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NOVEL *dnaA* ALLELES OF *Escherichia coli* THAT ARE HYPERACTIVE IN INITIATION OF CHROMOSOMAL DNA REPLICATION

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NOVEL *dnaA* ALLELES OF *Escherichia coli* THAT ARE HYPERACTIVE IN INITIATION OF CHROMOSOMAL DNA REPLICATION

BY

Alec Jude Murillo

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ABSTRACT

NOVEL *dnaA* ALLELES OF *ESCHERICHIA COLI* THAT ARE HYPERACTIVE IN INITIATION OF CHROMOSOMAL DNA REPLICATION

By

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In the initiation of DNA replication in *Escherichia coli,* DnaA protein of 52 kDa recognizes and binds to 5 asymmetric 9-mer sequences called DnaA boxes in the chromosomal replication origin, leading to the assembly of the replication fork machinery. Because the activity of DnaA protein affects the frequency of initiation of DNA replication; previous work in the laboratory tested the hypothesis that DnaA protein controls the frequency of initiation. In support of this idea, Lyle Simmons, a former graduate student, developed a genetic method and was able to isolate seven ***dnaA*** alleles that were unresponsive to regulatory elements. In this work we examine these novel *dnaA* alleles by genetic and biochemical methods. We show that these alleles are dominant-negative to the wild type allele, that they are hyperactive for initiation and that two of the alleles, *H202Y* and *V292M*, appear to be non-responsive to the regulatory components Hda, the β–clamp and the *datA* locus.

DEDICATION

To

Kim

Mom and Dad M

Mom and Dad P

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`Images in this thesis are presented in color`

List of Abbreviations

bp	base pair(s)
Dam	deoxyadenosine methyltransferase
DnaA box	asymmetric 9-mer sequence bound by DnaA
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	(ethylenedinitrilo) tetraacetic acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
kDa	kilodalton
SDS	Sodium dodecyl sulfate
Tris	Tris (hyroxymethyl) aminomethane
13-mer	a sequence in *oriC* that is A+T rich and is unwound during initiation

Chapter I: Literature Review

Introduction

The accurate and timely duplication of an organism's genome is one of the most critical steps in the cell cycle. In both prokaryotic and eukaryotic organisms, intricate cellular mechanisms function to ensure complete replication of the genome. This process requires the precise regulation and coordinated interaction of a diverse group of proteins. Although the molecular details of this process vary among organisms, the basic fundamental aspects are similar. An origin of replication must first be recognized and bound by an initiator protein that induces a localized unwinding of the DNA. Opening of the origin(s) of replication is followed by assembly of the enzymatic machinery that acts at each replication fork. The replicative helicase progressively unwinds the dsDNA; Primase generates RNA primers, and a DNA polymerase extends the primers to synthesize the complementary strands using each separated parental DNA strand as a template. Under normal conditions, these events are highly regulated to ensure timely and accurate replication of the genome. However, defects in regulatory pathways lead to abnormal replication, causing cell death and/or uncontrolled cellular proliferation.

The regulatory mechanisms that control the timing and frequency of initiation are an area of intense study. In eukaryotic organisms, the unregulated replication and increased DNA damage can lead to cell apoptosis (Nagata 2002) or tumorogenesis (Bergoglio, Pillaire et al. 2002). In prokaryotic organisms, the unregulated replication can be lethal due to massive amounts of dsDNA breakage resulting from the collision of replication forks (Michel, Ehrlich et al. 1997; Simmons, Breier et al. 2004). These processes are

currently studied in both prokaryotic and eukaryotic model systems where regulatory pathways have been identified. Of all the organisms in which chromosomal replication is being studied, *Escherichia coli* is the most well understood. Basic research into chromosomal replication in *E. coli* began in the first half of the last century and continues today. Proteins directly involved in the replication of the *E. coli* chromosome have been identified and characterized biochemically, providing a solid foundation from which mechanisms that regulate this process can be studied and understood at the molecular level

The Cell Cycle of *Escherichia coli*

The *E. coli* cell cycle is composed of two distinct parts, the C and D periods. The C period represents the time interval from the initiation, to the termination of chromosomal DNA replication. Regardless of the growth rate, the C period is approximately 40 minutes. The D period represents the time required for cell division. This period overlaps the C-period, and requires an additional 20 minutes following the termination of chromosomal replication. Thus, when cell division occurs every 60 minutes, initiation of chromosomal replication occurs shortly after the segregation of daughter cells. However, cell division in *E. coli* cells can be accomplished in shorter or longer periods than a generation time of 60 minutes. When cell division requires an interval longer than 60 minutes, an additional period has been identified, named the B period. This period represents the time between the birth of the new cell and the initiation of chromosomal replication (Niki, Yamaichi et al. 2000). When cellular division occurs in less than 60

minutes, new rounds of chromosomal replication begin prior to the completion of the ongoing replication event to ensure that complete copies of the genome will be available for the daughter cells.

An intricate relationship exists between cell growth and chromosomal replication. As mentioned above, chromosomal replication requires 40 minutes, which can be longer than the doubling time of the cell in a rapidly growing culture. The "Initiation Mass" hypothesis has been put forth to describe this coordination of cell growth to DNA replication. The hypothesis states that initiation of chromosomal replication occurs whenever the cell mass reaches a certain threshold, which is designated the initiation mass (Donachie 1968; Donachie 1993; Donachie and Blakely 2003). This ensures the average number of copies of *oriC* to cell mass remains constant. In addition to reaching the initiation mass, the intracellular concentration of DnaA-ATP must also reach a threshold to initiate replication. Experimental evidence suggests that initiation of chromosomal replication occurs when the intracellular concentration of DnaA-ATP reaches 300-400 nM (Speck and Messer 2001).

Chromosomal Replication in the Model Organism *Escherichia coli*

The *Escherichia coli* genome consists of a single circular chromosome of 4700 kilobase pairs (McMacken, Silver et al. 1987; von Meyenburg and Hansen 1987). Chromosomal replication initiates at a discrete site of the chromosome termed *oriC,* which is located at 84.3 minutes on the chromosomal linkage map (von Meyenburg, Hansen et al. 1978).

Through biochemical and genetic characterization, the minimal origin of replication has been shown to be 245 base pairs in length (Meijer, Beck et al. 1979; Oka, Sugimoto et al. 1980), and contains several essential structural elements (see Figure 1.1). One element is an asymmetric 9-mer sequence, termed the DnaA box, to which DnaA protein binds (Fuller, Funnell et al. 1984). The replication origin carries five DnaA boxes whose position and orientation are essential for initiation to occur. A second DNA element is the Duplex Unwinding Element (the DUE) (Kowalski and Eddy 1989), consisting of 3 AT-rich 13-mers (Bramhill and Kornberg 1988). Binding sites for HU and IHF, which function in initiation either by stabilizing DnaA protein bound to *oriC* (Chodavarapu *et al*, unpublished results) or by altering the DNA structure of *oriC*, are also essential (Craig and Nash 1984; Torheim and Skarstad 1999).

As mentioned above, initiation of *E. coli* chromosomal replication requires the recognition and binding of five asymmetric 9-mer sequences within *oriC* by DnaA protein, the replication initiator. These DnaA boxes are nearly identical in sequence, with TT(A/T)TNCACA representing the consensus sequence. The binding of a DnaA-ATP monomer to each of these boxes induces a localized bending of the DNA, with each binding event bringing about a 40 degree bend (Schaper and Messer 1995). The binding of ATP-DnaA to these boxes occurs in a sequential manner beginning with box R4 followed by binding to R1, R2, R3 and lastly M (see Figure 1.1) (Margulies and Kaguni 1996). Analysis of the DnaA boxes through mutagenesis, sequence inversion or a reordering of the boxes, indicates that all five boxes are required for *oriC* function (Langer, Richter et al. 1996). It is believed the conformational change within *oriC,*

induced by the bending, facilitates the opening of the AT-rich region to the left end of *oriC* (see Figure 1.1). This open region is approximately 28 nucleotides and is extended to 44-52 nucleotides by the binding of single stranded DNA binding (SSB) protein (Meyer, Glassberg et al. 1979; Krause and Messer 1999). In addition to the R boxes found within *oriC*, three additional 9-mer sequences have been identified that are specifically recognized by DnaA-ATP (Ryan, Grimwade et al. 2002; McGarry, Ryan et al. 2004). These sequences, named I-sites (I1, I2, I3), differ by 3-4 bases from the R-boxes, and I-sites are highly conserved among origins of *Enterobacteriaceae* (Zyskind, Cleary et al. 1983). These sites are bound with weak affinity and require cooperative binding to strong R boxes for stable complex formation.

Once DnaA-ATP unwinds the AT-rich region within *oriC,* forming an intermediate named the open complex, DnaA recruits two $DnaB_6$-$DnaC_6$-ATP complexes to the open region and positions them in a head-to-head fashion at opposite ends of the unwound region (Bramhill and Kornberg 1988; Marszalek and Kaguni 1994; Marszalek, Zhang et al. 1996; Fang, Davey et al. 1999). Hydrolysis of ATP by DnaC and the subsequent dissociation of DnaC from DnaB unmasks the helicase function of DnaB. The unwound DNA is stabilized through the binding of SSB. Primase, the product of the *dnaG* gene (Rowen and Kornberg 1978; Ogawa, Baker et al. 1985; van der Ende, Baker et al. 1985), recognizes the complex of DnaB bound to the ssDNA, and then begins to synthesize RNA primers. The RNA primer annealed to DNA is recognized by the clamp loader of DNA Polymerase III (Pol III), which displaces primase from the DNA-primer sequence and places the β-clamp onto the newly primed DNA by opening the ring of the clamp

(Yuzhakov, Kelman et al. 1999). The association of the β-clamp with the Pol III core forms the Pol III holoenzyme complex that is incredibly fast (~750ntd/s) and processive (>50Kb). The Pol III holoenzyme then extends the primers to begin semi-discontinuous, bidirectional replication of the genome (Glover and McHenry 2001). The extension of DNA/RNA-primer complexes occurs such that the growing chain is elongated in the 5' to 3' fashion. Synthesis of the leading strand proceeds in an uninterrupted fashion, while synthesis of the lagging strand occurs in short 1-2kb fragments known as Okazaki fragments, which are each primed by primase. This process is coordinated by a centrally located clamp loader that remains bound to DnaB and a dimer of DNA Polymerase III holoenzyme to support the concurrent synthesis of leading and lagging strands (Gao and McHenry 2001; Gao and McHenry 2001).

DNA replication proceeds bidirectionally around the circular chromosome until the replication machinery at each fork meets in the terminus region of the chromosome which is located opposite of *oriC*. When replication forks meet, from opposite directions, the enzymatic machinery dissociates to terminate DNA replication. The terminus region contains several copies of a specific DNA sequence, called *ter*, which is specifically recognized by Tus protein (Hill and Marians 1990). When bound by Tus, the *ter* sequences halt replication forks from escaping the terminus region by blocking the replicative helicase, DnaB, in a directionally dependent manner (Lee, Kornberg et al. 1989). These loci are oriented such that replication of overlapping regions of the chromosome occurs. The loss of the replicative helicase halts DNA synthesis by DNA polymerase III holoenzyme. Following termination, daughter chromosomes are

separated and then segregated to separate positions of the elongating cell before septum formation and cell division.

DnaA: A AAA$^+$ Replication Initiator

AAA$^+$ Proteins

DnaA is a member of the large AAA+ superfamily of proteins. The AAA$^+$ proteins, (ATPases associated with diverse cellular activities) represent a large, functionally diverse group of nucleotide binding proteins found across all kingdoms of life. This superfamily of proteins encompasses those that were originally classified as AAA proteins, and includes additional members that are not active as an ATPase and others that do not bind ATP but form complexes with active ATPases. This superfamily of proteins is functionally diverse, acting in membrane fusion, proteolysis, assembly and disassembly of protein complexes, DNA replication, recombination and transcriptional regulation (Neuwald, Aravind et al. 1999; Ogura and Wilkinson 2001). Proteins in this superfamily share a conserved region of approximately 220-240 amino acids, referred to as the AAA domain or nucleotide binding domain, which contains several conserved motifs including those necessary for ATP binding and hydrolysis.

Based on the crystal structure of several AAA$^+$ proteins, the common fold has two domains. One is an N-terminal domain containing a RecA-like α/β fold and the nucleotide binding pocket. The second is a C-terminal α-helical domain. The RecA-like

Figure 1.1. Organization of *oriC*

The *E. coli* origin of replication is located between the *gidA* and *mioC* genes at 84.3 minutes on the chromosome. The *oriC* region contains five DnaA boxes named R1-R4 and M, and three additional sequences, named I-sites, bound only by DnaA-ATP. *E. coli oriC* also contains binding sites for IHF and Fis proteins, both of which are important factors for replication initiation. The left end of *oriC* is composed of three AT-rich 13-mers (blue ovals L, M, R) which become single-stranded during the remodeling process that occurs following DnaA binding.

L
M
R
AT rich DUE
R1
IHF
M'
I1 I2
R2
FIS
R3
I3
R4
gidB/A
oriC
mioC

N-terminal domain is composed of a five stranded β-pleated sheet that is flanked on one end by 2 α–helices and 3 α–helices on the other. Located within the N-terminal domain are the Walker A, Walker B and Sensor I motifs. The C-terminal domain consists primarily of α–helices but varies in size and is less structurally conserved than the N-terminal domain. Although the C-terminal domain shows little structural conservation, the domain is positioned diagonally above the base of the bound nucleotide. A conserved residue, typically arginine, found within the C-terminal domain has been termed the Sensor II motif and interacts with the bound nucleotide. This interaction appears to provide energy for binding rather than sensing the difference between ATP and ADP (Hattendorf and Lindquist 2002).

Electron micrographs of AAA+ proteins reveal that these proteins form oligomeric structures, typically hexameric. Adjacent monomers interact primarily through the α/β domains of the AAA+ modules, which assume a wedge-like conformation ideally suited for the construction of hexameric ring structures, to orient the nucleotide binding pocket at the interface between neighboring subunits. The bound adenine base sits in close proximity to the rim of the circular assembly with the phosphates of the nucleotide descending towards the center. At the interface, the N-terminal region of $\beta 5$ of the β sheet from one subunit points towards the bound nucleotide in the preceding subunit, positioning an arginine residue to the preceding active site. This arginine has been termed the "arginine finger" by analogy to the equivalent residue in the G-protein-GAP complex, and plays a cooperative role in ATP hydrolysis (Tomoyasu, Yuki et al. 1993; Karata, Inagawa et al. 1999; Hattendorf and Lindquist 2002; Zhang, Chaney et al. 2002).

All structurally characterized oligomeric AAA^+ proteins share this common mode of assembly, in which one protomer projects an arginine finger residue from a conserved AAA^+ motif, known as Box VII, into the nucleotide binding cleft of a neighboring protomer, forming a bipartite ATP-interaction site. The interaction of the conserved arginine finger with the γ-phosphate of the bound nucleotide in the adjacent monomer is of special importance.

The AAA+ superfamily of proteins has been divided into seven sub-groups or "clades," based upon function (Iyer, Leipe et al. 2004). The initiator clade, of which DnaA is a member, contains all of the origin processing proteins along with the eukaryotic, archeal and eubacterial helicase loading proteins, and is distinct from the other clades by an additional α-helix inserted between the second and third β-strands in the nucleotide binding pocket (Iyer, Leipe et al. 2004). Within prokaryotes and archaea, these initiator proteins exist in a monomeric state until they bind to specific DNA sequences within the origin of replication (Cunningham and Berger 2005) at which point they form a hexameric complex. Structures have been established for archeal and prokaryotic initiators in both the monomeric ADP-bound (Liu, Smith et al. 2000; Erzberger, Pirruccello et al. 2002; Siddiqui, Sauer et al. 2004) and in the oligomeric ATP-bound states (Erzberger, Mott et al. 2006), providing insight into how the binding of ATP induces a conformational change within the monomers that drives the assembly of the oligomeric structure at the origin of replication.

These structures have been modeled for *E. coli* DnaA based upon the crystal structure of

DnaA from *Aquifex aeolicus*. The conformation changes observed within DnaA upon ATP binding have been suggested to produce a helical hexamer at *oriC*, which positions DnaA Domain IV, the DNA binding domain, on the outside of the helical filament (Erzberger, Mott et al. 2006). This possible orientation of the DNA binding domains suggests that a right handed helical wrap may occur within the origin, possibly facilitating the melting of the AT-rich region of the origin.

The helical structure proposed for the DnaA hexamer leaves two AAA+ interaction surfaces exposed at opposing ends of the filament. A nucleotide binding pocket is unbound at one end, while an arginine finger protrudes from the other (Erzberger, Mott et al. 2006). These exposed surfaces are speculated to interact with additional replication factors that also contain AAA+ domains. One such protein, Hda, has been shown to regulate initiation of chromosomal replication negatively through a proposed interaction of the exposed nucleotide binding pocket of DnaA and the arginine finger of Hda when Hda is in complex with the β-clamp (Kato and Katayama 2001; Su'etsugu, Shimuta et al. 2005). Although evidence for a direct interaction of the DnaA helical filament with Hda and the β-clamp has yet to be shown, similar interactions have been shown in other AAA+ proteins to support the model of negative regulation of DnaA by Hda and the *β*-clamp.

The helical wrap model proposed by the Berger lab provides a structural framework for the molecular events during initiation at *oriC*. However, there are some potential areas of concern with this model. First, the model is based upon the crystal structure of DnaA

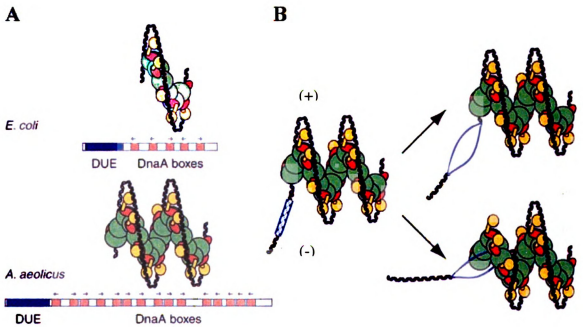
domains III and IV from *Aquifex aeolicus* (Erzberger, Pirruccello et al. 2002; Fujikawa, Kurumizaka et al. 2003; Erzberger, Mott et al. 2006) and the crystal structure of *E. coli* DnaA domain IV bound to DNA (Fujikawa, Kurumizaka et al. 2003). Although these structures provide insight into the conformational changes observed within DnaA and the orientation of DNA when bound to domain IV, they do not describe the structure of the entire protein. Thus, the model is speculative. Second, the organization and orientation of DnaA boxes within the origins of replication for both *A. aeolicus* and *E. coli* are dissimilar (Figure 1.2). By means of sequence inversion, the orientation of the DnaA-boxes within *oriC* has been shown to be essential for initiation of replication (Langer, Richter et al. 1996). The helical structure proposed by Ezrberger *et al* requires individual DnaA monomers be oriented in the same direction. Thus if a helical structure is to be formed at *oriC*, the DnaA monomers would need to align in the same direction to allow the interaction necessary for oligomerization.

The orientation of DnaA-boxes within *oriC* poses a problem for the helical filament model put forth by the Berger lab. However, evidence supporting the Berger model has been presented by the Botchan lab. Clarey *et al* 2006 have compared the proposed helical structure of DnaA at *oriC* to the structure of *Drosophila melanogaster* Cdc6/Orc1 (Clarey, Erzberger et al. 2006). Similar to what is seen with DnaA, electron microscopy reconstruction of the *D. melanogaster* ORC complex reveals a nucleotide dependent conformation change occurring within the components of the *D. melanogaster* Orc1-5

Figure 1.2. DnaA assembly at *oriC*

Panel A. Modulation of filament size enables the engagement of origins of different lengths with highly diverse numbers of DnaA boxes (shown in red; orientations indicated by arrows), exemplified by the *E. coli* and *Aquifex* origins. Domain IIIa is pictured in green. Domain IIIb is pictured in red and Domain IV is pictured in yellow.

Panel B. Filament formation generated by positive supercoiling may destabilize the origin unwinding element through compensatory negative supercoiling strain (top arrow). Coincident with or after opening, the axial channel of ATP-DnaA may directly engage the unwound DUE (bottom arrow).



Erzberger *et al*, Nat Struct Mol Biol (2006) 13(8): 676-83

complex. The helical structure proposed by the Berger lab has been modeled into the EM structure determined for the ATP bound Orc1-Orc5 complex. The spiral organization of the EM structure permitted the placement of five DnaA monomers into the core of the Orc structure (Clarey, Erzberger et al. 2006). Comparisons of the curvature, dimensions and center of mass for both models suggest a match between the core densities of the two structures. The results of this work in addition to the work from the Berger lab suggest that AAA+ initiators may assume a common helical assembly when interacting with their origin targets.

DnaA: Structure and Function

Although multiple proteins are required for the stages of initiation, elongation and termination of *E. coli* chromosomal replication, DnaA performs a unique role at the initiation stage. The *dnaA* locus is the first gene of an operon that also contains the *dnaN* gene, which encodes the β-clamp. This operon is located at approximately 83.6 minutes on the chromosomal linkage map. DnaA is a 52.5 kDa protein (Hansen, Hansen et al. 1982) composed of four distinct domains (Kaguni 1997; Sutton and Kaguni 1997). Extensive biochemical characterization and sequence alignment work has been performed to establish these domains and their associated functions. Domain I, corresponding to the N-terminal region (residues 1-90), is involved in self-oligomerization and the retention of DnaB in the prepriming complex. Residues 91-130 comprise a linker region, named Domain II, to which no functions have been assigned. Domains IIIa and IIIb (residues 131-296, 297-347) function in ATP binding and also in

the interaction of DnaA with the pSC101 encoded RepA. Domain IV (residues 348-467) functions in DNA binding (Erzberger, Pirruccello et al. 2002).

DnaA Domain I: Self Oligomerization and DnaB Retention

The interaction of DnaA Domain I with DnaB was determined from the analysis of a series of deletion mutants. Deletion of amino acid residues 1-62 and 1-129 both inactivated replication, while still allowing for binding to and unwinding of *oriC*. Although the mutant proteins retained the ability to bind DnaB, as measured by surface plasmon resonance, deletion mutants failed to load DnaB at *oriC* and thus were inactive in initiation of replication (Sutton, Carr et al. 1998). Additional mutational analysis of Domain I showed that a region near the N-terminus is involved in self-oligomerization, which is required to load DnaB into the open complex. These results indicate that a specific DnaA oligomeric structure at *oriC* is required for the loading of DnaB (Simmons, Felczak et al. 2003).

The recruitment of DnaB by DnaA to *oriC* also requires a separate region within residues 111-148 of DnaA, which overlaps Domains II and IIIa. The interaction of this region with DnaB was demonstrated through the use of a monoclonal antibody, M7, that recognizes a conformational epitope of DnaA within residues 111-148 (Marszalek, Zhang et al. 1996). Addition of the M7 antibody to *in vitro oriC* plasmid replication assays and to assays that depend on the binding of DnaA to a hairpin structure of a single stranded DNA, combined with results from surface plasmon resonance and ELISA experiments

that measure a direct interaction between DnaA and DnaB demonstrated the necessity of this region in the loading of DnaB to *oriC* (Marszalek, Zhang et al. 1996).

DnaA Domain III: Nucleotide Binding, Hydrolysis and Self Oligomerization

Domain III has been divided into two sub-domains named IIIa and IIIb. Domain IIIa is composed of a five stranded β-sheet flanked by helices α1 and α2 on the left and helices α3 through α8 on the right. This region contains the Walker A, Walker B and Sensor I motifs and adopts a conformation very similar to that of the nucleotide binding domain of RecA, which is characteristic of the AAA family of proteins. Domain IIIb folds into three anti-parallel α helices (α9-11) and contains the Box VII and Sensor II motifs. The combination of Walker A and B and Sensor I and II motifs coordinate the binding of a Mg^{2+} ion and either ADP or ATP at the nucleotide binding site.

As with other AAA+ proteins, mutations within the elements essential for ATP binding and hydrolysis can dramatically affect either process. Mutations of highly conserved residues within the Walker A and Walker B motifs, specifically lysine-178 to isoleucine and aspartic acid-235 to asparagine, abolished nucleotide binding, while leaving DNA binding unaltered (Mizushima, Takaki et al. 1998). These mutant proteins were shown to have less than one-tenth the specific activity of wild type DnaA in *in vitro* replication assays and were incapable of supporting replication in a strain with chromosomally encoded *dnaA46*(Ts). Additional mutagenesis work in Domain IIIa has shown the importance of ATP hydrolysis on regulation of replication initiation.

Figure 1.3. Four Functional Domains of DnaA

The functional domains of DnaA were determined through a genetic assay designed to identify mutations that inactivated the *dnaA* gene product (Sutton and Kaguni 1997). The missense mutations identified (triangles) were localized to distinct regions of the *dnaA* gene. The effects of these mutations on the function of DnaA were determined through biochemical characterizations. The identification of the various defects allowed for the determination of different functional domains of DnaA. The N-terminal region of DnaA, Domains I and II, are essential for interactions with the replicative helicase, DnaB, and self oligomerization. Domain IIIa contains the Walker A and B motifs in addition to the Sensor I motif. Within Domain IIIb the Sensor II and Box VII motifs can be found. The combination of elements within Domains IIIa and IIIb are essential for ATP binding and hydrolysis. Structural elements within Domain IV are essential for DNA binding.

Lyle Simmons, Doctoral Dissertation 2003

Site directed mutation of glutamic acid-204 to glutamine decreased the ATPase activity of DnaA to one third the activity of the wild type protein. The decrease in ATPase activity was further shown to have a lethal effect on host cells resulting from hyperactive initiation of replication. (Mizushima, Nishida et al. 1997)

Several other mutant DnaA proteins have been identified encoding mutations within Domain IIIa and IIIb. A group of these mutants, *dnaA5*, *dnaA46*, *dnaA601*, *dnaA604* and *dnaAcos*, all share the same alanine-184 to valine substitution in close proximity to the Walker A motif (Braun, O'Day et al. 1987; Hansen, Koefoed et al. 1992). In addition to the A184V mutation, each of these alleles bears at least one additional mutation within the gene, suggesting that the A184V mutation by itself is not well tolerated. The common substitution causes a cold sensitive phenotype(Hansen, Koefoed et al. 1992), which corresponds with a reduced affinity for ATP (Hansen, Koefoed et al. 1992; Carr and Kaguni 1996). Alleles in which glycine-177 has been replaced with aspartic acid also exhibit a cold sensitive phenotype (Sutton and Kaguni 1997). Each of the above mentioned alleles encode mutations within or near the Walker A motif, suggesting that these mutations alter the ability to bind to or hydrolyze ATP.

In addition to the Walker A and B motifs found within Domain III, the Box VII motif has been shown to be of great importance both in ATP sensing and in formation of an active nucleoprotein complex. Substitution of arginine-281 to alanine did not alter DNA binding or unwinding of *oriC*, but impaired the formation of the DnaA oligomer at *oriC* (Felczak and Kaguni 2004). These results suggest that formation of a stable DnaA-*oriC*

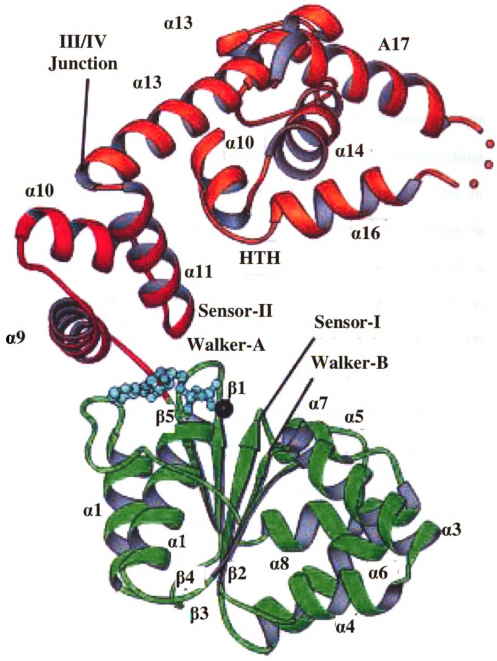
complex relies upon an interaction between adjacent monomers in addition to the interaction involving Domain I. It has been suggested that R281 may function as an arginine finger to sense if ATP is bound to the adjacent DnaA monomer, thereby stabilizing the oligomeric complex (Felczak and Kaguni 2004; Erzberger, Mott et al. 2006). This arginine finger interaction with adjacent proteins is a characteristic of AAA+ proteins.

DnaA Domain IV: DNA Binding

Binding of DnaA to *oriC* and additional sites throughout the chromosome is accomplished through interactions of Domain IV with the 9-mer DnaA box motif. The requirement of this region for DNA binding has been established through the work of several groups. In one study (Sutton and Kaguni 1997), mutations of residues 379 to 467 near the C-terminus of DnaA, rendered the protein inactive for sequence specific DNA binding. Additionally, threonine 435 was shown to be required to confer the specificity in recognizing the 9-mer DnaA box. Structural evidence determined by Fujikawa *et al*, also supports the interaction of Domain IV with DNA. Located within Domain IV, the helix-turn-helix motif, a characteristic of many DNA binding proteins, has been shown to interact with both the major and minor groove of the DnaA box sequence. One helix and loop interact with the major groove by forming hydrogen bonds and van der Waal's interactions with 5 base pairs of the DnaA box. Additional hydrogen bond interactions between arginine-399 and 3 base pairs have been identified within the minor grove

Figure 1.4. Crystal structure of DnaA protein from *Aquifex aeolicus*

A crystal structure of DnaA Domains IIIa, IIIb and IV has been determined by the Berger lab (Erzberger, Pirruccello et al. 2002). Domains I and II were deleted from the full length protein to facilitate structural determination by eliminating the oligomerization domain which had complicated previous crystallographic work. Domains IIIa, IIIb and IV have been represented as a ribbon diagram in this figure. Domain IIIa is a five stranded β-sheet (β1- β5) flanked by two sets of α-helices. The conformation of this region is similar to the RecA type fold that has been observed in many nucleotide binding proteins. The Walker A and B and Sensor I motifs are found within Domain IIIA. Domain IIIb is an anti-parallel three helix bundle that contains the Sensor II motif, common among the AAA^+ family. The Walker A and B boxes in conjunction with the Sensor I and II motifs are responsible for the coordinate binding of ADP and the Mg^{2+} ion. Domain IV begins at helix12, the acidic phospholipids binding region. The DNA binding domain is a helix-turn-helix motif that is very similar to the Trp repressor DNA binding fold. *E. coli* DnaA shares a 35% amino acid identity and has a 65% amino acid similarity with *A. aeolicus* DnaA. As such, the structure determined for *A. aeolicus* may significantly contribute to work being performed on *E. coli* DnaA.



Erzberger *et al.*, EMBO J.(2002) 21(18): 4763-4773

(Fujikawa, Kurumizaka et al. 2003).

Regulating the Initiation of Chromosomal Replication

Chromosomal replication in *E. coli* is tightly regulated and synchronized to the cell cycle to ensure that the genome is accurately replicated and then distributed to daughter cells at cell division. Three distinct mechanisms have been identified. One involves the sequestration of the *oriC* when it is in the hemi-methylated state following chromosomal replication (Lu, Campbell et al. 1994). The second involves the titration of excess DnaA (Kitagawa, Mitsuki et al. 1996; Kitagawa, Ozaki et al. 1998). The third requires the Regulatory Inactivation of DnaA (RIDA) (Katayama, Kubota et al. 1998). These pathways ensure that initiation occurs in a synchronized manner at all origins within the cell (Donachie 1968; Donachie 1993; Speck and Messer 2001; Donachie and Blakely 2003). Although each functions independently, a link can be seen between the three processes.

Origin Sequestration

Sequestration of the origin is achieved through the activity of SeqA protein, which preferentially binds to hemimethylated GATC sites throughout the chromosome. These hemi-methylated sequences are a byproduct of DNA replication. The newly synthesized daughter strands, which are not methylated, annealed to the methylated parental DNA generates the hemi-methylated DNA. Methylation of these sites is accomplished by Dam

methyl-transferase, which recognizes the hemi-methylated GATC sequences and then methylates the adenine residues of these sites. Eleven such sites are present within the 245 bp of *oriC,* with an additional eleven sites proximal to *oriC.* The high concentration of GATC sequences in the *oriC* region of the chromosome contributes greatly to the effectiveness of the sequestration of the DNA, when hemi-methylated, by SeqA. After replication of the *oriC* region, SeqA binds to these hemi-methylated GATC sites. The association of SeqA with the inner membrane of the cell is thought to localize the newly replicated *oriC* to this cellular compartment (von Freiesleben, Krekling et al. 2000). SeqA sequesters *oriC* for approximately one third of the cell cycle, and then dissociates. The previously sequestered GATC sites are then methylated by Dam methyltransferase allowing DnaA-ATP to bind the DnaA boxes within *oriC* and initiate the replication process.

The importance of *seqA* in the regulation of DNA replication has been demonstrated through several experimental approaches. Deletion of *seqA* causes extra initiations, which are asynchronous (von Freiesleben, Rasmussen et al. 1994). Extra initiations observed under other conditions can lead to replication fork collapse and inviability (Simmons, Breier et al. 2004). Conversely, an increase in the gene dosage of SeqA causes delayed initiation of replication (Bach, Krekling et al. 2003). Additionally, Dam methyl-transferase mutants have been identified that prevent further initiation events from occurring at *oriC*, following the initial round of replication (Messer, Bellekes et al. 1985; Smith, Garland et al. 1985).

Titration of DnaA-ATP

The titration of DnaA by its binding to DnaA box sequences also contributes to the regulation of replication initiation. Approximately 300 DnaA boxes are estimated to reside in the chromosome. Some DnaA boxes that are located upstream of genes function to regulate transcription of these genes, as is the case with *dnaA*. Others, such as those contained in the *datA* locus serve to titrate excess DnaA. This locus contains an equivalent number of DnaA boxes as does *oriC*, but *datA* is estimated to have an eightfold increased affinity for DnaA, thereby titrating active DnaA (Kitagawa, Mitsuki et al. 1996; Kitagawa, Ozaki et al. 1998). It is speculated that the binding of DnaA to this site ensures the timely initiation of replication. Several studies support this model. One showed that the deletion of the *datA* locus results in extra initiations (Kitagawa, Ozaki et al. 1998). In contrast, increasing the copy number of *datA* with a multi-copy plasmid (Kitagawa, Ozaki et al. 1998; Morigen, Lobner-Olesen et al. 2003) decreased the frequency of replication initiation and increased the cellular concentration of DnaA-ATP. Although these results suggest that the *datA* copy number is important, the chromosomal relocation of *datA* from its natural location near *oriC* to distal sites does not affect the frequency of initiation. Therefore, an early duplication of *datA* compared to the later copying is not critical to the control of initiation (Kitagawa, Ozaki et al. 1998).

Both *datA* and *oriC* have similar numbers of DnaA boxes, yet *datA* has been shown to bind a much larger number of DnaA monomers than *oriC*. The amount of DnaA bound to *datA* has been estimated through an indirect manner. Immunoblot analysis of cells

carrying a plasmid bearing the *datA* locus or the empty vector were used to estimate the number of DnaA monomers bound to both *oriC* and *datA*. The immunoblot results estimate that 370 DnaA monomers bind to *datA*, while only 45 DnaA monomers bind to *oriC*. These results suggest that extensive DnaA oligomerization occurs at *datA* (Kitagawa, Mitsuki et al. 1996). In addition to estimating the number of DnaA monomers bound by *oriC* and *datA*, these experiments showed an increase in DnaA transcription, presumably due to titration of DnaA monomers away from the DnaA promoter region decreasing the ability of DnaA to regulate its own transcription.

The titration of DnaA by *datA* has been shown to be involved in the regulation of the initiation of chromosomal replication. Deletion of the locus has been shown to result in increased initiation frequency, while increasing the number of *datA* loci present in the cell delays initiation. These results suggest that the *datA* locus regulates initiation frequency by titrating DnaA away from *oriC*. Although *datA* clearly titrates DnaA, it is not essential for cell viability (Kitagawa, Ozaki et al. 1998; Morigen, Molina et al. 2005). Flow cytometry analysis of cells lacking the *datA* locus showed a similar number of chromosomal equivalents as wild type cells (Morigen, Molina et al. 2005). These results also show that cells lacking the *datA* locus initiated chromosomal replication at a lower mass than wild type cells.

Regulatory Inactivation of DnaA-ATP (RIDA)

The third mechanism that regulates the initiation of chromosomal replication is the

Regulatory inactivation of DnaA (RIDA). Although both ADP- and ATP- bound DnaA can bind to DnaA boxes, only DnaA-ATP is active for replication (Sekimizu, Bramhill et al. 1987). Following initiation of replication, DnaA is stimulated to hydrolyze bound ATP, rendering DnaA inactive for initiation. DnaA is a weak ATPase and requires stimulation from external sources to hydrolyze the bound nucleotide. Early investigations into the mechanism of inactivation identified two factors, IdaA and IdaB, that were required for RIDA activity (Katayama, Kubota et al. 1998). N-terminal sequencing of purified IdaA identified the protein as the β-clamp of DNA polymerase III holoenzyme. Further work on IdaB showed that the partially purified protein fraction could be replaced by Hda protein (homologous to DnaA) (Kato and Katayama 2001). Hda, another member of the AAA^+ superfamily, shares significant homology with Domain III of DnaA. Hda and the β-clamp have been shown to form a complex, which promotes the hydrolysis of DnaA-ATP. Hda interacts with the β-clamp through a β-clamp binding motif (QL[SP]LPL) near the N-terminus of the protein (Dalrymple, Kongsuwan et al. 2001; Kurz, Dalrymple et al. 2004). Although Hda and β-clamp form a stable complex (Kawakami, Su'etsugu et al. 2006), hydrolysis of ATP bound to DnaA occurs when the Hda-β complex is loaded onto dsDNA. Additionally, no direct interactions between the Hda-β complexes and DnaA-ATP have been observed, which suggests that the binding of both DnaA-ATP and the Hda-β complex to dsDNA is a necessary step in the RIDA process.

DnaA-ATP can bind to DNA in a sequence-specific manner as well as nonspecifically (Fuller, Funnell et al. 1984; Roth and Messer 1995; Weigel, Schmidt et al. 1997;

Fujikawa, Kurumizaka et al. 2003), which stimulates the ATPase activity of DnaA (Sekimizu, Bramhill et al. 1987). These observations suggest a model in which DnaA-ATP first binds to dsDNA and then interacts with the DNA bound Hda-β-clamp complex. The binding of DnaA-ATP to DNA may induce a conformational change to permit an interaction of DnaA with the Hda-β-clamp complex, followed by ATP hydrolysis. Although it is attractive to consider that this process occurs with DnaA-ATP bound to the DnaA boxes of *oriC*, experimental evidence suggests that DnaA-ATP bound to DnaA boxes resists RIDA activity (Su'etsugu, Takata et al. 2004). Thus, one possibility is that passage of the replisome along the chromosome displaces DnaA-ATP bound to the DnaA boxes, allowing the displaced DnaA-ATP to non-specifically bind dsDNA and interact with bound Hda-β-clamp complexes. Alternatively, DnaA-ATP bound to DnaA boxes in the chromosome may interact with DNA-bound β-clamps complexed to Hda (Su'etsugu, Takata et al. 2004). Although the exact mechanism has yet to be determined, evidence shows that Hda and the β-clamp form a stable complex composed of two Hda dimers bound to one β-clamp (Su'etsugu, Takata et al. 2004; Su'etsugu, Shimuta et al. 2005; Kawakami, Su'etsugu et al. 2006), which interacts with DnaA-ATP (Su'etsugu, Shimuta et al. 2005; Kawakami, Su'etsugu et al. 2006).

Hyperactive Initiation of Chromosomal Replication

Several mechanisms have been identified that contribute to the regulation of chromosomal replication, but of essential importance is the presence of a functional DnaA. As has been mentioned above, ATP-DnaA initiates chromosomal replication in *E.*

coli by binding to the five DnaA boxes within *oriC*. Three mechanisms have been identified that regulate this process. However, these regulatory pathways can be bypassed resulting in increased initiation frequency from *oriC*. One such condition resulting in an increased frequency of initiation events at *oriC* is thru an over-supply of DnaA protein by use of plasmids bearing *dnaA* expressed from an inducible promoter (Atlung, Lobner Olesen et al. 1987; Xu and Bremer 1988). This condition is not lethal, but dramatically slows the growth rate. A second condition that results in increased frequencies of initiation from *oriC*, is the loss of functional Hda (Camara, Breier et al. 2005). Earlier work on Hda suggested that the protein was essential for viability (Kato and Katayama 2001). However, more recent studies in which a tetracycline resistance cassette was inserted into *hda* is viable, grows normally and initiates replication in a synchronous manner. Only when the *hda* allele is deleted does initiation occur in an asynchronous manner (Camara, Breier et al. 2005). A third method to alter the frequency of initiation is by use of specific *dnaA* mutations such as *dnaAcos*, which encodes a mutant DnaA protein that is hyperactive for initiation.

DnaAcos

The *dnaAcos* allele was isolated as a spontaneous temperature-resistant revertant of the *dnaA46* allele (Kellenberger-Gujer, Podhajska et al. 1978). This intragenic suppressor was isolated due to its ability to restore viability to a temperature-sensitive *dnaA46* mutant at the non-permissive temperature 42°C. At 30°C *dnaAcos* is cold sensitive, a phenotype shown to be linked to overinitiation from *oriC* (Braun, O'Day et al. 1987).

Both *dnaA46* and *dnaAcos* encode A184V and H252Y substitutions and both alleles exhibit hyperactive initiation at 30°C, although DnaA46 requires elevated levels of the molecular chaperones GroEL and GroES to display the hyperactive phenotype (Katayama and Nagata 1991). Biochemical analysis of a mutant DnaA bearing the A184V mutation has revealed a defect in ATP binding. This defect causes the hyperactivity of DnaAcos in initiation (Carr and Kaguni 1996).

In addition to the A184V and H252Y substitutions that are shared by DnaA46 and DnaAcos, DnaAcos also carries two additional substitutions, Q156L and Y271H. Recently it has been shown that the A184V and Y271H substitutions are responsible for the increase in initiations which are asynchronous (Simmons and Kaguni 2003). The increased abundance of replication forks progressing away from *oriC* are suggested to collide from behind with replication forks that have stalled when they encounter pyrimidine dimers, proteins frozen on the DNA, or collisions with RNA polymerase (Sandler 2000; Sandler and Marians 2000). These collision events result in an increased abundance of double stranded breaks, which if left unrepaired are lethal to the cell (Simmons, Breier et al. 2004).

We have isolated seven novel *dnaA* alleles that appear to be hyperactive for initiation of chromosomal replication. The mutations encoded by these alleles span the length of the DnaA protein, and affect three of the four domains. The goal of this work is to genetically, molecularly and biochemically characterize these alleles.

Chapter II: Molecular and Genetic Characterization of Novel *dnaA* Alleles With Altered Frequencies for Initiation of Chromosomal Replication

Introduction

Chromosomal replication in *Escherichia coli* is initiated through the sequence-specific binding of DnaA to the five DnaA boxes within the origin of replication, *oriC*. The frequency of initiation has been shown to be dependent upon the availability of ATP-bound DnaA. When cellular DnaA levels increase via induced expression from a vector bearing the *dnaA* allele, more frequent initiations occur (Atlung, Lobner Olesen et al. 1987; Xu and Bremer 1988). Several mechanisms have been identified that regulate the ability of DnaA to initiate replication. Immediately following replication of the *E. coli* origin of replication, *oriC*, the SeqA protein recognizes and binds to hemi-methylated GATC sites within the *oriC* region to sequester the origin to the inner membrane of the cell (von Freiesleben, Rasmussen et al. 1994). DnaA-ATP complexes are then stimulated to hydrolyze the bound nucleotide through interactions with the Hda-β-clamp complex, thus inactivating DnaA (Katayama, Kubota et al. 1998). Although both the ADP- and ATP-bound form of DnaA bind DNA, only the ATP-bound form is active for initiation of replication. As replication progresses around the circular chromosome, newly synthesized *dnaA-boxes* are recognized and bound by ATP-complexed DnaA, effectively lowering the pool of active DnaA (Ogawa, Yamada et al. 2002; Morigen, Lobner-Olesen et al. 2003). These three events regulate the process of chromosomal replication to ensure the accurate and timely duplication of the genome for distribution into daughter cells.

Experimental evidence suggests that initiation of chromosomal replication in *E. coli* is

regulated through the activities of DnaA. One would expect that mutations within the *dnaA* allele could produce proteins that are either deficient for initiation of replication or proteins that elevate the frequency of initiation. Both of these situations have been observed. Mutant forms of DnaA have been identified that are unable to initiate replication (Sutton and Kaguni 1995; Sutton and Kaguni 1997; Sutton and Kaguni 1997; Sutton and Kaguni 1997), as well as mutants that are hyperactive for initiation of chromosomal replication (Kellenberger-Gujer, Podhajska et al. 1978). Characterization of DnaA protein by the isolation and analysis of mutants has identified several biochemical functions (Sutton and Kaguni 1997; Sutton and Kaguni 1997; Simmons, Felczak et al. 2003). The loss of any of these functions can result in a DnaA protein that is incapable of initiating replication from *oriC*. Additionally, mutants that initiate replication with an increased frequency have also been identified. The *dnaAcos* allele was isolated as an intragenic suppressor of the *dnaA46(*Ts*)* allele (Kellenberger-Gujer, Podhajska et al. 1978). This mutant has been shown to increase the frequency of replication asynchronously from *oriC* (Braun, O'Day et al. 1987; Katayama, Akimitsu et al. 1997) at the non-permissive 30°C and also under conditions of its induced expression from plasmid vectors (Simmons and Kaguni 2003).

Other *dnaA* alleles, in addition to *dnaAcos*, have been identified that cause hyperactive initiation. Models to explain the increased frequency of initiation are either a constitutively active conformation that does not require ATP binding for activation (*dnaAcos*), or the inability of the mutant protein to hydrolyze bound ATP (DnaA R334A; see (Nishida, Fujimitsu et al. 2002)). It has been shown that the A184V and Y271H

mutations of DnaAcos result in a defect in ATP binding yet the mutant protein is active for initiation of replication (Carr and Kaguni 1996; Simmons and Kaguni 2003). A deficiency of ATP hydrolysis leads to the hyperactivity seen with the R334A mutation in DnaA (Nishida, Fujimitsu et al. 2002).

DnaAcos and DnaA R334A are examples of mutant DnaA proteins that escape the normal regulatory mechanisms either through loss of ATPase activity or through a conformational change resulting in constitutive activation. Based on the properties of these mutant proteins, the importance of ATP binding and hydrolysis on the process of regulating initiation of chromosomal replication is evident. In this study we sought to further characterize novel *dnaA* alleles that appear to be hyperactive for initiation of chromosomal replication. These alleles were isolated by Lyle Simmons, a former graduate student in the Kaguni laboratory. He used the *dnaAcos* allele as a control for the genetic method to isolate hyperactive alleles, and we were able to identify several novel *dnaA* alleles that appear to be dominant negative to chromosomally encoded wild type DnaA. The dominant negative phenotype has been associated with hyperactive initiation as determined by marker frequency analysis. We present here genetic, molecular and biochemical assays that have allowed us to separate the novel alleles into two specific groups: those that behave like *dnaAcos* and those that are hyperactive yet respond to the known regulatory mechanisms that control the frequency of initiation.

Materials and Methods

Bacterial Strains and Plasmids

E. coli K-12 strains used in the work have been listed in Table 1. Plasmid DNAs were prepared by either equilibrium centrifugation or by column chromatography (Qiagen, Midi Kit) and are listed in Table 2. All novel *dnaA* alleles (except S146Y originally isolated in pRB100) encoded by derivatives of pDS596 were isolated by Lyle Simmons, who analyzed the DNA sequence of each *dnaA* allele to determine the position of the respective mutation and the amino acid substitution.

Lethality of induced expression of novel *dnaA* alleles

E. coli MC1061 (relevant genotype *araD*139 Δ*araC)* electrocompetent cells were transformed with derivatives of pDS596 bearing the seven novel alleles, wild type *dnaA* and *dnaAcos* (plasmids listed in Table 2). Transformants were selected on LB media with 100 mg/ml ampicillin or LB plates with 100 mg/ml ampicillin, with or without 0.5% arabinose (v/v). Incubation was overnight at 30°C, 37°C and 42°C. The ratios of transformation efficiencies were calculated from the number of colonies obtained on media supplemented with arabinose divided by the number of colonies obtained in the absence of arabinose.

Table 1: *E. coli* strains used

Strain	Genotype	Source
MC1061	*araD*139, Δ(*ara*, *leu*)7697 Δ(*lac*)X74 *gal*U *gal*K *hsd*R2(r_k^-,m_k^+) *str*A *mcr*A *mcr*B1	Lab stock
SK002	*araD*139 Δ(*ara*, *leu*)7697 Δ(*lac*)X74 *gal*U *gal*K *hsd*R2(r_k^-,m_k^+) *str*A *mcr*A *mcr*B1 Δ*recB*	Korrapati and Kaguni (Unpublished)
BL21(DE3) pLysS	*E. coli* B F^- *dcm ompT hsdS*(r_B^- m_B^-) *gal* λ(DE3) [pLysS Cam^r]	Lab stock

Table 2: List of Plasmids

Plasmid	Properties	Source
pDS596	*dnaA* expression regulated by the ***araBAD*** promoter, Amp^r,	Hwang and Kaguni, 1988
pLS120	*dnaAcos* carried in pDS596 instead of *dnaA*$^+$	Simmons and Kaguni, unpublished results
pLS125	*dnaAG79D* carried in pDS596 instead of *dnaA*$^+$	Simmons and Kaguni, unpublished results
pLS126	*dnaAS146Y* carried in pDS596 instead of *dnaA*$^+$	Simmons and Kaguni, unpublished results
pLS127	*dnaAH202Y* carried in pDS596 instead of *dnaA*$^{++}$	Simmons and Kaguni, unpublished results
pLS128	*dnaAE244K* carried in pDS596 instead of *dnaA*$^{++}$	Simmons and Kaguni, unpublished results
pLS129	*dnaAV292M* carried in pDS596 instead of *dnaA*$^{++}$	Simmons and Kaguni, unpublished results
pLS130	*dnaAV303M* carried in pDS596 instead of *dnaA*$^{++}$	Simmons and Kaguni, unpublished results
pLS131	*dnaAE445K* carried in pDS596 instead of *dnaA*$^{++}$	Simmons and Kaguni, unpublished results
pACYC184	Cm^r, Tet^r	Laboratory Stock
pACMF1	pACYC184, *dnaN*, Cam^r	Felczak and Kaguni, unpublished results
pACMF41	pACYC184, *hda*, Cam^r	Felczak and Kaguni, unpublished results
pACMF57	pACYC184, *seqA*, Cam^r	Felczak and Kaguni, unpublished results
pACMF84	pACYC184, *datA*, Cam^r	Felczak and Kaguni, unpublished results
pKC597	*dnaA* expression regulated by T7 RNA polymerase; DnaA fused at it's N-terminus to polyhistidine, Ap^r	Walker et al, 2006
pKCG79D	*dnaAG79D* carried in pKC597 instead of *dnaA*$^+$	Simmons and Kaguni, unpublished results
pKCS146Y	*dnaAS146Y* carried in pKC597 instead of *dnaA*$^+$	Simmons and Kaguni, unpublished results
pKCH202Y	*dnaAH202Y* carried in pKC597 instead of *dnaA*$^+$	Simmons and Kaguni, unpublished results
pKCE244K	*dnaAE244K* carried in pKC597 instead of *dnaA*$^+$	Simmons and Kaguni, unpublished results
pKCV292M	*dnaAV292M* carried in pKC597 instead of *dnaA*$^+$	Simmons and Kaguni, unpublished results
pKCV303M	*dnaAV303M* carried in pKC597 instead of *dnaA*$^+$	Simmons and Kaguni, unpublished results
pKCE445K	*dnaAE445K* carried in pKC597 instead of *dnaA*$^+$	Simmons and Kaguni, unpublished results

Lethality suppression Assays

MC1061 (*araD*139 Δ*araC*) electrocompetent cells were co-transformed with derivatives of the plasmid pDS596 and derivatives of pACYC184 bearing ***datA***, ***hda***, ***seqA***, or ***dnaN*** (See Table 2). Transformants were selected for on LB media supplemented with 100 ug/ml ampicillin and 35 ug/ml chloramphenicol with or without 0.5% arabinose. After overnight incubation at 30°C, 37°C and 42°C, the ratio of colonies on media with arabinose divided by number of colonies in the absence of arabinose was determined.

Quantitative Real Time PCR of *oriC* and *relE*

E. coli MC1061 (relevant genotype *araD*139 Δ*araC*) was transformed with pDS596 or its derivatives bearing the *dnaAcos* or the novel ***dnaA*** alleles as listed in Table 2. Transformants were plated on selective media (100 ug/ml ampicillin) and incubated at either 30°C, 37°C or 42°C. Following overnight incubation, single colonies from each plate were transferred to 5 ml of LB media supplemented with 100 ug/ml ampicillin, and 1% glucose and grown overnight at the respective temperature. The overnight cultures were used to inoculate 100 ml of the above media such that the starting $OD_{(595nm)}$ was approximately 0.002. The cultures were incubated at 30°C, 37°C and 42°C with aeration to about 0.15 $OD_{(595nm)}$, at which point each culture was divided and a sample named "Time zero" was saved. Cells from the divided culture were collected by centrifugation (14k rpm for 2 minutes) and resuspended in the media supplemented with either 1% glucose or 0.5% arabinose, and then incubated at 30°C, 37°C or 42°C with aeration. At

time intervals of 30, 60, 90, 120 and 180 minutes, samples (3x 1.5 ml) were collected by centrifugation (1.5 minutes at 14k rpm), and immediately frozen in liquid N_2.

The concentration and purity of genomic DNA from these samples, isolated using Qiagen DnEasy Tissue kits, was measured by absorbance at 260 and 280 nm. Genomic DNA samples were diluted to approximately 1 ng/ul and used as the template for quantitative Real-Time PCR analysis in which each sample was analyzed in triplicate (25 µl reactions) following the supplier's (Applied Biosystems) guidelines and compared to a DNA standard of genomic DNA isolated from a stationary phase culture of *E. coli* MC1061. Primers for the amplification of *oriC* were GAGATCTGTTCTATTGTGATCTCTTATTAGGAT and CAGTTAATGATCCTTTCCAGGTTGT and those used in the amplification of *relE* were AGACCGGAGCTTAATCTTGTAACAA and ACAGTTGAAAAAGAAGCTGGTTGA. Real Time PCR was conducted with an Applied Biosystems 7500 Fast Real-Time PCR System. The default setting was used for amplification and detection.

Protein Quantitation

One of the three samples removed at 0, 30, 60, 90, 120 and 180 min from the induced and uninduced cultures was analyzed by quantitative western blotting analysis. These cell pellets were resuspended in SDS-PAGE sample buffer at a ratio of 10 µl per 0.1 $OD_{(595nm)}$ of culture. The whole cell lysates were separated electrophoretically in a 10% SDS-polyacrylamide ge. In parallel increasing amounts of purified wild type DnaA were used

to prepare a standard curve. Following electroblotting, the membranes (Protran, Schleicher & Schuell) were probed with M43 monoclonal antibody (Marszalek, Zhang et al. 1996) as the primary antibody and horseradish peroxidase-conjugated Goat anti-mouse antibody as the secondary antibody. After incubation with horseradish peroxidase-conjugated secondary antibody (BioRad), the chemiluminescence (Supersignal, Pierce) was detected with Xray film (X Omat, Kodak).

Protein Expression and Purification

E. coli strain BL21 (DE3) (pLysS) treated with the $CaCl_2$ method was transformed with derivatives of pKC597 bearing the novel *dnaA* alleles listed in Table 2. Transformants were selected on LB media supplemented with 100 μg/ml ampicillin and 35 μg/ml chloramphenicol followed by overnight incubation. Single colonies were used to inoculate of 5 ml of the above media to prepare overnight cultures, which were used to inoculated 50 ml of the above media to confirm overproduction of the mutant protein essentially as described below, or flasks containing a total of 6 L of the media. At an $OD_{(595nm)}$ of 0.6 – 0.8, IPTG was added to a final concentration of 0.2 mM. After two hours of induced expression, the cells were pelleted, and resuspended in 50 mM Tris-HCl pH 7.0 and 10% sucrose to an $OD_{(595nm)}$ of approximately 200. To obtain lysis, NaCl, $SpCl_3$, and imidazole were added to final concentrations of 0.5 M, 20 mM and 5 mM, respectively. The whole cell lysate was centrifuged in a 45Ti rotor at 40k rpm at 4°C for 20 minutes. The supernatant, Fraction I, was collected and frozen in liquid nitrogen.

NTA resin (Invitrogen) was charged with $Ni(SO_4)_2$ according to the manufacturer's instructions. The column was then equilibrated with 5 column volumes of buffer IMAC-A (20 mM Tris- HCl pH 7.6, 5 mM imidazole, 0.5 M NaCl, and 15% glycerol (v/v)). The chromatography resin was removed from the column and mixed with Fraction I for 1 hour. The slurry was then returned to the column followed by a wash with 10 column volumes of IMAC-B (20 mM Tris-HCl pH 6.0, 20 mM imidazole, 0.5 M NaCl, and 15% glycerol (v/v)). The column was then washed with 10 column volumes of IMAC-A to re-equilibrate the column to pH 7.6. The Histidine-tagged proteins were eluted from the column using IMAC-C (20 mM Tris-HCl pH 7.6, 400 mM imidazole, 0.5 M NaCl, and 15% glycerol (v/v)). The column was then stripped with 5 column volumes of IMAC-E (20 mM Tris-HCl pH 7.6, 100 mM EDTA, 0.5 M NaCl, and 15% glycerol (v/v)). Protein purification was monitored by SDS-PAGE of 4 µl samples of alternating column fractions. Fractions containing the mutant DnaA protein were pooled and dialyzed against Buffer C (50 mM Hepes-KOH pH 7.6, 1 mM EDTA, 2 mM DTT and 20% glycerol (v/v)). The precipitate following dialysis was resuspended in Buffer C+ (50 mM Hepes-KOH pH 7.6, 1 mM EDTA, 2 mM DTT, 20% glycerol (v/v), 0.6 M $(NH_4)_2$ SO_4, 10 mM magnesium-acetate and 4 M guanidine HCl). Gel filtration was performed using an Amersham Biosciences AKTA FPLC with a Superose 12 column equilibrated in Buffer D (50 mM Hepes-KOH pH 7.6, 1 mM EDTA, 2 mM DTT, 20% glycerol (w/v), 0.2 M $(NH_4)_2$ SO_4 and 10 mM magnesium-acetate).

In vitro oriC plasmid replication assay

Reaction mixtures (25 µl) contained HEPES-KOH (pH 7.5), 25 mM Tris-HCl (pH 7.5), 4% sucrose, 2 mM ATP, 0.5 mM CTP, UTP, and GTP, 100 µM dATP, dCTP, dUTP, and (^{3}H)dTTP (30 cpm/pmol), 11 mM MgOAc, 2 mM phosphocreatine, 5 mM DTT, 100 µg/ml creatine kinase, 0.08 mg/ml BSA, SSB, Hup A, Gyr A, Gyr B, DnaA, DnaB, Primase, Pol III*, and β-clamp at optimal levels, and 200 ng of supercoiled M13*oriC*2LB5 DNA (*oriC* plasmid). After the addition of increasing amounts of purified DnaA$^+$, DnaA H202Y and DnaA V292M, the reactions were incubated for 30 minutes at 30°C, and then 1 ml of 10% TCA and 0.1M NaPPi was added to stop the reactions and to precipitate the DNA. The precipitated DNA was collected by filtration through glass fiber filters and the incorporation of ^{3}H-dTTP was quantified by liquid scintillation counting.

ATP binding assays

Reactions (25 µl) to measure ATP binding by DnaA protein (Sekimizu et al., 1987) contained 2 pmol (100 ng) of protein and the indicated amounts of [α-^{32}P]-ATP in buffer containing 0.5 mM magnesium acetate, 15% glycerol, 0.01 % Triton X-1 00, and 50 mM Tris-HCI pH 8.0. Incubations were at 0°C for 20 min followed by filtration through nitrocellulose filters (Millipore HAWP; 0.22 µm, 13 mm) which were then washed with 500 µl of the above buffer. Radioactive ATP bound to the filters was quantified by liquid scintillation counting.

Two stage replication assay

The assay was performed essentially as described above for the *in vitro oriC* plasmid replication assay, however the assay was separated into two stages. Initially a reaction mixture was prepared containing HEPES-KOH (pH 7.5), 25 mM Tris-HCl (pH 7.5), 4% sucrose, 2 mM ATP, 0.5 mM CTP, UTP, and GTP, 100 μM dATP, dCTP, dUTP, and (^{3}H)dTTP (30 cpm/pmol), 11 mM MgOAc, 2 mM phosphocreatine, 5 mM DTT, 100 μg/ml creatine kinase, 0.08 mg/ml BSA, Pol III*, and β-clamp at optimal levels, and 200 ng of supercoiled M13*oriC*2LB5 DNA (*oriC* plasmid). To this, increasing amounts of Ni-NTA fractions containing Hda was added. The reactions were incubated at 30°C for 20 minutes at which point the remaining components (reaction mixture 2), SSB, HU (α dimer), Gyr A, Gyr B, DnaA, DnaB and Primase, were added to the reactions. Incubation at 30°C continued for an additional 20 minutes at which point the reaction was stopped and the DNA was precipitated with 1 ml of 10% TCA and 0.1M NaPPi. The precipitated DNA was collected by filtration through glass fiber filters and the incorporation of ^{3}H-dTTP was quantified by scintillation counting.

RESULTS

Induced expression of specific *dnaA* alleles causes lethality

Lyle Simmons, a former graduate student, developed a genetic method to identify *dnaA* alleles, that like *dnaAcos* interfered with growth when expression was induced in a

dnaA46(Ts) strain at 42°C. This growth interference, which was not observed with *dnaA*$^+$, has been shown to result from genomic damage induced by the collision from behind of a new replication fork with one ahead that has stalled or collapsed (Simmons and Kaguni 2003; Simmons, Breier et al. 2004). Knowing that the *dnaAcos* allele encodes a protein that is hyperactive for initiation, the novel alleles isolated by the approach may also encode hyperactive initiators.

As described above, Lyle's observations relied on a *dnaA46*(Ts) strain. To confirm and extend these results, we transformed a wild type strain of *E. coli* (MC1061, relevant genotype: *araD139* Δ(*ara, leu*)7697 with derivatives of pDS596 bearing the seven novel alleles, and also wild type *dnaA* and *dnaAcos* as controls (plasmids listed in Table 2). Transformants were plated on LB media (100 μg/ml ampicillin) with and without 0.5% arabinose followed by incubation at 30°C, 37°C and 42°C. Colonies were counted the following day and the ratio of the transformation efficiencies under induced to uninduced conditions was calculated for each *dnaA* allele.

It has been previously shown that induced expression of *dnaAcos* but not wild type *dnaA* was lethal to the host at both 30°C and 42°C (Simmons and Kaguni 2003; Simmons, Breier et al. 2004). To confirm these results, the effect of induced expression of *dnaAcos* encoded by pLS120 was compared to elevated *dnaA*$^+$ expression in cells carrying pDS596. The *dnaAcos*-dependent lethality at all temperatures (Table 3) contrasts with viability when *dnaA*$^+$ was induced.

Table 3. Lethality of induced expression of novel *dnaA* mutations in MC1061

	Ratio of colony formation		
Plasmid (allele)	**30°C**	**37°C**	**42°C**
pDS596 (*dnaA*$^+$)	1.1	1.2	0.9
pLS120 (*dnaAcos*)	1.5×10^{-7}	1.6×10^{-7}	1.1×10^{-7}
pLS125 (*G79D*)	3.7×10^{-7}	2.9×10^{-7}	3.1×10^{-7}
pLS126 (*S146Y*)	1.4×10^{-7}	4.0×10^{-4}	6.3×10^{-4}
pLS127 (*H202Y*)	7.8×10^{-7}	2.8×10^{-3}	4.8×10^{-3}
pLS128 (*E244K*)	1.0	0.5	4.1×10^{-3}
pLS129 (*V292M*)	1.6×10^{-7}	1.4×10^{-7}	1.6×10^{-7}
pLS130 (*V303M*)	0.60	0.8	3.0×10^{-3}
pLS131 (*E445K*)	0.8	0.6	5.8×10^{-3}

E. coli MC1061 cells transformed with the indicated derivatives of pDS596 (see Table 2) were plated on media with and without arabinose. The number of colonies observed when *dnaA* expression was induced compared to the number without induced expression is expressed as a ratio for each respective temperature.

In comparison, the *dnaA* alleles, *G79D*, *S146Y*, *H202Y* and *V292M* interfered with growth upon their induced expression at 30°C, 37°C and 42°C. The remaining three alleles (*E244K*, *V303M* and *E445K*) were temperature-sensitive, showing increased lethality at 42°C. These results are not wholly unexpected. The increased lethality seen at 42°C for *E244K*, *V303M* and *E445K* may be an artifact of the genetic method used to isolate the novel alleles. The assay was based upon the observation that induced expression of *dnaAcos* at 42°C caused growth interference, but its uninduced expression did not. It is possible that DnaAcos encoded by pLS120 forms mixed complexes with DnaA46 at *oriC* to maintain viability at the non-permissive temperature for the *dnaA46*(Ts) host strain. Accordingly, a mutant DnaA protein may interfere with viability at 42°C but this effect may be reduced at 30°C and 37°C. The results for *E244K*, *V303M* and *E445K* suggest that these alleles, like *dnaA46ts*, are also temperature sensitive. Complementation of *dnaA46ts* by these alleles may be the result of mixed oligomeric complexes forming at *oriC* similar to those formed by DnaAcos and DnaA46.

RT-PCR as a method to measure the initiation frequency

It has been shown that increased levels of DnaA-ATP (Atlung, Lobner Olesen et al. 1987) or hyperactive mutant DnaA proteins (Kellenberger-Gujer, Podhajska et al. 1978; Katayama and Kornberg 1994) lead to more frequent initiations. In the first case, the elevated levels of DnaA-ATP stimulate an increase in initiations, which does not interfere with viability. In the second, *dnaA* mutations may encode proteins that escape the normal regulatory pathways. To obtain direct evidence that the *dnaA* alleles promote extra

Figure 2.1. Establishment of baselines for Real-Time PCR analysis of *oriC/relE* ratios

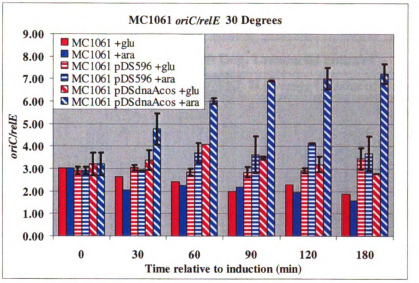
Panel A. *oriC/relE* ratios for cultures without a plasmid, or carrying pDS596 (*dnaA*) or pLS120 (*dnaAcos*)

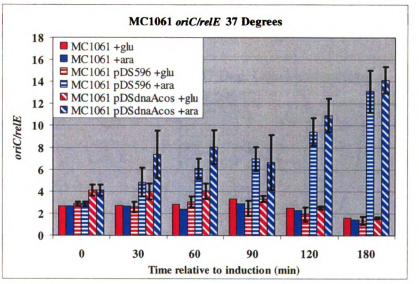
MC1061 (*araD139* Δ*araC*) without a plasmid, or with pDS596 (*dnaA*$^+$) or pLS120 (*dnaAcos*) were grown as described in "Materials and Methods." Samples were collected from both the induced and uninduced cultures at the time points indicated to determine the *oriC/relE* ratios from genomic DNA obtained from both the induced (arabinose) and mock-induced (glucose) cultures. Three cultures of MC1061 carrying pDS596 and pLS120 were grown at 37°C and analyzed, and two cultures each were grown at 30°C and 42°C and analyzed.

Panel B. Uninduced *oriC/relE* averages of cultures bearing pDS596 and its derivatives

Quantitative Real-Time PCR analysis was performed on samples collected from the uninduced cultures bearing either pDS596 or its derivatives (Table 2). The average of the *oriC/relE* ratios and the standard deviation for each time point were calculated.

A


MC1061 oriC/relE 30 Degrees
MC1061 +glu
MC1061 +ara
MC1061 pDS596 +glu
MC1061 pDS596 +ara
MC1061 pDSdnaAcos +glu
MC1061 pDSdnaAcos +ara
oriC/relE
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0
30
60
90
120
180
Time relative to induction (min)


MC1061 oriC/relE 37 Degrees
MC1061 +glu
MC1061 +ara
MC1061 pDS596 +glu
MC1061 pDS596 +ara
MC1061 pDSdnaAcos +glu
MC1061 pDSdnaAcos +ara
oriC/relE
18
16
14
12
10
8
6
4
2
0
0
30
60
90
120
180
Time relative to induction (min)

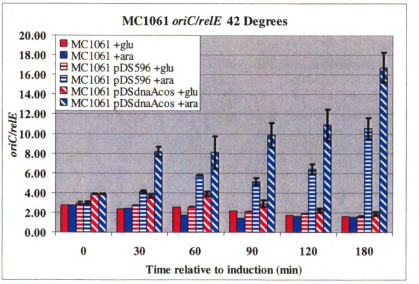
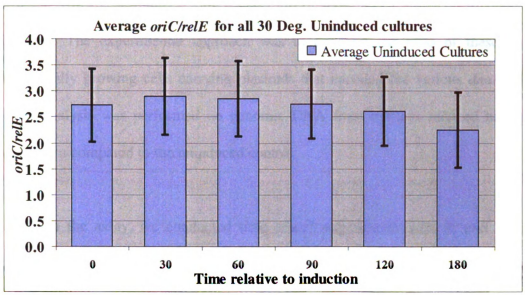
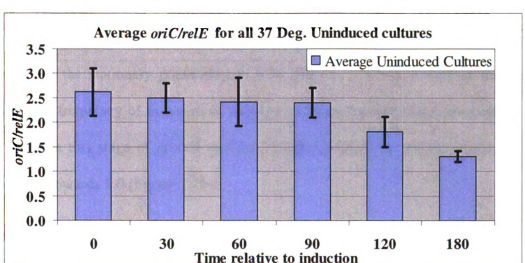
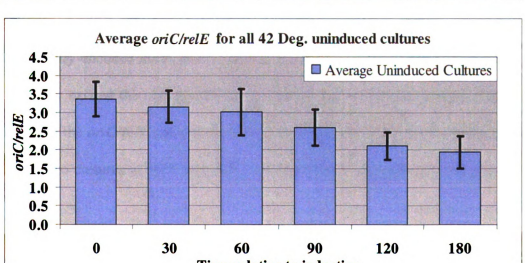

MC1061 oriC/relE 42 Degrees
MC1061 +glu
MC1061 +ara
MC1061 pDS596 +glu
MC1061 pDS596 +ara
MC1061 pDSdnaAcos +glu
MC1061 pDSdnaAcos +ara
oriC/relE
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
0
30
60
90
120
180
Time relative to induction (min)

Figure 2.1 continued

B







initiations, we developed a Real-Time PCR assay to quantify the abundance of *oriC* and *relE*, a locus in the terminus region. The ratio of these loci reflects the frequency of initiations. The experimental approach was to compare the ratio of these loci in logarithmically growing cells carrying plasmids that encoded the various *dnaA* alleles. RT-PCR analysis was performed on genomic DNA from cultures induced to express DnaA protein compared to the uninduced control.

To establish the assay, we conducted time course experiments with *E. coli* MC1061 bearing no plasmid. As expected, the analysis shows essentially no difference between the uninduced and induced cultures grown at the indicated temperatures. Additionally, the *oriC/relE* ratios decreased (Figure 2.1A) as the cultures progressed from exponential growth into the stationary phase (Figure 2.2). As the generation time of the culture slowed, the frequency of initiation diminished. Consistent with other observations that most cells at this stage of growth contain a single genome, we see the *oriC/relE* ratio decreasing towards 1.0 (Figure 2.1B).

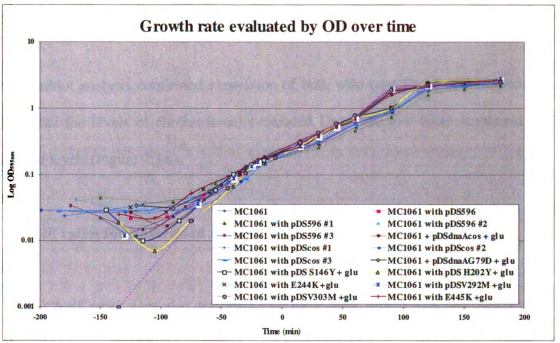
We also analyzed MC1061 with plasmids bearing the *dnaA*$^+$ and *dnaAcos* alleles under control of an arabinose-inducible promoter. Although the host strain has the chromosomally-encoded *dnaA* gene, induced expression from the plasmid-encoded gene should vastly exceed the chromosomally encoded level (Grigorian, Lustig et al. 2003). We averaged the *oriC/relE* ratios at the indicated time points from three cultures grown at 37°C, and two cultures at 30°C and 42°C. The *oriC/relE* ratios for the induced cultures

Figure 2.2. Growth rate of MC1061 cultures as a function of time

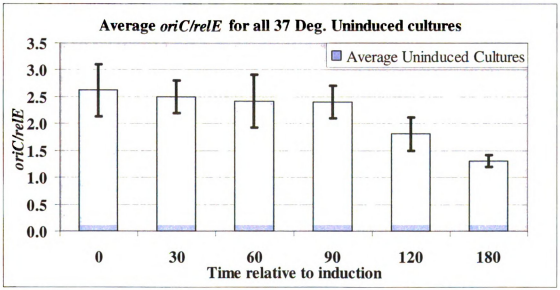
Panel A. Culture growth rate was monitored by determining the OD595nm at the indicated time points, prior to and following induced expression from pDS596 or its derivatives. When evaluated with the Real-Time PCR data a relationship between growth rate and *oriC/relE* ratio can be established. The curves shown here represent the data collected from the cultures grown at 37°C.

Panel B. *oriC/relE* ratios were averaged for all cultures grown at 37°C. As the cultures transition into stationary phase the *oriC/relE* ratios decrease towards one. The transition from logarithmic growth to the stationary phase occurred between 90 and 120 minutes.

A


Growth rate evaluated by OD over time
Log OD600nm
10
1
0.1
0.01
0.001
-200
-150
-100
-50
0
50
100
150
200
Time (min)
MC1061
MC1061 with pDS596
MC1061 with pDS596 #1
MC1061 with pDS596 #2
MC1061 with pDS596 #3
MC1061 + pDSdmaAcos + glu
MC1061 with pDScos #1
MC1061 with pDScos #2
MC1061 with pDScos #3
MC1061 + pDSdmaAG79D + glu
MC1061 with pDS S146Y + glu
MC1061 with pDS H202Y + glu
MC1061 with E244K +glu
MC1061 with pDSV292M +glu
MC1061 with pDSV303M +glu
MC1061 with E445K +glu

B


Average oriC/relE for all 37 Deg. Uninduced cultures
Average Uninduced Cultures
oriC/relE
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
30
60
90
120
180
Time relative to induction

bearing the wild type *dnaA* and *dnaAcos* plasmids, grown at 30°C and 42°C, show that DnaAcos induces more initiations than wild type DnaA (Figure 2.1A). However, the ratios at 37°C are not very different.

Immunoblot analysis confirmed expression of both wild type DnaA and DnaAcos, and show that the levels of chromosomally-encoded DnaA are low when compared to the induced levels (Figure 2.3A).

oriC/relE ratios for Novel *dnaA* alleles

The *oriC/relE* ratios were also determined for each of the seven novel *dnaA* alleles from induced and uninduced cultures grown at 30°C, 37°C and 42°C (Figure 2.4). Figure 2.4 compares the ratios for each allele at the same time point. As a control, we determined the ratios of all uninduced cultures at a given temperature (Figure 2.1B). Determining these ratios served two purposes. First we were able to verify that induced expression from the arabinose promoter did affect the frequency of replication initiation. Second we confirmed that in the absence of arabinose, the frequency of initiation was comparable to the MC1061 cells without plasmid.

The *oriC/relE* ratio data for the 30°C cultures shows that most of the alleles display some hyperactivity for initiation at 30°C with increasing time after induced expression. The level of initiation is comparable or exceeds that observed with DnaAcos. However S146Y did not follow this same pattern. After an initial increase in initiations up to the

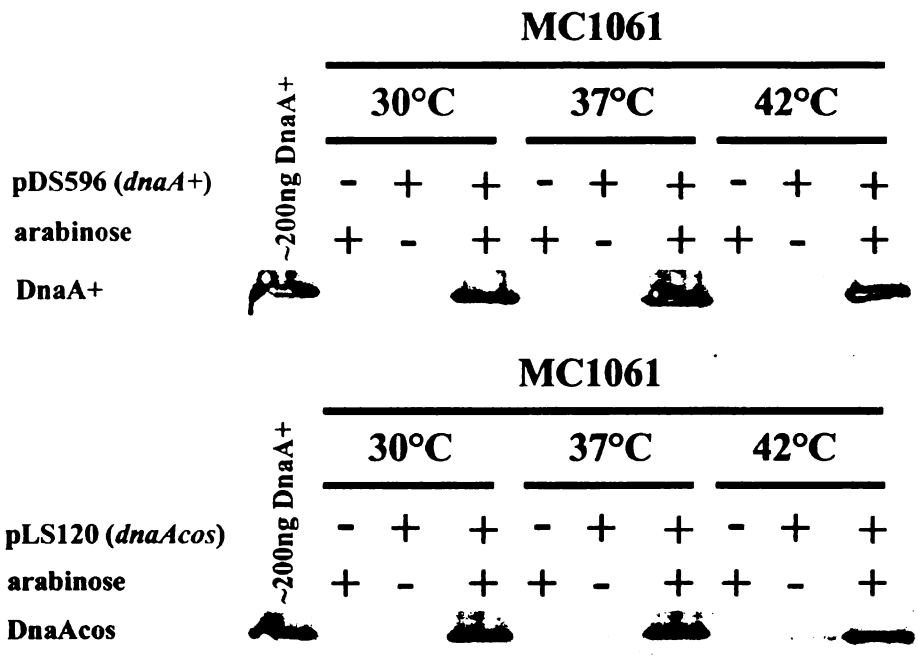
Figure 2.3. Immunoblot analysis of DnaA expression

Panel A. Whole cell lysates were prepared from MC1016 cells collected from induced and uninduced cultures grown at 37°C bearing either pDS596 (*dnaA*$^+$) or pLS120 (*dnaAcos*) at the times indicated. Approximately 10^8 cells from each sample were analyzed via SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblot results confirm the induced expression of the encoded DnaA protein following addition of arabinose to the media. Cultures grown in the absence of arabinose show low levels of DnaA, representing the endogenous levels of the protein. The nitrocellulose membrane was probed with the DnaA polyclonal C-terminal antibody.

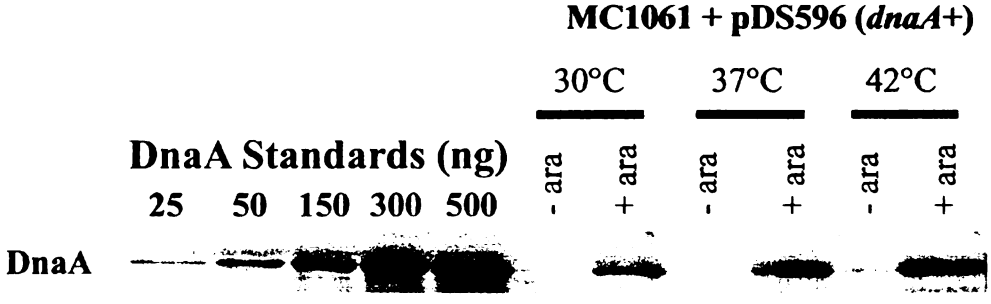
Panel B. Whole cell lysates were prepared from MC1061 cells bearing pDS596 collected 60 minutes post induction from cultures grown at 30°C, 37°C, and 42°C. Increasing amounts of purified wild type DnaA were used to prepare a standard curve. Approximately 10^8 cells were analyzed for both the 60 min uninduced and induced samples from cultures grown at 30°C, 37°C or 42°C. The nitrocellulose membrane was probed with the DnaA polyclonal C-terminal antibody.

Panel C. Whole cell lysates were prepared from MC1061 cells bearing the pDS596 derivatives listed in Table 2 collected 60 minutes post induction. Purified wild type DnaA (220 ng) was used as a marker for analysis of 10 µl of whole cell lysate, approximately 10^8 cells, prepared for each allele. The nitrocellulose membrane was probed with the DnaA polyclonal C-terminal antibody.

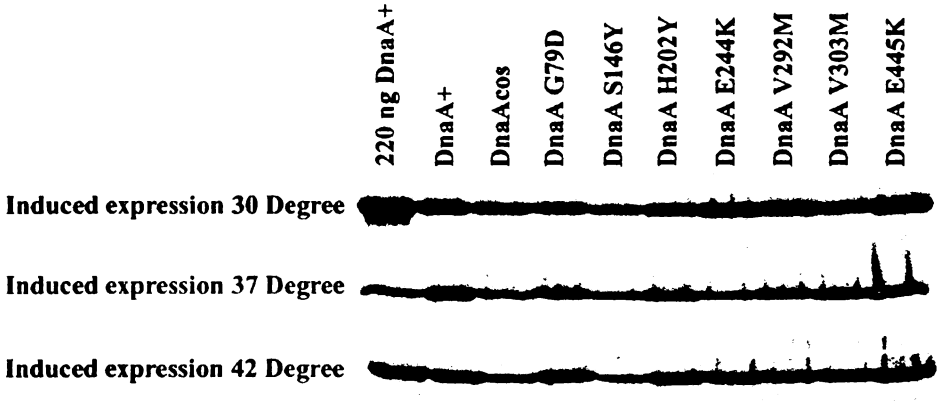
A


MC1061
30°C
37°C
42°C
~200ng DnaA+
pDS596 (dnaA+)
arabinose
DnaA+
MC1061
30°C
37°C
42°C
~200ng DnaA+
pLS120 (dnaAcos)
arabinose
DnaAcos

B


MC1061 + pDS596 (dnaA+)
30°C
37°C
42°C
DnaA Standards (ng)
25 50 150 300 500
- ara
+ ara
DnaA

C


220 ng DnaA+
DnaA+
DnaAcos
DnaA G79D
DnaA S146Y
DnaA H202Y
DnaA E244K
DnaA V292M
DnaA V303M
DnaA E445K
Induced expression 30 Degree
Induced expression 37 Degree
Induced expression 42 Degree

60 minute time point, the *oriC/relE* ratios were comparable to those of the wild type allele.

The data collected for the cultures carrying the plasmid-borne ***dnaA*** mutations at 42°C does not provide the same uniform results. Induced expression of the *S146Y* allele did not cause increased initiation. The *oriC/relE* ratios observed for this allele peak at 30 minutes and then gradually decrease to approximately half of the ratio seen when *dnaA*$^+$ was induced. *G79D*, *H202*, *V292M* and *E445K* all show elevated levels of initiation compared to wild type *dnaA* whereas *E244K* and *V303M* were similar to wild type *dnaA*.

The ratios calculated for the seven novel alleles, *dnaA*$^+$ and *dnaAcos* confirm that their induced expression leads to more frequent initiations. However, with the exception of S146Y, we are unable to correlate the increased initiation frequency with the phenotype of growth interference. As was seen with the *S146Y* allele at 30°C and 42°C, only minor increases in the frequency of initiations occurs following induced expression of the *S146Y* allele at 37°C.

Temperature dependent activity of DnaA

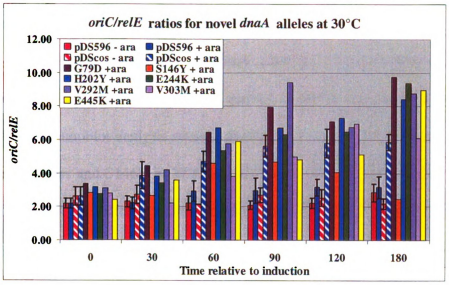
As described earlier, our experimental approach relied upon induced expression of the various *dnaA* alleles carried in a plasmid. Because of their dominant negative

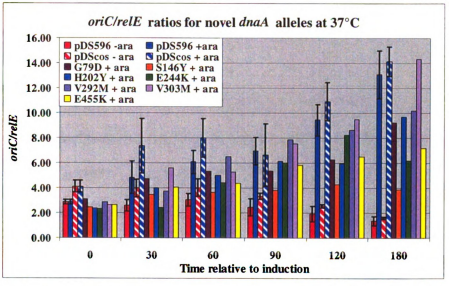
phenotypes, the *dnaA* alleles were under control of the *araBAD* promoter. Thus we were able to suppress expression of the mutant alleles using glucose-supplemented media until the culture density reached early log-phase growth, and then to induce expression by transferring the bacterial cells to liquid media containing arabinose. Unexpectedly, the *oriC/relE* data collected for the MC1061 cultures bearing pDS596 (*dnaA*$^+$) grown at 30°C showed only a minor increase in the frequency of initiation following induced expression (Figure 2.4). In contrast, the ratios observed for the plasmid bearing strain grown at 37°C and 42°C, increased with time after induced expression. This observation suggests two possibilities: either DnaA$^+$ has lower activity at 30°C than at 37°C and 42°C, or the level of protein synthesized following induction is substantially lower at 30°C that at the higher temperatures.

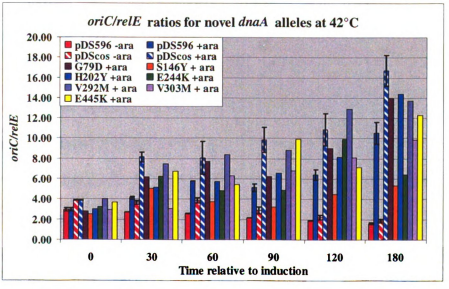
To distinguish between these possibilities, we compared protein expression levels by quantitative immunoblotting of whole cell lysates. Using an equivalent numbers of cells, we compared DnaA expression levels in MC1061 cultures bearing pDS596 grown at 30°C, 37°C and 42°C after one hour of induced expression. Our results show that the abundance of DnaA protein increased slightly with temperature (Figure 2.3B), but the high level of DnaA overproduction at 30°C does not correlate with the modest increase in the *oriC/relE* ratio for the same sample analyzed by RT-PCR. These results suggest that DnaA activity is inherently lower at 30°C or that it, and not DnaAcos, responds to an unknown regulatory factor at this temperature.

Figure 2.4. *oriC/relE* ratios for mutant alleles at 30°C, 37°C and 42°C

E. coli MC1061 (*araD139 ΔaraC)* was transformed with the *dnaA* plasmids listed in Table 2. Transformants were plated on selective media and single colonies were used to inoculate overnight cultures. Experimental cultures were then grown to an OD between 0.15 and 0.20, at which point the culture was split into two equal volumes. One-half was treated with glucose and the other half treated with arabinose. Samples for quantitative Real-Time PCR were collected at the time of induction and thereafter at 30, 60, 90, 120 and 180 minutes.


oriC/relE ratios for novel dnaA alleles at 30°C
pDS596 - ara
pDS596 + ara
pDScos - ara
pDScos + ara
G79D +ara
S146Y + ara
H202Y +ara
E244K +ara
V292M +ara
V303M +ara
E455K +ara
oriC/relE
12.00
10.00
8.00
6.00
4.00
2.00
0.00
0
30
60
90
120
180
Time relative to induction


oriC/relE ratios for novel dnaA alleles at 37°C
pDS596 -ara
pDS596 +ara
pDScos - ara
pDScos + ara
G79D + ara
S146Y + ara
H202Y + ara
E244K + ara
V292M + ara
V303M + ara
E455K + ara
oriC/relE
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
0
30
60
90
120
180
Time relative to induction


oriC/relE ratios for novel dnaA alleles at 42°C
pDS596 -ara
pDS596 +ara
pDScos -ara
pDScos +ara
G79D +ara
S146Y + ara
H202Y +ara
E244K +ara
V292M + ara
V303M + ara
E445K +ara
oriC/relE
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
0
30
60
90
120
180
Time relative to induction

Having shown that protein expression from pDS596 is roughly comparable at the three temperatures, we wanted to compare expression levels of the mutant DnaAs. As in Figure 2.3C, equivalent numbers of cells were analyzed via immunoblot compared to wild type DnaA expressed from pDS596 and to a known amount of purified DnaA protein. The immunoblot analysis reveals similar levels of most of the mutant proteins except for DnaAcos and S146Y at 37°C and 42°C (Figure 2.3C). These results support the conclusion that the differences in *oriC/relE* ratios are caused by the mutant DnaAs, and not by variations in their expression levels.

Temperature-dependent lethality of a strain deficient in the repair of double stranded breaks

The small increase in the *oriC/relE* ratio for cultures bearing pDS596 (*dnaA*$^+$) grown at 30°C led to an experiment to better understand the effects of induced expression of the wild type protein at 30°C. *E. coli* SK002 (Δ*recB*), which is deficient in the repair of double strand breaks, was transformed with pDS596 (*dnaA*$^+$) and pLS120 (*dnaAcos*), and plated on selective media with and without arabinose (0.5%). After incubation at 30°C, 37°C or 42°C, the frequency of colony formation was measured on rich media lacking or containing arabinose (Table 4). We found induced expression of *dnaAcos* was lethal at all temperatures. Induced expression *dnaA*$^+$ was lethal at both 37°C and 42°C but not 30°C. However, the colonies on media containing arabinose were smaller in size than those on media without arabinose at the lower temperature. These results combined with the RT-PCR and immunoblot results suggest that DnaA activity is reduced at 30°C, resulting in less frequent initiations.

Table 4: Effects of temperature on lethality in recombination deficient host

	Ratio of cfus (+ara/-ara)		
Plasmid (dnaA allele)	**30°C**	**37°C**	**42°C**
pDS596 (*dnaA*$^+$)	1.1	2.0×10^{-3}	1.0×10^{-3}
pLS120 (*dnaAcos*)	1.2×10^{-3}	7.9×10^{-3}	7.0×10^{-3}

The respective plasmids were used to transform *E. coli* SK002 (relevant genotype Δ*recB*). Transformants were plated on selective plates with and without arabinose and grown at 30°C, 37°C and 42°C. Ratios represent the number of colonies/ml on the induced plates divided by the number of colonies/ml on the uninduced plates.

Suppression of the lethal effect caused by elevated expression of the mutant DnaAs with multicopy plasmids carrying *datA*, *hda*, *dnaN* or *seqA*.

Initiation of chromosomal replication is regulated by three independent mechanisms. One involves the sequestration of hemi-methylated *oriC* by SeqA (von Freiesleben, Rasmussen et al. 1994; von Freiesleben, Krekling et al. 2000). The second is the titration of DnaA-ATP through its binding to DnaA boxes at the *datA* locus (Kitagawa, Mitsuki et al. 1996; Kitagawa, Ozaki et al. 1998). The regulatory inactivation of DnaA (RIDA) by the hydrolysis of bound ATP through interactions with Hda and the β-clamp (*dnaN*) of DNA polymerase III holoenzyme is the third (Katayama, Kubota et al. 1998; Kato and Katayama 2001; Kawakami, Su'etsugu et al. 2006). Magdalena Felczak in our laboratory developed a genetic method to measure the effect of each of these mechanisms on DnaA function. To summarize, elevated levels of wild type DnaA causes lethality if the host strain is defective in the repair of double strand breaks. The presence of multicopy plasmids carrying *datA*, *hda, dnaN* or *seqA* suppresses this lethal effect. We used this method to determine if the mutants DnaAs were hyperactive in initiation because they failed to respond to one or more of these regulatory mechanisms.

Previous work has evaluated the effects of the various regulatory components in cells expressing the wild type or hyperactive DnaA. The presence of a multicopy plasmid encoding the *seqA* gene has little or no effect on a wild type strain but restores viability to cells bearing the *dnaAcos* allele at the non-permissive 30°C (Lu, Campbell et al. 1994). In cells bearing wild type DnaA, the presence of additional copies of *datA* slows the rate of growth, but does not drastically alter the viability of the cells (Morigen, Lobner-Olesen

et al. 2003). When extra copies of *datA* are present in cells bearing the *dnaAcos* allele cell viability is not restored. The presence of functional Hda and β–clamp has been shown to be a necessary component of the Regulatory Inactivation of DnaA-ATP (Katayama, Kubota et al. 1998; Kato and Katayama 2001). These two proteins interact with DnaA and enhance the ATPase activity of DnaA. It is known that the mutations within the *dnaAcos* allele result in a protein that has a greatly reduced affinity for ATP. The hyperactive phenotype of DnaAcos likely results from a conformational change that mimics the ATP bound state. This conformational change renders the protein constitutively active and as such is not affected by the regulatory inactivation of DnaA-ATP by the Hda-β-clamp complex. As a result, elevated levels of either Hda or the β-clamp should have no effect on the lethal effects seen upon induced expression of DnaAcos. Unlike DnaAcos, wild type DnaA responds to the components of RIDA and as such elevated levels of Hda have been shown to decrease the frequency of initiation of chromosomal replication.

However, because the *dnaA* alleles under study cause lethality in strains that are capable of double strand break repair, we used *E. coli* MC1061 (*araD139* Δ*araC*). The strain was co-transformed with plasmids bearing the *dnaA* alleles and derivatives of pACYC184 bearing *dnaN*, *hda*, *seqA* or *datA* (pACMF1, pACMF41, pACMF57 and pACMF84). Expression of the latter set of genes was not under the control of their native promoters, whereas *datA* is a chromosomal DNA site. Transformants were plated on selective media, with and without arabinose, and grown overnight at 30°C, 37°C and 42°C.

Table 5. Suppression of novel DnaA mutants with initiation regulation components

We cotransformed *E.coli* MC1061 cells with the plasmids bearing the novel *dnaA* alleles and plasmids pACYC184, pACMF1, pACMF41, pACMF57 and pACMF 84. Equal volumes of the transformants were plated on selected media with and without arabinose. The ratios listed in Table 4 represent the number of colonies from the induced plate divided by the number of colonies from the uninduced plate.

[a] Compared to the colony size of MC1061 carrying the *dnaA*$^+$ plasmid pDS596, colonies of this plasmid-bearing strain observed on arabinose enriched media were pinpoint in size (<1/10 the size of the control colonies).

Table 5.

Allele	Temp	*pACYC184*	*datA*	*hda*	*dnaN*	*seqA*
dnaA	30°C	1.1	1.4	1.0	1.4	1.1
	37°C	0.97	1.1	1.1	1.0	0.91
	42°C	0.95	1.0	1.2	1.1	1.0
dnaAcos	30°C	6.0×10^{-4}	4.0×10^{-4}	4.0×10^{-4}	3.0×10^{-4}	0.53
	37°C	6.0×10^{-4}	3.0×10^{-4}	4.0×10^{-4}	3.0×10^{-4}	0.47
	42°C	1.0×10^{-3}	6.0×10^{-4}	4.0×10^{-4}	6.0×10^{-4}	0.62
G79D	30°C	1.0×10^{-2}	0.93[a]	1.0	0.73[a]	1.10[a]
	37°C	4.0×10^{-3}	0.67[a]	0.86	0.62[a]	0.24[a]
	42°C	3.0×10^{-3}	0.70[a]	0.88	0.18[a]	0.27[a]
S146Y	30°C	3.0×10^{-3}	0.67	1.01	1.1×10^{-3}	0.97
	37°C	1.0×10^{-3}	0.15	0.66	1.1×10^{-3}	1.0
	42°C	1.0×10^{-3}	4.7×10^{-3}	0.60	1.0×10^{-3}	0.86
H202Y	30°C	1.0×10^{-3}	2.9×10^{-3}	5.0×10^{-3}	1.8×10^{-3}	0.40
	37°C	3.7×10^{-4}	4.2×10^{-3}	2.4×10^{-3}	2.1×10^{-3}	0.88
	42°C	7.2×10^{-4}	2.7×10^{-3}	8.0×10^{-4}	1.6×10^{-3}	0.47
E244K	30°C	0.73[a]	1.36	0.95	0.67	1.1[a]
	37°C	0.73[a]	0.49[a]	1.1	0.83	0.73[a]
	42°C	2.0×10^{-3a}	0.66[a]	1.0	0.65	0.94[a]
V292M	30°C	5.7×10^{-4}	9.0×10^{-4}	2.5×10^{-3}	1.2×10^{-3}	0.59
	37°C	6.0×10^{-4}	4.0×10^{-4}	1.0×10^{-3}	6.0×10^{-4}	0.22
	42°C	3.1×10^{-4}	5.0×10^{-4}	1.2×10^{-3}	1.1×10^{-3}	0.27
V303M	30°C	0.81[a]	0.83[a]	0.93	0.83[a]	0.72[a]
	37°C	0.69[a]	1.3[a]	1.03	0.46[a]	1.1[a]
	42°C	0.37[a]	0.63[a]	0.90	9.3×10^{-2a}	1.3[a]
E445K	30°C	1.1[a]	0.78	0.95[a]	0.70[a]	1.1[a]
	37°C	0.75[a]	0.27[a]	0.79[a]	0.95[a]	0.29
	42°C	0.22[a]	9.9×10^{-2a}	0.93[a]	1.0[a]	0.13

MC1061 transformants bearing pDS596 (*dnaA*$^+$) and pLS120 (*dnaAcos*) behaved as expected. Compared to the control of pACYC184, cotransformation of MC1061 with pDS596 and derivatives of pACYC184 carrying *datA*, *hda*, *dnaN* or *seqA* gave rise to a comparable number of colonies whether on not *dnaA*$^+$ expression was induced. In contrast, only *seqA* carried in pACYC184 suppressed the lethal effect caused by the induced expression of *dnaAcos*. Our observations that *seqA* in a multicopy plasmid suppresses the lethal phenotype of *dnaAcos* are in line with previously published results (Lu, Campbell et al. 1994).

The results from the genetic method suggest that the *dnaA* mutations can be separated into two groups. The *H202Y* and *V292M* alleles are in one group based on their similarity to *dnaAcos* (Table 2). Like *dnaAcos*, *H202Y* and *V292M* show extremely poor levels of suppression by plasmids carrying *datA*, *hda* and *dnaN*, and varying degrees of suppression by *seqA*. These results indicate that *H202Y* and *V292M* escape RIDA. Like *dnaAcos*, these alleles may be hyperactive due to either poor binding of ATP or they may be deficient in ATP hydrolysis.

The remaining five alleles are in the second group because they respond to *datA* or *hda* when carried in pACYC184. Hda is a key component in the regulatory inactivation of DnaA. We suggest that elevating the level of Hda via a multicopy plasmid counteracts the increase capacity for more frequent initiations that would occur at an elevated level of DnaA. If so, the mutant DnaAs may bind ATP and hydrolyze the nucleotide as well as wild type *dnaA*.

The G79D and E445K substitutions lie outside of Domain III, which functions in ATP binding and hydrolysis. These results suggest that the substitutions affect the ability of the protein to hydrolyze the bound ATP. In the case of *G79D* this may be due to an impaired interaction with Hda or the β-clamp. The E445K substitution is located next to the DnaA signature sequence, which recognizes the DnaA box sequence. Although this domain appears to be far removed from the region of DnaA involved in ATP hydrolysis, crosslinking experiments have shown that this domain can be close to the bound ATP (Kubota, Ito et al. 2001).

Mutant DnaA proteins are active for replication from *oriC* in vitro

The replication activity of purified $DnaA^+$ protein was compared to that of purified DnaA H202Y and DnaA V292. As a negative control, we included a reaction without any DnaA protein. No replication activity was observed for this sample, indicating that the stimulation of *oriC* plasmid replication is due to the DnaA protein. By comparison, the mutant proteins displayed levels of replication activity nearly identical to that of the wild type protein (Figure 2.5A). These results indicate that the substitutions encoded by these alleles do not alter the ability of the proteins to initiate *oriC* plasmid replication.

Mutant DnaA proteins have a decreased affinity for ATP

Having determined that H202Y and V292M are both active for replication of an *oriC*

Figure 2.5. Diminished affinity for ATP does not affect *oriC* plasmid replication

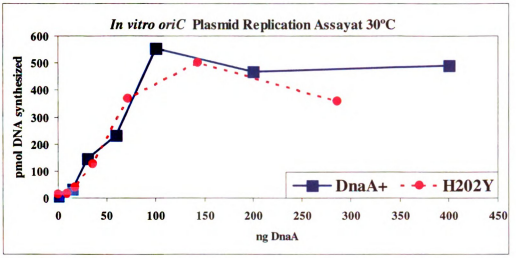
Panel A. *oriC* plasmid replication assay

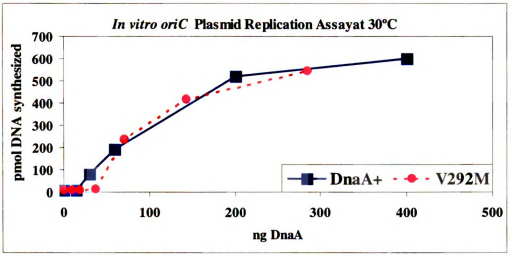
Replication activity was measured as described in "Materials and Methods" by incubation for 20 min at the indicated temperatures. In both panels, the activity of purified DnaA protein is represented by blue squares. The red circle represents V292M in the left panel and H202Y in the right panel. This experiment was performed by Dr. Sundari Chodavarapu in Dr. Jon M. Kaguni's laboratory.

Panel B. ATP Binding Assay

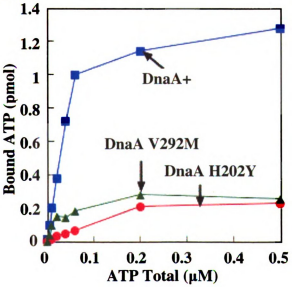
ATP binding was measured as described in "Materials and Methods." Reactions were incubated on ice for 20 min, then filtered through nitrocellulose filters, and the incorporation of radioactive nucleotide was measured by liquid scintillation counting. This experiment was performed by Dr. Sundari Chodavarapu in Dr. Jon M. Kaguni's laboratory.

A


In vitro oriC Plasmid Replication Assayat 30°C
pmol DNA synthesized
ng DnaA
DnaA+
H202Y


In vitro oriC Plasmid Replication Assayat 30°C
pmol DNA synthesized
ng DnaA
DnaA+
V292M

B


Bound ATP (pmol)
ATP Total (μM)
DnaA+
DnaA V292M
DnaA H202Y

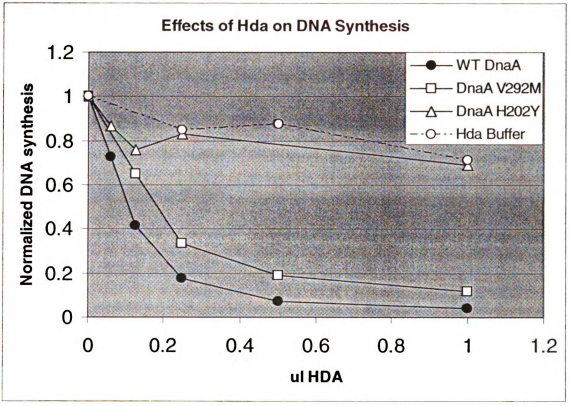
plasmid, we wanted to determine if these substitutions alter the ability of the protein to bind to ATP. The ATP binding activity of purified DnaA$^+$ was compared to that of DnaA H202Y and DnaA V292M. The binding curves indicate that both H202Y and V292M have a reduced affinity for ATP when compared to DnaA$^+$ (Figure 2.5B). These substitutions appear to alter the ability of the proteins to interact with ATP or the magnesium ion required for the positioning and hydrolysis of the nucleotide.

Novel mutants show mixed results to Hda stimulation of ATP hydrolysis

It has been observed that DnaA$^+$ activity is negatively regulated *in vitro* when in the presence of Hda protein or a crude fraction referred to as IdaB. Since the H202Y and V292M mutants both are as active as DnaA$^+$ for replication of an *oriC* plasmid, and both have a reduced affinity for ATP, we wanted to evaluate the ability of these proteins to respond to the RIDA component, Hda. We used a two stage replication assay to measure the ability of Hda to inhibit replication of an *oriC* plasmid. With DnaA$^+$, we saw that its preincubation with Hda decreased the amount of DNA synthesized by 95 percent (Figure 2.6). We also saw that the mutant protein V292M responded to Hda, with a reduction in DNA synthesis directly proportional to the amount of Hda added to the reaction reaching a maximum of approximately 90 percent (Figure 2.6). However, unlike DnaA$^+$ and V292M, the H202Y mutant did not respond to preincubation with Hda to the same extent as DnaA$^+$. We saw a decrease in synthesis of approximately 30 percent (Figure 2.6). As a control, we included reactions with increasing amounts of the buffer in which Hda is suspended. It is possible that the decrease in synthesis observed with H202Y

Figure 2.6. Inhibition of *oriC* plasmid replication by preincubation with Hda

A two stage replication assay, described in methods, was used to evaluate the ability of the H202Y and V292M mutants to react to stimulation by Hda. Replication assays containing either $DnaA^+$, DnaA H202Y or DnaA V292M, were preincubated with increasing amounts of Hda. Reactions, to which only the buffer used during the purification of Hda, were included as controls for analysis of the effects of Hda. This experiment was performed by Dr. Sundari Chodavarapu in Dr. Jon M. Kaguni's laboratory.


Effects of Hda on DNA Synthesis
Normalized DNA synthesis
1.2
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
1.2
ul HDA
WT DnaA
DnaA V292M
DnaA H202Y
Hda Buffer

is a result of the Hda buffer, and not due to Hda itself (Figure 2.6).

Discussion

Because DnaA protein regulates the frequency of initiation during the cell cycle, the protein has been studied intensely since the gene was first discovered (Kornberg and Baker 1992). Relying on a genetic approach to characterize this essential protein, many missense mutations have been isolated and characterized, but very few have been shown to stimulate function. Such mutations may bypass the regulatory mechanisms control the frequency of initiations. In this project started by Lyle Simmons, we sought to characterize *dnaA* alleles he isolated to better understand the regulatory mechanisms that govern the activity of DnaA protein.

Genetic screen and characterization of novel *dnaA* alleles

Lyle Simmons devised a genetic method to isolate novel hyperactive *dnaA* alleles. The method involved two steps. First, he transformed a *dnaA46* strain at the nonpermissive temperature with a plasmid carrying a randomly mutagenized *dnaA* gene, and selected for *dnaA* alleles that complemented the temperature sensitive phenotype of the *dnaA46* strain in the absence of induced expression. Second, he screened transformants for growth interference upon induced expression at 42°C. As the genetic assay was based on the behavior of the known hyperactive initiator *dnaAcos,* which can complement the *dnaA46* mutant at nonpermissive temperature when *dnaAcos* expression is not induced, whereas

its induced expression but not *dnaA*$^+$ causes lethality at 42°C, we expected to obtain other *dnaA* alleles with properties similar to *dnaAcos*. The genetic screen yielded seven alleles that are dominant-negative to chromosomally encoded ***dnaA***$^+$ and required the presence of *oriC* for growth interference (Simmons and Kaguni, unpublished results). My work shows that these phenotypes are not due to an increase in the level of protein expression (Figure 2.3C). The unique single amino acid substitutions of these alleles presumably affect specific biochemical functions of DnaA protein. Compared to a structural model of the *E. coli* DnaA protein, these amino acid substitutions may affect ATP binding, its hydrolysis or the interaction of DnaA with proteins that regulate its activity (Figure 2.7).

Hyperactive initiation *in vivo*

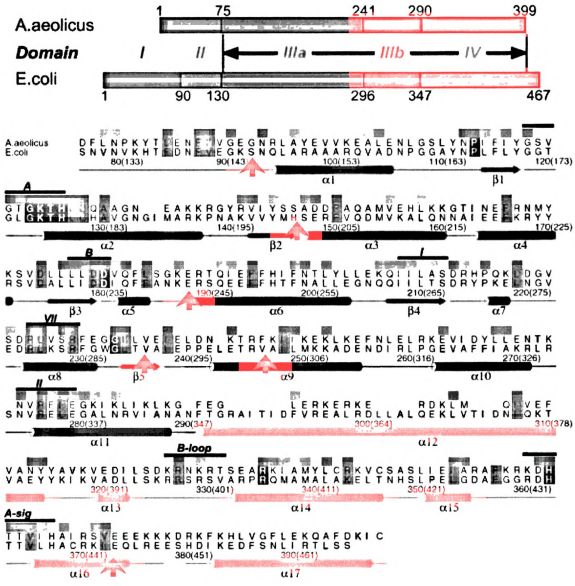
To demonstrate directly that the mutant DnaAs cause an elevated frequency of initiation, we performed quantitative Real-Time PCR analysis to measure the abundance of *oriC* to *relE*, a locus in the replication terminus region. The ratio of these loci reflects the frequency of initiations. Previous work established conditions for quantitative PCR analysis by the end-point method, utilizing radio labeled nucleotides that measured the abundance of specific DNA sequences in the bacterial chromosome (Simmons and Kaguni, 2003). To measure the abundance of these sequences more accurately, we used a Real-Time PCR method instead. We evaluated the alleles at 30°C, 37°C and 42°C to determine if the effects of these alleles were temperature-dependent. Plating data showed that *E244K*, *V303M* and *E445K* display a temperature-sensitive phenotype when

Figure 2.7. Locations of Amino Acid substitutions for novel *dnaA* alleles

Panel A. The locations of the amino acid substitutions encoded by the seven novel alleles have been indicated with red arrows on the figure from Erzberger et al 2002. The substitutions map to 3 of the 4 domains within DnaA.

Panel B. The predicted structure of *E. coli* DnaA protein is depicted by the blue ribbon diagram superimposed onto the known structure of *Aquifex aeolicus* DnaA, shown in red. Amino acid substitutions of the mutant proteins are shown in yellow. Domains IIIa, IIIb and IV, the Walker A box and the portion involved in binding to the DnaA box sequence are also shown. The position of the G79D substitution is not pictured because structural information for this portion of *A. aeolicus* DnaA is not available.

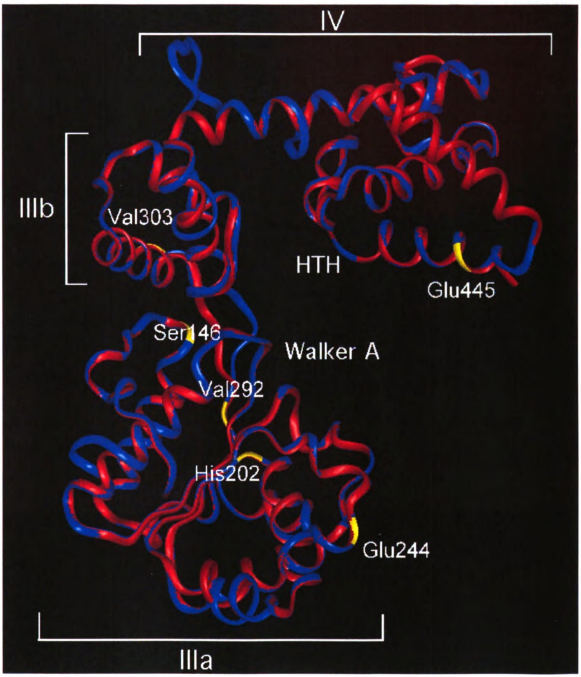
Panel A



Erzberger *et al.*, EMBO J.(2002) 21(18): 4763-4773

Figure 2.7 continued.

Panel B



Lyle Simmons, Doctoral Dissertation 2003

expression was induced. Using quantitative Real-Time PCR analysis, we determined that at 30°C six of the alleles induce more frequent initiation compared to wild type *dnaA*, and that initiations continue to increase with time after the onset of induced expression. The *oriC/relE* ratios are comparable or exceed those of *dnaAcos*. At 42°C, we observed that the alleles also elevate initiation frequency, but the distinction between their behavior and *dnaA*$^+$ is much less than that seen at 30°C. The *oriC/relE* ratios at 37°C generally appear to be intermediate between the ratios at 30°C and 42°C. For *S146Y*, at this temperature, this allele clearly does not increase the frequency of initiation. Part of the problem with this analysis is that although RT-PCR analysis is a quantitative method, its sensitivity to experimental variation requires statistical analysis of replicate samples.

Restoration of cell viability through supplementation of regulatory components

We have shown that the induced expression of the novel alleles is lethal to the host strain. For the *E244K*, *V303M* and *E445K* alleles, lethality is temperature dependent. In isolating novel *dnaA* alleles hyperactive for initiation of chromosomal replication, the goal is to characterize them via genetic and biochemical methods to understand how these alleles escape the regulatory mechanisms that control the frequency of initiation. Hence we utilized a genetic assay that measured the effect of *datA*, Hda, the β–clamp or SeqA on the *dnaA* alleles to identify those that failed to respond to the respective regulatory pathways. Our results suggest that the H202Y and V292M proteins are like DnaAcos in their failure to respond to *datA*, or *hda* and *dnaN*. However, SeqA suppresses the inviability associated with overproduction of these proteins. The

remaining alleles are in a second group, which can be suppressed at all temperatures by a multicopy plasmid encoding *hda*. Because Hda functions in concert with the β–clamp encoded by *dnaN* to stimulate the hydrolysis of ATP bound to DnaA, the results suggest that the mutant proteins have a diminished capacity for ATP hydrolysis.

Temperature dependent activity of *DnaA*

The slight increase in initiations observed following induced expression of wild type DnaA at 30°C suggests either a reduction in the level of expression or a decrease in the activity of the protein. To distinguish between these possibilities we compared equivalent amounts of cells isolated from MC1061 cultures bearing pDS596 grown at 30°C, 37°C and 42°C via western blot analysis. The immunoblot analysis indicated that the induced level of expression varied within a four-fold range. These results suggest that the activity of DnaA is reduced at 30°C. To substantiate this conclusion, we transformed the *E. coli* SK002 (relevant genotype: *recB268::Tn10*), which is defective in double strand break repair, with either the pDS596 (*dnaA*$^+$) plasmid or pLS120 (*dnaAcos*). The frequency of transformation on antibiotic-supplemented media with and without arabinose was measured, which showed that and elevated level of DnaAcos causes lethality at all temperatures, whereas the increased abundance of wild type DnaA is lethal at 37°C and 42°C but not 30°C. We conclude that a lower activity of DnaA at 30°C despite its increased abundance leads to less frequent initiations than expected.

Effects of the H202Y and V292M substitutions on DnaA activity

We have shown that the novel *dnaA* alleles *H202Y* and *V292M* are lethal when induced in a host bearing the wild type allele, these mutants elevate the frequency of initiation in the host, and that like *dnaAcos*, these alleles don't respond to the regulatory inactivation of DnaA or increased amounts of *datA*. Having shown that these alleles appear to be hyperactive through *in vivo* experiments, we wanted to better understand the effects of these alleles through *in vitro* assays. We compared $DnaA^+$ to the H202Y and V292M proteins in *oriC* plasmid replication, ATP binding and Hda inhibited two stage replication assays to develop a clearer picture as to how these mutants are escaping regulation.

The results of the *oriC* plasmid replication assay indicate that the substitutions encoded by *H202Y* and *V292M* do not affect the ability of the proteins to initiate replication. Both proteins displayed replication activity nearly identical to that seen with $DnaA^+$. Next we evaluated the effects of the substitutions on the ability to bind ATP. Both DnaA H202Y and DnaA V292M show a decreased ability to bind ATP when compared to $DnaA^+$. These two substitutions are located within 4 Å of the nucleotide binding pocket and as a result may perturb the ability of the protein to either bind or hydrolyze ATP. The H202Y substitution is positioned such that the tyrosine side chain may project into the nucleotide binding pocket and alter the proteins ability to coordinate the magnesium ion or bind ATP. Additionally, this substitution may affect the ability of the arginine finger or the adjacent DnaA monomer to accurately sense the state of the bound nucleotide. The side chain could also rotate and become solvent exposed, possible altering the ability of the

protein to interact with Hda or the β–clamp. The V292M substitution is located in a β-strand immediately adjacent to the P-loop. It is possible that the projection of the methionine side chain towards the P-loop pushes the loop towards the ATP binding cleft, altering the conformation such that ATP binding is hampered due to steric hindrance.

We completed our analysis of the H202Y and V292M proteins by conducting a two-stage *oriC* plasmid replication assay. In this assay we preincubated the *oriC* plasmid, DnaA, PolIII*, the β–clamp and increasing amounts of Hda for 20 minutes and then added the remaining proteins required for replication from *oriC*. Preincubation of these reactions with Hda was intended to stimulate the ATPase activity of DnaA, inactivating DnaA thus inhibiting its ability to initiate replication. $DnaA^+$ behaved as we expected and showed a dramatic decrease in the amount of DNA synthesized. Unlike DnaA+, the H202Y protein does not respond to the stimulus of Hda, and as a result we see little difference between the Hda buffer control and the H202Y reactions. The V292M protein behaves similarly to DNA^+, with synthesis decreasing with increasing amounts of Hda. This result contradicts what was observed in the lethality suppression assay, in which the presence of a multicopy plasmid bearing the *hda* allele did not suppress the lethality of V292M. We believe that the conditions used for the replication assay account for this discrepancy. Under normal physiologic conditions, the concentration of ATP within an *E. coli* cells is approximately 1.0 mM, however the concentration of ATP in the replication assays is 3.0 mM. Under these conditions we could be forcing the binding of ATP to the V292M protein and as a result altering the activities of the protein.

Chapter III: Summary and Perspectives

The analysis of the *dnaA* gene and its protein product via genetic and biochemical methods has provided important details revealing the biochemical functions of DnaA protein required for initiation of DNA replication (Hansen, Koefoed et al. 1992; Sutton and Kaguni 1995; Sutton 1996; Sutton and Kaguni 1997; Sutton and Kaguni 1997; Sutton and Kaguni 1997; Sutton, Carr et al. 1998). Although many alleles have been identified, very few have been shown to encode a more active form of the protein. The most well characterized hyperactive allele is *dnaAcos,* which encodes four amino acid substitutions, (Q156L, A184V, H252Y and Y271H). The A184V and H271Y substitutions have been shown to be responsible for the hyperactive phenotype associated with *dnaAcos* (Simmons and Kaguni 2003). The A184V substitution has been shown to dramatically reduce ATP binding (Katayama and Kornberg 1994; Katayama, Crooke et al. 1995; Carr and Kaguni 1996; Simmons and Kaguni 2003) while the H271Y mutation stabilizes the protein (Simmons and Kaguni 2003). In addition to *dnaAcos*, an additional allele, *dnaA219*, has been identified that also exhibits a hyperactive phenotype. Like *dnaAcos*, *dnaA219* contains the A184V and H252Y substitutions found in the parental allele *dnaA46*, but unlike *dnaAcos* contains an additional substitution R342C. As with *dnaAcos*, *dnaA219* causes lethal levels of overinitiation. We have completed a series of experiments that have characterized novel alleles that are hyperactive for initiation of chromosomal replication.

Lyle Simmons identified seven novel *dnaA* alleles believed to be hyperactive for initiation of replication. DNA sequence analysis showed that they encode single amino acid substitutions that map to Domains I, III and IV, with five mutations within Domain

III that forms the ATP binding pocket and contains the structural elements essential for ATP hydrolysis. The remaining two mutations are located within Domains I, the DnaA oligomerization, DnaB and proposed Hda interaction region, and Domain IV, the DNA binding region. Homology modeling revealed that several of the substitutions are in close proximity to the ATP binding site. These substitutions may reduce ATP hydrolysis which is stimulated by Hda and the β– clamp, thus favoring the active ATP-bound form of DnaA. In addition to the cluster of substitutions located around the ATP binding motif, the *G79D* and *E445K* alleles encode amino acid substitutions near the N-terminus or in the DNA binding motif of the DnaA protein, respectively. The G79D substitution within Domain I may reduce the ability of the mutant protein to interact with Hda and the β–clamp in their ability to simulate ATP hydrolysis. The E455K mutation within the DNA binding domain may either increase the affinity of DNA for its target sequence or confer a conformational change mimicking the active form of the protein. My experiments were intended to determine the biochemical mechanisms that cause hyperactive initiation.

We have examined these alleles both genetically and biochemically. Our work showed that all are dominant-negative to *dnaA*$^+$ and with the exception of *S146Y* alter the frequency of initiation from the bacterial origin of replication, *oriC*. We were able to separate the alleles into two distinct groups: those that fail to respond to suppression by elevated levels of regulatory elements *(H202Y* and *V292M)*, and those that respond to elevated levels of *datA* and Hda *(G79D, S146Y, E244K, V303M* and *E445K).*

In my work, we focused on the two mutations that display phenotypes similar to *dnaAcos*. The *H202Y* and *V292M* alleles increase the frequency of initiation, and fail to respond to increased abundance of the regulatory elements *datA*, Hda or the β-clamp, like *dnaAcos*. The remaining alleles all respond to suppression by the various regulatory components supplied on multicopy plasmids. These alleles, specifically G79D and E445K, merit further examination as they do not appear to directly alter the conformation of the ATP binding pocket or the ATPase activity of DnaA.

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