cat** (Figure 5, H, E, G, and I).

Of note is that **diaA::FRT kan** and **dnaAΔ96-120** mutations both suppress **seqA::FRT cat**. This suggests that the two work similarly, and share a common mechanism, as positive regulators to control DNA replication.

Doubling times were calculated to determine the extent to which suppression of **seqA::FRT cat** occurred as a result of **dnaAΔ96-120** and **diaA::FRT kan** mutations.

**Table 2.- Doubling Times**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>Doubling Time 25°C (min)</th>
<th>Doubling Time 37°C (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655</td>
<td>Wild-type</td>
<td>105</td>
<td>51</td>
</tr>
<tr>
<td>STL7222</td>
<td>seqA::FRT cat</td>
<td>353</td>
<td>125</td>
</tr>
<tr>
<td>STL12692</td>
<td>dnaAΔ96-120</td>
<td>118</td>
<td>47</td>
</tr>
<tr>
<td>STL12697</td>
<td>dnaAΔ96-120 seqA::FRT cat</td>
<td>127</td>
<td>56</td>
</tr>
<tr>
<td>STL12846</td>
<td>diaA::FRT kan</td>
<td>117</td>
<td>54</td>
</tr>
<tr>
<td>STL12848</td>
<td>diaA::FRT kan seqA::FRT cat</td>
<td>147</td>
<td>73</td>
</tr>
<tr>
<td>STL12851</td>
<td>dnaAΔ96-120 diaA::FRT kan</td>
<td>109</td>
<td>51</td>
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<tr>
<td>STL12854</td>
<td>dnaAΔ96-120 seqA::FRT cat diaA::FRT kan</td>
<td>125</td>
<td>60</td>
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Table 2.- Doubling times show decreased generation time caused by mutant **seqA::FRT cat** is alleviated by mutant **dnaAΔ96-120** and **diaA::FRT kan** genotypes. Doubling times in LB media at 25°C and 37°C were calculated by averaging doubling times determined using a BioScreenC from Oy Growth Curves AB Limited. A semi-log plot was constructed of O.D.490 measurements versus time in hours and generation time was determined from 2/slope during exponential phase. This calculation was then converted into minutes.
Doubling times were calculated at 25°C and 37°C in LB media (table 2).

Doubling times vary to a degree and are dependent on both media conditions and slight variations in temperature and aeration. As such, MG1655 is provided as a control and is always done in conjunction with the strains being examined. The wild-type strain had a doubling time of 51 minutes. As expected, seqA::FRT cat had a much longer doubling time than wild-type, approximately 3.5x and 2.5x at 25°C and 37°C respectively.

dnaAΔ96-120 and diaA::FRT kan as well as the double mutant were similar to wild-type. When coupled with seqA::FRT cat, the mutations restore doubling times to those comparable to wild-type at both temperatures.

Complementation testing was completed by transforming vectors containing either his-tagged DnaA or his-tagged DnaAΔ96-120 into dnaA446, a high temperature sensitive mutant allele of dnaA. If complementation occurs, the plasmid copy of DnaA should alleviate temperature sensitivity.

Table 3.- Complementation Testing

<table>
<thead>
<tr>
<th>Strain background</th>
<th>Plasmid</th>
<th>37°C</th>
<th>42°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655</td>
<td>none</td>
<td>9.86E-1</td>
<td>1.42E0</td>
</tr>
<tr>
<td>dnaA46</td>
<td>none</td>
<td>8.33E-1</td>
<td>2.50E-6</td>
</tr>
<tr>
<td>dnaA46</td>
<td>6x-hisDnaA</td>
<td>1.22E0</td>
<td>1.27E-2</td>
</tr>
<tr>
<td>dnaA46</td>
<td>6x-hisDnaAΔ96-120</td>
<td>8.49E-1</td>
<td>5.32E-4</td>
</tr>
</tbody>
</table>

Table 3.- Transformation of vectors containing DnaA and DnaAΔ96-120 into temperature sensitive dnaA446. Survivability was calculated to determine complementation.

Transformation of dnaA446 with vectors expressing wild-type DnaA reveal a decrease in lethality at 42°C from dnaA446 with no vector of 10,000 fold.

Transformation of dnaA446 with vectors expressing DnaAΔ96-120 decreased lethality 100 fold at 42°C indicating partial complementation of plasmid dnaA with dnaA446 as it did not return the strain to levels like that of wild-type (table 3).
DNA damage assays were completed using azidothymidine (AZT), a thymidine analog that terminates elongation activity during DNA synthesis through the replacement of a hydroxyl functional group with an azido group inhibiting the addition of nucleotide groups to the developing DNA. This inhibitory action results in massively elongated cells due to the arrest of DNA polymerization (Elwell, LP et al., 1987).
DNA damage assays were completed at 25° C and 37° C on LB media containing AZT doses of 0 ng, 25 ng, 50 ng, or 75 ng (figure 6). Data for assays completed on LB
plate medium at 25°C and 37°C demonstrates the effects of slowing down the rate of replication through temperature on viability when exposed to DNA damaging agents. For both temperatures, wild-type *E. coli* was relatively unaffected by low doses of AZT, however, at high doses of AZT the wild-type strain showed sensitivity with the fraction survival dropping. Consistent and dramatic sensitivity of *seqA*:FRT *cat* to AZT was observed at both temperatures, with low fraction survivals even at small doses. *dnaAΔ96_120* showed trends similar to that of wild-type and had fraction survival rates much higher than *dnaA*(Sx), a different mutant *dnaA* allele, at 37°C. As evidence of suppression of the *seqA*:FRT *cat* mutant phenotype by *dnaAΔ96_120* and *diaA*:FRT *kan*, *dnaAΔ96_120* *seqA*:FRT *cat*, *diaA*:FRT *kan* *seqA*:FRT *cat*, and *dnaAΔ96_120* *diaA*:FRT *kan* *seqA*:FRT *cat* strains show consistent survival at high doses of AZT at low temperature. *dnaAΔ96_120* *seqA*:FRT *cat* and *diaA*:FRT *kan* *seqA*:FRT *cat* respond similarly to AZT and show alleviation of the *seqA*:FRT *cat* sensitivity to AZT. Suppression effects were most profound at lower temperatures with the fraction survival of *dnaAΔ96-120 seqA*:FRT *cat*, *diaA*:FRT *kan* *seqA*:FRT *cat*, and *dnaAΔ96-120 diaA*:FRT *kan* *seqA*:FRT *cat* consistently greater than that of wild-type at high doses of AZT. *dnaAΔ96-120* and *diaA*:FRT *kan* mutants seemed to respond equivalently to AZT on rich media as did the double mutant *dnaAΔ96-120 diaA*:FRT *kan*.

Strains were analyzed using flow cytometry before and after the addition of replication inhibitors rifampicin and cephalexin to determine DNA content, ploidy, and synchrony of replication.
Figure 7a.- Flow cytometric analysis of DNA content in dnaA, diaA, and seqA derivatives. Samples grown in LB medium at 37°C are shown in Log Phase A) Wildtype B) seqA::FRT cat C) dnaAΔ66-120 D) dnaAΔ66-120 seqA::FRT cat E) diaA::FRT kan F) diaA::FRT kan seqA::FRT cat G) dnaAΔ94-120 diaA::FRT kan H) dnaAΔ94-120 diaA::FRT kan seqA::FRT cat
Figure 7b.- Flow cytometric analysis with "run-out" conditions

Figure 7b.- Flow cytometric analysis of DNA content in dnaA, diaA and seqA derivatives. Samples grown in LB medium at 37°C are shown treated with runout conditions: I) Wildtype J) seqA::FRT kan K) dnaAD96_126 L) dnaAD96_126 seqA::FRT cat M) diaA::FRT kan N) diaA::FRT kan seqA::FRT cat O) dnaAD96_126 diaA::FRT kan P) dnaAD96_126 diaA::FRT kan seqA::FRT cat
Table 4.- Median DNA content

<table>
<thead>
<tr>
<th>Strain</th>
<th>Median (a.u) 25° C</th>
<th>Median (a.u) Run out 25° C</th>
<th>Median (a.u) 37° C</th>
<th>Median (a.u) Run out 37° C</th>
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</thead>
<tbody>
<tr>
<td>MG1655</td>
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<td>74</td>
<td>145</td>
<td>172</td>
</tr>
<tr>
<td>seqA::FRT cat</td>
<td>140</td>
<td>120</td>
<td>305</td>
<td>306</td>
</tr>
<tr>
<td>dnaAΔ56-120</td>
<td>60</td>
<td>41</td>
<td>161</td>
<td>159</td>
</tr>
<tr>
<td>seqA::FRT cat</td>
<td>89</td>
<td>111</td>
<td>183</td>
<td>215</td>
</tr>
<tr>
<td>diaA::FRT kan</td>
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<td>171</td>
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<tr>
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</tr>
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<td>dnaAΔ56-120</td>
<td>56</td>
<td>65</td>
<td>162</td>
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</tr>
<tr>
<td>diaA::FRT kan</td>
<td>84</td>
<td>70</td>
<td>163</td>
<td>213</td>
</tr>
</tbody>
</table>

Table 4.- a.u – arbitrary units. Flow cytometric analysis was completed at 25° C and 37° C. C. Cephaloxin 32 µg/ml and Rifampicin 300 µg/ml were used to set “run-out” conditions. Median DNA content was determined from resultant histograms using FlowJo software.

Flow cytometry revealed that seqA::FRT cat had consistently much greater DNA content than wild-type (table 4) as a result of over-initiation due to a lack of sequestration of the origin of replication. It was also seen that dnaAΔ56-120 and diaA::FRT kan had similar median DNA content lower than that of wildtype and that the double mutant was similar to both single mutants suggesting under-initiation of DNA replication and epistasis of dnaA and diaA. When seqA::FRT cat was paired with dnaAΔ56-120 or diaA::FRT kan, and both in the case of the triple mutant, median DNA content resembled that of wild-type indicating that alleviation of over-initiation had occurred.

Following run-out, resultant histograms revealed that wild-type resolved to 2 main peaks and a smaller third corresponding to 2n, 4n, and 8n genome equivalents (figure 7b). seqA::FRT cat did not run-out and was identical to the histogram obtained prior to setting run-out conditions (figure 7a). This was true of its derivatives as well as a
result of the gross over-initiation and excessive number of replication forks. dnaAΔ96-120, diaA::FRT kan, and the double mutant resolved to primarily 2n with smaller 3n peaks denoting a loss of synchrony (figure 7b).

From the results of phenotypic assays such as colony morphology studies, flow cytometry and from the results of DNA damage assays it was suggested that domain II of DnaA was perhaps involved in the interaction with DiaA. To test this hypothesis the protein-protein interaction was tested by pull-down experiments.

Figure 8.- Binding of DiaA to DnaAΔ96-120

A. B. C. D. E. F. G. H.

Figure 8.- Protein pull-down assay shows binding of DnaA to DiaA: (A) Crude lysate BBD-DiaA (B) Crude lysate HisDnaA (C) Crude lysate HisDnaAΔ96-120 (D) Washed BBD-DiaA-streptavidin beads (E) Washed BBD-DiaA-streptavidin beads HisDnaA (F) Protein standard (G) Washed BBD-DiaA-streptavidin beads HisDnaAΔ96-120 (H) Washed HisDnaA-streptavidin beads

Pull-down experiments showed that both wild-type DnaA and DnaAΔ96-120 bind to DiaA (figure 8). Employing a bait and prey system, DiaA and DnaA proteins respectively, biotinylated DiaA was immobilized on streptavidin beads and HisDnaA or HisDnaAΔ96-120 were pulled down. If interaction between the DiaA and DnaA proteins occurred the His tagged proteins would bind to the immobilized DiaA and could be visualized using western blot analysis. It was thus determined that mutations in domain II of DnaA do not impact binding to DiaA (figure 8, E and G).
As stated before, DnaA is an essential regulatory protein and binds to high and low affinity DnaA boxes at oriC. Its mechanism of binding to DNA at the origin is vital to DNA replication and as such we were interested in determining if a deletion of residues 96-120 in domain II of DnaA affected DNA binding at the origin.

In order to determine if domain II played a role in the binding of DnaA to the origin of replication a DNA binding experiment was completed. Primer pairs that mimic the region spanning OriC were washed over immobilized DnaA or DnaAΔ96-120 bound to DnaA with the addition of ATP to promote binding. Initially, this experiment was difficult to optimize though after several attempts we concluded that the radiolabeled oligonucleotide of OriC did not bind to either wild-type DnaA or DnaAΔ96-120. Thus, we were unable to obtain significant data on the DNA binding capacities of DnaAΔ96-120 at the origin as our method was unsatisfactory (data not shown).

DISCUSSION

The replication initiator protein DnaA is essential in *Escherichia coli*. Its functionality is integral to ensuring that replication occurs correctly and efficiently. In light of the essential nature of dnaA, it was surprising to discover cells in which a deletion in domain II was not only tolerated, but improved viability in the presence of DNA damaging agents such as AZT. A deletion of residues 96-120 of DnaA was identified by the suppression of a double mutant in *obgE* and *seqA*.

In *seqA* mutants, the sequestering ability of seqA is inhibited allowing DnaA to bind prematurely to low affinity sites at the origin. The result of such mutations is a premature initiation of replication, which is indicated by high levels of DNA content
during flow cytometric analysis (table 4). Additionally, through sequestration of the origin, SeqA restraints replication forks from converging onto sites of DNA damage caused by agents such as AZT. Thus, seqA mutants show increased sensitivity to AZT, which is relieved at low temperatures by a deletion of residues 96-120 in DnaA as a result of decreased replication initiation (figure 6). Under initiation of DNA replication by dnaAAΔ96-120 was shown by flow cytometric analysis (table 4).

Colony morphology and temperature sensitivity studies as well as calculations of doubling time reveal that dnaAAΔ96-120 and diaA::FRT kan both suppress mutant seqA::FRT cat. The dnaAAΔ96-120 phenotype resembles that of diaA::FRT kan (figure 4). Both suppress AZT sensitivity caused by seqA::FRT cat and correct the over-replication of seqA::FRT cat as revealed by median DNA content restored to a level similar to that of wild-type in flow cytometry. dnaAAΔ96-120 and diaA::FRT kan mutations alone and in tandem result in slightly decreased DNA content from that of wild-type and asynchronous replication indicated by 3N peaks (figure 7b). Epistasis of dnaA and diaA is suggested by the fact dnaAAΔ96-120 and diaA::FRT kan produce phenotypes almost identical to each other and the double mutant in colony morphology, doubling time, AZT sensitivity and flow cytometric analysis. AZT sensitivity conferred by seqA::FRT cat is suppressed slightly better by dnaAAΔ96-120 than diaA::FRT kan at 37°C indicating that they may perhaps have properties independent of each other (figure 6).

Our initial impression was that a deletion of residues 96-120 in DnaA would inhibit binding of DiaA though this was shown to be incorrect through protein pull-down experiments (figure 8). Additionally, we were unable to adequately test the hypothesis that DnaAΔ96-120 would affect the binding of DNA at the origin of replication as our
current method was inadequate as we could not show that wild-type DnaA binds radiolabeled DNA sequences equivalent to that at OriC, a well documented fact.

As results of AZT survivability assays and flow cytometry reveal that dnaAΔ96-120 and diaA::FRT kan cause under-initiation of replication and relieve over-initiation caused by seqA::FRT cat it is probable that the non-essential domain II of DnaA plays a regulatory role in the initiation of DNA replication and that DiaA, through its interaction with DnaA, may act to control this regulatory function.

CONCLUSION

As the under-initiation of DNA replication by dnaAΔ96-120 has been shown, it is possible that domain II of dnaA perhaps serves as a necessary dynamic clement in the binding of DnaB helicase. The known role of domain II as a linker region in conjunction with our data suggest that residues 96-120 play a role in DNA replication initiation by potentially allowing for a necessary conformational change in DnaA that may optimize the binding of DnaB helicase. This change is perhaps initiated by DiaA, suggested by the fact that diaA::FRT kan behaves similarly to the dnaAΔ96-120 mutant.

In order to demonstrate the role of amino acid residues 96-120 of DnaA in binding to DnaB helicase protein pull-down experiments of the nature conducted for DiaA and DnaA would need to be carried out using DnaA and DnaB to test their interaction given a deletion in domain II.
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REFERENCES


