Regulation of Chromosomal DNA Replication in *Escherichia coli*: I. Function of an N-terminal Domain in DnaA Oligomer Formation; II. Biochemical and Genetic Studies of Hyperactive *dnaA* Alleles

By

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ABSTRACT

Regulation of Chromosomal DNA Replication in *Escherichia coli*:
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The initiation of DNA replication in *Escherichia coli* is a tightly regulated process that results in the completion of one round of replication per cell cycle. DnaA is a 52 kDa sequence-specific DNA binding protein that is required for initiation of replication from the bacterial origin, *oriC*. DnaA protein has been studied intensely over the last several decades to determine the biochemical functions that are required for initiation of replication. Despite these efforts, several questions remain concerning the biochemical functions of DnaA protein that are required for replication initiation and mechanisms that might exist in the cell to limit the frequency of initiation.

The N-terminal region of DnaA protein has been implicated in two biochemical activities (e.g. oligomerization and replicative helicase interaction). In this study, amino acids in the N-terminal region of DnaA protein are identified that are required for oligomerization. These results are the first to show that specific amino acids of DnaA protein are required for oligomerization and that DnaA protein oligomerization is a required activity for initiation from *oriC*.

Because DnaA protein is responsible for initiation of DNA replication, it is expected that *dnaA* alleles could be obtained that are unresponsive to elements that regulate initiation. The *dnaAcos* allele is an example of a *dnaA* allele that causes hyperactive initiation and is inert to negative regulation. In this work, *dnaAcos* and

several novel hyperactive *dnaA* alleles are examined genetically. We show that many activities of DnaA protein function to set the rate of origin firing during the cell cycle. This work also demonstrates that the wild type protein is negatively regulated *in vivo,* and that hyperactive *dnaA* alleles are unresponsive to these regulatory elements.

The study of hyperactive initiation was extended to visualize the genome-wide distribution of replication forks by microarray analysis. Hyperactive initiation was induced and the array pattern obtained clearly indicates that replication forks are stacked near the origin, suggesting that replisomes are stalled randomly under these conditions. Cell viability is compromised and absolutely requires homologous recombination, and replication fork restart proteins (RecB and PriA respectively). This data is consistent with Holliday junction formation and RuvC cleavage at stalled replication forks.

To
Kristi, my wife
and my inspiration

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List of Abbreviations

bp	base pair(s)
Dam	deoxyadenosine methyltransferase
DnaA box	asymmetric 9-mer consensus sequence bound by DnaA
DTT	dithiothreitol
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
E. coli	*Escherichia coli*
B. subtilis	*Bacillus subtilis*

Chapter I

Literature Review

Introduction

The process of genome duplication is a fundamental pathway that is required for the perpetuation of all living organisms. One of the model organisms used to study the mechanisms that regulate DNA replication is the bacterium *Escherichia coli*. This organism contains a single circular duplex DNA molecule of 4,700 kilobase pairs (von Meyenburg and Hansen 1987). The genome contains a genetic locus, *oriC*, where replication begins and replication forks proceed bidirectionally (Prescott and Kuempel 1972). After replication has proceeded 180° around the chromosome, replication is terminated, the chromosomes are segregated, and cell division ensues, producing two daughter cells each with a single copy of the genetic material.

The cell cycle

The *E. coli* cell cycle is composed of two distinct parts, the C period and the D period. The C period represents the time required for events including initiation and termination of DNA replication. The D period represents the time required to complete cell division (Cooper and Helmstetter 1968). A total of 60 minutes is required for the completion of the bacterial cell cycle (40 minutes C period and 20 minutes D period) after its onset. It has certainly been noticed that rapidly growing cultures are able to double in less than 60 minutes. To allow for doubling intervals that are less than 60 minutes, new rounds of replication must be initiated before the first round of replication has been completed.

Analysis of DNA content using flow cytometry has revealed that *E. coli* growth is synchronous, resulting in the number of genomes to be 2^n ($n \geq 1$ and an integer). This observation indicates that initiation is tightly regulated, even in rapidly growing cultures (Boye, Lobner-Olesen et al. 1988; Skarstad, von Meyenburg et al. 1988). Analysis of

Figure 1. Localization of chromosomal DNA sequences during different periods of the cell cycle. (adapted from Niki and Hiraga, 1998) This diagram represents a cell growing with a doubling time of 60 minutes. The circle (O) located at the left nucleoid border represents *oriC*. The circle located (●) at the right nucleoid border represents the terminus. Early in replication *oriC* is duplicated and one segment begins migrating toward the right nucleoid border while the other segment remains. The terminus migrates to the midcell after one segment of *oriC* has reached the other nucleoid border. After the terminus is duplicated the chromosomes are decatenated and a septum forms at the midcell. This results in the formation of two daughter cells each with *oriC* and the terminus located at the nucleoid borders. A Model for the migration of *oriC* and the terminus to their respective cellular positions has been proposed. A putative *cis*-acting centromere sequence may be located with *oriC* in a loop of the nucleoid. This loop is pulled to the other nucleoid border after *oriC* has been duplicated. This diagram indicates that the bacterial nucleoid has a specific arrangement with in *E. coli.* This result also suggests that the subcellular localization of *oriC* and the replication terminus are important functionally during the cell cycle.

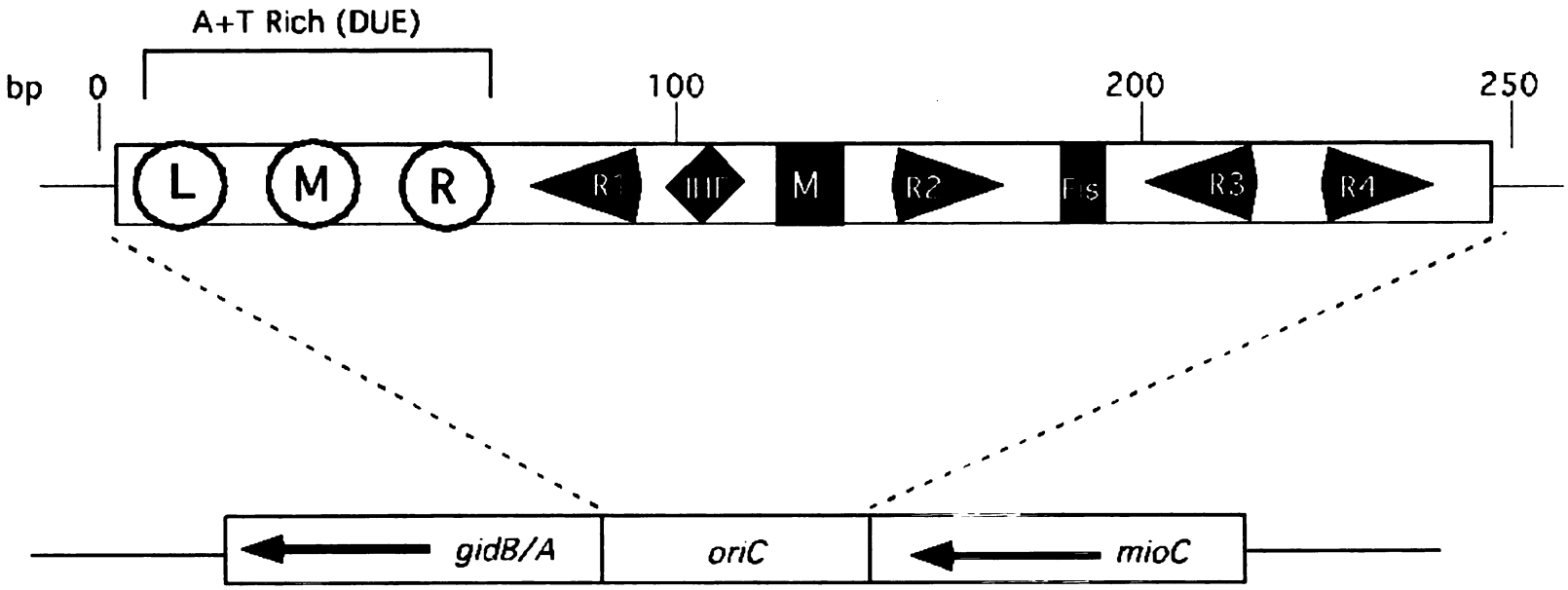
temperature sensitive *E.coli* strains have revealed initiation to be asynchronous at the permissive temperature, and at the restrictive temperature DNA replication has failed to initiate (Skarstad, von Meyenburg et al. 1988). These studies were the first to demonstrate that mutations could result in aberrant replication control. More recently, the use of fluorescently labeled proteins, and the hybridization of fluorescently labeled DNA probes have indicated that the bacterial nucleoid is well organized (Figure 1). These results support a model for origin migration from one nucleoid border to the other subsequently after duplication of *oriC* (Niki, Ichinose et al. 1988; Niki, Jaffe et al. 1991; Niki and Hiraga 1997; Webb, Teleman et al. 1997; Niki and Hiraga 1998; Hiraga 2000; Hiraga, Ichinose et al. 2000). These studies not only indicate tight regulation of replication but they also verify a specific arrangement of replication proteins and the bacterial nucleoid during the cell cycle.

The origin of replication

The *E.coli* origin of replication is the site for initiation of bidirectional replication (Prescott and Kuempel 1972), located at 84.3 minutes on the chromosomal linkage map (von Meyenburg, Hansen et al. 1978). The minimal sequence required to confer replication of a plasmid is 245 bps (Meijer, Beck et al. 1979; Oka, Sugimoto et al. 1980). The *oriC* sequence is highly conserved indicating structural significance (Zyskind, Cleary et al. 1983), and *oriC* contains several motifs (Figure 2) common among enteric bacteria (Buhk and Messer 1983). The origin contains five 9-mer sequences that serve as binding sites for the replicative initiation protein, DnaA (discussed in more detail below) (Fuller, Funnell et al. 1984). The origin also contains other binding sites for proteins involved in activation or inhibition of initiation (Craig and Nash 1984; Torheim and Skarstad 1999). There are three A+T rich 13-mers [also known as the duplex unwinding element (DUE)]

Figure 2. Structural organization of *oriC*

The origin of replication (Matsui et. al, 1985; Gille and Messer, 1991; Kano et. al, 1991) *oriC* is located between genes *gidA* and *mioC* with the direction of transcription indicated by the black arrows. The *oriC* region contains five DnaA protein binding sites (R1-R4, M). The directionality of these DnaA boxes are indicated. The origin also contains binding sites for IHF and Fis proteins ,which are important for the initiation of DNA replication. The left end of *oriC* contains three A+T rich 13-mer regions which are denatured during processing of the origin by DnaA protein, to form the open complex. The 13-mers are represented by circles and as labeled (L,M,R).


A+T Rich (DUE)
bp
0
100
200
250
L
M
R
R1
IHF
M
R2
Fis
R3
R4
gidB/A
oriC
mioC

located at the left border of *oriC*. The DUE constitutes a *cis*-acting element (Kowalski and Eddy 1989) subsequently denatured during initiation to form the open-complex. The open-complex then serves as the site for assembly of the replication fork machinery (Bramhill and Kornberg 1988; Bramhill and Kornberg 1988). The origin also contains the highest number of GATC sequences for a DNA segment of this size in the *E. coli* genome. The GATC sequence is repeated nine times in *oriC* (Zyskind, Harding et al. 1981). This sequence is the recognition site for Dam methyltransferase, which methylates the adenine base. Methylated *oriC* can be specifically bound by proteins that regulate initiation.

DnaA binding to oriC

Five 9-mer sequences (R1, R2, R3 R4, and M) which contain a consensus sequence TTATCCACA (or slight variation) are specifically bound by DnaA protein when DnaA is complexed with ATP (DnaA-ATP) (Fuller and Kornberg 1983; Fuller, Funnell et al. 1984) The binding of DnaA to *oriC* has been analyzed using gel shift mobility assays, DNase I foot-printing and surface plasmon resonance (Matsui, Oka et al. 1985; Roth and Messer 1995; Speck and Messer 2001). Comprehensive mutagenesis of the DnaA boxes indicates that all five are required for DNA replication, although box R3 appears to be dispensable under certain conditions (Langer, Richter et al. 1996). For most DnaA boxes, inversion of the sequence, point mutations or position exchange causes a phenotype indicating the important architecture of this region (Langer, Richter et al. 1996). It has been suggested that DnaA-ATP has an affinity for single stranded DNA and the ATP-bound form of DnaA can bind directly to the A-T rich 13-mer region of *oriC*. This activity aids in the unwinding of the origin and allows for sequential assembly of essential replication proteins (Speck and Messer 2001).

Other oriC *binding proteins*

In addition to specific binding of DnaA to *oriC,* many other essential proteins bind to the origin and regulate this process. Integration host factor (IHF) was originally identified for its role in lambda phage integration into the *E. coli* genome (Katayama, Takata et al. 1997). Analysis of IHF binding to a specific site within *oriC* revealed IHF assists in initiation (Hwang and Kornberg 1992). IHF is known to bend DNA upon binding (Moitoso de Vargas, Kim et al. 1989). It is thought that this topological change in the DNA is required for initiation (Hwang and Kornberg 1992).

Factor for inversion stimulation (Fis) is another accessory protein that binds to *oriC.* Like IHF, Fis also assists in initiation by inducing topological distortion of the DNA upon binding (Gille, Egan et al. 1991; Gille and Messer 1991). Fis protein binds in close proximity to DnaA box R3, although the binding is not mutually exclusive to DnaA protein binding to R3 (Margulies and Kaguni 1998). Biochemical data indicates that high concentrations of Fis protein inhibit replication from an *oriC* plasmid. This result reveals that a limited amount of Fis is required for initiation (Hiasa and Marians 1994).

Both IHF and Fis, under physiological conditions, are *oriC* accessory binding proteins that contribute to initiation. Other *oriC* binding proteins have been isolated which negatively regulate the initiation process. Inhibition of chromosomal initiation (IciA) protein was identified as a protein that specifically binds the 13-mer region (DUE) of *oriC* (Hwang and Kornberg 1990; Hwang and Kornberg 1992; Hwang, Thony et al. 1992). This 33 KDa protein has the ability to block open-complex formation leads to a decrease in the formation of ssDNA in P1 endonuclease sensitivity assays correlates with increasing amounts of IciA *in vitro* (Katayama, Takata et al. 1997). SeqA protein is known for its ability to sequester *oriC* to the *E .coli* membrane to stop untimely initiation. SeqA protein can also bind to the 13-mer region and this activity prevents open-complex formation (Brendler, Abeles et al. 1995).

Plasmids which depend solely on *oriC* as the site where replication is initiated are referred to as minichromosomes. Minichromosomes have served as useful models for the study of replication both *in vitro* and *in vivo*. *In vivo* replication of these plasmids is bidirectional (Meijer and Messer 1980) and replication is coordinated with the cell cycle (Leonard and Helmstetter 1986; Koppes and von Meyenburg 1987), highlighting that its regulation is similar to that of the chromosomal origin. Minichromosomes are randomly distributed at cell division unless a partition sequence is present (Ogura and Hiraga 1983). When the copy number reaches a critical elevation, these plasmids have been observed to integrate into the chromosome suggesting some level of incompatibility with chromosomal *oriC* (Lobner-Olesen 1999). In *B. subtilis,* the incompatibility between chromosomal *oriC* and plasmid *oriC* is more severe. Minichromosomes carrying *B. subtilis oriC* are maintained for short periods of time with a copy number of one (Moriya, Fukuoka et al. 1988; Moriya, Atlung et al. 1992). Differences between chromosomal replication and minichromosomal replication have been described. Strains carrying HU and IHF null mutations do survive, although they are unable to maintain minichromosomes, indicating differences in accessory protein requirements between the two systems (Kano, Ogawa et al. 1991). With these differences in mind, minichromosomes have been widely used to distinguish the order of events that take place during initiation *in vitro* and have been successful in determining protein requirements *in vivo*.

In vitro *replication assay*

The replication process has been efficiently studied using *in vitro* replication assays dependent on either a crude extract or the reconstitution of the replication machinery with purified components (Fuller, Kaguni et al. 1981; Kaguni and Kornberg 1984). Both *in vitro* replication assays require exogenously supplied supercoiled *oriC* plasmid (Fuller, Kaguni et al. 1981; Kaguni and Kornberg 1984). Replication of minichromosomes proceeds bidirectionally which has also been observed during replication from the chromosomal origin [(Kaguni and Kaguni 1992) for review; (Prescott and Kuempel 1972)]. The reconstituted *in vitro* assay requires many purified proteins to carry out three distinct stages of replication (initiation, replication fork progression, and suppression of nonspecific initiation) and all of these stages are required for propagation of the minichromosome *in vitro* (Kaguni, Fuller et al. 1982; Kaguni, Bertsch et al. 1985). One of the more effective ways to use the *in vitro* assays is to measure the replication activity of mutant DnaA proteins (Hwang and Kaguni 1991; Hupp and Kaguni 1993; Hupp and Kaguni 1993). This assay has also been used to assess the effect of mutations present in other replication proteins (DnaC) that may compromise activity (Ludlam, McNatt et al. 2001).

DnaA protein

DnaA is a 52.5-kDa protein that is required for DNA replication in *E. coli* (Skarstad, von Meyenburg et al. 1988). DnaA is also required for initiation of replication from many plasmid origins (pSC101, F, R6K). Several plasmids contain one or more DnaA boxes but do not require DnaA protein for replication (Molin, Diaz et al. 1980; Molin and Nordstrom 1980; Fuller, Funnell et al. 1984; Hansen and Yarmolinsky 1986; Kline, Kogoma et al. 1986; Wu, Goldberg et al. 1992).

Table 1. Summary of *dnaA* mutations cloned or isolated by classical genetics

Mutation (*dnaA* allele)	Replication phenotype	Biochemical or genetic mechanism	Reference
Domain I (1-90)			
M1V	Defective *in vivo*	Unknown	Sutton and Kaguni, 1997
L3S, L10S, L17S (*dnaA413*)	Defective *in vitro* and *in vivo*	Failure of DnaA-DnaA interaction	Mima *et. al*, 2002
C9Y	Defective *in vivo*	Unknown	Sutton and Kaguni, 1997
L17S (*dnaA427*)	Defective *in vitro* and *in vivo*	Failure of DnaA-DnaA interaction	Mima *et. al*, 2002
E21K	Defective *in vivo*	Unknown	Sutton and Kaguni, 1997
W25S	Defective *in vivo*	Unknown	Sutton and Kaguni, 1997
I26S	Defective *in vitro*	Failure to unwind *oriC*	Mima *et.al*, 1999
A31T	Defective in pSC101, active in *oriC in vivo*	Unknown	Sutton and Kaguni, 1995
L40S	Defective *in vitro*	Failure to unwind *oriC*	Mima *et.al*, 1999
Δ62	Defective *in vitro* and *in vivo*	Failure to retain DnaB in prepriming complex	Sutton *et. al*, 1999
Domain II (91-130)			
Δ129	Defective *in vitro* and *in vivo*	Failure to interact with DnaB	Sutton *et. al*, 1999
Domain IIIa (131-296)			
V157E (*dnaA167*)	Defective 42°C *in vivo*	Unknown	Hansen *et. al*, 1992
G175D	Defective *in vivo*	Poor ATP-binding	Sutton and Kaguni, 1997
A184V, H252Y (*dnaA46*)	Defective, 42°C *in vitro*	Poor ATP-binding	Carr and Kaguni, 1996

Table 1. Continued

Domain IIIa (131-296)			
A184V, P296Q (*dnaA601/602*)	Defective 42°C *in vivo*	Unknown	Hansen, *et. al*, 1992
A184V, A347V (*dnaA604/606*)	Defective, 42°C *in vivo*	Unknown	Hansen *et. al*, 1992
V198M	Defective in pSC101, and *oriC in vivo*	Unknown	Sutton and Kaguni, 1995
M210L	Defective(cold sensitive) *in vivo*	Unknown	Guo *et. al*, 1999
A213D (*dnaA73*)	Cold Sensitive defective	Suppressor of *dnaX*(ts)	Gines-Candelaria
I219N	Defective(cold sensitive) *in vivo*	Unknown	Guo *et. al*, 1999
F222L	Defective(cold sensitive) *in vivo*	Unknown	Guo *et. al*, 1999
D235N	Non-complementing	Poor ATP-binding and *oriC* unwinding	Mizushima *et, al*, 1998
F240I	Defective (cold sensitive) *in vivo*	Unknown	Guo *et. al*, 1999
R245Q	Defective(cold sensitive) *in vivo*	Unknown	Guo *et. al*, 1999
T253A	Defective (cold sensitive) *in vivo*	Unknown	Guo *et. al*, 1999
E279V	Defective (cold sensitive) *in vivo*	Unknown	Guo *et. al*, 1999
G287S	Defective in pSC101, active in *oriC in vivo* and *In vitro*	Unknown	Sutton and Kaguni, 1995
Domain IIIb (297-347)			
T301I	Defective in pSC101, active in *oriC in vivo* and *in vitro*	Unknown	Sutton and Kaguni, 1995

Table 1. Continued

R302C	Defective (cold sensitive) *in vivo*	Unknown	Guo *et. al*, 1999
R334H	Defective (cold sensitive) *in vivo*	Unknown	Guo *et. al*, 1999
R342H	Defective (cold sensitive) *in vivo*	Unknown	Guo *et. al*, 1999
L340P	Decreased activity *in vitro*	Decreased DNA-binding activity	Blaesing *et. al*, 2000
Domain IV (348-467)			
E361G	Defective (cold sensitive) *in vivo*	Unknown	Guo *et. al*, 1999
L363K, L373R	Suppresses acidic phospholipid deficient growth arrest *in vivo*	Unable to interact with phospholipids	Zheng *et. al*, 2001
L366K	Suppresses acidic phospholipid deficient growth arrest *in vivo*	Unable to interact with phospholipids	Zheng *et. al*, 2001
K372E	Decreased activity *in vitro*	Decrease in Cardiolipin interaction	Yamaguchi *et. al*, 1999
V383M	Defective *in vivo*	Unknown	Sutton and Kaguni, 1997
E385K	Defective (cold sensitive) *in vivo*	Unknown	Guo *et. al*, 1999
I389N (dnaA203/204)	Defective 42°C *in vivo*	Unknown	Hansen *et. al*, 1992)
S396F	Defective *in vitro*	DNA-binding defect	Blaesing *et.al*, 2000
K397E	Defective (cold sensitive) *in vivo*	Unknown	Guo *et. al*, 1999
R401A	Defective *in vitro*	DNA-binding defect	Blaesing *et. al*, 2000

Table 1. Continued

Domain IV (348-467)	Continued		
V403A	Defective *in vitro*	DNA-binding defect	Blaesing *et. al*, 2000
R407C	Decease in activity *in vitro*	DNA-binding defect	Blaesing *et. al*, 2000
R407A	Defective *in vitro*	DNA-binding defect	Blaesing *et. al*, 2000
R407H	Defective *in vivo*	Unknown	Sutton and Kaguni, 1997
Q408R	Defective *in vitro*	DNA-binding defect	Blaesing *et. al*, 2000
K415A	Defective *in vitro*	DNA-binding defect	Blaesing *et. al*, 2000
K415E	Defective *in vitro*	DNA-binding defect	Blaesing *et. al*, 2000
L417P	Decrease in activity *in vitro*	DNA-binding defect	Blaesing *et. al*, 2000
S421N	Decrease in activity *in vitro*	DNA-binding defect	Blaesing *et. al*, 2000
S421G	Decease in activity *in vitro*	DNA-binding defect	Bleasing *et. al*, 2000
T426A	Defective *in vitro*	DNA-binding defect	Blaesing *et. al*, 2000
G426D	Defective *in vivo*	Unknown	Sutton and Kaguni, 1997
R432L (*dnaA721*)	*dnaX*(Ts) suppressor *in vivo*	Unknown	Gines-Candelaria *et. al*, 1995
H434A	Decease in *activity in vitro*	DNA-binding defect	Blaesing *et. al*, 2000
H434Y	Decrease in activity *in vitro*	DNA-binding defect	Blaesing *et. al*, 2000
T435A	Defective(cold sensitive) *in vivo*	Unknown	Guo *et. al*, 1999

Table 1. Continued

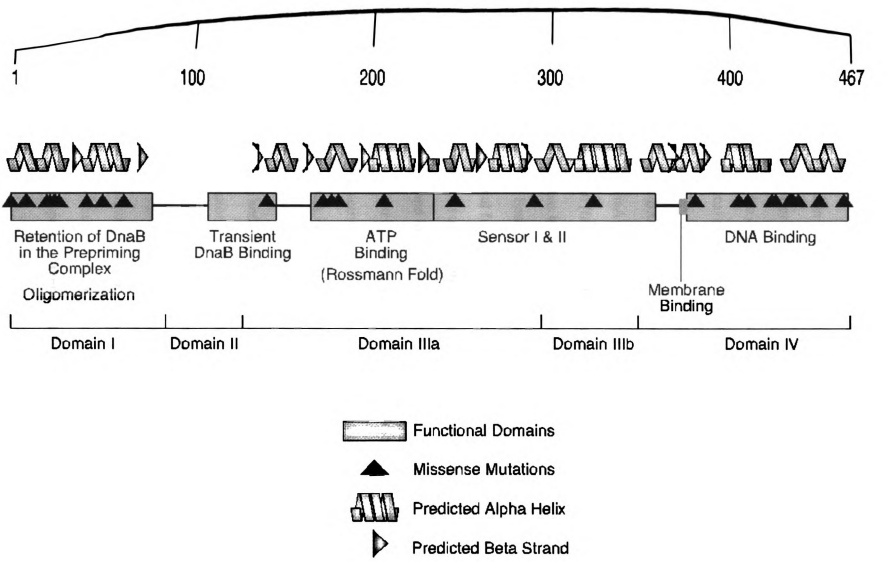
Domain IV (348-467)	Continued		
T435K	*dnaX*(Ts) suppressor *in vivo*	Unknown	Gines-Candelaria *et. al*, 1995; Blaesing *et. al*, 2000
T435M	Defective *in vivo*	DNA-binding defect	Sutton and Kaguni, 1997
T435P	Defective *in vitro*	DNA-binding defect	Blaesing *et. al*, 2000
T436A	Defective *in vitro*	DNA-binding defect	Blaesing *et. al*, 2000
V437M	Defective *in vivo*	Unknown	Sutton and Kaguni, 1997
A440T	Defective *in vivo*	DNA-binding defect	Sutton and Kaguni, 1997
A440V	Defective *in vivo*	DNA-binding defect	Sutton and Kaguni, 1997; Blaesing *et. al*, 2000
K443E	Defective *in vitro*	DNA-binding defect	Blaesing *et. al*, 2000
L447W	Defective in pSC101, active in *oriC in vivo*, and *in vitro*	Unknown	Sutton and Kaguni, 1995

The information in this table is compiled from the work of many laboratories. Deletion mutants were omitted from this table unless the study of the mutant protein or allele was essential for understanding the role of the domain effected by the deletion. Alleles with multiple mutations were also omitted unless the allele has been well studied and the resulting phenotype clearly indicated.

Alleles of the *dnaA* gene that are hyperactive or cause a phenotype that correlates with hyperactive initiation are listed in Table 2.

Figure 3. Schematic representation of DnaA functional domains

A genetic assay was developed to select for mutations that inactivated the *dnaA* gene (Sutton and Kaguni, 1997). The missense mutations obtained (triangles) were localized to distinct regions of the *dnaA* gene. These mutant proteins were assayed to determine the biochemical defect resulting in a failure of replication activity. The defects observed allowed for the identification of the functional domains described in this diagram. The N-terminal region (Domains I and II) of DnaA protein is important for both interactions with the replicative helicase DnaB and for self interaction (oligomerization). Domain IIIa contains a Rossman fold (Walker A box) and a Sensor I motif. These elements are important for ATP binding. Domain IIIb contains the Walker B box and the Sensor II motifs (Sensor I and II motifs are shared among AAA^+ family members) which are important for ATP binding and hydrolysis. Domain IV of DnaA protein contains a region which is required for DNA binding activity.


1
100
200
300
400
467
Retention of DnaB in the Prepriming Complex
Oligomerization
Transient DnaB Binding
ATP Binding (Rossmann Fold)
Sensor I & II
DNA Binding
Membrane Binding
Domain I
Domain II
Domain IIIa
Domain IIIb
Domain IV
Functional Domains
Missense Mutations
Predicted Alpha Helix
Predicted Beta Strand

More than 100 mutations in DnaA protein have been isolated which span the entire protein [many are listed in Table 1 (Hansen, Koefoed et al. 1992; Mima, Yamagachi et al. 1999; Yamaguchi, Hase et al. 1999; Mima, Makise et al. 2002)]. Many of these alleles have been assayed *in vivo* or the mutant protein has been assayed *in vitro* and a phenotype or biochemical defect has been described (Kellenberger-Gujer, Podhajska et al. 1978; Sutton and Kaguni 1995; Sutton and Kaguni 1997; Guo, Katayama et al. 1999; Blaesing, Weigel et al. 2000). The phenotype caused by most of these mutations results from inactivation of DnaA protein, although a few mutations have been described (Table 2) that activate function [for review (Katayama, Fujimitsu et al. 2001)]. The study of these mutant proteins has been pursued to elucidate the functional domains of DnaA protein, and determine how DnaA protein is regulated (Figure 3).

Two biochemical functions have been ascribed to the N-terminal region of DnaA. This region (also referred to as Domain I and II) has been shown to be important for DnaA self-interaction and interaction with the replicative helicase, DnaB. The first 86 amino acids of DnaA, can be used to construct a λcI repressor chimera which dimerizes and retains repression activity. Solid phase protein-binding assays have also been used to demonstrate DnaA-DnaA interaction (Weigel, Schmidt et al. 1999).

Two different portions of the DnaA N-terminal region are responsible for interaction with DnaB, and these two interactions regulate separate steps of prepriming complex formation (Sutton, Carr et al. 1998; Seitz, Weigel et al. 2000). Residues 1-110 are important for transient interaction (or recruiting) with DnaB resulting in DnaB binding to the 13-mer region of *oriC* to form the prepriming complex. Residues 130-168 are responsible for retention of DnaB in the prepriming complex (Sutton, Carr et al. 1998). Based on a few studies that have been designed to define the area of DnaA-DnaA and DnaA-DnaB protein-protein interaction, it appears that the functional domains

overlap, and specific amino acid residues have not been identified to be important or required for either interaction.

DnaA-ATP binding

DnaA protein binds ATP (Domain IIIa) and this activity is required for activation (Sekimizu, Bramhill et al. 1987; Sekimizu, Bramhill et al. 1988). DnaA-ATP is required for open-complex formation at *oriC*. DnaA-ADP is unable to form the open-complex; however, DnaA-ATPγS does facilitate *oriC* unwinding, suggesting that DnaA-ATP provides a conformational change that is required for initiation, but hydrolysis of ATP is not necessary (Sekimizu, Bramhill et al. 1987). Two *dnaA* alleles which contain the *A184V* (*dnaA*46 and *dnaA*5) missense mutation have reduced ATP binding activity and are temperature sensitive for initiation (Hwang and Kaguni 1991; Hupp and Kaguni 1993; Hupp and Kaguni 1993; Hupp, Meek et al. 1993). This mutation is solely responsible for the thermolability of these proteins (Carr and Kaguni 1996). The A184V missense mutation is located near a P-loop motif (Walker A box, GXXGXGKT) which is common among many nucleotide binding proteins. The Walker A box is responsible for interactions with the triphosphate moiety, whereas the Walker B box is important for chelating the Mg^{2+} ion (Walker, Saraste et al. 1982; Saraste, Sibbald et al. 1990). Site-directed mutagenesis of the ATP binding region of DnaA protein has shown that Lys178 and Asp 235 are critical for ATP binding and activation of DnaA protein for open-complex formation (Mizushima, Takaki et al. 1998). Immuno-precipitation of DnaA protein from soluble extracts during the C and D period of the cell cycle has revealed that the highest level of DnaA-ATP is observed just before the onset of replication. The level of DnaA-ATP drops rapidly after replication is initiated (Kurokawa, Mizushima et al. 1998). This data indicates that the cellular level of DnaA-ATP is closely tied to the state of replication as the abundance of DnaA-ATP coincides with the cell cycle. It has also

been shown that DnaA-ATP has a higher affinity for certain DNA sequences. A 6-mer consensus sequence (AGATCT) has been identified that is only recognized by the ATP bound form of DnaA protein. One of these DnaA-ATP boxes (6-mer) is located in the promoter region of the *dnaA* gene adjacent to the more well characterized 9-mer DnaA box that is bound by DnaA protein to repress transcription of the *dnaA* gene (Braun, O'Day et al. 1985; Lee and Hwang 1997). DNase I protection patterns clearly indicate that DnaA-ATP protects this region more effectively than DnaA-ADP resulting in increased repression of *dnaA* gene expression (Speck, Weigel et al. 1999). Interestingly, DnaA-ATP is only able to occupy the 6-mer binding site when it is adjacent to a 9-mer site suggesting coopertivity in DnaA-ATP binding to the 6-mer sequence.

DnaA-RepA (pSC101) *interaction*

Novel alleles of the *dnaA* gene have been isolated that are defective for pSC101 maintenance but active for replication from *oriC* (Sutton and Kaguni 1995). These mutations are located in the region of DnaA protein contained by residues 237-360 (Domain IIIa-b). It has been suggested that this region may be important for protein-protein contact with the pSC101 initiation protein RepA, although this interaction has not been demonstrated directly. Forward and reverse yeast-two hybrid assays have shown interaction between DnaA and RepA utilizes the N-terminus and the C-terminus of DnaA protein (Sharma, Kachroo et al. 2001). Seitz and coworkers confirmed this observation by constructing and internal deletion of amino acid residues 137-376 and determined that the mutant protein still functioned in pSC101 maintenance. Based on the data described above, it seems likely that the novel *dnaA* alleles (V198M, G287S, T301I) are unable to assemble a functional initiation complex at the pSC101 origin due to a defect in some required activity other than interaction with RepA protein.

Anionic phospholipid regulation of DnaA protein

Disruption of the *pgs*A coding region (encoding phosphatidylglycerol phosphate synthase catalyzing the step of commitment of phosphatidylglycerol biosynthesis) causes a decrease in the levels of anionic phospholipids in the *E. coli* cell membrane, which leads to growth arrest (Heacock and Dowhan 1987; Heacock and Dowhan 1989; Xia and Dowhan 1995). It was observed that activation of DNA replication through a DnaA independent pathway (constitutively stable DNA replication, described later) suppressed the growth arrest previously observed (Xia and Dowhan 1995).

It was demonstrated later that DnaA protein interacts physically with anionic phospholipids (Garner and Crooke 1996) and this interaction is important for the reactivation of DnaA protein by stimulating the release of ADP allowing DnaA protein to bind ATP to activate it for initiation of replication (Garner and Crooke 1996). Deletion of this region (360-373) and (or) single amino acid substitutions have been shown to suppress growth arrest of *E. coli* strains deficient in anionic phospholipid synthesis (Zheng, Li et al. 2001). These results are interesting because they describe a direct role for phospholipids in the initiation of DNA replication. Recent results however, place this observation under scrutiny (see below).

A careful analysis of *pgsA* null mutants demonstrated that the cells are viable when DNA replication proceeds normally through *oriC* and is *dnaA* dependent (Kikuchi, Shibuya et al. 2000). The viability of this strain requires inactivation of the *lpp* gene (encoding lipoprotein, see below). It was observed that the *pgsA* null mutants were able to grow in rich media, but were not viable in minimal media or temperatures over 40°C. Other experiments revealed that in a *pgsA* null strain there is an accumulation of prolipoprotein in the periplasm. One of the important steps in prolipoprotein maturity is a diacylglycerol group donated from phosphatidylglycerol (a product of the *pgsA* pathway of acidic phospholipid biosynthesis). Apparently, the unmodified prolipoprotein

accumulates in the periplasm and is able to make covalent linkages with the outer membrane resulting in a disruption of the cell surface. This has now been attributed to the growth defects that are caused in *pgsA* null mutants, and confirms the dependence on deletion of the *lpp* gene. The role of acidic phospholipids in DNA replication is rather unclear at this time. Further experiments will be required to strengthen or weaken the role of DnaA protein and membrane attachment in the regulation of initiation.

DnaA C-terminus

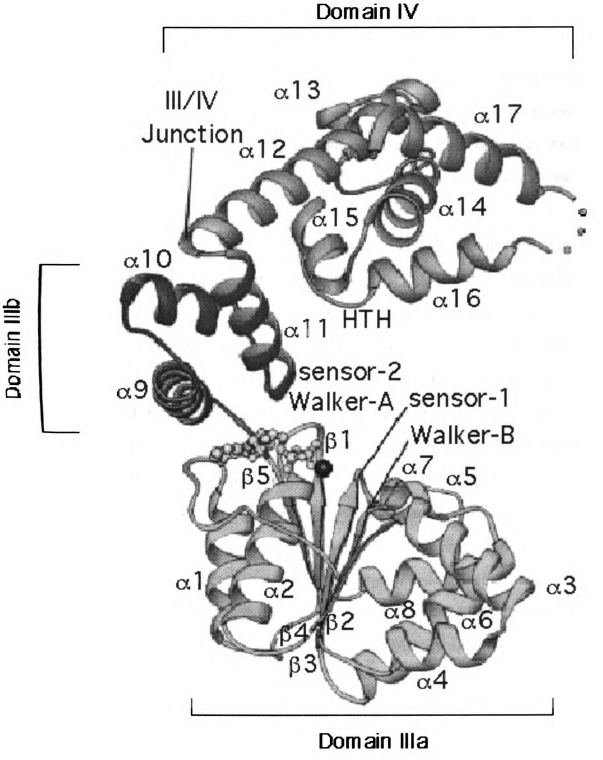
DnaA protein binds specifically to its cognitive 9-mer sequences. This interaction takes place in the C-terminal domain (also known as Domain IV) residues 387-467 (Sutton and Kaguni 1997). The sequence specific binding of DnaA to *oriC* is conferred by threonine residue 435 (Sutton and Kaguni 1997). The 94 amino acid DNA binding domain has the capability to bind DNA *in vivo,* random mutations throughout the 94 amino acid region cause a decrease or abolish DNA binding activity (Blaesing, Weigel et al. 2000). To date no known DNA binding domain mutations cause an increase in the binding affinity of DnaA protein to *oriC* or other DNA sequences.

Structure of DnaA (Domains IIIa-IV) protein from Aquifex aeolicus

Several labs have tried to crystalize DnaA protein with the goal of elucidating the folded of the macromolecule. These failed efforts included the purification of DnaA protein from *E. coli* as well as from bacterial thermophiles. Recently the

Figure 4. Crystal structure of DnaA protein from *Aquifex aeolicus*

The structure of DnaA (from Erzberger et. al, 2002) includes Domains IIIa, IIIb and IV. Domains I and II were deleted from the full-length protein to circumvent the problem of oligomerization, which has complicated the crystallographic attempts of others. This figure is composed of a ribbon diagram containing Domains IIIa, IIIb and IV. Domain IIIa is a five-stranded β-sheet (β1-β5) flanked by two sets of α-helices on each side. This fold is similar to the RecA-type fold that is observed in many nucleotide binding proteins. Domain IIIb is an antiparrallel three-helix bundle. This contains the Sensor II motif which is common among AAA^+ family members. Domain IIIa contains the Walker A and B boxes and the sensor I (AAA^+) motif. 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 helix 12, which is the acidic phospholipid binding region of DnaA protein. The actual DNA binding domain is a helix-turn-helix and is very similar to the Trp repressor DNA-binding fold. *A. aeolicus* and *E. coli* DnaA protein share 35% amino acid identity and 65% amino acid similarity, indicating the structure will be important for studies of *E. coli* DnaA protein.


Domain IV
III/IV
Junction
α13
α17
α12
α15
α14
α10
α16
HTH
α11
sensor-2
α9
Walker-A
sensor-1
Walker-B
β1
β5
α7
α5
α1
α2
α3
α8
α6
β2
β4
β3
α4
Domain IIIb
Domain IIIa

structure was solved for Domains IIIa-IV (see below) to a 2.7A resolution with ADP bound to DnaA protein (Erzberger, Pirruccello et al. 2002). Domain I and II were deleted during the cloning procedure to circumvent potential problems caused by DnaA protein oligomerization. DnaA protein from *E. coli* and *A. aeolicus* share 35% sequence identity and 65% sequence similarity over the aligned region (Erzberger, Pirruccello et al. 2002). Because *E. coli* and *A. aeolicus* share strong sequence identity, the structural information obtained bears significantly on work with *E. coli* DnaA protein (Figure 4).

Domain IIIa of DnaA protein is composed of a five β-strand bundle flanked by two α-helices (α1- α2) on the left and five α helices on the right (α3-α8). Domain IIIa is a version of the RecA-type fold which is often observed among nucleotide-binding proteins. Domain IIIb is organized with three anti-parallel α-helices (α9-α11) connected to Domain IIIa by a short linker segment. The region contained by Domain IIIa-IIIb comprises the Walker A / B boxes and the Sensor-1 and 2 motifs (common to AAA^+ proteins). Domain IV contains DNA-binding activity, and is connected to Domain IIIb by a long α-helix (α12). This helix is presumed to be used as the site for membrane attachment based on *E. coli* DnaA protein studies. This connector helix is linked to a helix-turn-helix (HTH) motif which is with in a structural fold almost identical to the DNA-binding domain fold of the Trp repressor (Erzberger, Pirruccello et al. 2002). This recent advancement should enable more direct mutagenic studies of *E. coli* DnaA protein, and will help facilitate the dissection of DnaA protein's regulatory role.

Molecular chaperones and the activation of DnaA

The temperature sensitive mutant proteins DnaA46 and DnaA5 are activated for replication by molecular chaperones (Hwang and Kaguni 1988; Hwang and Kaguni 1988; Hupp and Kaguni 1993; Hupp and Kaguni 1993; Hupp and Kaguni 1993; Carr and Kaguni 1996). Both DnaA5 and DnaA46 contain the A184V missense mutation. This

causes a reduction in the affinity for ATP, and ATP binding is almost abolished at the restrictive temperature. Overexpression of these mutant proteins results in the formation of DnaA aggregates which are inactive in biochemical assays. It is thought that these temperature sensitive DnaA mutant proteins have a greater propensity to form aggregates because of the nucleotide binding defect. Even though chaperones (DnaK, GrpE) have been demonstrated to have a role in activation of temperature sensitive DnaA proteins, a clear role for chaperone activity in $DnaA^+$ protein activation has not been established. The synthesis of DnaK protein does not change during the cell cycle in *E. coli*. ClpB and DnaK proteins have been shown to be important for activation of the initiation protein (TrfA) for plasmid RK2 replication but the action of these chaperones is not required for *oriC* dependent replication (Konieczny and Liberek 2002).

Hyperactive initiation of DNA replication

According to the model that DnaA protein is responsible for initiation of replication, it would be expected that the frequency of initiation could be increased by either an over-supply of DnaA protein or by isolating mutations in DnaA protein that cause an increase in the frequency of initiation. Both of these expectations have been documented. Increasing the level of DnaA protein in the cell results in unscheduled initiation. In these studies, *dnaA* gene expression is under the control of an inducible promoter, resulting in elevated DnaA protein expression (Atlung, Lobner Olesen et al. 1987). This synthetic situation is not lethal to the host, but results in a decreased growth rate. The idea that the cell prefers a steady-state DnaA protein level is confirmed by results describing a sequence near *oriC* containing several DnaA-boxes (*datA*). The *datA* locus is required for normal growth (Ogawa, Yamada et al. 2002). When this segment is deleted, cells undergo increased initiation. Based on this observation, it has been proposed that the *datA* locus serves to titrate the initiation potential of excess DnaA

protein under normal growth conditions. The other well-characterized condition involves a mutant form of DnaA protein, DnaAcos (see below), which causes a cold sensitive phenotype to the host. The cold sensitive phenotype associated with DnaAcos is due to hyperactive initiation, which is lethal to the host strain at the nonpermissive temperature (for other hyperactive *dnaA* allele see Table 2).

DnaAcos

The *dnaAcos* allele was isolated as an intragenic suppressor of the temperature sensitive *dnaA46* allele (Kellenberger-Gujer, Podhajska et al. 1978). Marker frequency experiments have measured the relative amount of the *oriC* locus to be 3-5 fold greater than the *terC* locus after the temperature shift from 42°C to 30°C (Katayama and Kornberg 1994) indicating that DnaAcos induces hyperactive initiation of DNA replication. Cloning and sequencing of the *dnaAcos* allele revealed four nucleotide substitutions which result in four amino acid changes in the protein (Q156L, A184V, H252Y and Y271H). Elevated expression of strains harboring DnaAcos have a lethal phenotype at both 30°C and 42°C when the *dnaAcos* allele is encoded on a plasmid and linked downstream of the *lac*UV5 promoter (Braun, O'Day et al. 1987). The *A184V* mutation of *dnaAcos* does cause hyperactive initiation of replication at 30°C when plasmid-borne; however, the *A184V* mutation alone does not reconstitute the plasmid-borne *dnaAcos* phenotype of lethality at 30°C and 42°C, indicating that other mutations are required (Nyborg, Atlung et al. 2000). DnaAcos protein has been purified to homogeneity and assayed *in vitro*. As expected DnaAcos binds ATP poorly (K_d100 μM), and has more replication activity than DnaA in crude extracts. Interestingly, DnaAcos has poor replication activity in replication assays reconstituted with purified components.

This result suggests that DnaAcos requires a soluble factor for activation that is present in the crude extract and absent from the reconstituted system. This result

prompted others to try and identify proteins in the soluble fraction that regulate DnaA protein activity. The beta clamp of DNA polymerase III holoenzyme (Katayama, Kubota et al. 1998) and Hda (Hda is a novel protein which negatively regulates DnaA protein) (Kato and Katayama 2001) were purified as components of a soluble fraction (RIDA regulatory inactivation of DnaA) that causes DnaA protein to hydrolyze ATP quickly.

Some researchers have tried to isolate mutations in other genes or to determine if overexpression of other proteins will suppress DnaAcos. An increase in the expression of the *cedA* gene product suppresses DnaAcos. Strains carrying the *dnaAcos* allele are filamentous suggesting an inhibition or a reduction in cell division. Overexpression of CedA protein restores cell morphology but did not inhibit over-replication. The *cedA* dependent pathway is thought to be independent of FtsZ (FtsZ oligomerizes to form a ring during cell division), as filamentation of cells bearing *dnaAcos* is independent of the FtsZ inhibitor SfiA protein (Katayama, Takata et al. 1997). This conclusion seems imprecise as SfiC (*e14* gene product) protein can also inhibit cell division through interaction with FtsZ.

Deletion of the *dam* gene also suppresses DnaAcos. It was observed that *dam*⁻ strains fail to overinitiate replication. If Dam methylase is supplied in *trans* the over-replication of DnaAcos was restored. This result indicates that DnaAcos may depend on the activation of *oriC* by Dam methylase to over-initiate replication (Katayama, Akimitsu et al. 1997). The only replication-related protein that is known to suppress DnaAcos is SeqA. It was observed that a moderate over-expression of SeqA protein allowed for

Table 2. Hyperactive *dnaA* alleles

Mutation and allele	Replication Phenotype	Mechanism	Reference
Q156L, A184V, H252Y, Y271H (dnaAcos)	Hyperactive 30°C, *in vivo* and *in vitro*	Assumed to be caused by nucleotide binding defect	Kellenberger-Gujier *et. al*, 1978
V157E	Hyperactive *in vivo*	Unknown	Hiraga and Saitoh, 1974
G175D	Dominant Negative to *dnaA*$^+$	Unknown	Sutton and Kaguni, 1997
P431L, G177D	Dominant Negative to *dnaA*$^+$	Unknown	Sutton and Kaguni, 1997
G177D	Dominant Negative to *dnaA*$^+$	Unknown	Sutton and Kaguni, 1997
G177D, E248K, E249K	Dominant Negative to *dnaA*$^+$	Unknown	Sutton and Kaguni, 1997
A184V	Hyperactive 30°C *in vivo*	Poor ATP binding	Nyborg *et. al*, 2000; Carr and Kaguni, 1996
A184V+H252Y (dnaA46)	Hyperactive at 30°C when GroE is overexpressed	ATP binding	Katayama and Nagata, 1991
A184T	Dominant Negative to *dnaA*$^+$	Unknown	Sutton and Kaguni, 1997
A184V, H252Y, R342C	Hyperactive *in vivo*	Unknown	Weigel *et. al*, 1999
R334A	Hyperactive *in vitro*	Crippled ATPase activity	Nichida *et. al*, 2002

The *dnaA* alleles listed in this table are either known to cause hyperactive initiation or it has been suggested based on genetic evidence. Most of these alleles encode a mutation near the Walker A box to correlate hyperactive initiation to a defect in nucleotide binding. In the case of DnaAcos it has been demonstrated that ATP binding activity is very poor and DnaAcos is hyperactive *in vitro* and *in vivo*. The A184V mutant protein is hyperactive *in vivo* but not *in vitro* at 30°C.

colony formation of a *dnaAcos* strain at 30°C (Lu, Campbell et al. 1994). Although the mechanism of this suppression is unknown, an increase in the amount of SeqA protein is probably inhibitory for initiation of replication as it may decrease the availability of *oriC* for DnaAcos, preventing hyperactive initiation.

Alternative modes of chromosomal replication in *Escherichia coli*

The primary focus of this review is to summarize DnaA-dependent initiation of DNA replication. Other pathways of chromosomal replication do exist in *E. coli* and these pathways are important because they allow *E. coli* to survive under abnormal circumstances, and they utilize many of the proteins that are required for *oriC*-dependent replication.

The SOS response in *E. coli* is induced after DNA damage, ethanol treatment or thymine starvation. During the SOS response chromosomal DNA must be repaired and replicated. The mode of chromosomal replication that is activated during the SOS response is termed iSDR {(inducible stable DNA replication, reviewed in (Kogoma 1997)} and is referred to as being stable because this pathway is not inhibited by protein synthesis inhibitors. In contrast, DNA replication from *oriC* requires an unstable factor as new rounds of replication require protein synthesis (Kogoma, Torrey et al. 1981). The pathway of iSDR replicates from two known origins (*oriM1* and *oriM2*) (Magee, Asai et al. 1992). The origin *oriM1* is located in part of *oriC* and *oriM2* is located at *terC* where normal chromosomal replication is terminated. Replication through iSDR is independent of DnaA protein, as *dnaA* null strains are able to support iSDR. This pathway is dependent on many of the homologous recombination proteins (Asai, Sommer et al. 1993) and is a form of recombination-dependent replication (RDR). It has been suggested that replication activity used for iSDR resembles that required for recombination. It is thought that RecA* protein (an activated form of RecA) promotes

strand invasion to form a D-loop. Strand separation facilitated by this D-loop (Asai and Kogoma 1994) provides a site for the loading of DnaB helicase by the primosome (Masai, Asai et al. 1994) of which all the components are required for iSDR activity.

The other method of DnaA independent chromosomal duplication is termed constitutively stable DNA replication (cSDR). This mode of replication is activated in strains that lack a functional *rnhA* gene (Torrey and Kogoma 1987). The *rnhA* gene codes for RNase H protein, which specifically recognizes DNA-RNA hybrids. RNase H recognizes and degrades DNA-RNA hybrids that arise outside of *oriC*, and confers specificity of replication to *oriC*. Deletion of the *dnaA* gene has no effect on cSDR (Ogawa, Pickett et al. 1984). Initiation of replication through cSDR (like iSDR) is dependent on RecA protein (Torrey and Kogoma 1987) and assembly of the primosome. The initiation of DNA replication takes place at random sites around the chromosome termed *oriK* (de Massy, Fayet et al. 1984). The strand opening at these sites is thought to be facilitated by R-loop formation of the DNA-RNA hybrids (Kogoma 1997). Both iSDR and cSDR do not require concomitant protein synthesis (von Meyenburg, Boye et al. 1987), unlike DnaA dependent replication (Kogoma 1978). Although iSDR and cSDR may appear similar, they are distinct pathways of chromosomal replication.

The pathways of iSDR and cSDR are examples of replication in the absence of DnaA protein and these pathways are either activated in response to adverse conditions (iSDR) or mutations (cSDR). Other modes of DnaA independent replication have been described. These pathways involve integrative suppression, where the need for DnaA is suppressed by integration of a plasmid origin which replicates independently of DnaA protein. The most well-characterized example involves the integration of the mini R1 replicon into *oriC* (Koppes and Nordstrom 1986). Although DnaA protein is not required for chromosomal replication, other replication proteins are required.

Replication fork collapse and replication fork restart

As described earlier, the *E. coli* cell cycle constitutes two distinct parts, the C and D periods. The C period is the time required for the faithful replication of the *E. coli* genome. During the C period, replication fork collapse, DNA repair and replication fork restart occur in response to DNA damage or other events that block replication fork progression. Pyrimidine dimers, frozen proteins on the DNA, collisions with RNA polymerase have all been cited as frequent events that result in replication fork demise (Sandler 2000; Sandler and Marians 2000). After replication fork collapse the replication fork must be restarted to complete replication and ensure survival of the cell. Replication fork restart is a process that is dependent on proteins that constitute the primosome, and those that support homologous recombination (Marians 2000).

The primosome requires the biochemical activities of PriA, PriB, DnaT, PriC, DnaC, DnaB and DnaG proteins. The primosome is assembled in an ordered manner with the initial binding of PriA, followed by PriB, DnaT and PriC (Ng and Marians 1996a; Ng and Marians 1996b). It has been observed that PriA binding is stabilized by the binding of PriB, which facilitates the binding of DnaT. PriA also directs the binding of the $DnaC_6$-$DnaB_6$ complex. After DnaC releases DnaB in an ATP-dependent reaction, DnaG (primase) transiently associates with the primosome to prime replication (Ng and Marians 1996; Ng and Marians 1996).

It has been observed that *priA* null mutations are viable. These mutations, however, are lethal when DNA damage is significantly increased due to UV treatment or growth in the presence of a DNA damaging agent. PriA null mutants are also inviable when the *rep* coding region is disrupted (Sandler and Marians 2000). Because Rep helicase moves ahead of the replication fork and acts as a "bulldozer" clearing the path for the replication machinery, it is not surprising that *priA rep* double mutants are not viable (Sandler, McCool et al. 2001). It was also observed that *priA* null strains have

constitutive expression of the SOS response and are impaired for homologous recombination, suggesting an important link between replication fork restart and homologous recombination.

Similarities between prokaryotic and eukaryotic initiation of DNA replication

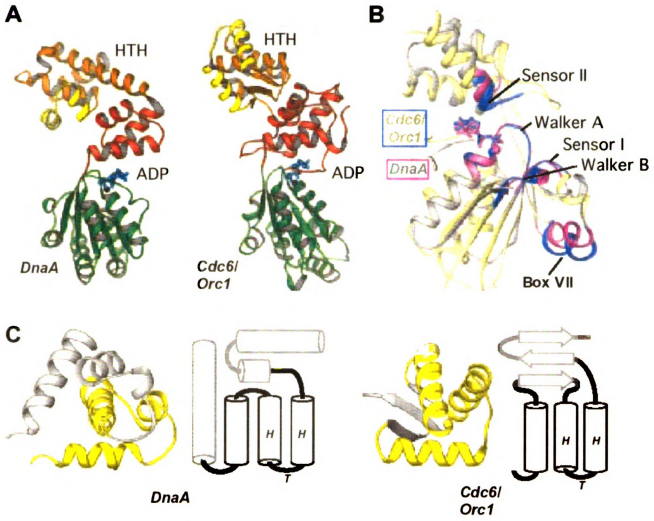
E. coli has served as a model system for the study of many biochemical pathways in other organisms. In studies of DNA replication, the extent of information known regarding the steps of initiation and elongation are well beyond that which is known regarding eukaryotic replication. Certainly, there are many limitations associated with studying eukaryotic replication that are overcome rather easily in *E. coli* (see below). Despite the obvious differences between these two systems, it is apparent that many commonalities exist between prokaryotic and eukaryotic initiation.

DnaA protein promotes the initiation of DNA replication in *E. coli*. The analogous functioning complex in yeast, and metazoans is the origin recognition complex (ORC) (Bell and Stillman 1992). ORC is composed of six polypeptides (Orc1-6) which are required for origin binding activity (Bell and Stillman 1992). *S. cerevisiae* ORC must bind ATP prior to binding the replication origin for stable association. ATP hydrolysis by ORC is very slow, which is analogous to the slow rate of ATP hydrolysis by DnaA (Klemm, Austin et al. 1997). ATP binding affinity for DnaA is very similar to that observed for ORC (Orc1 and Orc5 20-100 nM, DnaA 30 nM). Like DnaA, ORC also remains bound to the replication origin throughout the cell cycle, although the DNase I protection pattern is different for cells that are in G_1 versus the pattern observed for S and M phase cells (Brown, Holmes et al. 1991; Diffley and Cocker 1992; Diffley, Cocker et al. 1994). Similarly, DnaA protein remains bound to three of the five DnaA boxes throughout the cell cycle, with the fourth and fifth boxes becoming occupied just prior to

initiation. DnaA protein seems to have a similar functional role to ORC, although only modest amino acid sequence similarity between ORC subunits and DnaA are observed.

The homology that does exist between replication proteins of both systems comes in the form of the AAA$^+$ family (Neuwald, Aravind et al. 1999) of ATPases (ATPases associated with a variety of cellular activities). DnaA protein is a member of this extensive collection of proteins. This family also includes, but is not limited to, DnaC protein, proteins of the γ complex (Clamp loader) in prokaryotic initiation, Cdc6/Cdc18 (Cdc18 is *S. pombe* Cdc6), Orc1,4,5, MCM2-7, and RFC family members from eukaryotic replication. Many of the proteins in this group form hexamers (specifically, the γ complex), suggesting that DnaA and other members of this group may do the same. Recent crystal structure data has revealed significant similarities between eukaryotic and prokaryotic initiation proteins (Erzberger, Pirruccello et al. 2002). Many replication and transcription proteins in archaea are homologous to proteins involved in these processes in eukaryotic cells. Interestingly, the crystal structure of *A. aeolicus* DnaA is very similar (Figure 5) to the structure of Cdc6 (Liu, Smith et al. 2000), which may be the most DnaA-like eukaryotic replication protein. The role of Cdc6 protein *in vivo* has been shown to be important for loading a complex of MCM proteins (4,6,7) a complex speculated to function as a helicase to support replication fork movement (Chong, Hayashi et al. 2000). However, the helicase activity measured *in vitro* is weak (You, Komamura et al. 1999; Ishimi and Komamura-Kohno 2001; Lee and Hurwitz 2001). DnaA protein has been demonstrated to be important for the loading of DnaB to form the prepriming complex in *E. coli*. In *S. cerevisae* two *cdc6*

Figure 5. Structural comparison of bacterial and archaeal replication proteins
Archaeal Cdc6/Orc1(from Liu et. al, 2000; Erzberger et. al, 2002) and bacterial DnaA protein have very similar structures even though the sequence homology is limited to 15%. **(A)** A ribbon diagram of the overall crystal structure and domain organization of DnaA protein and the closest known homologue to DnaA protein archaeal Cdc6/Orc1 protein. The green and orange regions of each structure corresponds to the ATP binding and processing domain. The yellow region corresponds to the domain containing DNA binding activity. Both structures contain helical domains fused to AAA^+ motifs. **(B)** The light gray and pale yellow areas correspond to regions of DnaA and Cdc6/Orc1 that are important for the ATP binding cleft. This picture represent a superimposition of the AAA^+ regions of the two proteins. The Sensor I (S-I) Sensor II (S-II) Walker A (W-A) and Walker B (W-B) are labeled and colored in magenta for DnaA, and blue corresponding to Cdc6/Orc1. The arrangement of the AAA^+ motif and the Box VII segment are strongly correlated between these two proteins. **(C)** A comparison of the DNA binding Domains of DnaA and Cdc6/Orc1. A ribbon diagram is located next to the secondary structure topology diagram. In the topology diagram the bold and yellow regions correspond to the conserved area of the fold; the gray region indicates the differences between the two folds. The DNA binding domain of both proteins is comprised of a HTH (helix-turn-helix) motif. The remaining fold of the C-terminal domains are arranged differently suggesting an evolutionary relationship may exist that is connected by conversion or a rearrangement of modules.


A
HTH
ADP
DnaA
HTH
ADP
Cdc6/
Orc1
B
Sensor II
Cdc6/
Orc1
Walker A
Sensor I
DnaA
Walker B
Box VII
C
H
H
T
DnaA
H
H
T
Cdc6/
Orc1

alleles (*cdc6-2* and *cdc6-3*) were isolated that result in "persistent" initiation. It was determined that the cause of this activity was the constant association of MCM proteins with chromatin, suggesting a constitutively active form of Cdc6 protein continually loads the "helicase" (Liang and Stillman 1997).

The molecular cause of hyperactive initiation in *E. coli* is the major subject of the work presented below. The objectives of this research are to identify amino acid changes in DnaA protein that result in an increased frequency of initiation. This work will be pursued to better understand the regulatory mechanisms that exist to limit the frequency of replication initiation in *E. coli*. Based on the work of others, we know that hyperactive initiation results in growth interference. The mechanism that results in growth interference after hyperactive initiation has never been identified. Experiments are also presented to examine the fate of replication forks after hyperactive initiation, and mechanisms responsible for lethality are discussed. The work presented in this dissertation are designed to understand the role of DnaA protein in the regulation of initiation or DNA replication.

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Chapter II

DnaA Protein of *E. coli*: Initiation of DNA Replication Requires DnaA Oligomerization and Specific N-terminal Amino Acids

Abstract

DnaA box sequences present in multiple copies within the replication origins of bacteria and prokaryotic plasmids are recognized by the replication initiator, DnaA protein. At the *E. coli* chromosomal origin, *oriC*, DnaA is speculated to oligomerize in order to initiate chromosomal DNA replication. We developed an *in vivo* assay of oligomer formation at *oriC* that relies on complementation between two *dnaA* alleles that are inactive by themselves. One allele is *dnaA46*; its inactivity at the nonpermissive temperature is due to a specific defect in ATP binding. The second allele, *T435K*, does not support DNA replication because of its inability to bind to DnaA box sequences within *oriC*. We show that the *T435K* allele carried in a plasmid can complement the temperature sensitivity of the *dnaA46* allele. The results support a model of oligomer formation in which DnaA46 is bound to the DnaA box sequences of *oriC*, and the mutant protein carrying the T435K substitution interacts with the bound DnaA46 to form a complex that is active in initiation. Relying on this assay, leucine 5, tryptophan 6 and cysteine 9 in a predicted alpha helix near the N-terminus of DnaA were identified that, when altered, interfere with oligomer formation. Glutamine 8 and proline 28 are additionally needed for oligomer formation when oriC is carried in a plasmid, suggesting that the structure of the DnaA-*oriC* complex at the chromosomal *oriC* locus is similar but not identical to that assembled on a plasmid. These results provide direct evidence that DnaA oligomerization at *oriC* is required for initiation to occur.

Introduction

Initiation of DNA replication involves the recognition of the replication origin by an origin binding protein, followed by the stepwise assembly of other proteins to build the replication fork machinery. In *E. coli*, the origin binding protein is DnaA, recognizing a DNA motif termed the DnaA box (Fuller and Kornberg 1983). In a complex with ATP, DnaA then opens the parental duplex and recruits the replicative helicase, DnaB, to this site to form the prepriming complex (Sekimizu, Bramhill et al. 1987; Bramhill and Kornberg 1988; Marszalek and Kaguni 1994). Two helicase molecules are recruited, one for each replication fork to promote bidirectional fork movement (Fang, Davey et al. 1999; Carr and Kaguni 2001). A similar process occurs in eukaryotes; the ORC proteins (Orc1-6) recognize the replication origin sequence to recruit Mcm2-7, the eukaryotic counterpart to *E. coli* DnaB (reviewed in (Bell 2002)). Recent results describing the crystal structure of a truncated form of DnaA protein from A. aeolicus reveal a remarkable similarity to that of archaeal Cdc6/Orc1 (Erzberger, Pirruccello et al. 2002). The similarities in structure and function of these proteins at the stage of initiation suggest that a common biochemical pathway is followed in all organisms.

The *E. coli* chromosomal origin, *oriC*, is defined as a 245 base pair minimal DNA sequence, based on mutational analysis of this sequence carried in a ColE1-derived plasmid vector (Oka, Sugimoto et al. 1980). In combination with comparative DNA sequence analysis, conserved sequences were identified that include five DnaA box motifs named R1, M, R2, R3 and R4 (Fuller and Kornberg 1984; Zyskind, Cleary et al. 1983; Matsui, Oka et al.1985). The idea that nonconserved sequences act as spacers to maintain the proper orientation between sites bound by proteins is supported by other mutational studies. Insertion of 4 or 8 base pairs or deletion of 4 base pairs between DnaA boxes R3 and R4 inactivated the cloned replication origin sequence (Woelker and

Messer 1993). However, insertion or deletion of one helical turn (10-12 base pairs) between these DnaA boxes was tolerated. These findings support a model of helical phasing, involving the interaction among DnaA molecules bound to *oriC*.

The conclusions drawn from these studies contrast with those of Bates et al. (Bates, Asai et al. 1995). They described that insertion of a 2 kilobase pair fragment carrying the spectinomycin resistance gene (the omega fragment) between DnaA boxes R3 and R4, or deletion of R4 did not interfere with *oriC* function when these alterations were present in the bacterial chromosome. However, these modifications were inactivating when carried in a plasmid. Although DNA replication of the bacterial chromosome was inefficient and asynchronous, the results suggest that the nucleoprotein complex assembled at the chromosomal *oriC* locus is different from that which is assembled on an *oriC* plasmid.

We previously described a mutational approach that identified functional domains within DnaA protein of 467 amino acids (Sutton and Kaguni 1997). A set of mutations clustered near the N-terminal coding region were obtained, using a genetic selection that yielded mutants defective in DNA replication activity. These mutants were also impaired in autoregulation of dnaA expression (Sutton and Kaguni 1997); results from other studies suggest that repression of *dnaA* transcription involves oligomerization of DnaA molecules bound to the *dnaA* promoter region (Lee and Hwang 1997). More recent results show that this region (residues 2-86) of DnaA protein can replace the dimerization domain of CI repressor encoded by bacteriophage lambda (Weigel, Schmidt et al. 1999). Because an N-terminal domain within the first 62 amino acids of DnaA also interacts with DnaB in loading of the helicase at *oriC* (Sutton, Carr et al. 1998; Seitz, Weigel et al.1999), we wanted to identify amino acid residues that function solely in oligomerization of DnaA to distinguish this functional domain from that which interacts with DnaB. As another objective, we sought to demonstrate that oligomerization of

DnaA at *oriC* is a required function for initiation to occur. At *oriC*, oligomer formation among DnaA monomers is thought to be required during initiation, but little evidence exists to support this conclusion.

To address these objectives, we speculated that we may be able to demonstrate oligomer formation in vivo by using two *dnaA* alleles that were defective in mutually exclusive functions. Whereas each allele alone is inactive, their combined presence in the same bacterial cell may then result in the formation of mixed complexes that are active in initiation. We chose the *dnaA46* allele as one of the pair because the mutant protein is active in binding to oriC, at 42°C, but is inactive in DNA replication due to a defect in ATP binding (Carr and Kaguni 1996).

The second allele of the pair was chosen because it is defective in binding to *oriC*. In other work, we showed that threonine 435 near the C-terminus of DnaA protein is involved in recognition of the DnaA box sequence (Sutton and Kaguni 1997). Based on the crystallographic structure of a truncated form of *A. aeolicus* DnaA protein, this amino acid resides at the end of an alpha helix and before the start of a loop in a region designated the DnaA signature sequence (Erzberger, Pirruccello et al. 2002). A mutant protein carrying a methionine substitution at this position (T435M) is inactive in DNA replication because it fails to bind to *oriC* (Sutton and Kaguni 1997). However, T435M can augment limiting levels of wild type DnaA in DNA replication of an *oriC* plasmid in vitro presumably by the formation of mixed oligomers of T435M and $DnaA^+$. In the study described below, we used a different dnaA allele encoding a lysine substitution at threonine 435 (T435K). As expected, this mutant protein is active in ATP binding, but defective in recognition of the DnaA box sequence (Severson et al., manuscript in preparation). We demonstrate allelic complementation between T435K and *dnaA46* at a temperature that inactivates the function of DnaA46 protein when assayed alone. Using an approach to combine the T435K mutation with other mutations that substitute amino

acids near the N-terminus of DnaA, we then assayed these alleles for intergenic complementation of the *dnaA46* allele. Because *C9Y* and *P28L* alleles are known to inactivate the function of DnaA protein in DNA replication (Sutton and Kaguni 1997), we focused on amino acid substitutions near the N-terminus of DnaA protein to determine if these and other unique missense mutations (*L5A, W6A, Q7A* and *Q8A*) were inactive in oligomer formation. Substitutions L5A, W6A, Q7A, Q8A and C9Y affect consecutive residues that reside in a predicted α-helix (Sutton, Carr et al. 1998) proximal to the N-terminus of DnaA, and within Domain I, a larger conserved sequence shared among bacterial DnaAs (Fujita, Yoshikawa et al. 1990; Messer 2002).

Experimental Procedures

Strains and plasmids-The *E. coli* K-12 strains used in this study are listed in Table 1, and were grown in LB media (Miller 1992). Plasmid DNAs were purified by cesium chloride-ethidium bromide density gradient centrifugation or by chromatography (Plasmid Midi Kit, Qiagen). Plasmid pRB100 expresses the *dnaA*$^+$ gene from its native *dnaA* promoters and is a derivative of pBR322 (Braun, O'Day et al. 1985). Plasmid pAB2 expresses the T435K mutation from the native *dnaA* promoters and is a derivative of pACYC177 (Gines, Blinkova et al. 1995). Plasmids carrying both N terminal mutations and the *T435K* mutation are derivatives of pAB2. Plasmid pKC596 encodes the *dnaA*$^+$ gene under control of the T7 RNA polymerase promoter (Carr and Kaguni 1996). The *oriC* plasmid pCM959-CmR is a derivative of pCM959 (Buhk and Messer 1983) constructed by insertion of the chloramphenicol acetyltransferase gene (Simmons and Kaguni, 2003; Hupert-Kocurek and Kaguni, unpublished results).

Bacterial transformation-Transformations were by electroporation (*E. coli* Pulser, BioRad) following the recommendations of the manufacturer (BioRad) with competent cells resuspended at a cell density of ~2 x 10^{10} cells/ml, and stored at -70°C. Unless otherwise indicated, 10 ng of plasmid DNA was mixed with 40 µl of competent cells. After electroporation, the cells were quickly resuspended in LB or SOC medium and incubated in the absence of antibiotic selection for 20 min under slight agitation. The cells were diluted and plated on the appropriate selective media and incubated overnight at the temperatures indicated.

Site-directed mutagenesis-The N-terminal mutations were introduced into plasmid pAB2 by PCR mutagenesis with overlapping primers carrying the desired mutations as indicated by the underlined sequences. For brevity, only one of the two complementary primer sequences is shown: L5A, GTGTCACTTTCGGCTTGGCAGCAGTGT; W6A, GTCACTTTCGCTTGCGCAGCAGTGTCTTG; Q7A,

CTTTCGCTTTGGGCGCAGTGTCTTGCC; Q8A, TCGCTTTGGCAGGCGTGTCTTGCCCGA; C9Y, TGGCAGCAGTATCTTGCCCGA; and P28L, TGTGGATACGCCTATTGCAGGCGGA. Both the N-terminal and C-terminal portions of the dnaA gene were sequenced to verify presence of the respective N-terminal mutation and to confirm the presence of the T435K mutation.

Glutaraldehyde Crosslinking-Protein crosslinking was in 25 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.01% Nonidet P-40, 100 mM NaCl, and 1 mM DTT with purified DnaB (200 ng), DnaAΔ62 (180 ng), or a derivative of wild type DnaA carrying a polyhistidine tag at its N-terminus (190 ng) in a final volume of 25 µl. The DnaA derivative is comparable in activity to wild type DnaA protein *in vivo* and in DNA replication activity *in vitro* (Walker et al., manuscript in preparation). Glutaraldehyde was added to a concentration of 0.01%, followed by incubation at room temperature for the times indicated. Reactions were quenched by the addition of 5 µl of 50% glycerol, 0.2 M Tris, 1% SDS, 0.1% bromophenol blue and 5 mM DTT. Samples were incubated at 86°C for 5 min, electrophoresed alongside prestained molecular weight markers (Gibco-BRL) in an SDS-polyacrylamide gel, and transferred to a membrane (Protran, Schleicher & Schuell). Protein transfer was confirmed by the presence of prestained molecular weight markers on the membrane. Detection of DnaA protein complexes was with rabbit antiserum prepared against the DNA binding domain of DnaA (residues 370-467) unless noted otherwise, or with affinity-purified antibody that is specific for DnaB. After incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (BioRad), the chemiluminescence (Supersignal, Pierce) was detected with Xray film (X Omat, Kodak).

Table 1. List of Strains

Strain	Genotype[a]	Parent	Source
LS1073	*araD139* Δ*araC leu7697* Δ(*lac*)X174 *galU galK hsdR2*(r_k^-,m_k^+) *strA mcrA mcrB1 tna*::Tn*10 dnaA46*	MC1061	Simmons and Kaguni, 2003
LS1065	*araD139* Δ*araC leu7697* Δ(*lac*)X174 *galU galK hsdR2*(r_k^-,m_k^+) *strA mcrA mcrB1 tna*::Tn*10 dnaA46* Δ*oriC*::pKN1562(clockwise) Km^r,	MC1061	This work
LS1061	*araD139* Δ*araC leu7697* Δ(*lac*)X174 *galU galK hsdR2*(r_k^-,m_k^+) *strA mcrA mcrB1* Δ*oriC*::pKN1562(clockwise) Km^r	MC1061	Simmons and Kaguni, 2003
LS1062	*araD139* Δ*araC leu7697* Δ(*lac*)X174 *galU galK hsdR2*(r_k^-,m_k^+) *strA mcrA mcrB1* Δ*oriC*::pKN1562(clockwise) Km^r *dnaA*850::Tn*10*	LS1061	Simmons and Kaguni, 2003
MC1061	*araD139* Δ*araC leu7697* Δ(*lac*)X174 *galU galK hsdR2*(r_k^-,m_k^+) *strA mcrA mcrB*		Lab stock

[a] The strains listed are derivatives of MC1061 and were constructed by P1 transduction (Simmons and Kaguni, 2003).

Results

Intragenic complementation as an in vivo *assay for DnaA oligomerization.* The notion that oligomerization of DnaA at *oriC* is required for initiation is based initially on estimates from electron microscopy which suggest that 20-40 DnaA monomers are bound to *oriC* (Fuller, Funnell et al. 1984; Crooke, Thresher et al. 1993). Other results indicate that an N-terminal domain of DnaA is involved in oligomerization, and that this activity may be required for initiation from *oriC* (Sutton and Kaguni 1997; Weigel, Schmidt et al. 1999). To obtain direct evidence to support this concept, we chose the *T435K* and *dnaA46* alleles for reasons described above. The experimental approach was to construct merodiploid strains in which the host carried the *dnaA46* locus, and different plasmid-borne *dnaA* alleles were introduced by transformation. When the *T435K* allele (plasmid-borne) was introduced into the *dnaA46* strain, complementation was observed at the higher temperature, confirming that oligomer formation occurs between the two mutant proteins. Likewise, the *dnaA*$^+$ plasmid complemented the *dnaA46* allele at the elevated temperature, in agreement with previous observations (Hansen, Atlung et al. 1984). However, the latter results do not support the formation of mixed oligomers as DnaA$^+$ protein alone at *oriC* may support initiation in the *dnaA46* host strain. In contrast, the vector for the *dnaA*$^+$ plasmid (pBR322) or that for the *T435K* allele (pACYC177) were ineffective. Double mutations carrying the *T435K* substitution combined with different missense mutations near the start of the *dnaA* coding region were assayed in parallel. *Q7A, Q8A* and *P28L* were active in complementation whereas *L5A, W6A* and *C9Y* were comparable to the empty vector controls (Table 2). These results suggest that the latter group of mutations are inactive due to a defect in oligomerization. Oligomerization of DnaA at *oriC* is thought to involve interactions among monomers bound to the different DnaA boxes at this site. Because T435K does not bind to the DnaA

Table 2. *L5A*, *W6A*, and *C9Y* alleles are defective in allelic complementation when combined with *dnaA46*

Plasmid	*dnaA* allele	Plating Efficiency[a]
dnaA$^+$	pRB100	1.0
pBR322	None	$2.5X10^{-3}$
pAB2	*T435K*	1.0
pACYC177	None	$1.9X10^{-4}$
pLS2	*L5A+T435K*	$2.0X10^{-3}$
pLS3	*W6A+T435K*	$6.8X10^{-3}$
pLS4	*Q7A+T435K*	1.1
pLS5	*Q8A+T435K*	1.0
pLS1	*C9Y+T435K*	$4.9X10^{-4}$
pLS6	*P28L+T435K*	0.97

[a]The respective plasmids were transformed into LS1073 (*dnaA46*), followed by incubation in SOC media for 20 min before plating on LB media containing 100 μg/ml ampicillin. After overnight incubation at 30°C or 42°C, plating efficiencies were determined as the ratio of transformants obtained at the elevated temperature over those obtained at 30°C. Transformation frequencies of the various plasmids at 30°C ranged from 10^6 to 10^7 transformants per μg of plasmid DNA.

boxes but only retains nonspecific DNA binding activity, the results of Table 2 suggest that monomers of T435K bind to DnaA46 monomers complexed to respective DnaA box sequences. In the experiment of Table 2, the activity of the *dnaA* alleles was measured by initiation from the chromosomal *oriC* locus.

As studies show that DnaA box R4 is required for replication from *oriC* when this sequence is contained in a plasmid, but that *oriC* in the bacterial chromosome remains functional when R4 is deleted (Bates, Asai et al. 1995; Weigel, Messer et al. 2001), the possibility arises that the oligomeric DnaA complexes assembled at the chromosomal *oriC* locus may be different from those assembled on an *oriC* plasmid. To address the above possibility, allelic complementation between the chromosomal *dnaA46* allele and the different plasmid-encoded alleles was measured upon transformation of an *oriC* plasmid (pCM959-CmR) (Table 3). The host strain chosen for this experiment (LS1065, relevant genotype: Δ*oriC*::pKN1592, *dnaA46*) lacked a functional *oriC* locus, and was integratively suppressed by an R1 derivative (pKN1592). The reason for selecting this host strain was because its viability is independent of *dnaA* function. Thus, the frequency of obtaining transformants at the nonpermissive temperature for the *dnaA46* strain reflects the ability of DnaA46 protein to oligomerize with the form of DnaA encoded by the plasmid on an *oriC* minichromosome. Interestingly, the results of Table 3 are similar but not identical to those of Table 2. The *T435K* mutation alone and combined with *Q7A* were active in maintaining the *oriC* plasmid. All other mutations including *Q8A* and *P28L* (combined with *T435K*) were inactive. These results suggest that residues involved in DnaA oligomerization at *oriC* are conditionally dependent on whether this locus is contained in a plasmid or the bacterial chromosome.

As a control for the experiment of Table 3, the *oriC* plasmid was co-transformed with plasmids that carried either the *dnaA*$^+$ or *T435K* alleles, but into two isogenic strains

Table 3. Allelic complementation by *dnaA46* and *T435K* in *oriC* plasmid maintenance demonstrates the formation of mixed oligomers.

Resident Plasmid	*dnaA* gene	Plating efficiency[a] 42°C/30°C
pRB100	*dnaA*$^+$	1.0
pBR322	None	$9.9X10^{-4}$
pAB2	*T435K*	0.97
pACYC177	None	$6.7X10^{-4}$
pLS2	*L5A+T435K*	$5.4X10^{-4}$
pLS3	*W6A+T435K*	$6.2X10^{-4}$
pLS4	*Q7A+T435K*	1.0
pLS5	*Q8A+T435K*	1.0
pLS1	*C9Y+T435K*	$9.0X10^{-3}$
pLS6	*P28L+T435K*	0.98

[a]LS1065 (*dnaA46* Δ*oriC*::pKN1592) was transformed with the indicated plasmids harboring either the indicated *dnaA* allele or the vector as a control, and the transformation mixture was plated at 30°C and 42°C (see Table 5). These competent cells were transformed with *oriC* plasmid pCM959-CmR and the transformation was plated at 30°C and 42°C on LB media supplemented with 50 μg/ml ampicillin and 25 μg/ml chloramphenicol to select for both plasmids.

Table 4. The *T435K* allele is defective in maintenance of an *oriC* plasmid.

Plasmid[a] co-transformed with pCM959-Cm^R	LS1062 (Δ*oriC*, ΔdnaA)[b]	LS1061 (Δ*oriC*)[b]
pRB100 (*dnaA*$^+$)	1.0	1.1
pBR322	$1.7X10^{-3}$	1.0
pAB2 (T435K)	$4.4X10^{-2}$	1.0
pACYC177	$2.9X10^{-3}$	1.1

The indicated plasmids (100 ng each) were separately co-transformed with pCM959-Cm^R (200 ng) into LS1062 (Δ*oriC*::pKN1562, *dnaA850*::Tn*10*), LS1061 (Δ*oriC*::pKN1562), or MC1061 as described in "Experimental Procedures." After electroporation, the transformants were grown in SOC media for 20 min, and dilutions were plated on LB media containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol, and plates were incubated for 24 hr at 37°C. In separate experiments, 100 ng of pRB100 DNA was determined to be near saturation for transformation of MC1061. The amounts of the respective plasmids were chosen to favor uptake of both plasmids on co-transformation.

The number of co-transformants obtained for the indicated strains was divided by the number of co-transformants obtained (5×10^5 per reaction) with the control strain MC1061.

that differed from the *dnaA46* host strain used in Table 3. One strain was disrupted at the *dnaA* locus (dnaA850::Tn10) whereas the other was *dnaA*$^+$. Both strains lacked essential sequences within *oriC* (hence Δ*oriC* and integratively suppressed by the R1 derivative pKN1562). Because T435K is defective in initiation from *oriC* due to its inability to bind to the DnaA box sequence (Walker et al., manuscript in preparation), the result that *T435K* failed to maintain the *oriC* plasmid in the null *dnaA* host was expected (Table 4). In comparison, the wild type *dnaA* gene maintained the *oriC* plasmid when either plasmid-borne (in the Δ*oriC*, Δ*dnaA* host strain) or encoded by the bacterial chromosome (in the Δ*oriC*, *dnaA*$^+$ host strain). These results confirm that the activity of *T435K* in allelic complementation is due to a physical interaction between T435K and DnaA46 protein.

Immunoblot analysis indicates that the mutant proteins are expressed at levels sufficient to support initiation. The assumption in the above experiments is that the steady state levels of the plasmid-encoded mutant proteins are sufficient to sustain DNA replication. Because we have found that some mutant forms of DnaA protein are exquisitely sensitive to proteolysis ((Sutton and Kaguni 1997), data not shown), we performed immunoblot analysis to determine the steady state levels of the N-terminal mutant proteins. To exclude the contribution of chromosomally encoded DnaA protein in the analysis, a Δ*dnaA* host strain was used. Control immunoblot experiments were performed with plasmid-free strains, showing that the *dnaA*$^+$ strain (LS1061) expressed DnaA but that the isogenic Δ*dnaA* strain (LS1062) did not (data not shown). These results confirm that the levels of the gene products being measured in the Δ*dnaA* host strain are indeed plasmid-encoded. Expression of the different plasmid-borne *dnaA* alleles in the Δ*dnaA* host strain (LS1062) was then analyzed. Compared to the level of wild type DnaA, the level of T435K was elevated (Table 5). Inasmuch as expression of

Table 5. Steady state levels of mutant DnaA Proteins

Plasmid	Protein	Relative level[a]
pRB100	DnaA$^+$	1.0
pAB2	T435K	1.7
pLS2	L5A+T435K	0.5
pLS3	W6A+T435K	0.6
pLS4	Q7A+T435K	0.9
pLS5	Q8A+T435K	0.6
pLS1	C9Y+T435K	0.2
pLS6	P28L+T435K	0.6
pKC596	DnaA$^+$	0.07

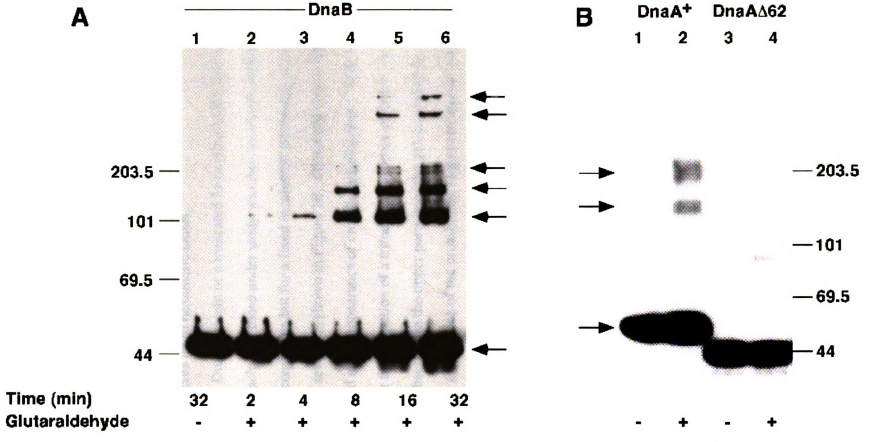
[a] The steady state levels of mutant proteins were determined by immunoblot analysis as described (Simmons and Kaguni, 2003). Whole cell lysates of LS1062 (*dnaA850*::Tn*10*) bearing the respective plasmid-borne *dnaA* alleles were analyzed by immunoblotting. Purified DnaA protein was analyzed in parallel to confirm that the immunoreactive species was indeed DnaA. As the primary antibody, a monoclonal antibody (M43) that is specific for DnaA protein (Marszalek and Kaguni, 1996) was incubated with the membrane. Other steps of the immunoblotting procedure were as described in "Experimental Procedures," but with horseradish peroxidase-conjugated goat anti-mouse antibody as the secondary antibody. The Xray film that captured the chemiluminescence signal was then analyzed quantitatively with Kodak EDAS 120 software, and normalized to the amount of DnaA$^+$ protein encoded by pRB100. The pBR322 vector of pRB100 is reported to have a comparable copy number compared to the pACYC177 vector for pAB2, and its derivatives that encode two missense mutations. Plasmid pKC596 is a pET11a derivative in which expression of *dnaA* is from a T7 RNA polymerase promoter.

the *dnaA* gene is autoregulated (reviewed in Messer 2002), the higher level of T435K is consistent with its defect in binding to the DnaA box motif. The difference is not due to the number of the plasmids carrying the *T435K* or *dnaA*$^+$ alleles. Both parental plasmids pBR322 and pACYC177 are reported to have comparable copy numbers (Chang and Cohen 1978; Covarrubias, Cervantes et al. 1981).

Immunoblot analysis established that the levels of the other mutant proteins relative to wild type DnaA ranged from 0.2 (C9Y + T435K) to near wild type levels (Q7A + T435K) (Table 5). Because the steady state level of C9Y + T435K was the lowest of all of the mutant proteins, its inactivity in complementation may be due to its reduced abundance. To address this possibility, we examined the amount of DnaA protein expressed from a plasmid (pKC596) in which the wild type gene but without the natural *dnaA* promoter region was placed downstream and under control of the T7 gene 10 promoter (Carr and Kaguni 1996). Compared to the level encoded by pRB100 (in which *dnaA*$^+$ expression is from the *dnaA* promoters) (Braun, O'Day et al. 1985), the level of DnaA expressed by pKC596 was about 14-fold reduced (Table 5). As the host strain lacks T7 RNA polymerase, we presume that read-through transcription from an upstream promoter accounts for this level of DnaA protein. Given that this amount is sufficient to complement the temperature sensitivity of a *dnaA46* strain (LS1073; data not shown), the results indicate that the inactivity of the *C9Y+ T435K* allele in complementation is not due to its lower abundance, but its inability to oligomerize at *oriC* in the bacterial chromosome.

Glutaraldehyde crosslinking of DnaA. To measure oligomer formation biochemically, we relied on glutaraldehyde as a crosslinking agent. As DnaB in the presence of Mg^{2+} is well characterized to exist as a hexamer of identical subunits (Bujalowski, Klonowska et al. 1994), DnaB protein was used as a control to optimize the

Figure 1. Glutaraldehyde crosslinking of DnaA confirms that an N-terminal region is required for oligomerization. Panel A. DnaB protein was incubated with 0.01% glutaraldehyde as described in "Experimental Procedures" at room temperature for the indicated lengths of time. As a negative control, DnaB was incubated in parallel but without glutaraldehyde treatment (lane 1). Samples were analyzed by electrophoresis in a 10% polyacrylamide gel under denaturing conditions. After transfer to a membrane, DnaB was detected by immunoblotting. Panel B. Purified $DnaA^{+}$ and an N-terminal deletion (DnaAΔ62) were incubated at room temperature for 10 min with 0.01% glutaraldehyde, where indicated, as described in "Experimental Procedures." Samples were analyzed by electrophoresis in an 8% polyacrylamide gel under denaturing conditions. DnaA protein was detected by immunoblotting. The electrophoretic mobilities of molecular weight standards are indicated. Arrows indicate the crosslinked complexes.


A
DnaB
1
2
3
4
5
6
203.5
101
69.5
44
Time (min)
32
2
4
8
16
32
Glutaraldehyde
-
+
+
+
+
+
B
DnaA+
DnaAΔ62
1
2
3
4
203.5
101
69.5
44
-
+
-
+

method. Shown in Figure 1A, incubation of DnaB with 0.01% glutaraldehyde resulted in the time-dependent appearance of crosslinked oligomers. In the absence of glutaraldehyde, these oligomers were not observed. As judged by SDS-polyacrylamide gel electrophoresis, the electrophoretic mobilities of the different crosslinked species of DnaB correspond well to those expected of a dimer, trimer and higher order complexes up to hexameric DnaB.

DnaA protein or a truncated form (DnaAΔ62) lacking the N-terminal 62 amino acids including those under study was then incubated with glutaraldehyde under the above conditions but for a fixed incubation period (10 min). A representative experiment among several is shown in Figure 1B. Glutaraldehyde treatment of $DnaA^+$ protein resulted in the appearance of crosslinked oligomers. Their electrophoretic mobilities suggest the formation of a trimer, and a DnaA tetramer. No other crosslinked oligomers were observed in the upper portion of the gel, including the region of the wells. The diffuse appearance of the crosslinked oligomers may be due to intramolecular crosslinking that then influences the shape of the polypeptides and their relative electrophoretic mobilities under the conditions of denaturing electrophoresis. The negative control of DnaAΔ62 incubated without glutaraldehyde revealed a trace amount of a species expected for dimeric DnaA. We presume that its presence is due to its residual resistance to denaturation under reducing conditions because its appearance was variable among experiments. Glutaraldehyde treatment of the deletion mutant resulted in a slightly increased amount of dimeric protein, but other oligomers were not observed. In other experiments, crosslinking of $DnaA^+$ with ethylene glycobis (sulfosuccinimidylsuccinate) resulted in the formation of complexes whose electrophoretic mobilities were similar to those expected of a dimer, trimer and tetramer, but the bands were not diffuse (data not shown). DnaAΔ62 treated with this agent gave rise to a complex expected of a crosslinked dimer. These results indicate that a domain

within the N-terminal 62 amino acids that is lacking in the truncated protein is required to form oligomers more complex than dimers. These observations provide additional evidence to support the results from genetic assays that N-terminal amino acids function in DnaA oligomer formation.

Discussion

Oligomer formation of DnaA. The *E. coli* replication origin contains five DnaA boxes that are recognized by DnaA protein (Fuller and Kornberg 1983; Matsui, Oka et al. 1985). Estimates based on electron microscopy of 20-40 DnaA monomers bound to *oriC* have been recently refined by quantitative immunoblot analysis (Carr and Kaguni 2001). The latter studies indicate that ten DnaA monomers are bound to *oriC* in a replication intermediate termed the prepriming complex. Its formation involves the recruitment of DnaB from the DnaB-DnaC complex by DnaA bound to *oriC* . These ratios of DnaA monomers per *oriC* suggest an interaction among DnaA monomers. We now have direct evidence that oligomer formation at *oriC* is required for initiation to occur. Furthermore, the results indicate that leucine 5, tryptophan 6 and cysteine 9 participate in oligomer formation regardless of whether *oriC* is at its normal chromosomal location, or carried in a plasmid. In contrast, glutamine 8 and proline 28 function in oligomer formation only on an *oriC* plasmid. Apparently, the DnaA-*oriC* complex assembled on a plasmid is similar but not identical structurally to that assembled at *oriC* in the bacterial chromosome.

Other studies implicate N-terminal residues 2 through 86 in DnaA oligomerization (Weigel, Schmidt et al. 1999). Two independent approaches were used to support this conclusion. One relied on streptavidin beads to which DnaA or N-terminal deletions were attached via biotinylation, followed by the measurement of the binding of wild type DnaA to the protein-coated beads. The second utilized the *E. coli* one hybrid system that relies on transcriptional repression of the lambda P_L promoter. The cited study involved substitution of the C-terminal dimerization domain of lambda CI repressor with resides 2 through 86 from the N-terminal region of DnaA protein. This region was shown to replace the dimerization domain of CI repressor so that the hybrid protein is active in transcriptional regulation. However, the reverse is not true. The

dimerization domain of CI repressor cannot replace this region of DnaA to support pSC101 replication (Seitz, Weigel et al. 2000). Apparently, this region of DnaA has other functions in addition to oligomerization.

It has been suggested that leucine 3, leucine 10 and leucine 17 near the N-terminus form a leucine zipper motif, and that this structure functions in DnaA oligomer formation (Schaper and Messer 1997; Mima, Makise et al. 2002). Codons for these residues were mutated by Mima et al. to encode serine, and the respective alleles were assayed in complementation of the temperature sensitive phenotype of a *dnaA508* strain. The plasmid-borne *L3S* and *L10S* alleles were as active as *dnaA*$^+$, suggesting that these amino acids do not form a leucine zipper motif. The difficulty in interpreting these results is that it is not clear if the mutant proteins are active by themselves, or if complementation requires the formation of mixed complexes of either L3S or L10S and DnaA508[1]. As noted below, we suspect that the defect of the *dnaA508* allele is not in oligomer formation. The *L17S* mutation was not active in complementation, and the mutant protein bound poorly to GST-DnaA complexed to glutathione agarose beads (Mima, Makise et al. 2002). Interestingly, this substitution resides at the end of the predicted α-helix (residues 3-16; (Sutton and Kaguni 1997)) that L5, W6 and C9 reside in. As the α-helix is formed by 3.6 amino acids per helical turn, W6 and C9 are on the same face of this structure. L10 is on the opposing face. These results support the model that amino acids L5, W6 and C9, which are absolutely required for oligomer formation, reside in an α-helical structure, with L17 positioned at the end and possibly on the same side as W6 and C9. In this model, these amino acids in this structure participate directly in oligomer formation.

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[1] Individual *dnaA* alleles encoding L3A and L10A amino acid substitutions are active in initiation on an *oriC* plasmid *in vivo*, indicating that leucine 3 and leucine 10 (30% as active as *dnaA*$^+$) are not critical for the activity of DnaA protein (M. Felczak and J.M. Kaguni, unpublished results).

That this region also functions in interaction with DnaB is based on results with a truncated form of DnaA lacking the N-terminal 62 residues. This mutant protein failed to retain DnaB in the prepriming complex whereas wild type DnaA protein was active (Sutton, Carr et al. 1998). In support, Seitz et al. concluded that amino acids 24-86 of DnaA are required for interaction with DnaB (Seitz, Weigel et al. 2000). Earlier, we had isolated a collection of nonfunctional missense mutations of *dnaA* that mapped within or near this N-terminal region . Included in this collection are the *C9Y* and *P28L* alleles studied in this report. In our *in vivo* method to measure oligomer formation as reflected by intergenic complementation, we show that cysteine 9 is required, and proline 28 participates only when oligomers assemble on an *oriC* plasmid.

The *dnaA508* allele encodes P28L and T80I substitutions, which confer a temperature sensitive phenotype. We had previously shown that *P28L* by itself was temperature sensitive for pSC101 replication and, for replication of an *oriC* plasmid *in vivo* (M. Felczak and J. M. Kaguni, unpublished results). The results described above show that P28L is conditionally required for oligomer formation on an *oriC* plasmid. Weigel et al. had examined the function of the N-terminal region derived from the *dnaA508* allele by substitution of the dimerization domain of CI repressor with residues 2 through 86 of DnaA508 (Weigel, Schmidt et al. 1999). The hybrid protein bearing these substitutions was almost as active as a similar construct containing the corresponding region from wild type DnaA protein in transcriptional repression. Although the individual contribution of the T80I substitution was not examined in the cited study, one interpretation is that the biochemical defect of the P28L substitution is not in the failure to oligomerize.

T435K and suppression of dnaX(Ts). The d*naX* gene encodes the tau and gamma subunits of DNA polymerase III holoenzyme . These subunits function to load the beta clamp, also known as the DnaX complex, of the DNA polymerase, conferring processive

DNA synthesis. The *T435K* mutation was isolated originally as a cold-sensitive suppressor of the *dnaX2016* allele . Two models have been proposed to explain the suppression mechanism (Walker et al., manuscript in preparation). In the model of stabilization of tau and/or gamma subunit function, T435K is proposed to interact and sustain tau and/or gamma activity so that bacterial growth can occur. In the second model, T435K is reduced in its activity in initiation of DNA replication from *oriC*, resulting in less frequent initiations. The partially active tau and/or gamma can now keep up at a level sufficient to provide viability. Under either model, T435K is active in supporting initiation from chromosomal *oriC*. The results described in our study are consistent with in vitro results showing that T435K is defective in binding to the DnaA box motif, and can augment the activity of a limiting level of wild type DnaA protein via the formation of mixed complexes.

Model of DnaA at oriC. Our results presented here support a model that a monomer of DnaA46 bound to *oriC*, but impaired in ATP binding activity, interacts with an N-terminal domain of a T435K monomer to localize the latter to *oriC*. Whereas each mutant protein alone is inert, the mixed oligomer is active in sustaining DNA replication from *oriC*. Under this model, a separation of labor among monomers occurs in which the function of DNA binding is separated from ATP binding. However, the form of DnaA that is functional in initiation is likely to be more complex than a dimer of DnaA. Based on the glutaraldehyde crosslinking results which suggest that DnaA oligomerizes to form a tetramer (Figure 1), we speculate that each dimer at each DnaA box then interacts with another bound dimer to assemble a DnaA tetramer. Other results support the notion of a DnaA tetramer. In the one-hybrid system, cooperative binding of a pair of CI repressor dimers at two of the three CI operator sequences assembles a tetramer of CI that is required for transcriptional repression of the lambda P_L promoter . The ability of residues 2 through 86 of DnaA to substitute for the dimerization domain of CI repressor in

transcriptional repression is consistent with a DnaA tetramer as the active form in initiation at *oriC* (Weigel, Schmidt et al. 1999).

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Chapter III

The *dnaAcos* Allele of *E. coli*: Hyperactive Initiation is due to Substitution of A184V and Y271H, resulting in Defective ATP Binding and Aberrant DNA Replication Control

Abstract

Chromosomal DNA replication is regulated at the level of commitment to this biochemical pathway. In *E. coli*, DnaA protein appears to regulate this process. A mutant form, DnaAcos carrying four amino acid substitutions, is apparently defective in responding to regulatory signals because it induces hyperactive initiation from the bacterial replication origin (*oriC*). In this report, the phenotype of hyperactive initiation is shown to be the result of two specific amino acid substitutions. One (A184V) immediately adjacent to a Walker A box (P loop motif) results in a defect in ATP binding (Carr and Kaguni, *Mol. Microbiol.* 20:1307-1318, 1996). The second amino acid substitution (Y271H) appears to stabilize the activity of the mutant protein carrying the A184V substitution. The mutant protein carrying both amino acid substitutions (A184V + Y271H) is defective in modulating the frequency of initiation from *oriC*, as demonstrated by marker frequency analysis of *oriC* and a locus near the replication terminus. These results indicate that a defect in ATP binding results in aberrant control of DNA replication.

Introduction

Duplication of the genome in free-living organisms occurs once per cell cycle, and is a highly regulated event. In *E. coli*, this event occurs at a specific time in the bacterial cell cycle at a particular ratio of chromosomal origins to cell mass ((Donachie 1965) reviewed in ref. (Herrick, Kohiyama et al. 1996)). As evidence indicates that bacterial DNA replication is regulated at the level of initiation of DNA synthesis, the proposed models address the regulation of this process either at the stage prior to its onset, or after semi-conservative synthesis in order to block unscheduled rounds of DNA replication (Hansen, Christensen et al. 1991; Kitagawa Ozaki et al. 1998; Katayama 2001). In a model addressing the latter process, the availability of the chromosomal origin sequence (*oriC*) to the replication machinery is controlled by sequestration (Campbell and Kleckner 1990; Lu, Campbell et al. 1994). *E. coli* chromosomal DNA is normally methylated at GATC sites by DNA adenine methylase. As SeqA binds preferentially to hemi-methylated DNA (Brendler, Abeles et al. 1995; Slater, Wold et al. 1995), the product of one round of DNA replication, the chromosomal origin is occluded for a period of time to prevent another round of DNA replication until proper partitioning of daughter chromosomes has occurred.

Because DnaA protein initiates DNA replication from the bacterial replication origin, other models suggest that its activity is regulated so that DNA replication occurs at the proper time (Sekimizu, Bramhill et al. 1987; Katayama, Fujimitsu et al. 2001). Upon binding to specific sets of sequences termed DnaA boxes within *oriC*, the ATP-bound form of DnaA protein then opens the parental duplex DNA to form an intermediate required in the initiation process (Bramhill and Kornberg 1988). Because DnaA complexed to ADP, and mutants that bind poorly to ATP are defective in initiation from *oriC*, models have centered on the ATP binding activity of DnaA (Sekimizu, Bramhill et al. 1987; Carr and Kaguni 1996; Katayama, Fujimitsu et al. 2001). DnaA is a

weak ATPase. When bound to an adenine nucleotide, the cofactor is released on interaction with acidic phospholipids (Castuma, Crooke et al. 1993). Based on these observations, it has been suggested that the interaction of DnaA with the cytoplasmic membrane provides a mechanism whereby DnaA in a complex with ADP can be recycled to bind ATP, initiating another round of DNA replication (Sekimizu, Bramhill et al. 1987; Crooke 2001). A related model is based on the observation that ATP bound to DnaA is hydrolyzed on addition of the beta subunit of DNA polymerase III holoenzyme and a newly identified protein named Hda (Kato and Katayama 2001). Specifically, the interaction of DnaA at *oriC* with beta as a component of the replication fork machinery and Hda stimulates the hydrolysis of ATP bound to DnaA. This event marks the transition between the stages of initiation and elongation of DNA replication. Because the DnaA-ADP complex is less active in initiation, this mechanism provides a means to prevent initiation of DNA replication at an unscheduled time.

Another model is based on the availability of DnaA for initiation (Hansen, Christensen et al. 1991). Elevated *dnaA* expression results in an increased frequency of initiation from *oriC* (Atlung, Lobner Olesen et al. 1987; Lobner-Olesen, Skarstad et al. 1989; Nyborg, Atlung et al. 2000). This observation suggests that the level of DnaA protein is normally limiting. In its support, the *datA* locus appears to be a site that titrates excess DnaA protein. Deletion of either this site, or essential DnaA boxes within it results in unscheduled initiations from *oriC* (Kitagawa, Ozaki et al. 1998; Ogawa, Yamada et al. 2002).

If DnaA protein acts to regulate the frequency of initiation from *oriC*, a prediction is that *dnaA* mutations defective in this regulated pathway should be obtained. Indeed, the *dnaAcos* allele appears to fail to respond to a regulatory signal(s), resulting in hyperactive initiation (Braun, O'Day et al. 1987). This intragenic suppressor was isolated by its ability to restore growth to a *dnaA46* strain at the nonpermissive temperature (42

°C). At 30 °C *dnaAcos* is cold-sensitive, a phenotype linked to overinitiation from *oriC*. Biochemical studies of DnaAcos suggest that it has elevated DNA replication activity in a replication system composed of an *oriC*-containing plasmid as a template and a crude enzyme fraction as the source of other required replication proteins (Katayama and Kornberg 1994).

Structure-function studies correlate functional domains of DnaA protein to its primary structure (Figure 1). To understand the altered biochemical properties of DnaAcos, we sought to identify the mutation that gives rise to its hyperactive initiation activity. The *dnaAcos* allele encodes four amino acid substitutions (Q156L, A184V, H252Y, Y271H) (Braun, O'Day et al. 1987). Two substitutions are encoded by the *dnaA46* allele (A184V, H252Y) (Johanson, Haynes et al. 1986); *dnaAcos* was isolated as an intragenic suppressor of *dnaA46*(Ts) (Braun, O'Day et al. 1987). In this study, expression of plasmid-borne *dnaAcos* was placed under control of the native *dnaA* promoters. When the *dnaAcos* plasmid was introduced into a *dnaA*$^+$ host strain, we observed that *dnaAcos* is dominant-negative. This phenotype correlates with hyperactive initiation as demonstrated by a quantitative PCR assay used to measure the relative ratio of loci at *oriC* or near *terC*. Relying on the dominant-negative phenotype of *dnaAcos* as a genetic assay, each possible combination of the *dnaAcos* mutations was then tested. The results show that the combination of *A184V* and *Y271H* causes the *dnaAcos* phenotype and hyperactive initiation. We conclude that *A184V* alone results in overinitiation due to a defect in ATP binding. The Y271H substitution appears to confer stability at elevated temperature to the mutant protein carrying A184V.

Table 1. List of Strains

Strain	Genotype	Parent	Source
LK211	*asnA*$^+$, *asnB32*, *thi-1*, *relA*1, *spoT1*, λ^-, Δ*oriC*::pKN1562 (clockwise), Km^r	ER	Koppes and Nordstrom, 1986
LK348	*asn*A$^+$, *asnB32*, *thi-1*, *relA1*, *spoT1*, λ^- Δ*oriC*::pKN1562 (counterclockwise), Km^r	ER	Koppes and Nordstrom, 1986
AQ9433	*trpA9605*, *his-29*, *proB* (or *proC*), *ilv*, *metB1*, *thyA*, (*deoB* or *deoC*), *rnh*::*cat*, *dnaA850*::Tn*10*	AQ3519	T. Kogoma
W3110	F$^-$, λ^-, IN (*rrnD-rrnE*)1, *rph-1*		Lab Stock
LS211	*asnA*$^+$, *asnB32*, *thi-1*, *relA1*, *spoT1*, λ^- , Δ*oriC*::pKN1562 (clockwise), Km^r *dnaA850*::Tn*10*	LK211	This Work
LS110	F$^-$, λ^-, IN(*rrnD-rrnE*)1, *rph-1*, Δ*oriC*:: pKN1562 (clockwise), Km^r	W3110	This Work
LS778	F$^-$, λ^-, IN(*rrnD-rrnE*)1, *rph-1*,*tna*::Tn*10*, *dnaA*46	W3110	This Work
LS1073	*araD*139, Δ*araC*, *leu*7697, Δ(*lac*)X174, *galU*, *galK*, *hsdR*2(r_k^-,m_k^+), *strA*, *mcrA*, *mcrB*1, *tna*::Tn*10*, *dnaA*46	MC1061	This Work
LS1061	*araD*139, Δ*araC*, *leu*7697, Δ(*lac*)X174,*galU*, *galK*, *hsdR*2(r_k^-,m_k^+), *strA*, *mcrA*, *mcrB*1, Δ*oriC*::pKN1562(clockwise) Km^r	MC1061	This Work
LS1062	*araD*139, Δ*araC*, *leu*7697, Δ(*lac*)X174, *galU*, *galK*, *hsdR*2(r_k^-,m_k^+), *strA*, *mcrA*, *mcrB*1, Δ*oriC*::pKN1562(clockwise) Km^r, *dnaA*850::Tn*10*	LS1061	This Work
MC1061	*araD*139, Δ*araC*, *leu*7697, Δ(*lac*)X174, *galU*, *galK*, *hsdR*2($r_k^-$$m_k^+$), *strA*, *mcrA*, *mcrB*1		Lab Stock

Table 2. List of Plasmids

Plasmids	Characteristics	Vector or Parent Plasmid	Source
pRB100	Ap^r, *dnaA*$^+$, *dnaA* promoters	pBR322	Braun *et al.*, 1985
pLS103	Ap^r, *H252Y*	pRB100	This work
pLS104	Ap^r, *Q156L*	pRB100	This work
pLS105	Ap^r, *A184V*	pRB100	This work
pLS106	Ap^r, *Y271H*	pRB100	This work
pLS107	Ap^r, *Q156L+A184V*	pRB100	This work
pLS108	Ap^r, *Q156L+H252Y*	pRB100	This work
pLS109	Ap^r, *Q156L+Y271H*	pRB100	This work
pLS110	Ap^r, *A184V+H252Y*	pRB100	This work
pLS111	Ap^r, *A184V+Y271H*	pRB100	This work
pLS112	Ap^r, *H252Y+Y271H*	pRB100	This work
pLS113	Ap^r, *Q156L+A184V+H252Y*	pRB100	This work
pLS114	Ap^r, *A184V+H252Y+Y271H*	pRB100	This work
pLS115	Ap^r, *Q156L+A184V+Y271H*	pRB100	This work
pLS116	Ap^r, *Q156L+H252Y+Y271H*	pRB100	This work
pLS*dnaAcos*	Ap^r, *dnaAcos*	pRB100	This work
pLS118	Ap^r, *Y271H*, p*araBAD* [a]	pING1	This work
pLS119	Ap^r, *A184V+Y271H*, p*araBAD*	pING1	This work
pLS120	Ap^r, *dnaAcos*, p*araBAD*	pING1	This work
pLS121	Ap^r, *A184V*, p*araBAD*	pING1	This work
pLS122	Ap^r, *Q156L+A184V*, p*araBAD*	pING1	This work
pDS596	Ap^r, *dnaA*$^+$, p*araBAD*	pING1	Hwang and Kaguni, 1988
pBR322	Ap^r, Tc^r		Lab Stock
pRB37	*placUV5*'[b], *dnaAcos*	pRB25	Braun *et al.*, 1987[b]
pRB25	*placUV5*', *dnaA*$^+$	pBR322	Braun *et al.*, 1987
pKC596	Ap^r, T7^c, *dnaA*$^+$	pET 11a	Carr and Kaguni, 1996[c]
pKC215	Ap^r, T7, *dnaA*46	pET 11a	Carr and Kaguni, 1996
pKC-A184V	Ap^r, T7, *A184V*	pET 11a	Carr and Kaguni, 1996
pKC-H252Y	Ap^r, T7, *H252Y*	pET 11a	Carr and Kaguni,

pCM959-Cm^r	*oriC*, Cm^r	1996 unpublished results[d]

[a] p*araBAD* represents the *araBAD* promoter under which the respective *dnaA* alleles is regulated by AraC repressor. These plasmids were constructed by replacing the EcoNI-XhoI restriction fragment of wild type sequence contained in pDS596 with the corresponding DNA fragment (gel-purified) from the pRB100 derivative carrying the respective mutation.

[b] p*lacUV5*' denotes the *lacUV5* promoter which modulates the expression of the respective *dnaA* alleles.

[c] In pKC596 and related derivatives, *dnaA* expression is from a bacteriophage T7 RNA polymerase promoter.

[d]A DNA fragment carrying the gene encoding chloramphenicol acetyltransferase derived from pBR325 (nucleotides 3911 to 5487 of pBR325) was PCR amplified and used to replace an 837 base pair *Pvu*II DNA fragment (nucleotides 3069 to 3905) of pCM959 (Buhk and Messer 1983). The PCR primer sequences inserted in construction of pCM959-Cm^r introduced *Bam*H1 sites at the junctions between pCM959 and the DNA fragment carrying the chloramphenicol resistance gene (gGATCCGTC and gGACGGATC, respectively, in which the upper case letters represent primer sequences). The structure of the junctions was confirmed by DNA sequence analysis (K. Hupert-Kocurek and J.M. Kaguni, unpublished results).

Experimental Procedures

Bacteriological methods-Bacteriological media and methods including P1 transduction to construct the strains of Table 1 are as described (Mann, Carrington et al. 1992). Purified DNAs were quantitated spectrophotometrically and electrophoresed on agarose gels to verify that greater than 95% of the DNA was supercoiled. Transformation of the indicated strains was by electroporation (BioRad *E. coli* Pulser) with 10 ng of plasmid DNA. Various dilutions of the electroporated sample were plated on media containing the appropriate antibiotic and transformation frequencies were assessed after overnight incubation at the indicated temperature. *E. coli* LS1062 grows slowly, requiring 24-30 hr incubation at 30 °C.

Separation of dnaAcos *mutations*-The *dnaAcos* mutations were separated by subcloning and by PCR mutagenesis with overlapping primers containing the desired nucleotide changes. Briefly, *A184V*, *H252Y* and *dnaA46* were subcloned from pKC215 (*dnaA46*), pKC-*A184V* and pKC-*H252Y* (Carr and Kaguni 1996) by *Eco*R1 and *Ava*I cleavage to yield a 1.5 kb fragment containing the respective *dnaA* mutation(s). These DNA fragments were used to replace the corresponding DNA fragment, but of wild type sequence, carried in the *dnaA*$^+$ plasmid pRB100 (Braun, O'Day et al. 1985) to yield the plasmid derivatives in Table 2. *dnaAcos* was subcloned from pRB37 (Braun, O'Day et al. 1987) as described above in a 1.5 kb *Eco*R1-*Ava*I restriction fragment. In pRB37, *dnaAcos* is under *lacUV5* promoter control. Substitution of the wild type sequence in pRB100 by the *dnaAcos*-containing fragment places the allele under control of the natural *dnaA* promoters. At this point, the presence of the *A184V* mutation in *dnaAcos*, *dnaA46*, and the single *A184V* mutation was confirmed by resistance to *Sph*I cleavage; the mutation that gives rise to the A184V substitution destroys an *Sph*I site. The remaining mutations of *dnaAcos* were introduced into pRB100 using the following overlappping primers carrying the respective nucleotide substitutions: Q156L,

CG

AC

und

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and

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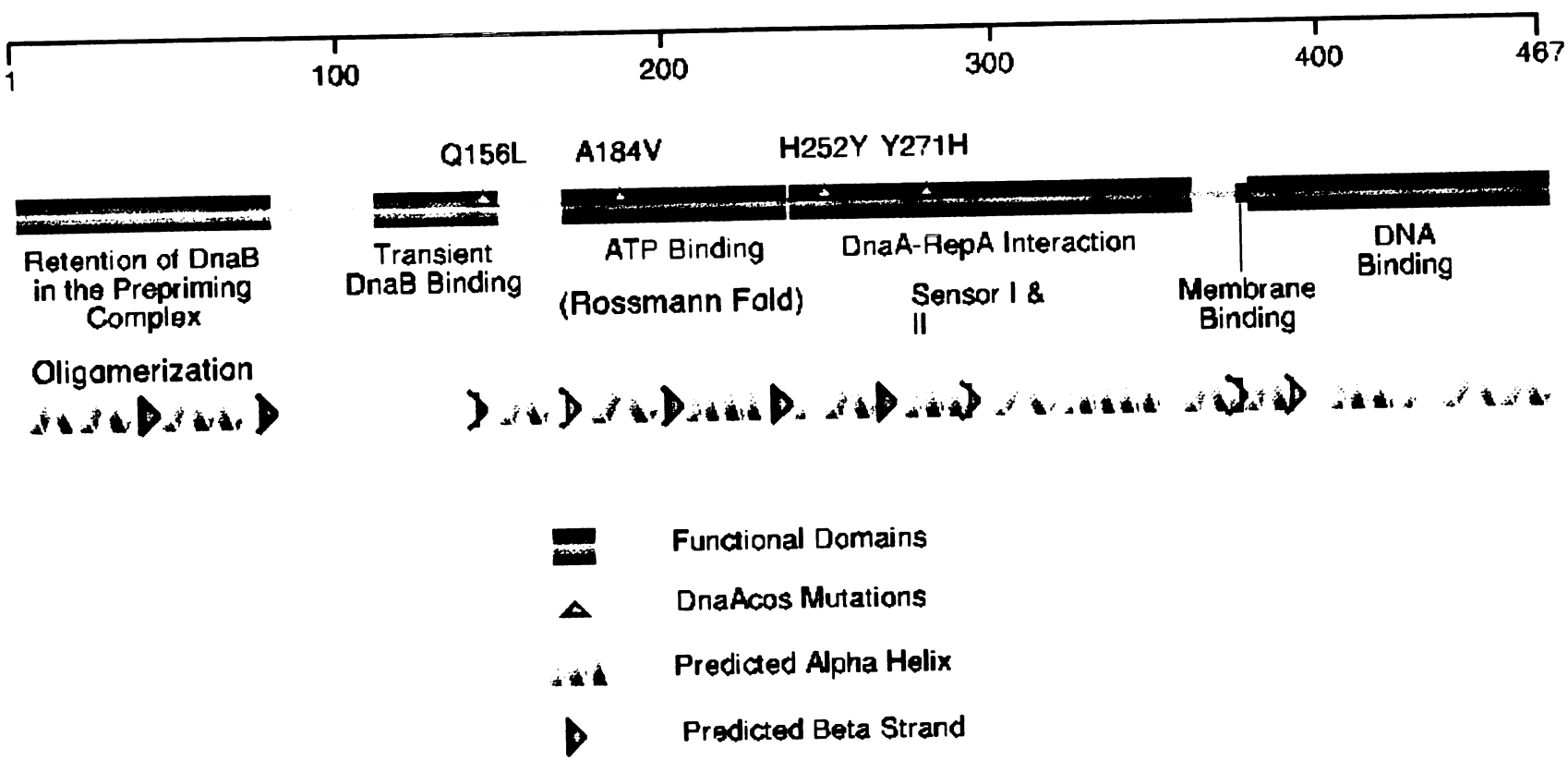
ACA

CGGCGGCTCGCCTGGTGGCGGATAA and the complementary sequence; Y271H, ACC TCGGATCGCCATCCGAAAGAG and the complementary sequence. The underlined nucleotide introduces the corresponding amino acid substitution. To construct the double mutant encoding Q156L and Y271H, the mutagenesis was done sequentially. To construct pLS113 encoding substitutions of Q156L + A184V + H252Y, pLS110 (*A184V + H252Y*) was used as the template with overlapping primers to introduce the mutation for Q156L. Alternatively, pLS110 served as the template for overlap extension PCR to insert the *Y271H* mutation, forming pLS114. pLS115 and pLS116 were constructed from pLS104 (*A184V*) and pLS103 (*H252Y*), respectively, by sequential PCR mutagenesis. DNA sequence analysis of the *dnaA* coding region including flanking sequences was performed to confirm that each plasmid of Table 2 contained the correct respective mutations.

Quantitative PCR assay-Cultures were grown at 30 °C to an optical density at 595 nm of 0.2, then arabinose was added to 1% (w/v) final concentration to induce expression of the indicated *dnaA* alleles. After continued incubation as indicated, the cells were harvested by centrifugation, frozen in liquid nitrogen, then stored at –70 °C. The isolated bacterial chromosomal DNA (DNeasy, Qiagen) was quantitated spectrophotometrically, and unless indicated 1 ng was used as the template in PCR reactions with respective primers to amplify *oriC* or *relE*. PCR reactions were assembled according to the manufacturer's recommendations (PCR Master mix, Promega), but contained [α^{32}P]-dATP (1.25 μCi per reaction, 3000 Ci/mmol, Perkin-Elmer), and amplification was for 23 cycles with Taq DNA polymerase (1.25 units). To measure the amount of PCR-amplified DNA, the radiolabelled product was acid precipitated with 10% (w/v) trichloroacetic acid onto glass fiber filters (GF/C, Whatman), and radioactivity was quantitated by liquid scintillation spectrometry. Primers to amplify *oriC* are ACAGCGTACAATACGCCACT and ACGGTGAGCACGACGGCTTT. To amplify

the *relE* region which is linked to *terC*, the primers are GTTGAAGTACTTGAGTCACC and CATTCAGACTTGAATGCGTG. These primers are identical to those used by Nyborg *et al.* (Nyborg, Atlung et al. 2000) and amplify *relE*, *relF*, and *rem*.

Figure 1. Functional domains of DnaA protein relative to mutations of *dnaAcos*. A domain near the N-terminus is involved in oligomerization of DnaA (Sutton and Kaguni 1997; Jakimowicz, Majka et al. 1998) and in retention of DnaB in the *oriC* prepriming complex (Sutton, Carr et al. 1998; Seitz, Weigel et al. 2000). A second domain is involved in interaction with DnaB (Sutton, Carr et al. 1998; Seitz, Weigel et al. 2000). A Rossmann fold constitutes the ATP binding domain (Sutton and Kaguni 1997) followed by a region carrying the Sensor I and II motifs of AAA^+ family proteins (Koonin 1992; Neuwald, Aravind 1999), and the membrane binding domain (residues 372-381; (Garner and Crooke 1996). The DNA binding domain is near the C-terminus (Roth and Messer 1995; Sutton and Kaguni 1997). Symbols within the functional domains denote amino acid substitutions of DnaAcos (Q156L, A184V, H252Y, and Y271H). The scale at the top indicates length in amino acid residues. The end points for respective domains and the secondary structure prediction by the PHD method have been described (Sutton and Kaguni 1997; Sutton, Carr et al. 1998).


1
100
200
300
400
467
Q156L
A184V
H252Y Y271H
Retention of DnaB in the Prepriming Complex
Transient DnaB Binding
ATP Binding
(Rossmann Fold)
DnaA-RepA Interaction
Sensor I & II
Membrane Binding
DNA Binding
Oligomerization
Functional Domains
DnaAcos Mutations
Predicted Alpha Helix
Predicted Beta Strand

Results

dnaAcos *is dominant-negative to* dnaA$^+$-The *dnaAcos* allele that confers hyperactive initiation of DNA replication was originally cloned under *lacUV5* promoter control in a pBR322 derivative as attempts to isolate this allele linked to its native *dnaA* promoters (plasmid-borne) failed (Braun, O'Day et al. 1987). When expression is from the native *dnaA* promoters, DnaAcos apparently interferes with growth but the wild type protein does not. Likewise, when *dnaAcos* is under control of the *lacUV5* promoter, its induced expression (but not *dnaA*$^+$) also interferes with viability. In contrast, an *oriC* null strain, in which constitutive stable DNA replication is activated by disruption of *rnh* (Rnase H), did not show growth interference when *dnaAcos* expression was induced by IPTG (Katayama and Kornberg 1994). Disruption of the *oriC* locus was required, presumably because of hyperactive initiation from *oriC*. Because of our interest in identifying the mutation(s) responsible for hyperactive initiation activity, we chose to propagate *dnaAcos* and combinations of mutations of this allele in an *oriC* null strain (LS1061, Table 1). In this host strain, DNA replication is independent of DnaA protein, minimizing the possibility of selecting for mutations in the host genome or in the plasmid-borne *dnaA* gene that alleviate the lethal phenotype of *dnaAcos*.

As *dnaAcos* carries multiple mutations, all combinations of them were constructed in a plasmid vector, and their presence verified by DNA sequence analysis (Table 2). These plasmids were then used to transform a wild type strain to assay for dominance over *dnaA*$^+$ (Table 3). The control plasmid (pRB100) harboring *dnaA*$^+$ (Braun, O'Day et al. 1985), or pBR322 (data not shown) transformed the *dnaA*$^+$ strain with approximately equal efficiency at both 30 °C and 42 °C, and did not show growth interference. In contrast, the *dnaAcos*-bearing plasmid (pLS*dnaAcos*, Table 2) transformed the *dnaA*$^+$ strain at almost 10^3-fold lower frequency at both temperatures, indicating that *dnaAcos* is lethal in a wild type strain when expressed from the *dnaA*

promoters. The dominant-negative effect of *dnaAcos* over chromosomally encoded *dnaA*$^+$ when expression of DnaAcos is at elevated levels confirms previous observations (Braun, O'Day et al. 1987; Katayama and Kornberg 1994).

A184V and Y271H are required for the overinitiation phenotype of dnaAcos-This dominant-negative phenotype provided a genetic assay to measure the activity of different combinations of mutations encoded by the *dnaAcos* allele. Except for the *A184V* mutation assayed at 30 °C, none of the single mutations conferred growth interference at either temperature (Table 3). The dominant-negative phenotype of the *A184V* mutation at 30 °C that correlates with overinitiation activity confirms previous observations (Nyborg, Atlung et al. 2000). Importantly, this phenotype was not observed at 42 °C to distinguish the behavior of the *A184V* mutation from that of *dnaAcos*, suggesting that other mutations contribute to the phenotype of *dnaAcos*. Analysis of the double mutants showed that *H252Y* combined with *A184V* no longer interfered with growth of the *dnaA*$^+$ host strain (compare lines 5 and 10 of Table 3 under the data for incubation at 30°C). This phenotype was also observed in a *dnaA46* strain at 30 °C (data not shown). In contrast, Nyborg *et al.* observed that a pBR322-derived plasmid carrying these two mutations conferred cold-sensitivity when introduced into either a *dnaA*$^+$ or *dnaA46* strain (Nyborg, Atlung et al. 2000). The reason for these differences is unknown. Of the double mutants, the *A184V + Y271H* mutant had the same dominant-negative phenotype at both temperatures as *dnaAcos*. Analysis of the triple mutants supports the conclusion that the combination of *A184V* and *Y271H* is responsible as only those triple mutants carrying the *A184V* and *Y271H* mutations interfered with growth at both temperatures. Growth interference at 30 °C was also observed with the *A184V + Y271H*

Table 3. Transformation of W3110 (*dnaA*$^+$, *oriC* $^+$) with plasmids carrying various *dnaA* mutations.

Plasmid	*dnaA* Allele	CFUs[a] at 30°C	CFUs at 42°C
pRB100	*dnaA*$^+$	3.3×10^7	3.2×10^7
pLS*dnaAcos*	*dnaAcos*	4.4×10^4 [b]	6.6×10^4 [b]
pLS103	*H252Y*	2.1×10^7	2.4×10^7
pLS104	*Q156L*	3.3×10^7	3.2×10^7
pLS105	*A184V*	2.8×10^4	2.3×10^7
pLS106	*Y271H*	1.8×10^7	2.2×10^7
pLS107	*Q156L+A184V*	3.0×10^4	3.0×10^7
pLS108	*Q156L+H252Y*	2.6×10^7	2.7×10^7
pLS109	*Q156L+Y271H*	2.4×10^7	2.6×10^7
pLS110	*A184V+H252Y*	2.3×10^7	1.9×10^7
pLS111	*A184V+Y271H*	4.0×10^4	4.4×10^4
pLS112	*H252Y+Y271H*	2.5×10^7	2.8×10^7
pLS113	*Q156L+A184V+H252Y*	1.6×10^5	2.4×10^7
pLS114	*A184V+H252Y+Y271H*	3.2×10^4 [b]	8.8×10^4 [b]
pLS115	*Q156L+A184V+Y271H*	2.4×10^4 [b]	6.8×10^4 [b]
pLS116	*Q156L+H252Y+Y271H*	3.3×10^7	3.2×10^7

[a] Colony forming units (CFUs) are normalized and expressed as the number of transformants observed per μg of plasmid DNA at the indicated temperature.

[b] Plates of these transformants contained two colony morphologies. Small colonies were assumed to have arisen by spontaneously obtained suppressors and were excluded in the calculation of transformation efficiencies.

double mutant and those triple mutants carrying these mutations in a host strain carrying the *dnaA46* allele (data not shown).

The dominant-negative phenotype requires oriC-We constructed a strain (LS110, Table 1) isogenic to the *oriC*$^+$ strain (W3110), but null at the *oriC* locus and integratively suppressed with a mini-R1 replicon. Such integratively suppressed strains replicate independently of *dnaA* function (Koppes and Nordstrom 1986; Blinkova, Gines-Candelaria et al. 2000). The different combinations of mutations comprising the *dnaAcos* allele were used to transform this strain to determine if the dominant-negative phenotype was dependent on initiation from *oriC* (Table 4). The observation that none of the plasmid-borne alleles affected growth of this strain demonstrates that the dominant-negative phenotype of *dnaAcos* and the double mutant carrying *A184V* and *Y271H* requires the presence of *oriC*. Shown below, overinitiation from *oriC* appears to be the reason for the dominant negative effect.

Expression of dnaAcos *and the* A184V + Y271H *double mutant results in hyperactive initiation*-The phenotype of *dnaAcos* and the *A184V + Y271H* mutant when present in the *oriC*$^+$ host strain suggests that hyperactive initiation activity from *oriC* is the cause. To confirm this expectation, a quantitative PCR assay was used. The objective was to determine the relative ratios of the chromosomal origin (*oriC*) to a locus distant from this site. We chose the *relE* locus because of its proximity to the replication terminus, *terC*. Hyperactive initiation with *dnaAcos* or with *A184V + Y271H* mutant should result in an elevated ratio of *oriC* to *relE* compared to the *dnaA*$^+$ control. We restricted the analysis to only two loci because Nyborg *et al.* had shown that overinitiation was from *oriC* as indicated by the reduced abundance of markers with increasing distance from *oriC* (Nyborg, Atlung et al. 2000). In the following experiments, expression of the respective alleles was induced from a regulated promoter (*araB* promoter) whereas the

Table 4. Transformation of LS110 (*dnaA*$^+$, Δ*oriC*) with plasmids carrying various *dnaA* mutations.

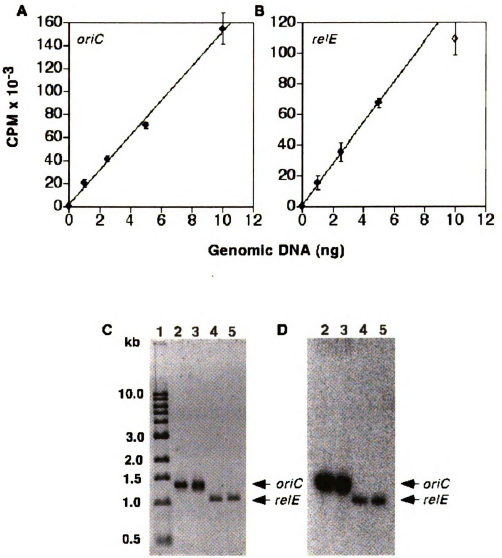
Plasmid	*dnaA* Allele	CFUs at 30°C	CFUs at 42°C
pRB100	*dnaA*$^+$	2.9×10^6	2.6×10^6
pLS*dnaAcos*	*dnaAcos*	2.0×10^6	2.4×10^6
pLS103	*H252Y*	1.1×10^6	1.1×10^6
pLS104	*Q156L*	1.2×10^6	1.4×10^6
pLS105	*A184V*	1.9×10^6	2.1×10^6
pLS106	*Y271H*	5.5×10^5	5.5×10^5
pLS107	*Q156L+A184V*	1.1×10^6	1.1×10^6
pLS108	*Q156L+H252Y*	1.4×10^6	1.4×10^6
pLS109	*Q156L+Y271H*	1.7×10^6	1.9×10^6
pLS110	*A184V+H252Y*	3.5×10^5	3.7×10^5
pLS111	*A184V+Y271H*	1.2×10^6	1.4×10^6
pLS112	*H252Y+Y271H*	1.0×10^6	1.0×10^6
pLS113	*Q156L+A184V+H252Y*	2.3×10^6	2.5×10^6
pLS114	*A184V+H252Y+Y271H*	1.0×10^6	1.0×10^6
pLS115	*Q156L+A184V+Y271H*	1.1×10^6	1.0×10^6
pLS116	*Q156L+H252Y+Y271H*	1.0×10^6	1.0×10^6

Colony forming units (CFUs) are expressed as the number of transformants observed per μg of plasmid DNA.

transformation experiments relied on elevated expression due to the copy number of the plasmid vector.

As one control, we demonstrated that the amount of amplified DNA is proportional to the amount of input template DNA (Figure 2). Both *oriC* and *relE* showed a linear relationship for amplification relative to the amount of chromosomal DNA template over at least a 6-fold range. In another control experiment, we found that with increasing time after induced expression of *dnaA*$^+$, the ratio of *oriC* to *relE* proportionately increased (data not shown). This observation also confirms previous observations that elevated *dnaA*$^+$ expression results in an increased frequency of initiation from *oriC* (Atlung and Hansen 1993). However, the replication forks do not continue to completion but stall at apparently random sites on the chromosome. As a third control, we determined that the ratio of *oriC* to *relE* in chromosomal DNA prepared from stationary phase cells was 1.3 (data not shown), consistent with other observations that most cells at this stage of growth contain a single genome. Finally, we showed that PCR amplification generated DNA fragments of the expected sizes (Figure 2). The inclusion of [α^{32}P]-dATP in the PCR reaction mixture resulted in a single radiolabelled DNA for each respective PCR reaction (*oriC*, or *relE)* as visualized by autoradiography after agarose gel electrophoresis. The amount of radioactivity measured by liquid scintillation spectrometry in the solubilized gel slice containing the DNA fragment (*oriC* or *relE*) was essentially identical to the amount of acid-insoluble radioactivity in an equivalent portion of the reaction after PCR amplification (data not shown). These results indicate that the PCR primers specifically amplify either *oriC* or *relE*, with no false priming. The results also show that the respective PCR products can be quantitated directly by the amount of acid-insoluble radioactivity.

Figure 2. PCR amplification of *oriC* and *relE* show that amplification is proportional to the amount of bacterial chromosomal DNA used as the template and is specific for these loci. Increasing amounts in duplicate of *E. coli* chromosomal DNA were PCR-amplified with primers specific for *oriC* (Panel A), or *relE* (Panel B). The levels of amplification that indicate a linear response over at least a 6-fold range were measured by liquid scintillation spectrometry of acid-insoluble radioactively-labeled DNA (see "Experimental Procedures"). Repeated experiments yielded similar results. In Panel B, the data at 10 ng was omitted in order for the curve derived by linear regression to intersect at zero. In Panels C and D, PCR amplification of *oriC* and *relE*, a locus near *terC*, was performed as described in "Experimental Procedures." Panel C is an ethidium bromide-stained agarose gel of electrophoretically separated DNAs amplified by PCR. Lane 1 contains DNA molecular weight markers with the sizes of relevant DNAs indicated in kb. Lanes 2 and 3 contain duplicate samples in which *oriC* was PCR-amplified, yielding the expected 1.3 kb fragment. Lanes 4 and 5 are duplicate samples of the PCR-amplified *relE* locus, resulting in the expected fragment of 1 kb. Panel D is the autoradiogram of the gel shown in Panel C. The only radioactive DNAs detected correspond to the PCR products visualized by ethidium bromide staining in Panel C.


A
B
oriC
relE
CPM x 10-3
Genomic DNA (ng)
C
D
kb
10.0
3.0
2.0
1.5
1.0
0.5
oriC
relE

Having validated the conditions for the quantitative PCR assay, cultures of the respective plasmid-bearing strains were grown to early log phase, and expression was induced for the times indicated in order to assess hyperactive initiation. A portion of each culture was analyzed by immunoblotting to confirm that expression of mutant or wild type DnaA protein was dependent on induction, and that the expression levels were roughly comparable (data not shown; see Table 5 legend). Bacterial DNA for quantitative PCR analysis was isolated from cultures grown at 30 °C or 42 °C (Table 5). Compared to the *oriC*-to-*relE* ratio after induced *dnaA*$^+$ expression, the *Y271H* allele was comparable to *dnaA*$^+$ in stimulating initiation at either temperature. The ratio of *oriC* to *relE* with *dnaAcos* was significantly higher by comparison to correlate its dominant-negative phenotype to hyperactive initiation. Results with *A184V* + *Y271H* were similar to those ratios for *dnaAcos* at both temperatures. The *A184V* mutation is well documented to result in elevated initiation at 30 °C (Nyborg, Atlung et al. 2000). Quantitative PCR analysis showed that this mutation was as active as *dnaAcos* in stimulating initiation at 30°C, but not at 42°C. Because the *Q156L* + *A184V* allele displayed a dominant-negative phenotype at 30°C but not at 42°C (Table 3), this allele was also examined. Compared to *dnaA*$^+$, this pair of mutations resulted in an elevated *oriC*-to-*relE* ratio, but this pattern was not observed at 42°C. These observations correlate the dominant-negative phenotype of respective *dnaA* alleles (Table 3) to elevated initiation activity (Table 5). The most striking result from this set of experiments is that the *A184V* and *Y271H* mutations when present together appear to be responsible for the hyperactive initiation activity of *dnaAcos*.

In vivo *initiation activity as measured by* oriC *plasmid maintenance*-The individual mutations of *dnaAcos* as well as the different combinations were examined to determine their *in vivo* function in initiation. Because the mutations are plasmid-borne, a

Table 5. Marker frequency analysis of *oriC* and *relE* by quantitative PCR.

Plasmid	allele	*oriC*/*relE* 30°C	*oriC*/*relE* 42°C
pDS596	*dnaA*$^+$	3.7±0.1	9.2±2.5
pLS118	*Y271H*	3.3±0.6	8.5±1.7
pLS120	*dnaAcos*	10.1±1.1	14.0±2.3
pLS119	*A184V+Y271H*	8.7±0.4	15.6±3.6
pLS121	*A184V*	11.4±1.2	6.3±0.2
pLS122	*Q156L+A184V*	6.0±0.6	4.4±1.1

The indicated plasmids were used to transform MC1061, and grown at 30°C or 42°C to an optical density at 595nm of 0.15-0.2. Arabinose was then added (1% final concentration) to induce expression, and incubation was continued for a period equivalent to three generations (240-300 min 30°C and 90 min at 42°C). Bacterial chromosomal DNA was isolated as described in "Experimental Procedures" for use as a template in quantitative PCR reactions. Statistical analysis to calculate the range was in duplicate for the samples from the 30°C cultures, and for 5-7 identically prepared samples from the 42°C cultures with the exception of *A184V* and *Q156L + A184V*. For the latter samples, statistical analysis at both temperatures was on duplicate samples.
As a control, *E. coli* MC1061 harboring the respective plasmid (pDS596, DnaA$^+$; pLS118, Y271H; pLS121, DnaAcos; and pLS119, A184V + Y271H) was grown in LB media supplemented with 100 μg/ml ampicillin to an optical density of 0.3 at 30 °C, induced by the addition of arabinose (1% w/v final concentration), then incubated further for 30 min. Whole cell lysates prepared from 0.4 optical density units (595 nm) of culture were analyzed by immunoblotting (see "Experimental Procedures") to demonstrate comparable levels of expression. Purified DnaA protein was analyzed in parallel as a control. As another control, LS1062 (relevant genotype: Δ*oriC*::pKN1562, *dnaA850*::*Tn*10) harboring pDS592 (*dnaA*$^+$) was grown essentially as described above and whole cell lysates from uninduced and induced cultures were analyzed by immunoblotting. DnaA protein was detected only in the sample from the induced culture, confirming that the host strain does not encode DnaA protein.

Table 6. Function of *dnaAcos* mutations in *oriC* plasmid maintenance.

Plasmid	*dnaA* Allele	CFUs[a] 42°C/30°C
pRB100	*dnaA*$^+$	1.0
pBR322	None	ND[b]
pLS*dnaAcos*	*dnaAcos*	1.0
pLS103	*H252Y*	0.94
pLS104	*Q156L*	1.0
pLS105	*A184V*	4.2×10^{-2}
pLS106	*Y271H*	1.1
pLS107	*Q156L+A184V*	0.92
pLS108	*Q156L+H252Y*	0.95
pLS109	*Q156L+Y271H*	1.0
pLS110	*A184V+H252Y*	5.2×10^{-3}
pLS111	*A184V+Y271H*	0.97
pLS112	*H252Y+Y271H*	1.0
pLS113	*Q156L+A184V+H252Y*	1.5×10^{-3}
pLS114	*A184V+H252Y+Y271H*	0.92
pLS115	*Q156L+A184V+Y271H*	1.1
pLS116	*Q156L+H252Y+Y271H*	1.0

[a]The host strain LS1062 (Δ*oriC*::pKN1562, *dnaA*850::Tn*10*) carrying the respective plasmids encoding the indicated *dnaA* alleles or pBR322 as a negative control was transformed with an *oriC*-containing plasmid (pCM959-Cm^r; 100 ng per transformation reaction). The ratio of colony forming units (CFUs) obtained after transformation and subsequent incubation at 42°C or 30°C is shown at the right. The transformation frequencies at 30°C were about 10^5 per μg of pCM959-Cm^r DNA. Transformants were selected on LB media supplemented with 100 μg/ml ampicillin and 25 mg/ml chloramphenicol.

[b] ND indicates that colonies were not observed when LS1062 harboring pBR322 was transformed with the *oriC*-plasmid. This control demonstrates that LS1062 is *dnaA* null and unable to maintain pCM959-Cm^r at either temperature.

host strain (LS1062, relevant genotype: *dnaA810*::*Tn*10, Δ*oriC*::pKN1562) was used that lacked a functional *dnaA* gene so that the effect of the mutation could be measured without the complication of host-encoded *dnaA*. An integrated miniR1 plasmid that does not depend on *dnaA* function for DNA replication serves as an alternate replication origin for the bacterial chromosome (Koppes and Nordstrom 1986). The different plasmid-encoded *dnaA* mutations were then introduced into this genetic background, and the activity of these alleles in initiation at *oriC* was assessed by maintenance of an *oriC* plasmid (Table 6). As controls, the plasmid encoding the wild type *dnaA* gene maintained the *oriC* plasmid at both 30 °C and 42 °C whereas pBR322 was inert. The *dnaA46*(Ts) allele comprises the *A184V* + *H252Y* mutations; the temperature sensitive-phenotype conferred by the plasmid encoding this allele is another control for the assay. For the *dnaAcos* allele and its separated mutations, they were functional at both temperatures with the exception of *A184V*, which was temperature-sensitive. As described above, the single *A184V* substitution gives rise to overinitiation activity *in vivo* only at 30°C (Table 5; (Nyborg, Atlung et al. 2000)). Correspondingly, the mutant protein carrying this substitution is active at 30°C but not at 42°C when assayed in a replication system with a crude protein fraction as the source of other essential replication proteins (Carr and Kaguni 1996).

Examination of pairwise combinations of mutations revealed that *Q156L* or *Y271H* suppressed the temperature-sensitive phenotype of *A184V* when combined with it (Table 6). As shown in Table 5, *Y271H* combined with *A184V* resulted in hyperinitiation activity at 42°C. In contrast, *Q156L* + *A184V* was even less active than *A184V* in initiation at the higher temperature. The explanation for the anomalous behavior of *Q156L* + *A184V* (active in *oriC* plasmid maintenance at 42°C, but poorly active in initiation at this temperature) may be due to limitations with the transformation assay.

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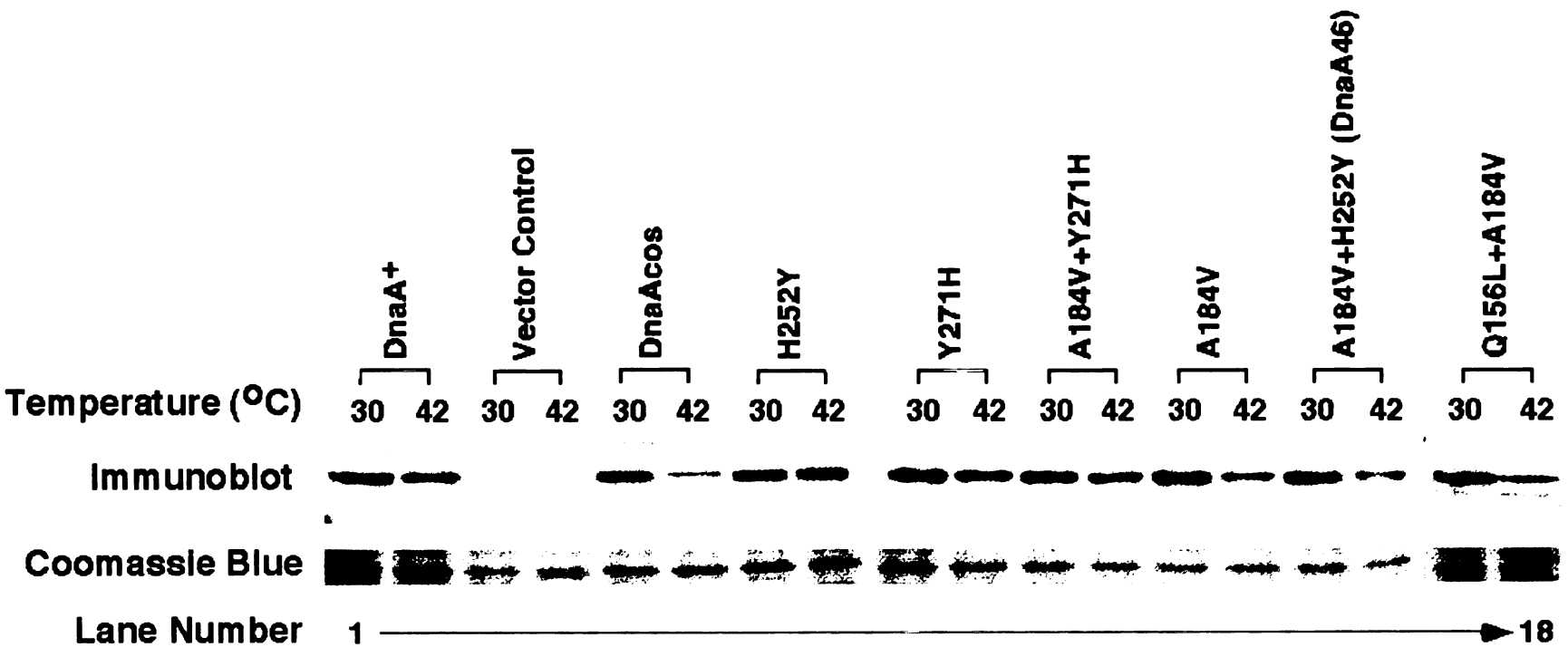
Figure 3. Relative stabilities of mutant DnaA proteins.
E. coli LS1062(Δ*oriC*::pKN1562, *dnaA810*::Tn*10*) was grown at 30 °C in LB media supplemented with 100 μg/ml ampicillin to an optical density (595 nm) of 0.1-0.15, then a portion of each culture was shifted to 42°C. After continued incubation for 2 hr, 1.5 ml of culture was removed. The resuspended cell pellets corresponding to 2.5 x 10^7 cells were lysed in Laemmli sample buffer by incubation at 86°C for 2 min, and whole cell lysates were electrophoresed in a 10% SDS-polyacrylamide gel. After transfer, the membrane (Protran, Schleicher & Schuell) was incubated with M43 monoclonal antibody (Marszalek, Zhang et al. 1996) as the primary antibody, and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (BioRad). Detection with X-ray film (Kodak X-Omat) was by chemiluminescence (SuperSignal, Pierce). Quantitation was with a Kodak EDAS 120 software, and normalized to the level of $DnaA^+$ protein at 30°C and 42°C.



Protein	Relative Stability 30°C	42°C	Protein	Relative Stability 30°C	42°C
DnaA	1.0	1.0	A184V	0.7	0.8
DnaAcos	0.7	0.4	A184V+Y271H	1.0	0.7
H252Y	0.9	1.1	A184V+H252Y (DnaA46)	0.9	0.4
Y271H	1.2	0.8	Q156L+A184V	1.0	0.4

Plasmids that are propagated by initiation from *E. coli oriC* are segregationally unstable, and show a propensity to become integrated into the chromosome upon antibiotic selection (unpublished observations; (Lobner-Olesen 1999)). In support, the *Q156L* mutation does not appear to suppress the temperature sensitivity of *A184V* when combined with *H252Y*.

The suppression of the temperature-sensitive phenotype of *A184V* for *oriC* plasmid maintenance by *Q156L* or *Y271H* suggests that either the physical form of A184V or its activity is stabilized at the elevated temperature. To discriminate between these two possibilities, immunoblot analysis was performed on the assorted *dnaA* alleles as they were expressed from the natural *dnaA* promoters. This method should be able to demonstrate if the steady-state level of A184V (a combination of its rates of synthesis and degradation) is lower at 42°C, and if Y271H counterbalances this instability when combined with A184V. The indicated plasmid-bearing strains were grown at 30°C to midlog phase, then a portion was shifted to 42°C. After two hours of continued growth at both temperatures, an equal number of cells from each culture was analyzed by Western blotting (Figure 3). The levels of the mutant proteins were quantitated and normalized relative to wild type DnaA. We did not detect DnaA protein with the strain carrying the vector , but lacking a cloned *dnaA* allele, confirming the null *dnaA* genotype of the host strain (LS1062 *dnaA850*::*Tn*10; data not shown). Compared to the level of DnaA$^+$ protein at either temperature, none of the mutant proteins was dramatically unstable. The relative ratio of DnaAcos (0.7 and 0.4 relative to DnaA$^+$ at 30°C and 42°C, respectively) confirms that its dominant-negative phenotype and elevated initiation activity (Tables 3, 5) is not due to oversupply but hyperactivity. These results also suggest that the inactivity of *A184V* (and *dnaA46*) in initiation at 42°C (Table 6) is not due to its physical instability because its steady-state level at 42°C is near that of wild type DnaA (0.8 relative to DnaA$^+$). Instead, its enzymatic activity appears to be labile. The relative physical

stability of the mutant protein carrying the A184V and Y271H substitutions compared to A184V alone suggests that the Y271H substitution acts by stabilizing an enzymatic function that leads to hyperactive initiation. Because of the resolution of denaturing polyacrylamide gel electrophoresis, we cannot exclude the remote possibility that a few amino acids have been proteolyzed from either A184V or DnaA46 at the higher temperature to result in inactivity.

As a control, the same set of samples was analyzed by SDS-polyacrylamide gel electrophoresis, followed by staining with Coomassie blue. An arbitrary portion of the gel is presented in Figure 3, showing that similar amounts of protein were loaded in each lane. The results from the control experiments indicate that, within experimental error, the differences in steady-state levels of mutant and wild type DnaA protein are as represented in Figure 3.

Discussion

DnaA is a member of the AAA$^+$ family of nucleotide binding proteins (Koonin 1992; Neuwald, Aravind et al. 1999). Other members of this group include the *E. coli* δ' and γ subunits of the DnaX clamp loader complex, the five subunits of the eukaryotic RFC clamp loader complex, Orc1, 4 and 5, Mcm 2-7 of the eukaryotic origin recognition complex, and Cdc6. Cdc6/Orc1 from *P. aerophilum* is structurally similar to the portion of *A. aeolicus* DnaA whose structure was recently solved (Erzberger, Pirruccello et al. 2002). The Sensor I and II motifs of AAA+ proteins are speculated to function in regulating enzyme activity by conferring the ability to distinguish whether ATP or ADP is bound. Because both DnaA and Cdc6/Orc1 bind ATP and also function to recruit the replicative helicase (DnaB and Mcm 2-7, respectively) to the replication origin (Marszalek and Kaguni 1994; Bell, 2002), the study of DnaA function may provide important insight into the general mechanism of initiation and its regulation. Due to the interesting phenotype of *dnaAcos* and the possibility that its study may be revealing, we sought to determine which mutations were responsible.

The *dnaAcos* allele was isolated as an intragenic suppressor of the *dnaA46*(Ts) allele (Braun, O'Day et al. 1987). Two mutations (*A184V* and *H252Y*) are encoded by *dnaA46* (Johanson, Haynes et al. 1986). The remaining two mutations in *dnaAcos* are *Q156L* and *Y271H* (Braun, O'Day et al. 1987). Each of these mutations was separated, and also combined in the different possible combinations to determine the substitution responsible for hyperactive replication activity.

Hyperactive initiation of the A184V + Y271H *mutant*-The genetic assay to measure hyperactive initiation relied on the dominant-negative effect of *dnaAcos* when its expression was from the *dnaA* promoters and in a *dnaA*$^+$ host strain. The assay demonstrated that *A184V* combined with *Y271H* accounts for the *dnaAcos* phenotype at both 30 °C and 42 °C. In contrast, *A184V* alone is dominant-negative to chromosomal

dnaA$^+$ only at 30 °C. Previous studies show that this single mutation results in increased initiations at *oriC* (Nyborg, Atlung et al. 2000).

We showed that the dominant-negative phenotype of the *A184V + Y271H* double mutant is dependent on the presence of *oriC*. Hyperactive initiation determined by a quantitative PCR assay correlates with the lethal effect observed with plating efficiencies. In these experiments, induced expression of either *dnaAcos* (as a positive control) or the *A184V + Y271H* mutation resulted in an increased initiation frequency as indicated by elevated ratios of *oriC* to *relE*.

The A184V substitution resides next to a P-loop motif (also known as a Walker A box; residues 172-179, GXXGXGKT; (Goud, Zahraoui et al. 1990; Gines-Candelaria, Blinkova et al. 1995)) that is absolutely conserved among nearly 100 *dnaA* orthologues. This sequence motif is found in proteins that bind nucleotide cofactors. For those proteins that have been studied at the structural level, amino acids of the motif interact with the β and γ phosphates of the bound nucleotide, an event that is presumably important in both nucleotide binding and hydrolysis (Reinstein, Brune et al. 1988; Reinstein, Schlichting et al. 1990; Rehrauer and Kowalczykowski 1993).

Genetic studies show that the mutation encoding the A184V substitution shared by classically obtained *dnaA* alleles (*dnaA5*, *dnaA46*, *dnaA601/602*, and *dnaA604/606*) (Hansen, Koefoed et al. 1992) is responsible for the temperature sensitive phenotype ((Carr and Kaguni 1996) Table 6). This behavior correlates with both the thermolabile replication activity of a mutant form of DnaA carrying the A184V substitution, and a defect in ATP binding activity. As expected, DnaAcos is also defective in ATP binding (Katayama and Crooke 1995). The defect in ATP binding of the protein carrying the A184V substitution correlates with overinitiation at 30 °C (Nyborg, Atlung et al. 2000). The failure to detect overinitiation at the elevated temperature is apparently due to

instability of its replication activity; the *Y271H* substitution stabilizes this activity at 42 °C, leading to overinitiation (Table 6).

How can a mutant protein defective in ATP binding result in overinitiation when current models invoke a requirement for ATP binding so that initiation can occur? Perhaps the asynchronous initiation phenotype of alleles encoding the A184V substitution provides a clue (Boye, Lobner-Olesen et al. 1988). These alleles give rise to odd numbers of chromosomes indicative of asynchronous initiation. In contrast, wild type strains display a pattern of 2^n chromosomes ($n \geq 0$ and an integer) under conditions where cell division and new rounds of initiation are inhibited, but ongoing rounds are allowed to proceed to completion. The failure to bind ATP apparently results in unscheduled initiations that occur additionally prior to and/or after the normal time of initiation during the bacterial cell cycle. Under steady state growth, these aberrant events (overinitiation) then lead to an abnormally elevated ratio of *oriC* to *ter* and lethality. The major conclusions from our study is that a DnaA mutant (A184V) with impaired ATP binding activity is active in initiation of chromosomal replication but displays aberrant replication control. The Y271H substitution stabilizes this activity at 42°C. Jointly, A184V and Y271H account for the overinitiation phenotype of *dnaAcos*.

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Chapter IV

Novel *dnaA* alleles are defective for regulation of initiation from the *E. coli* replication origin *oriC*

Abstract

Chromosomal replication in all organisms is a process that is carefully regulated to ensure that the genome is duplicated once and only once during the cell cycle. In *E. coli,* this process is regulated by DnaA protein. Many *dnaA* alleles have been examined that fail to initiate replication. The study of these alleles has allowed for the precise determination of the biochemical activities required for DnaA protein function. There are only two well characterized *dnaA* alleles that result in an increased frequency of origin firing. The first, DnaAcos, is unable to limit the frequency of initiation because of an ATP binding defect. The second, DnaA R334A, is defective in ATPase activity. Under normal growth conditions the intrinsic ATPase activity of DnaA protein negatively regulates initiation by ATP hydrolysis and formation of the inactive DnaA-ADP complex. The R334A mutant protein is inert to this type of regulation. To probe mechanisms of hyperactive initiation we sought to isolate novel *dnaA* alleles that are unresponsive to regulation. Many of the regulatory signals that trigger initiation have yet to be identified. In this study, we describe the isolation of *dnaA* alleles that have a similar phenotype to that of *dnaAcos*. We also determined that the lethality caused by these alleles appears to be due to increased initiation *in vivo*. The conservation of initiation protein function between DnaA protein (*E. coli*) and Cdc6 protein (*S. cerevisiae*) are discussed.

Introduction

DnaA protein is a sequence specific DNA-binding protein that occupies the origin of replication, *oriC* (Messer, Egan et al. 1991). There are several reported observations that confirm DnaA protein is the trigger that results in the initiation of DNA replication. When DnaA protein is overexpressed from a *lacUV5* promoter the frequency of initiation increases (Atlung, Lobner Olesen et al. 1987; Xu and Bremer 1988). A recently identified locus (*datA)* serves as a high affinity binding site that is occupied by a few hundred DnaA monomers (Kitagawa, Ozaki et al. 1998; Ogawa, Yamada et al. 2002). This site titrates the initiator protein lowering the pool of cellular DnaA protein available for origin binding. Further analysis demonstrated that deletion of the *datA* locus results in hyperactive initiation. Another important mechanism that regulates the frequency of initiation is the sequestration of the hemimethylated origin DNA to the cell membrane by SeqA protein (Slater, Wold et al. 1995). The action of SeqA protein prevents reinitiation for 8-10 minutes just after the onset of the first initiation event.

If DnaA is primarily responsible for modulating the frequency of initiation of DNA replication, then two expectations arise. First, mutant forms of DnaA protein should be isolated that result in a failure to begin the process of replication (Sutton and Kaguni 1995; Sutton and Kaguni 1997; Sutton and Kaguni 1997; Sutton and Kaguni 1997). Second, mutant proteins that exhibit an increase in the frequency of initiation should also be isolated (Kellenberger-Gujer, Podhajska et al. 1978). Indeed, both of these expectations are fulfilled. Extensive mutagenic analysis of the *dnaA* gene has revealed several biochemical functions that are required for replication from *oriC* (Sutton and Kaguni 1997; Sutton and Kaguni 1997). If any of these functions are crippled, the cell fails to initiate chromosomal replication. Likewise, a mutant form of DnaA protein (DnaAcos) has been isolated, and an increase in the frequency of initiation observed as measured by the abundance of *oriC* DNA compared to a control locus near the site of

replication termination (Kellenberger-Gujer, Podhajska et al. 1978; Katayama, Akimitsu et al. 1997). The increase in replication activity caused by this mutant protein is lethal to the host strain (Simmons and Kaguni 2003). The amino acid substitutions encoded by the *dnaAcos* allele has been carefully analyzed and it was determined that two amino acid substitutions (A184V+Y271H) are required for the increased activity of this mutant protein (Simmons and Kaguni 2003). Biochemical analysis of the A184V bearing-protein has revealed a defect in ATP binding, and it is this defect that results in a failure of DnaAcos to limit the frequency of initiation (Katayama, Crooke et al. 1995; Carr and Kaguni 1996). After a round of replication has been initiated DnaA protein hydrolyzes ATP, and generates the inactive ADP bound form (Sekimizu, Bramhill et al. 1987). It has been shown that the release of bound ADP is very slow *in vitro*, suggesting that formation of the ATP-bound DnaA protein requires its synthesis. The time required for transcription and translation of DnaA protein might serve as one of the mechanisms that limits initiation frequency. A mutant form of DnaA protein has been studied that does not readily convert to the ADP bound (inactive) form (Nishida, Fujimitsu et al. 2002). DnaA protein-bearing an R334A amino acid change has a defect in ATPase activity. The activity of this mutant protein, like DnaAcos, also results in an increased frequency of replication initiation (Nishida, Fujimitsu et al. 2002).

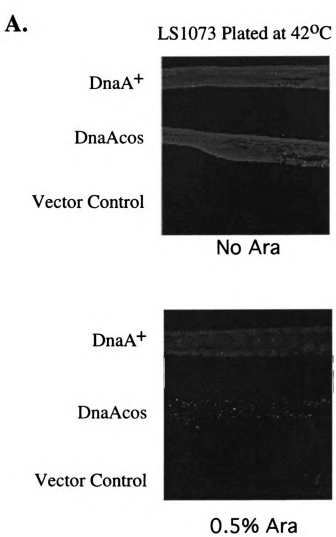
DnaAcos and R334A are examples of mutant DnaA proteins bearing amino acid substitutions that result in a failure of the protein to respond to regulatory signals. In these examples, either a defect in ATP binding or its hydrolysis results in aberrant replication control. These results underscore the importance of ATP binding and hydrolysis on the regulation of replication fork assembly. In our study, we sought to identify novel alleles of the *dnaA* gene that increase the frequency of initiation in attempt to characterize other regulatory mechanisms that modulate DnaA protein function. The *dnaAcos* allele was used as a control for the assay and we were able to identify seven

novel alleles that appear to be defective in regulating the frequency of initiation. These alleles are also dominant negative to chromosomally encoded *dnaA*$^{+}$. The dominant negative phenotype has been shown to result from hyperactive initiation as determined by marker frequency experiments (Simmons and Kaguni 2003). We also measured the replication activity of the corresponding mutant proteins *in vitro*. Based on a homology model of *E.coli* DnaA protein derived from the crystal structure of the *A. aeolicus* protein, the mutations are located in Domains I, IIIa, IIIb and IV. This information suggests we have obtained hyperactive alleles that are defective in ATP binding, ATP hydrolysis, DNA binding and possibly altered interactions with the replicative helicase DnaB. We also discuss hyperactive initiation of *S. cerevisiae* Cdc6 protein and the implications of our findings and how they relate to the regulation of eukaryotic initiation.

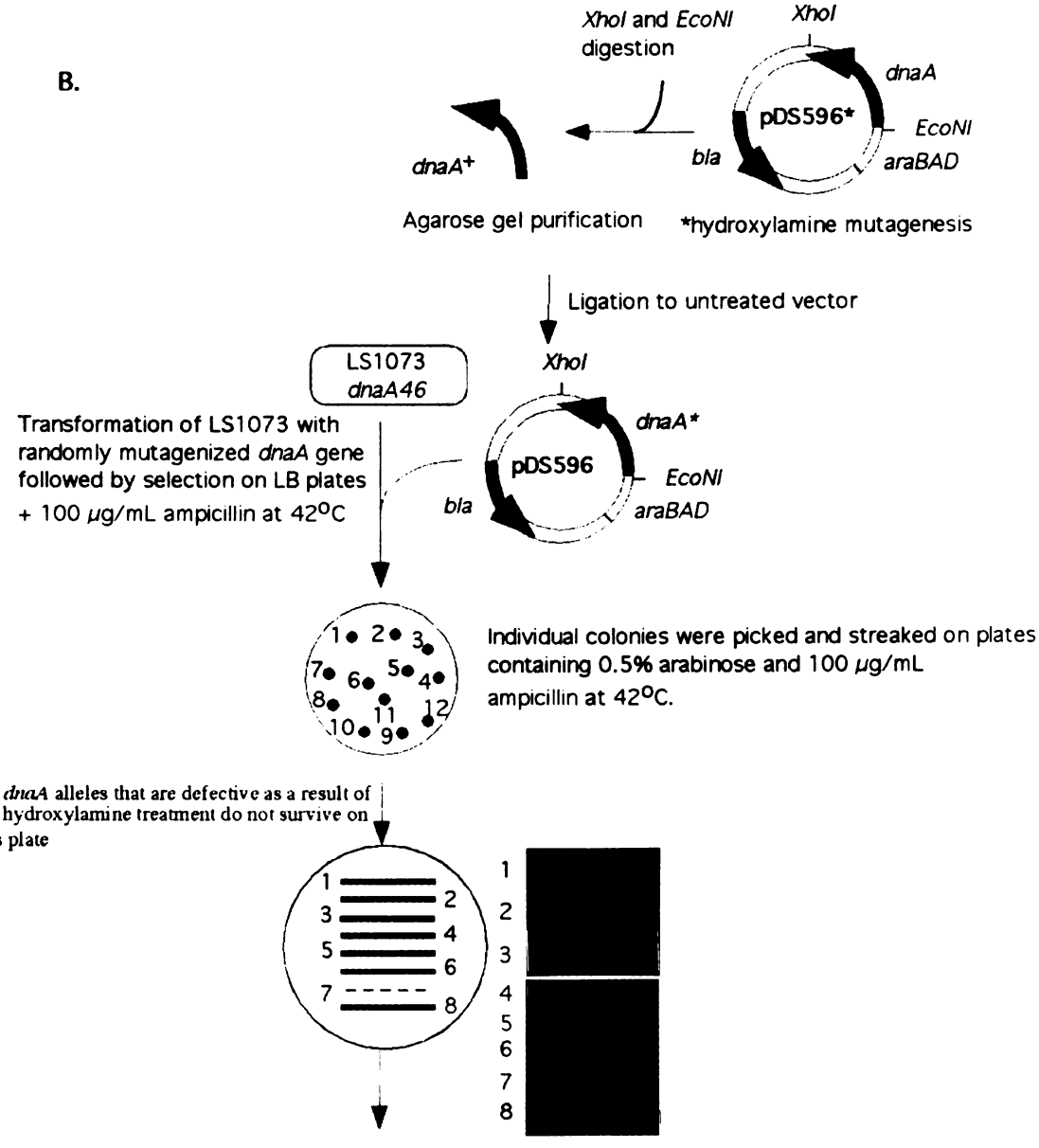
Table 1. List of plasmids

Plasmid	Characteristics	Source
pDS596	p*araBAD*, Ap^r, *dnaA*$^+$	Hwang and Kaguni, 1988
pDS319	p*araBAD*, Ap^r, *dnaA204*(ts)	Hwang and Kaguni, 1988
pLS120	p*araBAD*, Ap^r, *dnaAcos*	Simmons and Kaguni, 2003
pLS125	p*araBAD*, Ap^r, *G79D*	This work
pLS126	p*araBAD*, Ap^r, *S146Y*	This work
pLS127	p*araBAD*, Ap^r, *H202Y*	This work
pLS128	p*araBAD*, Ap^r, *E244K*	This work
pLS129	p*araBAD*, Ap^r, *V292M*	This work
pLS130	p*araBAD*, Ap^r, *V303M*	This work
pLS131	p*araBAD*, Ap^r, *E445K*	This work
pRB100	*dnaA* promoters, Ap^r, *dnaA*$^+$	Braun et. al, 1985
pLS*dnaAcos*	*dnaA* promoters, Ap^r, *dnaAcos*	Simmons and Kaguni, 2003
pLS132	*dnaA* promoters, Ap^r, *G79D*	This work
pLS133	*dnaA* promoters, Ap^r, *S146Y*	This work
pLS134	*dnaA* promoters, Ap^r, *H202Y*	This work
pLS135	*dnaA* promoters, Ap^r, *E244K*	This work
pLS136	*dnaA* promoters, Ap^r, *V292M*	This work
pLS137	*dnaA* promoters, Ap^r, *V303M*	This work
pLS138	*dnaA* promoters, Ap^r, *E445K*	This work
pCM959-Cm^r	*oriC*, Cm^r	Simmons and Kaguni, 2003
pKC596	T7 promoter, Ap^r, *dnaA*$^+$	Carr and Kaguni, 1996
pLS139	T7 promoter, Ap^r, *G79D*	This work
pLS140	T7 promoter, Ap^r, *S146*	This work
pLS141	T7 promoter, Ap^r, *H202Y*	This work
pLS142	T7 promoter, Ap^r, *E244K*	This work
pLS143	T7 promoter, Ap^r, *V292M*	This work
pLS144	T7 promoter, Ap^r, *V303M*	This work
pLS145	T7 promoter, Ap^r, *E445K*	This work
PLS145	T7 promoter, Ap^r, *A184V*	This work
pBR322	ColE1, Ap^r, Tc^r	Lab Stock

Figure 1. Genetic method to isolate hyperactive *dnaA* alleles.
LS1073 (relevant genotype, *dnaA*46) harboring either the plasmid vector or this DNA with the *dnaA*$^+$ or *dnaAcos* allele was plated on LB media with or without 0.5% (w/v) arabinose, as indicated. A genetic screen was developed to obtain *dnaA* alleles that are hyperactive in initiation based on the phenotype of growth interference upon induced expression of *dnaAcos*. As shown in Panel A, growth interference was not observed when *dnaA*$^+$ expression was induced. In Panel B, mutagenesis to increase the frequency of obtaining hyperactive mutations was by hydroxylamine treatment of pDS596 (Sutton and Kaguni, 1995). The *XhoI-EcoNI* restriction fragment carrying *dnaA* was gel-purified and ligated to the unmutagenized vector fragment, then used to transform LS1073 by electroporation. Transformants were selected at 42°C on LB media supplemented with 100 μg/mL ampicillin to obtain plasmid-encoded *dnaA* alleles that complement the temperature sensitive phenotype of the host-encoded *dnaA46* allele. Individual transformants were then plated on LB media supplemented with 100 μg/mL ampicillin and 0.5% (w/v) arabinose to identify those alleles that interfere with viability when expression was induced. Plasmid DNA was purified from these isolates by chromatography (Qiagen), and used to transform LS1073. The transformants were selected as described above and examined to confirm their phenotype of growth interference when plated on LB media containing 100 μg/mL ampicillin and 0.5% arabinose. DNA sequence analysis confirmed the presence and type of mutation. Approximately 1200 colonies were screened. Of these 37 colonies caused growth interference after re-transformation, and 15 putative mutants were analyzed by sequence analysis. After sequencing 7 were verified as mutant *dnaA* alleles.


A.
LS1073 Plated at 42°C
DnaA+
DnaAcos
Vector Control
No Ara
DnaA+
DnaAcos
Vector Control
0.5% Ara

B.



Plasmid isolation and retransformation to verify the phenotype. The *dnaA* gene was sequenced if the plasmid was linked to growth interference.

Experimental Procedures

Bacterial strains and plasmids *E. coli* K-12 strains have been described previously (Simmons and Kaguni 2003). *E. coli* LS1066 was constructed by P1 transduction of the *dnaA850*::Tn*10* allele from LS1061 into MS1062 (relevant genotype MC1061 but *zia*::pKN500). Plasmid DNAs were prepared by either equilibrium centrifugation or by column chromatography (Qiagen, Midi Kit) and are listed in Table 1. All novel alleles (except S146Y originally isolated in pRB100) were isolated in vector pDS596. The novel alleles were cloned into pRB100 by digestion of both pDS596 and pRB100 with restriction endonucleases *EcoNI* and *XhoI*. This digestion generates two fragments. The 1544 base pair DNA fragment contains the entire *dnaA* gene coding region. The larger DNA fragment from pRB100 (approximately 4000 base pairs) is the empty vector. These DNA fragments were ligated to link the novel alleles down stream of the native *dnaA* promoters. The DNA sequence of the *dnaA* coding region was determined for each recombinant plasmid to verify the presence of the respective mutations before use in the genetic assays.

Quantitative PCR

The method used for marker frequency analysis has already been described (Simmons and Kaguni 2003). Briefly, the indicated cultures were grown to early log phase (0.1-0.15, $O.D._{595nm}$) and 0.5% arabinose (w/v) was added to the cultures to induce *dnaA* expression. The cultures were then incubated for 3 hrs and the $O.D._{595nm}$ was determined to verify that the cultures were still in log phase growth. Cells were concentrated by centrifugation and frozen in liquid nitrogen. The number of cells processed for column chromatography (Qiagen, DNeasy) was in accordance with the manufactures recommendations ($\leq 2X10^9$ cells). The concentration of chromosomal

DNA was determined by absorbance at A_{260} and purity (A_{260}/A_{280})was measured between 1.7-1.9 to verify that the concentration determination was accurate. Quantitative PCR analysis was with 1 ng of chromosomal DNA in duplicate as described (Simmons and Kaguni, 2003). The amount of amplified DNA was quantitated by scintillation spectrometry after acid precipitation onto glass fiber filters (Whatman, GF/C).

Mutant protein overexpression in RTS

Each of the novel alleles was cloned downstream of an T7 RNA polymerase promoter for overexpression in the rapid transcription translation assay (RTS). The alleles were cloned into pKC596 (Carr and Kaguni 1996) by partial digestion with *Eco*R1 and complete digestion with *Rsr* II. Fragments were gel purified and ligated to construct the recombinant plasmid linking the novel alleles to a T7 promoter. DNA sequence analysis was performed to verify the presence of the respective mutations. RTS reactions were reconstituted according to the manufacturers recommendations (Roche). Reaction volumes were either 50 µl or 25 µl and the amount of plasmid DNA for wild type and mutant *dnaA* was 500 ng or 250 ng respectively. Samples were centrifuged for 5 minutes at 5000 rpm and the amount of protein that remained soluble was quantitated by western blot against known amounts of purified DnaA protein

In vitro replication assays

Complementation assays with a crude fraction (Fr II) prepared from WM433 (relevant genotype: *dnaA*204) were performed essentially as described (Fuller, Kaguni et al. 1981). Except for DnaA the crude fraction contains all of the proteins required to sustain *in vitro* replication of an *oriC* plasmid. The amounts of wild type or mutant DnaA protein added to the reaction are indicated.

Immunoblot analysis

Immunoblot analysis was performed essentially as described (Simmons and Kaguni 2003). Briefly, cells were grown to mid-log phase and expression of the various *dnaA* alleles was induced by addition of 0.5% arabinose (w/v) followed by incubation for 15 minutes. The optical density of the cultures was then determined. Cells were collected by centrifugation and frozen in liquid nitrogen. An equivalent number of cells were lysed and samples were electrophoresed on 10% SDS-polyacrylamide gels. Proteins were then transferred to nitrocellulose (Protran, Schleicher & Schuell) for immunoblot analysis as described (Simmons and Kaguni 2003).

Homology modeling

Was accomplished using the SWISS-MODEL web site (www.expasy.org/swissmod/SWISS-MODEL.html). The DnaA primary structure was submitted along with the coordinates for the crystal structure of *A. aeolicus* DnaA protein (J.M. Berger personal communication). The model was viewed and manipulated using the Insight II graphics program. Images in this dissertation are presented in color.

Table 2. Plating efficiencies of plasmid-borne *dnaA* alleles in LS1073[a] (*dnaA46*)

Plasmid	allele	Plating Efficiency 30°C	Plating Efficiency 42°C
pDS596	*dnaA*$^+$	1.0	0.9
pLS120	*dnaAcos*	2.2 X 10^{-3}	4.2 X 10^{-3}
pDS319	*dnaA204*(ts)	1.0	1.3 X 10^6
pBR322	None	1.0	ND

[a] *E. coli* LS1073 (relevant genotype:*dnaA46*) was transformed with pBR322 or the respective plasmids in which *dnaA* gene expression is under *araBAD* promoter control. Various dilutions were then plated on LB media supplemented with 100 μg/mL ampicillin, and with or without 0.5% (w/v) L-arabinose to measure colony formation at the indicated temperatures. The frequency of transformation in the absence of arabinose was 10^6-10^7 per μg of plasmid DNA. Plating efficiency was calculated as the ratio of colony forming units (CFUs) in the presence of arabinose divided by the CFUs observed in the absence of arabinose.

Results

Isolation of lethal dnaA *alleles*

We developed a genetic assay based on the following observations using a strain that carries the *dnaA*46(Ts) allele. We noticed that upon introduction of a plasmid in which the *dnaA*$^+$ or *dnaAcos* alleles is downstream of the inducible ara*BAD* promoter complementation of the *dnaA*46 defect at 42°C was observed in the absence of induced expression. When these strains were plated in the presence of arabinose the plasmid-borne *dnaA*$^+$ allele supported growth of the *dnaA*46 host at the nonpermissive temperature. In contrast, the elevated *dnaAcos* expression interfered with viability (Figure 1A, and Table 2 lines 1 and 2). These observations provided a genetic assay to screen for alleles with properties similar to *dnaAcos*.

We controlled for the assay by transforming the *dnaA46* host strain with plasmids-bearing the *dnaA*$^+$ and *dnaAcos* alleles. We show at 30°C and 42°C overexpression of DnaA$^+$ protein does not interfere with growth. In contrast, the overexpression of DnaAcos protein interferes with viability at 30°C and 42°C. We included a vector control in this assay to demonstrate the temperature sensitivity of the *dnaA46* host strain, and a *dnaA* allele encodes a mutant protein defective in DNA binding (*dnaA204*). We were concerned that defective *dnaA* alleles containing missense mutations affecting biochemical functions other than ATP binding could form active replication complexes with DnaA46 protein. The *dnaA204* allele provides a control for this possibility because the mutant protein is defective in DNA binding. This allele allows for normal growth at 30°C, but at 42°C it is unable to complement the *dnaA46* defect at low levels of expression. Interestingly, it does complement when expression is elevated suggesting oversupply of this mutant protein allows for formation of an active replication complex. This allele serves as an additional control for our assay, and it displays the opposite behavior compared with the alleles isolated in our screen (Table 2).

We treated a plasmid bearing the *dnaA*$^+$ gene with hydroxylamine and subcloned the DNA fragment encoding the mutagenized *dnaA* gene into the untreated plasmid vector. The ligation mixture was then used to transform the *dnaA*46 host followed by incubation at 42°C. The transformants that arose represent *dnaA* alleles that are active in complementation of the temperature sensitive growth of the *dnaA*46 allele. Individual colonies were then grown on LB media containing 0.5 % arabinose (w/v). Transformants able to grow in the presence of arabinose are similar in behavior to *dnaA*$^+$ and were not studied further. Those that did not grow under elevated expression were characterized in more detail (Figure 1B). Plasmid DNA was isolated and used to retransform the *dnaA46* host to verify the phenotype described above. Plasmids that clearly conferred the lethal phenotype were analyzed by DNA sequence analysis. We screened nearly 1200 transformants that apparently complemented the *dnaA46* defect in the absence of arabinose. From this collection (Table 3) we obtained seven novel *dnaA* alleles that each containing a single missense mutation.

Table 3. Nucleotide and amino acid substitutions in novel *dnaA* alleles

Nucleotide Position[a]	Nucleotide Change	Amino Acid Substitution	Frequency of Conservation[b]
236	G-A	*G79D*	5/30
604	C-T	H202Y	7/30
730	G-A	E244K	19/30
874	G-A	V292M	11/30
907	G-A	V303M	8/30
1333	G-A	E445K	12/30
437	C-A	S146Y	14/30
1067	C-T	Silent	

[a] The plasmid pDS596 which carries the *dnaA*$^+$ gene was mutagenized by hydroxylamine treatment essentially as described (Sutton and Kaguni, 1997). The 1.5 kbp restriction fragment carrying the *dnaA* coding region was then isolated and used to replace this same restriction fragment in pDS596 that was nonmutagenized. The ligation mixture was then used to transform LS1073 (relevant genotype: *dnaA46*(Ts). Transformants were selected at 42°C to obtain those *dnaA* alleles that are active in DNA replication and able to complement the nonfunctional *dnaA46* gene product. The transformants were then screened for their ability to grow on LB medium supplemented with 0.5% arabinose. The *dnaA*$^+$ allele in pDS596 does not interfere with viability when its expression is induced. We discarded those that remained viable under this condition to eliminate alleles that retained *dnaA*$^+$ function. In comparison, induced expression of *dnaAcos* interferes with viability. Hence, those transformants that failed to grow in the presence of arabinose were chosen for further characterization. The map positions of the missense mutations are relative to the first nucleotide of the *dnaA* coding sequence (Hansen et. al, 1982).

[b] The frequency of conservation was determined by comparison of 30 DnaA orthologs.

[c] The S146Y mutation was isolated from a screen (Simmons and Kaguni, unpublished data) intended to isolate *dnaA* alleles that failed to replicate the bacterial chromosome, but were active for replication of an *oriC* plasmid. In this assay the

Table 3. Legend continued
plasmid-borne allele under native *dnaA* promoter control failed to maintain a *dnaA46* bearing strain (LS1073) at 42°C, but was able to maintain an *oriC* plasmid in a Δ*oriC* host at the same temperature (LS1062). Further analysis of this allele revealed that growth interference was observed in the *dnaA46* host at 30°C. This result suggested that S146Y was hyperactive and not defective for replication from the chromosomal origin of replication, *oriC*. Mutagenesis of the *dnaA* gene to obtain this mutation was by error-prone PCR (Ludlam et. al, 2001).

Table 4. Novel *dnaA* alleles are lethal when overexpressed in an *oriC*$^+$strain

Plasmid	Allele	*oriC*$^+$ CFUs[a] 42°C Ara/No Ara	Δ*oriC* CFUs 42°C Ara/No Ara
pDS596	*dnaA*$^+$	1.0	1.0
pLS120	*dnaAcos*	$5.0X10^{-3}$	1.0
pLS125	*G79D*	$7.1X10^{-4}$	1.0
pLS126	*S146Y*	$6.8X10^{-4}$	0.9
pLS127	*H202Y*	$4.8X10^{-4}$	0.9
pLS128	*E244K*	$5.3X10^{-3}$	0.9
pLS129	*V292M*	$1.3X10^{-4}$	1.0
pLS130	*V303M*	$7.1X10^{-4}$	1.0
pLS131	*E445K*	$2.4X10^{-4}$	1.0

[a]Plating efficiency is expressed as the number of CFUs observed in the presence of 0.5% arabinose, divided by the CFUs observed in the absence of arabinose. Transformation efficiencies of the indicated plasmids was between 10^5-10^7 per μg of DNA. All colonies on 0.5% arabinose were smaller than those growing in the absence of arabinose but were similar in size to the wild type control. The Δ*oriC* strain contains a mini R1 integrated into *oriC* eliminating *oriC* dependent replication. The *oriC*$^+$ strain is LS1073 and is isogenic to the Δ*oriC* strain LS1062 used to obtain the data presented in the rightmost column. LS1073 was the host used to isolate the novel alleles described in this work.

Steady-state levels of protein expression

Previous studies showed that elevated DnaA$^+$ protein expression resulted in more frequent initiations, but this level did not interfere with viability of a wild type strain. Because of the possibility that even higher expression levels may interfere with viability, and that the phenotypes of the mutations we obtained is due to this reason, immunoblot analysis was performed (Figure 2A). To eliminate the contribution by the chromosomally encoded *dnaA* gene, a *dnaA* null strain was transformed with the respective plasmids, and expression of the mutant proteins at midlog phase growth was induced for 15 min. The analysis is of an equivalent number of cells revealing a comparable level of expression for most alleles. Whereas DnaAcos and S146Y proteins were observed at lower levels, the phenotype of these alleles is not due to an increase in protein abundance. Rather, it is the specific amino acid substitution that results in growth interference.

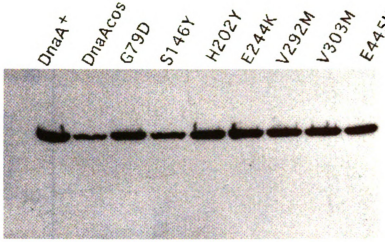
Lethality requires the presence of functional oriC

Experiments were performed to quantitate the level of growth interference when these plasmid-borne alleles were introduced into a *dnaA*$^+$ host strain (Table 4. column 3). All of the alleles showed a reduction in viability compared to *dnaA*$^+$ furthermore, the level of growth interference was more than that observed with *dnaAcos* except for E244K that was almost identical. This experiment reveals the potent effect of these novel alleles on cell viability. As a control for this assay, we transformed an isogenic strain that is null for both *oriC* and the *dnaA* gene. Indeed, the data (Table 4 column 4) indicates that the lethal phenotype is dependent on the presence of *oriC*. We were also able to suppress growth interference by the presence of a multi-copy plasmid-bearing

Figure 2. Relative expression levels of *dnaA* alleles

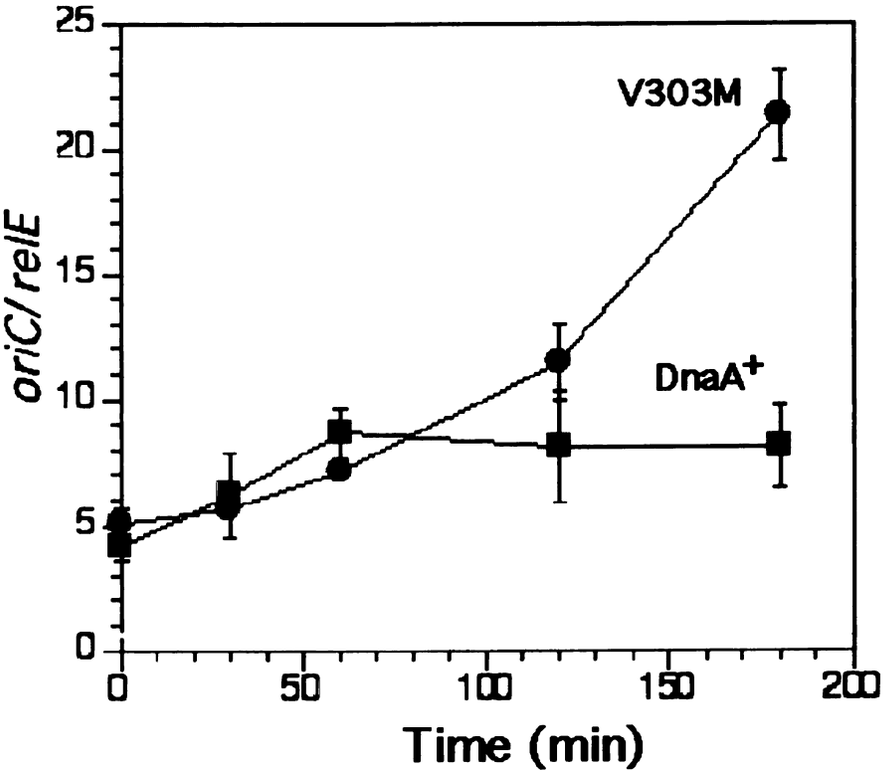
In Panel A, *E.coli* LS1062 (relevant genotype: *dnaA* 850::Tn*10*, Δ*oriC*::pKN1562) was transformed with the respective plasmids, and cultures were grown in LB media containing 100 μg/mL ampicillin to an O.D. $_{595nm}$ of 0.4-0.6. Arabinose was added to a final concentration of 0.5% (w/v) followed by incubation for 15 min to induce expression. Whole cell lysates were then prepared and analyzed by immunoblotting as described (Simmons and Kaguni, 2003). The relative abundance of the mutant proteins was normalized to DnaA$^+$. In Panels B and C, LS1066 (relevant genotype, *dnaA850*:: Tn*10*, *oriC*$^+$, zia::pKN500) carring the respective *dnaA* plasmids listed in Panel A were grown in LB media supplemented with 100 μg/mL ampicillin to early log phase (O.D.$_{595nm}$ ~0.15, 0.2). Then arabinose was added to 0.5% (w/v), followed by incubation for the times indicated in Panel B, or for 3 hrs in Panel C. Genomic DNA was purified (DNeasy, Qiagen) and quantitative PCR analysis was done to determine the relative abundance of *oriC* to *relE* as described (Simmons and Kaguni, 2003), with the abundance of *relE* normalized to 1. The range from duplicate samples is indicated by the error bars. For Panel B and C, we noticed two colony morphologies when LS1066 was transformed by pLS130 (*V303M*). Cells that gave rise to small colonies may lack the plasmid or the V303M mutation because the *oriC* to *relE* ratio was comparable to that of plasmid-free LS1066. Quantitative PCR analysis of DNA from normal size colonies of LS1066 carrying pLS130 yielded the data in Panel B and C.

A.



LS1062 Transformant	DnaA protein	Relative Protein Level
pDS596	DnaA+	1.0
pLS120	DnaAcos	0.4
pLS125	G79D	0.9
pLS126	S146Y	0.6
pLS127	H202Y	1.1
pLS128	E244K	1.1
pLS129	V292M	0.9
pLS130	V303M	1.0
pLS131	E445K	1.0

B.


25
20
15
10
5
0
oriC/ relE
V303M
DnaA+
0
50
100
150
200
Time (min)

C.

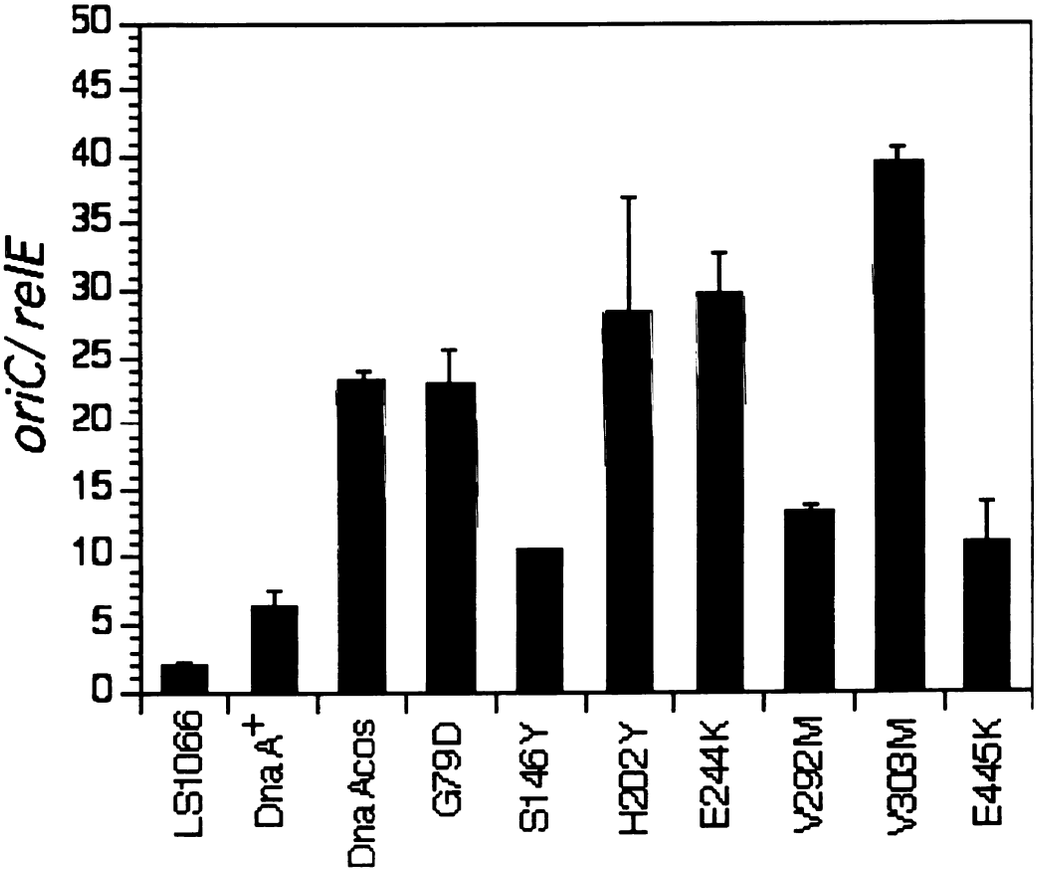

50
45
40
35
30
25
20
15
10
5
0
oriC/ relE
LS1066
DnaA+
DnaAcos
G79D
S146Y
H202Y
E244K
V292M
V303M
E445K

Table 5. Dominant-negative effect of *dnaA* alleles when expression is elevated in a *dnaA*$^+$ host strain

		Plating Efficiency	
Plasmid	Allele	30°C	42°C
pDS596	*dnaA*$^+$	1.0	1.0
pBR322	None	1.1	1.0
pLS120	*dnaAcos*	3.0 X 10^{-3}	1.5 X 10^{-2}
pLS125	*G79D*	1.5 X 10^{-3}	1.4 X 10^{-3}
pLS126	*S146Y*	9.2 X 10^{-4}	7.0 X 10^{-4}
pLS127	*H202Y*	2.7 X 10^{-3}	2.3 X 10^{-3}
pLS128	*E244K*	0.3[b]	3.6 X 10^{-4}
pLS129	*V292M*	2.8 X 10^{-4}	2.4 X 10^{-4}
pLS130	*V303M*	0.1[b]	4.9 X 10^{-2} [b]
pLS131	*E445K*	0.2[b]	6.6 X 10^{-4}

[a] The respective plasmids were used to transform MC1061 (*dnaA*$^+$) and plating efficiency on media with and without arabinose at 30°C and 42°C was determined as in Table 5.

[b] Compared to the colony size of MC1061 carrying the *dnaA*$^+$ plasmid pDS596, colonies of this plasmid-bearing strain were pinpoint in size.

Table 6. Novel *dnaA* alleles are active for *oriC* plasmid replication

LS1062 Harboring Plasmid	Allele	Plating efficiency 42°C/30°C[a]
pRB100	*dnaA*$^+$	1.0
pBR322	None	ND
pLS*dnaAcos*	*dnaAcos*	1.0
pLS132	G79D	1.4
pLS133	*S146Y*	2.4
pLS134	*H202Y*	0.7
pLS135	*E244K*	1.0
pLS136	*V292M*	1.0
pLS137	*V303M*	8.7×10^{-4} [b]
pLS138	*E445K*	2.1×10^{-3}

[a] *E. coli* LS1062 (relevant genotype: Δ*oriC*::pKN1562, *dnaA850*::Tn*10*) was transformed with the indicated plasmids followed by selection for growth on LB media supplemented with 100 μg/mL ampicillin. Plasmid DNA was then purified from randomly chosen transformants to confirm the presence of the properly sized plasmid. Electrocompetent cells of each plasmid-bearing strain are then prepared and transformed with the *oriC* plasmid pCM959-Cm^r, followed by selection on LB media containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol. Plating efficiency was calculated as the ratio of pCM959-Cm^r transformants that arose at 42°C divided by the number of transfomants at 30°C.

[b] The colonies observed at 30°C with the V303M allele were very small as compared to *dnaA*$^+$ and other *dnaA* alleles used in this assay.

oriC (data not shown), indicating that DnaAcos protein can be titrated with multicopies of *oriC*. These results suggest that growth interference is due to initiation from *oriC*.

Novel alleles are dominant negative to dnaA$^+$

We tested the alleles to determine if a dominant negative phenotype was observed at both 30°C and 42°C (Table 5). In this experiment we used an empty vector and a plasmid-bearing the *dnaA*$^+$ allele as negative controls. As expected, neither the *dnaA*$^+$ allele or the plasmid vector control (Table 5 lines 1 and 2) resulted in growth interference. The *dnaAcos* allele displayed growth interference at both 30°C and 42°C, which is consistent with previous observations. All of the novel alleles were dominant negative at both 30°C and 42°C. Three of the alleles (encoding E244K, V303M, E445K) were partially suppressed by the presence of the *dnaA*$^+$ gene at 30°C; however, a 10-fold reduction in colony formation is still observed suggesting hyperactive initiation. As an additional control we cloned all the *dnaA* alleles downstream of the native *dnaA* promoters plasmid-borne (see Table 1 for characteristics). In this experiment the alleles are expressed from the native *dnaA* promoters, so elevated *dnaA* expression results largely from a plasmid copy number effect. We demonstrated that all novel alleles were dominant to *dnaA46* (data not shown) and several (S146Y, H202Y, V292M) were dominant to *dnaA*$^+$. This experiment demonstrates that the inviability caused by these alleles does not require high overexpression, and can be achieved with a modest increase in protein abundance (data not shown).

dnaA alleles are active for oriC *plasmid replication*

We sought to determine the replication activity of these alleles when they are the sole source of DnaA protein in the cell. To achieve this objective we transformed a strain that is Δ*oriC* and Δ*dnaA* with vectors linking the alleles downstream of the native *dnaA*

promoters. We grew these transformants under selection for the *dnaA* plasmids and then transformed the resultant strain with an *oriC* plasmid (pCM959-Cm^r). We plated an equal number of cells from each transformation reaction at 30°C and 42°C to measure the replication activity of these alleles. The *dnaA*$^+$ control maintained the *oriC* plasmid well at both temperatures (Table 6). The host bearing the plasmid vector did not maintain the *oriC* plasmid, confirming that the host strain is *dnaA* null. All other strains were transformed at 42°C by the *oriC* plasmid with comparable efficiency except when the strains harbored the *V303M* and *E445K* alleles (Table 6). These results indicate all the alleles are active for *oriC* plasmid replication as the sole source of DnaA protein when assayed at 30°C. The observation that *V303M* and *E445K* are inactive for *oriC* plasmid maintenance at 42°C suggests that at 42°C, mixed complexes form with the chromosomally encoded *dnaA* allele causing the interference phenotype observed in previous experiments. Another explanation is that in a *oriC* plasmid there are different DnaA protomer assemblies that are required when compared to *oriC* in the bacterial chromosome. It has already been shown that certain *dnaA* alleles have different replication phenotypes at these two *oriC* locations (Simmons et. al, submitted). Experiments have also demonstrated directly that *oriC* plasmid maintenance has different DnaA box spacing requirements than chromosomal *oriC*. These results indicate that the replication requirements of an *oriC* plasmid are slightly different than that of the bacterial chromosome (Bates, Asai et al. 1995).

Marker frequency analysis of hyperactive initiation in vivo

It has been shown previously that an elevated frequency of initiation leads to stalled replication forks affecting sequences located near *oriC* (Kellenberger-Gujer, Podhajska et al. 1978; Nyborg, Atlung et al. 2000). We used a quantitative PCR assay (Simmons and Kaguni 2003) to demonstrate hyperactive initiation *in vivo*. We

transformed the host strains (LS1073 and LS1066) with plasmids bearing the novel alleles and induced expression of the mutant DnaA proteins. Induction was allowed to continue for the optimal time of 3 hours (Figure 2B) prior to harvesting chromosomal DNA for analysis. We included both of these genetic backgrounds in the analysis to determine the level of hyperactive initiation caused by the *dnaA* alleles in the original background (LS1073) and a background where *oriC* dependent replication can be driven without the complication of a *dnaA* gene background (LS1066, relevant genotype:pKN500::zia, *dnaA*850::Tn*10*). In these experiments we measured hyperactive initiation by harvesting chromosomal DNA and then PCR amplifying *oriC* and the *relE* locus for comparison. The PCR amplification was done in the presence of a α^{32}P labeled dATP and the incorporation of the radioactive material was determined after acid precipitation and isolation of the labeled material on glass fiber filters. We then quantified the amount of α^{32}P label by scintillation spectrometry. We observed hyperactive initiation in the *dnaA*46 background as all novel *dnaA* alleles resulted in at least a two-fold increase (10-fold for V303M) in the *oriC* to *relE* ratio compared to the *dnaA*$^+$ control and the strain growing without a plasmid (data not shown). We decided to examine the affect at 30°C only as the 42°C data may be compromised in light of the *oriC* plasmid maintenance results (Table 6, lines 9,10). As a control for the 42°C plating efficiency data we examined *dnaAcos* and observed a 5-fold increase in *oriC* to *relE* ratio at 42°C compared to the *dnaA*$^+$ gene control, and a 10-fold increase compared to the strain only control (data not shown).

After confirming an increase in *oriC* to *relE* DNA in the *dnaA*46 background we decided to assay the alleles for hyperactive initiation from chromosomal *oriC* when the

novel alleles are the sole source of DnaA protein in the cell. We performed a similar experiment but this time in a *dnaA* null host. This strain contains an R1 replicon integrated outside of *oriC* in the *zia* locus. Upon disruption of the *dnaA* coding region (*dnaA850*::Tn*10*) viability was maintained by initiation from the integrated R1 replicon. When DnaA protein is supplied by a plasmid-borne allele, initiation can occur from *oriC*. We did a time course experiment to identify the proper time point for analysis. In this experiment (Figure 2B) expression of the *dnaA* and *V303M* alleles was induced and time points were extracted from 0-3 hrs. This experiment revealed that wild type DnaA protein reaches peak *oriC* to *relE* values within the first 60 minutes of induction. In contrast, induction of V303M results in an increase in *oriC* to *relE* values during the entire time of induction (Figure 2B). This experiment is interesting because it suggests that DnaA protein responds to some negative regulator (perhaps Hda protein), and V303M is unresponsive to this factor. We examined the *dnaAcos* allele in the same experiment and obtained similar results (data not shown).

After establishing the proper induction time for this experiment (3 hrs) we analyzed all the novel *dnaA* alleles (Figure 2C). This experiment provided similar results to the previous experiment in the *dnaA46* background. All the novel alleles showed an increase in *oriC* to *relE* DNA compared to LS1066 and LS1066 bearing a *dnaA*$^+$ plasmid (Figure 2C). These results clearly establish an increase in initiation activity resulting from the activity of these mutant proteins *in vivo*. We obtained higher initiation activity in the *dnaA* null strain, suggesting *dnaA46* interferes to some extent.

Mutant DnaA proteins are active for replication from oriC in vitro

We cloned all seven novel alleles so that their expression is from a T7 RNA

Figure 3. Mutant DnaA proteins are active for *oriC* plasmid replication *in vitro*
The respective mutant proteins were produced *in vitro* in a coupled transcription-translation system (RTS100, Roche), and their concentration were determined by quantitative immunoblot analysis relative to proportional amounts of purified DnaA protein. Most mutant proteins were produced at a concentrations a of about 140 ng/µl. Replication activity was measured as described in "Experimental Procedures" by incubation for 20 min at the indicated temperatures. In the upper left panel, the activity of purified DnaA protein is represented by open symbols. The closed circle(●) corresponds to the indicated RTS samples at 42°C. The closed squares(■) correspond to RTS samples incubation at 30°C.

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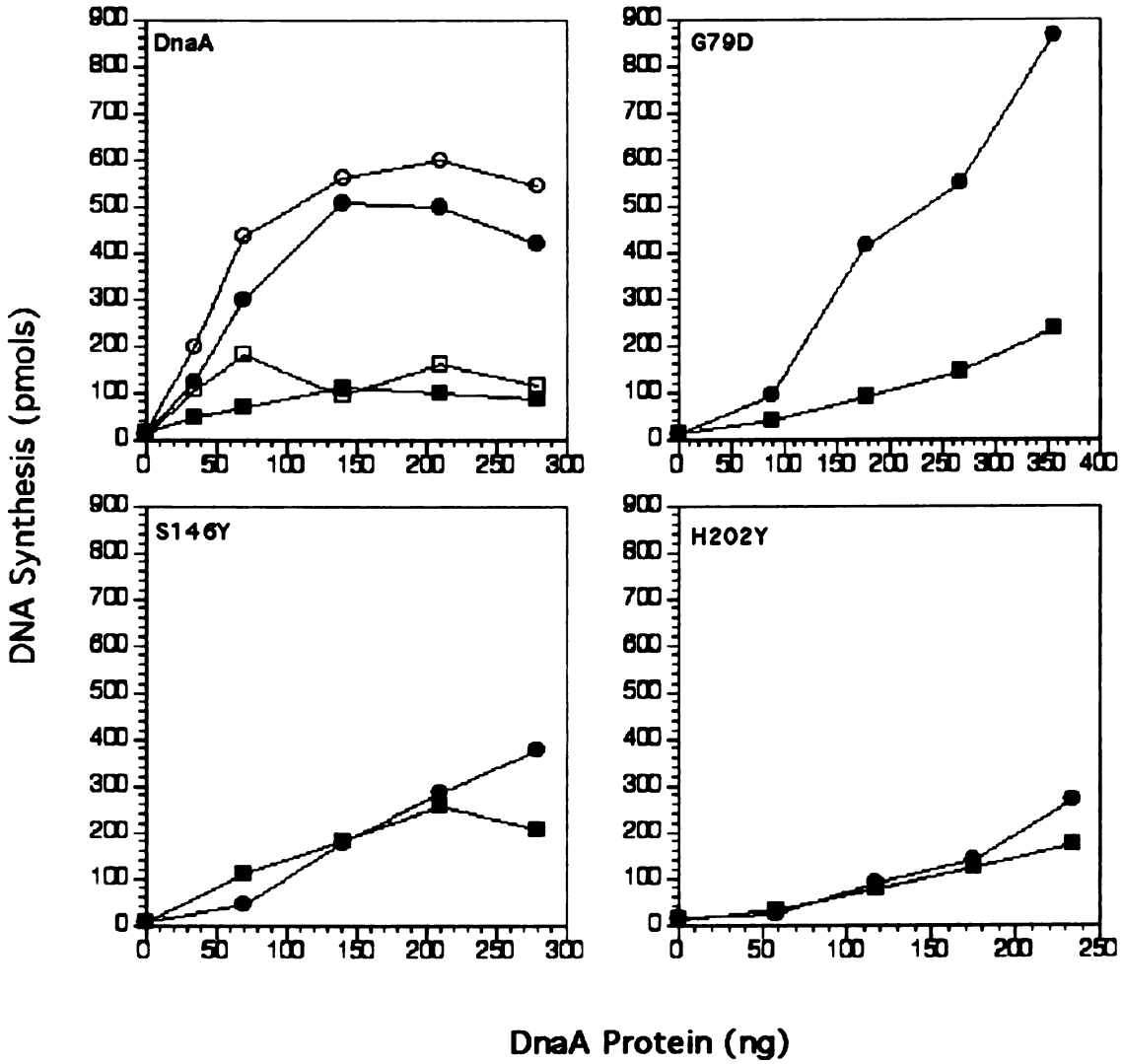
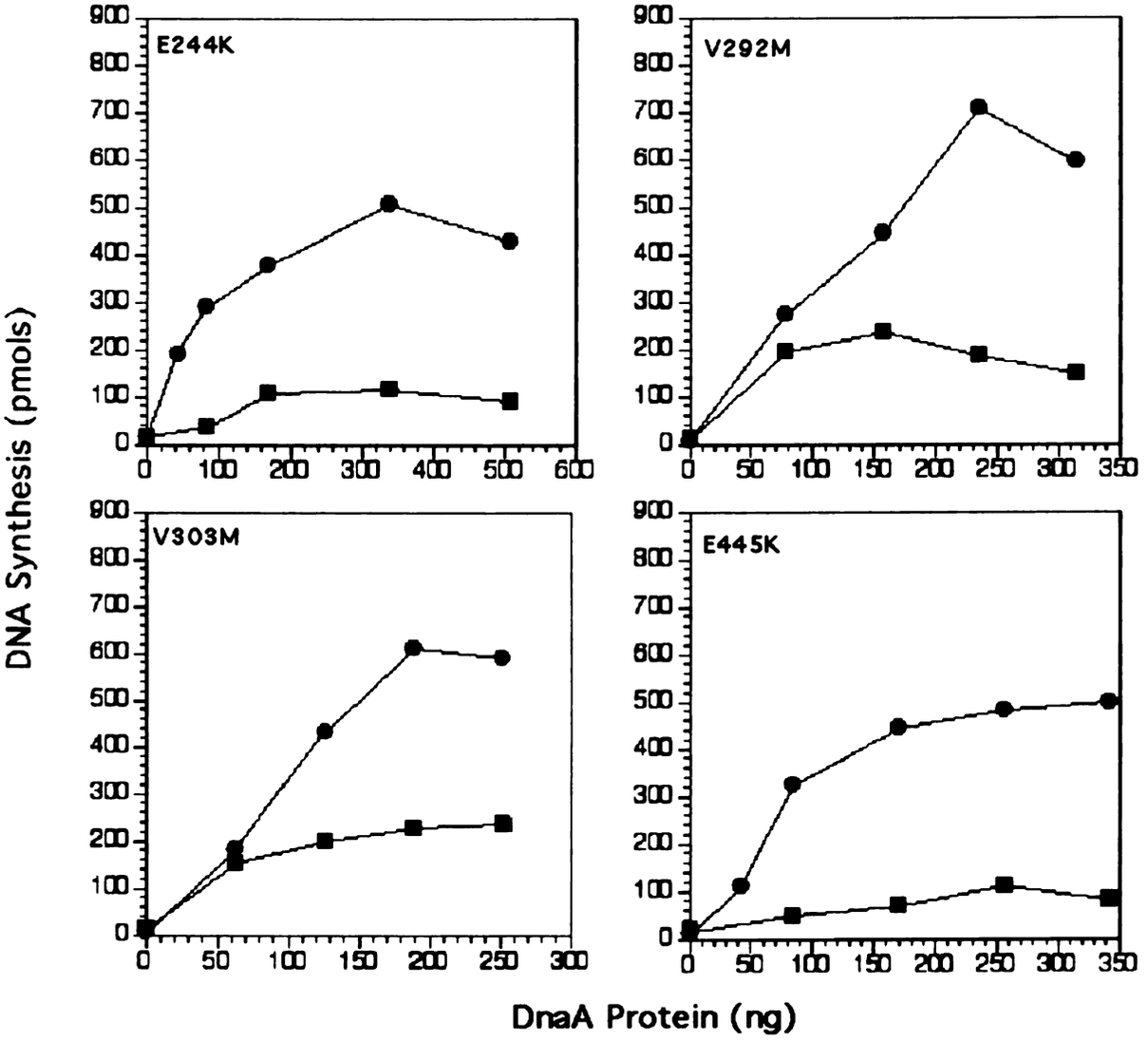
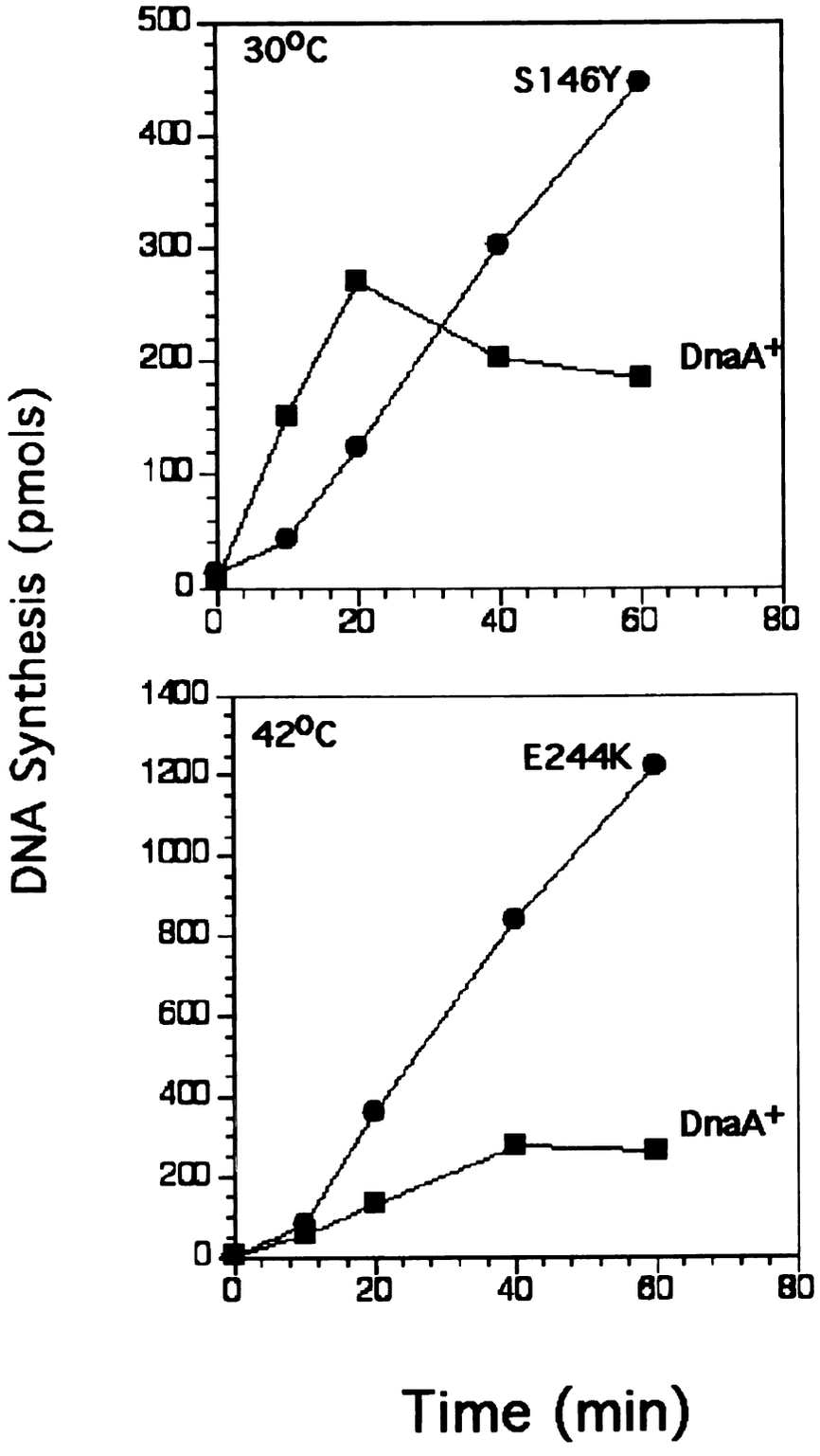



E244K
V292M
V303M
E445K
DNA Synthesis (pmols)
DnaA Protein (ng)

polymerase promoter in order to express the corresponding mutant proteins in an *in vitro* coupled transcription-translation system (Roche RTS). The concentration of the soluble protein was determined by western blot analysis compared to a known amounts of the wild type protein. The replication activity of purified DnaA$^+$ protein was compared to DnaA$^+$ obtained from the RTS system. As a negative control, we assayed a portion of the RTS material that was mock treated with a vector control plasmid for replication activity. This sample was devoid of replication activity, indicating that the stimulation of *oriC* plasmid replication is due to the DnaA protein in the RTS fraction (data not shown). By comparison, mutant proteins displayed varying levels of replication activity (Figure 3). The G79D mutant protein was very active whereas H202Y protein was about half as active as DnaA$^+$. We did not visualize a robust increase in replication activity in this assay, so we examined the mutant proteins in a time course experiment. Again, in this assay most of the mutant proteins did not show a robust increase in replication activity compared to the wild type control, although, most of the mutant proteins were as active or slightly more active than DnaA$^+$ protein (data not shown). We did however, observe a significant increase in replication activity when comparing DnaA$^+$ and E244K at 42°C and S146Y at 30°C (Figure 4). In both of these experiments DnaA protein reaches a peak of replication activity. In contrast, E244K and S146Y show an increase in replication activity in nearly a linear pattern. The data obtained in this experiment is similar to our observations of *oriC* to *relE* ratios from the *in vivo* time course experiment. DnaA$^+$ protein activity appears to be regulated negatively while the mutant proteins are unresponsive to this soluble factor. 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. All of the mutant proteins showed an increase in replication activity at both

Figure 4. Continued DNA replication activity with hyperactive mutant proteins
DnaA$^+$ or the respective mutant proteins were produced *in vitro* as described in Figure 3, and DNA replication activity was measured at 30°C or 42°C for the times indicated. The graphs corresponding to the mutant protein or the DnaA$^+$ control are also indicated. The DnaA$^+$ protein used in this experiment was generated in the RTS system. For each reaction the amount of DnaA protein was constant at 50 ng.


30°C
S146Y
DnaA+
42°C
E244K
DnaA+
DNA Synthesis (pmols)
Time (min)
0
100
200
300
400
500
0
200
400
600
800
1000
1200
1400
0
20
40
60
80

temperatures until at least the 40 min time point, but most increased linearly through the 60 min time point (data not shown).

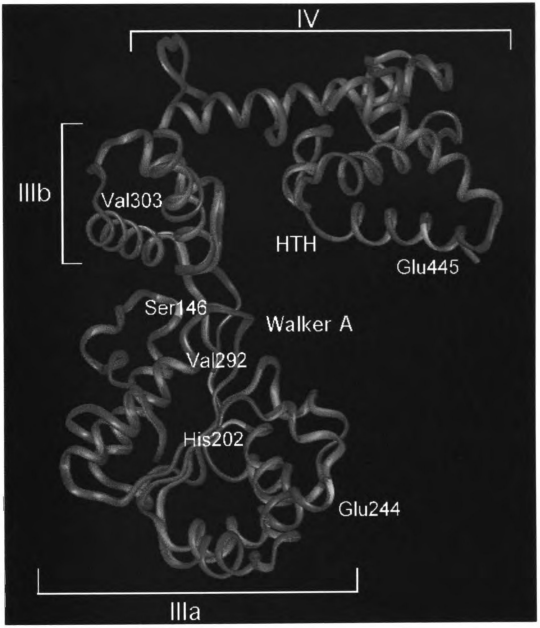
Location of missense mutations

The position of the missense mutations relative to the functional domains of DnaA protein suggest that the amino acid substitutions affect different functional domains. The *E. coli* primary structure shares almost 35% identity and 65% similarity over the aligned region [data not shown,(Erzberger, Pirruccello et al. 2002)]. Recently, the crystal structure of DnaA protein from *A. aeolicus* (Erzberger, Pirruccello et al. 2002) was reported. The structure was determined for Domain IIIa, which is a abbreviated RecA- type fold, Domain IIIb, which is comprised of an antiparallel three-helix bundle, and Domain IV, which is the DNA-binding domain and contains a helix-turn-helix (HTH) motif.

We constructed a homology model of the *E. coli* protein (Figure 5) based on the structure of *A. aeolicus* DnaA, and mapped the positions of the amino acid substitutions. The *E. coli* protein (Figure 5) almost completely overlaps the *A. aeolicus* structure. The amino acid substitutions S146Y, H202Y, V292M, and V303M are in close proximity to the ATP binding pocket. Based on the homology model, these mutations may affect ATP binding or hydrolysis. The mutation E445K is located in the HTH motif of the DNA binding domain, suggesting that aberrant complexes are formed at *oriC* to result in an increased frequency of initiation. The E244K substitution is located in Domain IIIa which contains the Walker A box and Sensor I motif. Because this substitution is located in an outer α-helix and is distant from the site of ATP binding, it seems unlikely that this residue perturbs ATP binding. This residue may be involved in a regulatory mechanism to control initiation. If this residue is solvent exposed in the full length protein, it may

Figure 5. Homology model of *E. coli* DnaA protein (Domains IIIa-IV)

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.


IV
IIIb
Val303
HTH
Glu445
Ser146
Walker A
Val292
His202
Glu244
IIIa

interact with proteins during assembly of the prepriming complex. We were unable to model the G79D substitution because Domain I and II are not part of the *A. aeolicus* crystal structure.

Discussion

DnaA protein has been studied intensely for the last twenty years (Kornberg and Baker 1992). Although many missense mutations have been isolated and characterized, only two appear to bypass a regulatory mechanism that might serve as a checkpoint to limit the frequency of initiation. We sought to isolate other *dnaA* alleles under the hypothesis that regulatory mechanisms function to limit DnaA protein activity to regulate the frequency of initiation during the cell cycle.

Genetic screen and characterization of novel dnaA *alleles*

We devised a genetic method to isolate novel hyperactive *dnaA* alleles. The method involved two steps. First, we 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 *dnaA46* defect. This step required that complementation was in the absence of induced expression. In the second step, transformants were screened for growth interference when the level of expression was elevated. As the genetic assay was based on the behavior of the *dnaAcos* allele that is hyperactive in initiation we expected to obtain other *dnaA* alleles with similar properties. These two genetic experiments have been used as valid assays to discriminate between *dnaA* alleles that are defective for replication or benign, compared with alleles that are hyperactive (Simmons and Kaguni, 2003). This assay yielded seven alleles that are dominant negative to chromosomally encoded $dnaA^+$ and required the presence of *oriC* for growth interference. As an additional control, we showed that these phenotypes are not due to an increase in the level of protein expression (Figure 2A). The alleles encoded unique single amino acid substitutions that may affect specific biochemical functions of DnaA protein, as indicated by the locations of the amino acid substitutions.

Hyperactive initiation in vivo

To demonstrate that the alleles we obtained result in an elevated frequency of initiation, we performed quantitative PCR analysis to measure the abundance of *oriC* and *relE*, a locus in the replication terminus region. In previous work, we established conditions for quantitative PCR analysis that accurately measured the abundance of specific DNA sequences in the bacterial chromosome (Simmons and Kaguni, 2003). In the cited study, we demonstrated that the ratio of *oriC* to *relE* reflects the frequency of initiation. In stationary phase cells, this ratio is near 1, whereas in logarithmically growing wild type cells, this ratio of 4 is consistent with other results that indicate that the majority of cells carry 4 copies of *oriC* in the replicating chromosome. Using this method, we confirmed that the *dnaA* alleles, when induced, indeed promote more frequent initiations. Some alleles such as H202Y, E244K and V303M are more active than *dnaAcos* as reflected by their higher *oriC*/*relE* ratios. The S146Y, V292M and E445K mutations are about half as active as *dnaAcos,* but more active than *dnaA*$^+$ in stimulating initiation when expression of these alleles is induced. The activity of the mutant proteins in sustaining *in vitro* DNA replication of an *oriC*-containing plasmid was measured, and compared to DnaA$^+$ protein. Assays were performed at 30°C, the standard incubation temperature and also at 42°C. The latter temperature was chosen because the mutant alleles were able to complement the *dnaA*46(Ts) allele at 42°C *in vitro*. Except for V303M and E445K the replication activity of the mutant proteins correlates with the ability of the corresponding plasmid-borne allele to maintain an *oriC* plasmid in the *dnaA*850::Tn*10* host stain. The reduced ability of V303M and E445K at 42°C in maintenance of the *oriC* plasmid contrasts with the ability of these plasmid-borne alleles to complement the host-encoded *dnaA*46(Ts) allele at 42°C. We do not have an explanation for this apparent discrepancy.

Hyperactive initiation in vitro

The genetics of these *dnaA* alleles, and the *in vivo* analysis indicates hyperactive initiation. We decided to examine the replication activity of the mutant proteins *in vitro* in a Fraction II complementation assay (Kaguni, Fuller et al. 1982). Because our mutant proteins behaved so poorly during preparation of the soluble fraction we decided to synthesize the proteins in a rapid transcription-translation coupled assay *in vitro*. A few of the mutant proteins showed an increase in replication activity, but all were active for *oriC* plasmid replication *in vitro* at both 30°C and 42°C confirming they are active for replication from *oriC*. Even the most well studied hyperactive mutant protein DnaAcos, only shows a modest increase in replication activity (Katayama and Kornberg 1994). Our *in vitro* replication data and the published data on DnaAcos activity prompted the time course replication experiment (Figure 4). We observed a robust increase in replication with time for the E244K and S146Y mutant proteins compared with the wild type control. This data is interesting because the activity of the mutant proteins continues linearly with time, whereas DnaA$^+$ proteins reaches a peak activity. This data suggests that a soluble factor in the crude WM433 extract is able to negatively regulate DnaA protein but not the mutant proteins. We suggest that this factor could be Hda protein (Kato and Katayama 2001), or possibly the beta subunit of DNA polymerase III holoenzyme (Katayama, Kubota et al. 1998). Both of these proteins have already been implicated in negative regulation of DnaA protein.

We constructed a homology model of *E. coli* DnaA protein from the crystal structure of DnaA protein of *A. aeolicus* (Erzberger, Pirruccello et al. 2002) to determine the relative locations of our amino acid substitutions in the predicted folded structure. To our surprise all of the mutations except for G79D and E445K were located in Domain IIIa-IIIb indicating ATP binding or hydrolysis may be affected (Figure 5). We performed the same analysis for DnaAcos and determined that the two missense mutations required

for the *dnaAcos* phenotype (data not shown) are located in Domain IIIa, confirming ATP binding and hydrolysis are important regulatory biochemical mechanisms limiting DnaA protein function. We do believe that G79D, E445K, and probably E244K cause an increase in origin firing by some other biochemical mechanism. This suggests origin activation is not only limited through the availability of DnaA-ATP.

Cdc6 protein and the frequency of initiation

The recent crystal structure of Domains III and IV of *A. aeolicus* DnaA protein reveals a remarkable structural similarity to archaeal Cdc6/Orc1 from *P. aerophilium* (Liu, Smith et al. 2000). In yeast, Cdc6 functions in initiation of DNA replication. Two *cdc6* alleles (*cdc6-2*, *cdc6-3*) have been isolated from *S. cerevisiae* that result in "persistent initiation" as determined by >2C DNA content, and the constant association of MCM proteins with chromatin (Liang and Stillman 1997). The increased frequency of initiation leads to a failure of these cells to divide, a feature that is also observed under hyperactive initiation in *E. coli*. We aligned the primary structure of Cdc6 from *S. cerevisiae* and *P. aerophilum* to determine the corresponding amino acids and the structural domains affected by these mutations. The *cdc6-2* allele encodes an A320S amino acid substitution (*P. aerophilum* I223) that affects Domain II and is located in α-helix 12. If we use the structure of DnaA protein for comparison, this mutation may affect ATP hydrolysis because it resides in that portion of DnaA protein involved in ATP hydrolysis. The *cdc6-3* allele encodes two amino acid substitutions. H144T does not align with a corresponding amino acid of *P. aerophilum* Cdc6. L258S is located in β strand 4 and may affect ATP binding, based on the location of this residue in the structure of DnaA protein. From the comparative analysis of DnaA and Cdc6 the location of relative missense mutations to functional domains suggests that both proteins may limit the frequency of initiation through ATP binding. In the case of Cdc6 protein, ATP

binding defects appear to result in a constant loading of the MCM helicase complex at the origin of replication. It is possible that hyperactive *dnaA* alleles are able to continually load DnaB when ATP binding and hydrolysis are reduced.

What is the cause of lethality?

In earlier studies, we showed that the induced expression of DnaA protein resulted in an elevated frequency of initiation from *oriC*. Two independent methods of microarray analysis that measured the relative abundance of essentialy every gene sequence of the *E. coli* chromosome, and quantitative PCR analysis of *oriC* and *relE* were used to obtain results supporting this conclusion. Interestingly, an elevated level of DnaA protein does not interfere with viability unless the cell is defective in the repair of double strand breaks. In contrast, *dnaAcos* expressed at a comparable level interferes with viablility, apparently because of an even higher frequency of initiation. This hyperinitiation results in arrested replication forks, and the accumulation of an unusually high level of double strand breaks. Apparently, the failure to repair these double strand breaks interferes with viability. As shown in Figure 2, some of the *dnaA* alleles result in even higher frequencies of initiation than *dnaAcos*. We presume that the growth interference associated with these alleles when expression is induced is due to the accumulation of double strand breaks and their failure to be repaired. Other alleles do not confer as high a frequency of initiation, but the initiation frequency is nevertheless elevated in comparison to the wild type *dnaA* gene. These alleles interfere with growth presumably for the same reason.

One model proposes that DnaAcos causes whole chromosome overreplication, and the cause of lethality is a cell division or chromosome partitioning defect (Katayama, Takata et al. 1997). This observation is supported by results identifying a novel protein CedA, that restores cell morphology (*dnaAcos* causes filamentation). It is not clear that

CedA protein affects cell division, this protein may suppress DnaAcos and restore normal morphology as a result. We propose that *dnaAcos* and other hyperactive *dnaA* alleles cause an increase in the frequency of initiation that leads to stalled replication forks, and Holliday junction formation at the site of replication fork collapse. It has been documented that Holliday junctions are cleaved to the point of double strand breaks (DSB) by a fully activated RuvC protein (Michel, Ehrlich et al. 1997). We have shown that *dnaAcos,* and many of the novel alleles described in this study cause a large increase in linear DNA observed *in vivo* by PFGE analysis (data not shown). We have also demonstrated that cell viability during $DnaA^+$ induced hyperactive initiation requires homologous recombination proteins (Simmons et. al, in preparation). Taken together, when hyperactive initiation is induced many replisomes are assembled which results in a stacking of replication forks near *oriC*. This causes many of the replisomes to stall, and DSB are generated by RuvC action (Seigneur, Bidnenko et al. 1998). If these breaks are not sufficiently repaired, and the replication forks are not restarted through a primosome dependent pathway, then the host is no longer viable. In the case of $DnaA^+$ induced hyperactive initiation the level of linear DNA apparently does not out compete the homologous recombination machinery, but the increase in replication caused by hyperactive *dnaA* alleles (up to 20 fold) causes more DSB in the cell than can be repaired.

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Chapter V

Hyperinitiation of DNA replication in *E. coli* leads to replication fork collapse and inviability

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Abstract

Induced *dnaA*$^+$ expression from an overproducing plasmid stimulates initiation of chromosomal replication from the *E. coli* replication origin, *oriC*, but does not interfere with viability. More frequent initiations are also observed with the *dnaAcos* allele encoded in single copy in the bacterial chromosome, but lethality is observed with *dnaAcos* despite the lesser level of expression. In this report, we show that hyperinitiation induced by elevated *dnaA*$^+$ expression results in replication forks that abort or collapse, the majority of which are within about 15 map units neighboring the replication origin (*oriC*). As fork collapse occurs at apparently random sites, nucleoprotein complexes such as *datA* bound by DnaA are not the cause. To maintain viability, the damaged replication forks must be repaired by replication restart proteins including PriA and RecBCD. Viability under *dnaA*$^+$-dependent hyperinitiation contrasts with the lethality of *dnaAcos* regardless of its induced expression. When the replication restart pathway is blocked by a *recB* mutation, the accumulation of collapsed forks, reflected by the level of broken linear chromosomal DNA, is substantially higher under elevated *dnaAcos* expression compared to *dnaA*$^+$. These and other results support the conclusion that the lethal phenotype of *dnaAcos* is due to untimely initiations from *oriC* and the insufficiency in repairing the elevated level of collapsed replication forks.

Introduction

DNA replication in *E. coli* initiates from the chromosomal origin, *oriC* (reviewed in Kornberg and Baker, 1992; Messer, 2002). Replication forks then progress around the chromosome, terminating in a region opposite the chromosomal origin. As the time required to duplicate the chromosome is relatively constant, one view is that replication forks progress at constant speed. Other recent work suggests that replication forks occasionally arrest or collapse, requiring reassembly of the replication fork machinery in order to complete duplication of the chromosome to maintain viability (reviewed in Cox, Goodman *et al.*, 2000; Jones and Nakai, 2000). A key protein involved in restarting collapsed replication forks is PriA, first discovered in the study of bacteriophage ϕX174 DNA replication (reviewed in Sandler and Marians, 2000). Although *priA* is not essential for viability, mutants grow poorly, suggesting that replication forks become arrested frequently and must be restarted to reach the terminus region.

Replication forks may arrest by several mechanisms. When replication forks fail to advance due to an upshift in temperature of an *E. coli dnaB*(Ts) or *holD*(Ts) mutant to the nonpermissive temperature, fork regression and collapse occur (Flores, Bierne *et al.*, 2001; Michel, Ehrlich *et al.*, 1997; Saveson and Lovett, 1997). As another general mechanism, DNA lesions in the template strand of the parental duplex may stall the replicative polymerase, leading to regression of replication forks, their collapse and potentially lethal double strand breaks. A third mechanism involves a protein-DNA barrier. Paused replication forks have been documented when the replication fork machinery collides with transcribing RNA polymerase or with proteins firmly bound to DNA (French, 1992; Liu and Alberts, 1995; Vilette,Uzest *et al.*, 1992) . Indeed, because it takes about twice as long for replication forks to be completed in *rep* mutants compared to wild type strains, Rep helicase has been proposed to act at the fork to remove proteins bound to DNA (Lane and Denhardt, 1975). In eukaryotic systems, the replication fork

barrier may be programmed as in the case with the rDNA gene cluster in *S. cerevisiae*. FOB1 participates in the arrest of replication forks at the 3'-end of the rDNA transcription unit so that DNA replication does not interfere with rRNA synthesis (Kobayashi and Horiuchi, 1996; Kobayashi, Heck, *et al.*, 1998) .

DnaA protein regulates the process of chromosomal replication by modulating the frequency of initiation at the chromosomal origin. Indeed, IPTG-dependent *dnaA*$^+$ expression under *lacUV5* promoter control stimulates initiation from *oriC*, but fully replicated chromosomes are not produced because the newly initiated replication forks appear to be arrested (Atlung and Hansen, 1993; Skarstad, Lobner-Olesen *et al.*, 1989). Elevated *dnaA*$^+$ expression under this condition, or under expression from the *araBAD* promoter (shown below) did not interfere with viability. In contrast, the *dnaAcos* allele, originally isolated as an intragenic suppressor of the *dnaA46*(Ts) allele present in single copy in the genome, interfered with viability at 30°C that was attributed to an increased frequency of initiation (Kellenberger-Gujer, Podhajska *et al.*, 1978). In either circumstance, (elevated *dnaA*$^+$ expression by *lacUV5* promoter induction, or chromosomal *dnaAcos* expression from the natural *dnaA* promoters), marker frequency analysis of various loci distributed around the chromosome confirmed elevated frequencies of initiation, based on the abundance of *oriC* and markers nearby compared to a site near the replication terminus. However, these results did not explain the apparent contradiction that the lethal effect of *dnaAcos* compared to the nonlethal effect of elevated *dnaA*$^+$ expression is due to the level of initiation. In the study described herein, we addressed whether the lethality caused by hyperactive initiation by *dnaAcos* (or *dnaA*$^+$ under specific conditions described below) is due to the level of hyperinitiation. The results obtained by genomic microarray analysis, quantitative PCR, and pulse field gel electrophoresis support a model in which elevated initiation activity

results in replication fork collapse. Lethality results from the failure to repair the collapsed forks.

Under conditions of elevated initiation, it was also unclear whether the replication forks were stalled at specific or random sites. We considered the possibility that specific protein-DNA complexes may impede replication fork movement. As just one example, six sites in the bacterial chromosome have been described that are bound by DnaA with high affinity (Roth and Messer, 1998). The *datA* locus is one of these sites; it is estimated that several hundred DnaA monomers bind to the 1 kb region surrounding *datA* (Kitagawa, Mitsuki et al., 1996; Kitagawa, Ozaki et al., 1998; Ogawa, Yamada et al., 2002). As DnaA bound to DnaA box sequences in the coding regions of genes has been described to impede transcription by RNA polymerase (Messer and Weigel, 1997). DnaA bound to specific sites in the bacterial genome may likewise induce replication forks to pause or permanently arrest. In the work described here, we also tested the hypothesis that specific sites in the bacterial genome cause replication forks to stop under conditions log-phase growth or hyperinitiation. The results indicate that replication forks do not encounter specific barriers except for the Tus-*ter* complex, in the terminus region, where cessation of fork advancement is expected.

Table 1. Strains and plasmids

Strain	Genotype	Source
MC1061	*araD*139 Δ*ara-leu*)7696 Δ(*lac*)X74 *galU* *galK* *hsd*R2(r_k^-, m_k^+) *str*A *mcr*A *mcr*B1	Lab stock
LS1064	*priA2*::*kan araD*139 Δ*ara-leu*)7696 Δ(*lac*)X74 *galU* *galK* *hsd*R2(r_k^-, m_k^+) *str*A *mcr*A *mcr*B1	This work
JJC315	*leu6 his4 argE3 lacY1 galK2 ara14 xyl5 mtl1 tsx33 rpsL31 supE44 hsdR recB268*::Tn*10*	B. Michel (Michel *et al.*, 1997)
KHG1007	F⁻ λ⁻ IN (*rrnD-rrnE*)1 *rph-1his tus*::Tn*3* *lacZ*::*terL* (Km^r) (nonblocking orientation)	Y. Fujimura (Horiuchi and Fujimura, 1995)
KHG1005	F⁻ λ⁻ IN (*rrnD-rrnE*)1 *rph-1 his tus*::Tn*3* *lacZ*::*terL* (Km^r) (blocking orientation)	Y. Fujimura (Horiuchi and Fujimura, 1995)
Plasmids	**Characteristics**	**Source**
pBR322	amp^r tet^r	Lab stock
pDS596	*dnaA*⁺ p*araBAD*	Lab stock (Hwang and Kaguni, 1988)
pLS120	*dnaAcos* p*araBAD*	Lab stock (Simmons and Kaguni, 2003)
pLST435M	*T435M* p*araBAD*	This work
pLSG426D	*G426D* p*araBAD*	This work
pTus	Tus p*araBAD*	(Peter *et al.*, 1998)

Experimental Procedures

Strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. LS1064 was constructed by P1 transduction with JC19008 (*priA2::kan*; (Sandler, Samra *et al.*, 1996) as the donor strain and MC1061 as the recipient. Transductants were selected at 37°C on LB media supplemented with 25 μg/ml kanamycin. Plasmids pLS*T435M* and pLS*G426D* were constructed by partial *Rsr*II and *Eco*N1 digestion of pDS596 (in which the *dnaA*$^+$ coding region is controlled by the *araBAD* promoter) to gel-purify the vector DNA fragment away from *dnaA* coding sequences. This DNA was ligated to 1.5 kb DNA fragments carrying the *T435M* and *G426D* mutations of *dnaA*, obtained by *Rsr*II and *Eco*N1 digestion of pACHA-49 and pACHA-79. The resulting plasmids are identical in structure to pDS596, but wild type *dnaA* is replaced by these specific alleles.

Preparation of ectopic ter*-blocked chromosomal DNA.* The plasmid pTus, encoding the *tus* gene placed downstream from the *araBAD* promoter, was used to transform strain KHG1005 which carries a *ter* site inserted into *lacZ* in an orientation that blocks replication forks originating from *oriC* when the site is bound by Tus protein (Figure 1). The resulting plasmid-bearing strain was grown at 37°C in a rotary shaker to early log phase in LB media supplemented with 0.2% glucose. Cells were collected by centrifugation and resuspended in LB media containing 0.2% arabinose to induce Tus expression, followed by incubation with shaking for one hr at 37°C . As a negative control, KHG1007, which is isogenic to KHG1005 but carries the ectopic *ter* site inserted at *lacZ* in the nonblocking orientation, was grown to stationary phase, and genomic DNA was prepared as above. Sodium azide was added to the culture to a final concentration of 0.2% (w/v), and the culture was frozen and stored in liquid nitrogen. After concentrating the cells by centrifugation, genomic DNA was prepared using a DNeasy Tissue kit (Qiagen) following the manufacturer's instructions. The yield and purity of the genomic

DNA was determined spectrophotometrically, then digested with *AluI* endonuclease (Promega). A portion of the sample was electrophoresed on a 1% agarose gel to confirm cleavage, as indicated by the appearance of a range of DNA fragments from about 0.5 to 3 kb in size. The remainder of the cleaved DNA was purified with a PCR Purification kit (Qiagen), and the amount of DNA recovered was determined spectrophotometrically. The DNA was then lyophilized and stored frozen until use.

Preparation of chromosomal DNA from cultures induced to express DnaA$^+$ protein. *E. coli* MC1061 bearing pDS596 that encodes *dnaA*$^+$ under *araBAD* promoter control was grown in LB media at 37°C to 0.2 O.D. at 595 nm then the culture was divided. Where indicated, L-arabinose was added to one portion to a final concentration of 1% (w/v) to induce *dnaA* expression. The second portion was transferred to a separate flask, and growth was continued at 37°C without arabinose addition. Both the induced and untreated cultures were harvested by centrifugation after 45 min of continued incubation with shaking, and chromosomal DNA was prepared as described above. To serve as a negative control in the experiment of Table 2 and Figure 1, one portion of the culture was removed just prior to the time of arabinose addition, and genomic DNA was isolated from this sample as described above. Analysis of this sample is shown in Figure 1A.

Quantitative PCR analysis. The samples of purified bacterial chromosomal DNA were then analyzed by microarray (see below), or by quantitative PCR analysis as described (Simmons and Kaguni, 2003). In the latter method, 1 ng of DNA was used as the template in PCR reactions with 40 pmol of each pair of respective primers to amplify *oriC* or *relE*. PCR reactions were assembled according to the manufacturer's recommendations (PCR Master mix, Promega), but contained [α^{32}P]-dATP (1.25 μCi per reaction, 3000 Ci/mmol, Perkin-Elmer), and amplification was for 24 cycles with Taq DNA polymerase (1.25 units). To measure the amount of PCR-amplified DNA, the

radiolabelled product was acid-precipitated with 10% (w/v) trichloroacetic acid onto glass fiber filters (GF/C, Whatman), and radioactivity was quantitated by liquid scintillation spectrometry (Simmons and Kaguni, 2003). Primers to amplify *oriC* are ACAGCGTACAATACGCCACT and ACGGTGAGCACGACGGCTTT. To amplify the *relE* region, the primers are GTTGAAGTACTTGAGTCACC and CATTCAGACTTGAATGCGTG. These primers are identical to those used by Nyborg (Nyborg, *et al.*, 2000) and amplify *relE*, *relF*, and *rem*.

Microarray procedures and data analysis. Microarrays carrying nearly every annotated coding region in the *E. coli* genome were prepared as described (http://www.microarrays.org/protocols.html). Of the 4403 coding regions encoded by the *E. coli* genome, 4184 were successfully amplified for printing. Labeling of genomic DNA and hybridization to microarrays were performed essentially as described (http://www.microarrays.org/protocols.html), except that labeling reactions were allowed to proceed overnight to increase the amount of labeled probe synthesized. Fluorescence intensities of microarray spots were measured using a Genepix 4000B scanner and Genepix 3.0 software (Axon Instruments). Spots were filtered to require that at least 40% of pixels in each spot be at least one standard deviation above background in both channels. Smoothed trajectories of gene dosage versus chromosome position were calculated as a moving median with a window of 101 loci. Filtering, smoothing, linear regression, and plotting of microarray data were performed using the R language and environment, version 1.3.0 (http://www.r-project.org). As some experiments involved induced expression of *dnaA* or tus from a multicopy plasmid, the signals due to annealing of these sequences to the printed array were not included in the analysis.

Pulse field gel electrophoresis. To measure the amount of linear chromosomal DNA that reflects the steady-state amount of double strand breaks in the indicated strains, pulse field gel electrophoresis (PFGE) was in 1% agarose gels (Chromosomal Grade

Agarose, BioRad) in buffer composed of 45 mM Tris-borate (pH 9) and 1 mM EDTA with a CHEF-DRII apparatus (BioRad). Electrophoresis was for 16-20 hrs at 160 or 180 volts with a switching time ramped from 3 to 30 sec, as described (Michel, Ehrlich *et al.*, 1997). Samples were prepared by adding 1.5% agarose (low melting point, Gibco) to an equal volume of cells (3×10^8; 0.4 O.D. at 595 nm) in 10 mM Tris-HCl pH 8, 0.1 M EDTA, 10 mM EGTA (TEE buffer). If the samples were not analyzed immediately, they were stored at 4°C and used within 7 days. *S. cerevisae* chromosomal DNA (BioRad) or a mixture of concatamers of λ DNA (lambda ladder PFG marker; New England Biolabs) were used as molecular weight standards for each PFGE experiment and also to confirm that the conditions of electrophoresis were appropriate. Gels were stained with 0.5 μg/ml ethidium bromide, extensively destained, then photographed with a Kodak DC290 camera. Image analysis was with Kodak EDAS 120 software and the data was replotted with a graphing program (DeltaGraph Pro 3). The data of Figure 3 is a quantitative analysis of fluorescence staining of the DNA with ethidium bromide, and the background is equivalent to a portion of the gel that lacks DNA.

Results

Do replication forks stall at specific sites? To test the hypothesis that specific sites in the bacterial genome may function to arrest replication fork movement under the conditions of hyperinitiation, microarray analysis was performed. This quantitative method measures the abundance of specific gene sequences, and is typically used to measure RNA expression levels under regulated conditions. In our experiments, microarray analysis was used to measure the relative copy number of essentially every open reading frame of the *E. coli* chromosome, offering the possibility of demonstrating in a population of cells whether replication forks pause or arrest at specific sites in the genome. Genomic DNA was purified from cultures of *E. coli* MC1061 (relevant genotype: Δ*araBAD*) harboring a plasmid with the *dnaA*$^+$ gene under *araB* promoter control (pDS596; Table 1). One sample was isolated from the culture at early log phase, just prior to induction (Figure 1A). The culture was then divided, and arabinose was added to one portion to induce expression of *dnaA*$^+$ (Figure 1B). The second was mock-treated to serve as a control (Figure 1C), and both were incubated for an additional 45 min. In other experiments (data not shown), this length of induced *dnaA*$^+$ expression gave rise to the highest frequency of initiation as reflected by the ratios of *oriC* to *relE* (a site near *terC*), measured by quantitative PCR analysis (see below). The DNAs were then annealed to microarrays of *E. coli* genes to measure the relative abundance of each open reading frame. The data from the induced and uninduced samples were normalized relative to data from genomic DNA isolated from stationary phase cells by dividing the abundance of each locus by that from the stationary phase sample. The data were further normalized by expressing the number of copies of a specific locus as a ratio to the averaged abundance of loci in the terminus region where *relE* is located (see Table 2 legend). The averaged value of markers in the terminus region was used to represent the

Figure 1. Elevated *dnaA* expression induces hyperinitiation. Microarray analysis of chromosomal DNA was performed as described in "Experimental Procedures." The horizontal axis in each panel represents the position on the chromosome in map minutes (one map minute is 1% of the genome, or 46.4 kb). Except where noted, *oriC* is at zero minutes to facilitate visualization of replication fork density, resulting from bidirectional fork movement from *oriC*. The terminus region is at both the left and right margins. The vertical axis represents relative gene dosage on a base 2 logarithmic scale, so that a difference of one unit indicates a two-fold change in abundance. Turquoise points are raw values for individual microarray spots, and plots of smoothed values from a moving median are in purple. Dashed orange lines are least-squares best fit lines to the purple traces, drawn to help show that the gene dosage patterns fall along a straight line. *E. coli* MC1061 carrying the *dnaA*$^+$ plasmid, pDS596, was grown at 37°C with aeration in LB media. The microarray used to determine fork movement was prepared from total *E. coli* DNA, and of the 4403 expressed genes of *E. coli*, 4184 (95%) were successfully amplified for printing. The abundance of loci from induced and uninduced samples was normalized relative to their abundance in genomic DNA from a stationary phase sample. These values were then normalized by dividing by the average gene dosage of loci in the terminus region within 92.8 kilobase pairs (2 map minutes) flanking the point on the chromosome diametrically opposite *oriC*. The mean abundance of loci in the terminus region was used to represent the abundance of *relE*. Because *oriC* is a cis-acting site, its abundance could not be determined. As an alternative, the *gidA* and *mioC* genes that directly flank *oriC* (see Figure 2) were measured. The ratios of *gidA* and *mioC* relative to the average gene dosage of loci in the terminus region were then calculated, with the *gidA*/*relE* and *mioC*/*relE* ratios for samples from the stationary phase culture normalized to 1.0. Panel **A** is the sample from the culture removed at early log phase prior to induced *dnaA* expression. The gene dosage pattern shows straight lines descending from the origin and is indicative of normal, exponential growth. After this sample was removed, the culture was divided and arabinose was added (1% w/v final concentration) to one portion of the culture followed by incubation for 45 min before the cells were harvested and genomic DNA isolated for analysis (Panel **B**). The second portion was mock-treated (no arabinose addition) and incubated with shaking at 37°C for an additional 45 min (Panel **C**). In Panel **D**, *E. coli* KH1005 (relevant genotype, *lacZ*::*terL*) carrying pTus was grown as above to midlog phase, and arabinose (1% w/v final concentration) was added to induce *tus* expression (see "Experimental Procedures"). After continued growth for 1 hr, cells were pelleted and DNA prepared as described in "Experimental Procedures" for microarray analysis. This strain has a *ter* site inserted into *lacZ* (25 minutes) oriented to block clockwise-moving replication forks. The gene dosage pattern shows clear deviations at the *lacZ* locus (red line) and the natural terminus region where the positions of *terB* and *terD* are indicated by black vertical lines. The

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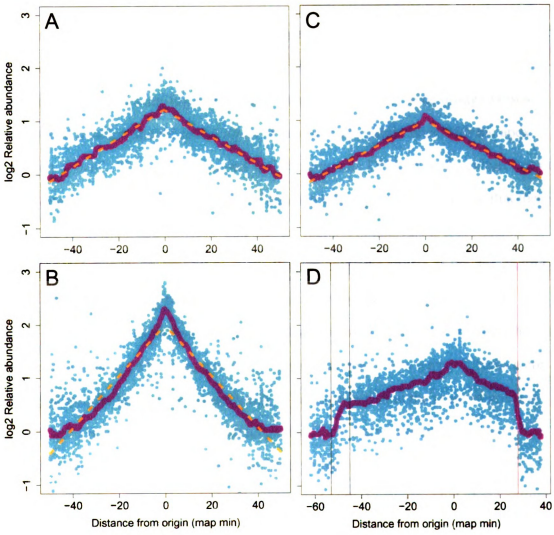
Figure 1. Legend continued

boundaries of this plot were shifted so that half of the blocked region between *lacZ* and the terminus region is shown on each side of *oriC*.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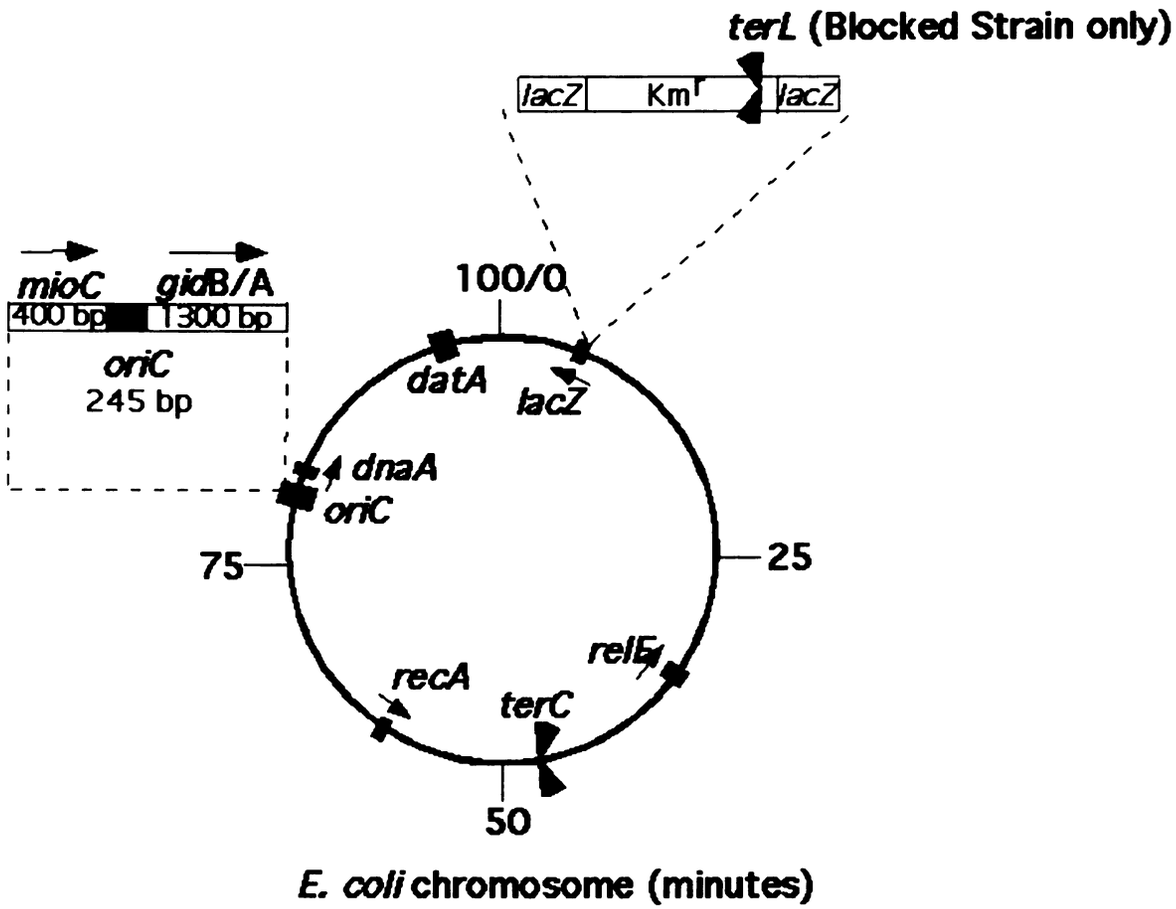
log2 Relative abundance
Distance from origin (map min)
Distance from origin (map min)

abundance of *relE*. Because *oriC* does not encode a gene product, adjacent genes (*mioC* and *gid* for *oriC*) were measured.

In wild type *E. coli* cultures under exponential growth, the relative abundance of loci diminishes exponentially with increasing distance from *oriC* (Cooper and Helmstetter, 1968). A semi-log plot of the relative abundance of gene dosage versus chromosomal position should reveal a pair of descending straight lines with the apex originating at *oriC*. The results obtained with the genomic DNA from the culture before induced *dnaA*$^+$ expression, or mock-treated confirm this expectation, showing that these samples are from log phase cultures, and that the method of analysis is reproducible (Figure 1). Relative to a vertical line drawn through *oriC*, the angles of the descending lines are similar, indicating that replication forks move bidirectionally at the same average speed. Of the samples in which DnaA$^+$ was overexpressed, we expected the distribution of loci to differ from that of the controls in one of two ways. If forks were arresting at specific sites, a downward break in the distribution at the point of arrest is expected (see Figure 1D). If sites of arrest are distributed across the chromosome, the distribution should show a global deviation from exponentiality. In Figure 1B, the abundance of loci no longer resembles that observed with the exponential phase cultures (Figure 1A, C), and the ratio of markers flanking *oriC* to *relE* is more than twice that of the uninduced culture. The results show that, at elevated DnaA, replication forks do not stall at *datA* or other specific sites, but appear to stop randomly throughout the chromosome. The steeper slope near *oriC* indicates that the replication forks induced by hyperactive initiation do not progress very far from this site. Indeed, the sequence abundance of markers near *terC* in this sample was comparable to that of the uninduced control, providing additional support to the view that replication forks fail to progress to the normal terminus region. Also, no

Figure 2. Map of the *E. coli* chromosome. The locations of *oriC*, *relE* and the ectopic *ter* site inserted at *lacZ* of strain KHG1005 (*lacZ*::*terL tus*::Tn*3*) are shown . This strain is a derivative of W3110, which carries a large chromosomal inversion between 73.8 and 90.7 min, placing *oriC* at 79.9 min. The distance from *oriC* to *lacZ* is 27.9 min. In KHG1007, *terL* is inserted at *lacZ* in the nonblocking orientation.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oriC
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datA
lacZ
dnaA
oriC
75
25
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terC
50
E. coli chromosome (minutes)

specific site appears to hinder replication fork movement when *dnaA*$^+$ expression was not induced.

In order to demonstrate that our method is capable of detecting forks stalled at a specific location, microarray analysis was performed with genomic DNA isolated from a strain bearing an ectopic *ter* site inserted at the chromosomal *lacZ* locus (Figure 2). Tus protein bound to *ter* sites in the vicinity of the replication terminus normally functions to block replication forks from escaping the terminus region (see Bussiere and Bastia, 1999 for a recent review). The ectopic Tus-*ter* complex is oriented to block replication forks at *lacZ* in a strain lacking the chromosomal *tus* gene and carrying a plasmid in which *tus* is regulated by the *araBAD* promoter. Genomic DNA isolated from the strain (KHG1005) induced for one hr to express Tus protein was annealed to the *E. coli* microarray, and the results were normalized as above to microarray data obtained from genomic DNA isolated from an isogenic strain (KHG1007), but carrying *lacZ*::*terL* in the nonblocking orientation, and grown in the absence of the *tus* plasmid to stationary phase. The plot of gene dosage versus chromosomal position (Figure 1D) reveals breaks at the ectopic *ter* site (red line) and the chromosomal terminus region (black lines). The results from these experiments confirm that the microarray method is capable of detecting forks stalled at specific sites on the bacterial chromosome.

The microarray results were confirmed by quantitative PCR assays in which we measured the abundance of *oriC* and *relE*, a locus near the replication terminus. In a recent report, we established conditions for quantitative PCR assays (Simmons and Kaguni, 2003). We showed that the DNA amplified was specific for *oriC* or *relE* with no false priming, that the level of amplification was proportional to the amount of bacterial chromosomal DNA, and that the ratio of *oriC* to *relE* on induced *dnaA*$^+$ expression

Table 2. Analysis by microarray and quantitative PCR of *oriC* or adjacent loci and *relE*.

Sample	Microarray[a] *gidA/relE*	Microarray[a] *mioC/relE*	Quantitative PCR[b] *oriC/relE*
stationary phase	1.0	1.0	1.0
early log phase no arabinose	2.4	2.4	2.3± 0.1
no arabinose 45 min growth	2.1	2.1	2.9± 0.3
1% arabinose 45 min induced expression	5.0	4.9	5.1±0.1

[a] Please see the legend to Figure 1.

[b]The *oriC/relE* values of genomic DNA isolated from MC1061 (pDS596) were normalized to the ratio of these loci in genomic DNA from this strain grown to stationary phase, normalized to 1.0. Each PCR reaction was performed in duplicate to calculate the standard deviation. The *oriC/relE* ratio for DNA from the stationary phase culture was 1.3. Cells in early stationary phase generally contain one complete chromosome, with a small fraction of cells carrying one partially replicated chromosome.

Table 3. Replication fork restart genes *priA* and *recB* are required for viability under conditions of hyperactive initiation

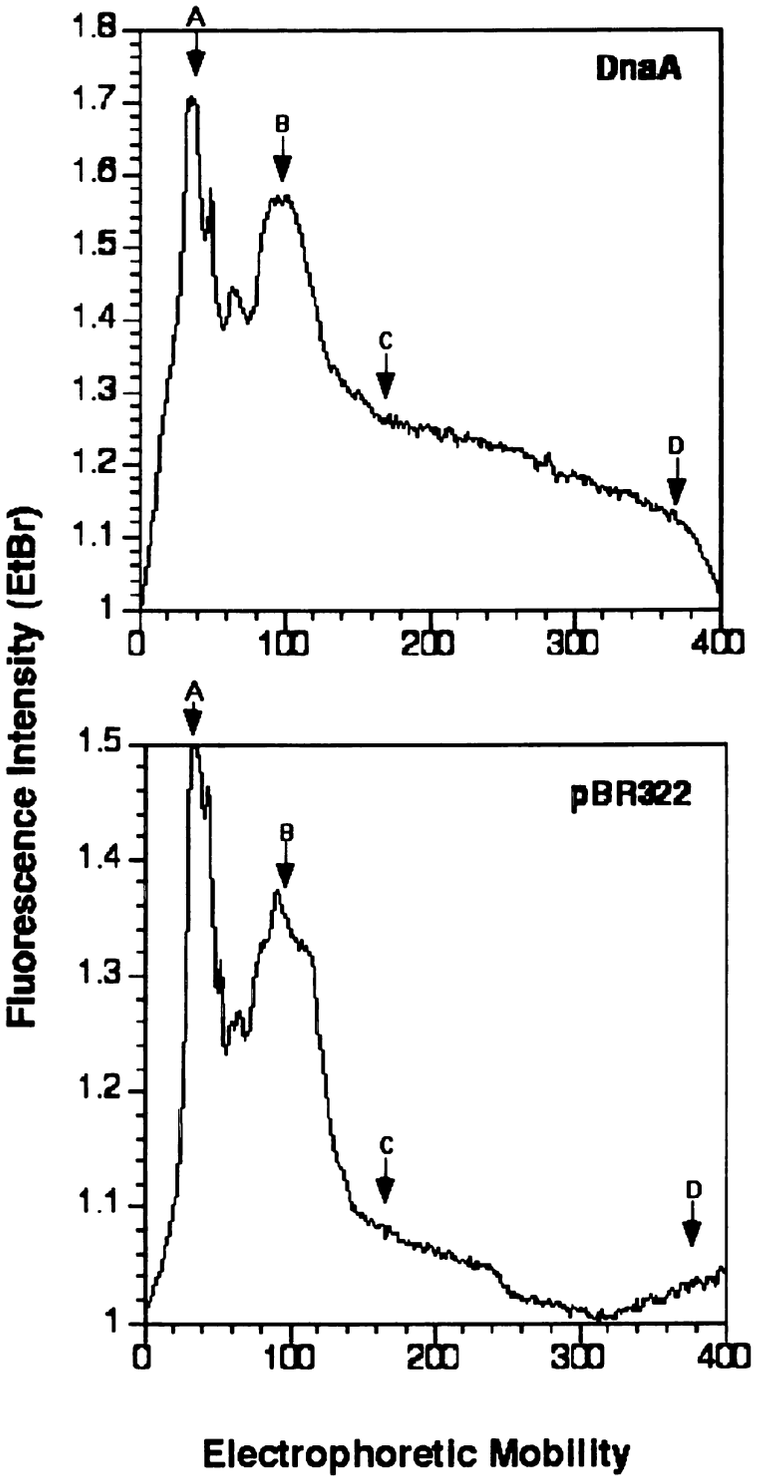
Plasmid	Allele	Plating Efficiency[a]		
		MC1061 (*recB*$^+$, *priA*$^+$)	LS1064 (*recB*$^+$, *priA2*::kan)	JJC315 (*recB*::Tn*10*, *priA*$^+$)
pDS596	*dnaA*$^+$	1.0	1.0×10^{-3}	2.7×10^{-3}
pBR322	None	0.98	0.97	1.2
pLS120	*dnaAcos*	9.5×10^{-3}	5.3×10^{-4}	4.4×10^{-4}
pLST435M	*T435M*	1.0	0.97	1.0
pLSG426D	*G426D*	1.1	1.2	1.0

[a]The indicated plasmids were used to transform the respective host strains by electroporation with a BioRad *E. coli* Gene Pulser following the manufacturer's instructions. Incubation was at 37°C until colonies were visible on the plates lacking arabinose (18-20 hr). MC1061 in the absence of arabinose was transformed with an efficiency ranging from 10^6-10^7 per μg of the various plasmid DNAs. The *recB* mutant was transformed with an efficiency of 2×10^5 per μg of the respective plasmid DNAs in the absence of arabinose. We confirmed the observations of others that strains lacking *priA* grow poorly on rich media compared to minimal media (Nurse, Zavitz *et al.*, 1991). We noted that transformation efficiency of the *priA2*::*kan* strain by the *dnaA*-encoding plasmids in the absence of induction on LB media (6×10^4 transformants per μg of plasmid DNA) was reduced compared to M9 media supplemented with glycerol (0.5% w/v) and casamino acids (0.5% w/v). Plating efficiency is described as a ratio of colony forming units obtained in the presence of arabinose to the number obtained in the absence of arabinose.

increased linearly with time up to 45 min then approached a plateau. The ratios of *gidA* and *mioC* (genes flanking *oriC*) to *relE* obtained by microarray were normalized by their division by the ratio of these loci in stationary phase cells (Table 2). The ratio of *oriC* to *relE* obtained by quantitative PCR analysis was similarly normalized. Their comparison reveals that the results are in good agreement to support the conclusions that the methods reflect the relative abundance of genetic loci, and that induced expression of *dnaA*$^+$ leads to hyperinitiation.

Hyperactive initiation interferes with viability in the absence of recombinational repair. The results described above are presented under the model that replication forks induced by hyperinitiation appear to be "arrested" as opposed to being transiently paused. The evidence that these forks do not progress to completion is based on the following observations. In experiments comparable to those of Figure 1 but under prolonged incubation after induced *dnaA*$^+$ expression (in MC1061 carrying the *dnaA*$^+$ expression plasmid, pDS596), we found that the array pattern was very similar to that of Figure 1B (data not shown). The gradient of loci from *oriC* toward the terminus region was only slightly reduced. In a control culture (MC1061 bearing pDS596) grown in parallel for the same time period but not induced, the culture had reached stationary phase as reflected by the lack of increase in culture turbidity, and by the microarray pattern. The latter revealed approximately equivalent abundance of all *E. coli* genes regardless of their chromosomal locations (data not shown). These results suggest that whereas some arrested forks induced by hyperinitiation can be resuscitated, a substantial fraction do not proceed to completion during this time period. Moreover, the absolute abundance of loci near the terminus region was comparable to the stationary phase sample, strongly suggesting that the forks are indeed arrested. The persistence of these arrested forks suggests that the replication restart pathway is inefficient. Inasmuch as viability as

Figure 3. Pulse field analysis of chromosomal DNA after induced expression of wild type or mutant DnaA protein. *E. coli* JJC315 (relevant genotype, *recB*::Tn*10*; (Michel, Ehrlich *et al.*, 1997) carrying the respective *dnaA* plasmids or pBR322 was grown as described in the legend to Table 4. Chromosomal DNA from 3 x 10^8 cells (0.4 O.D. at 595 nm) was analyzed by pulse field gel electrophoresis as described in "Experimental Procedures." DNA at "**A**" corresponds to the circular DNA that remains in the agarose plugs at the migration origin (Birren and Lai, 1993). DNA at " **B**" corresponds in size to 4-6 megabase pairs that has been attributed to spontaneous chromosome breakage observed in *recB* mutants . The region between arrows **C** and **D** is linear DNA in the size range from 750 to 40 kilobase pairs, respectively.



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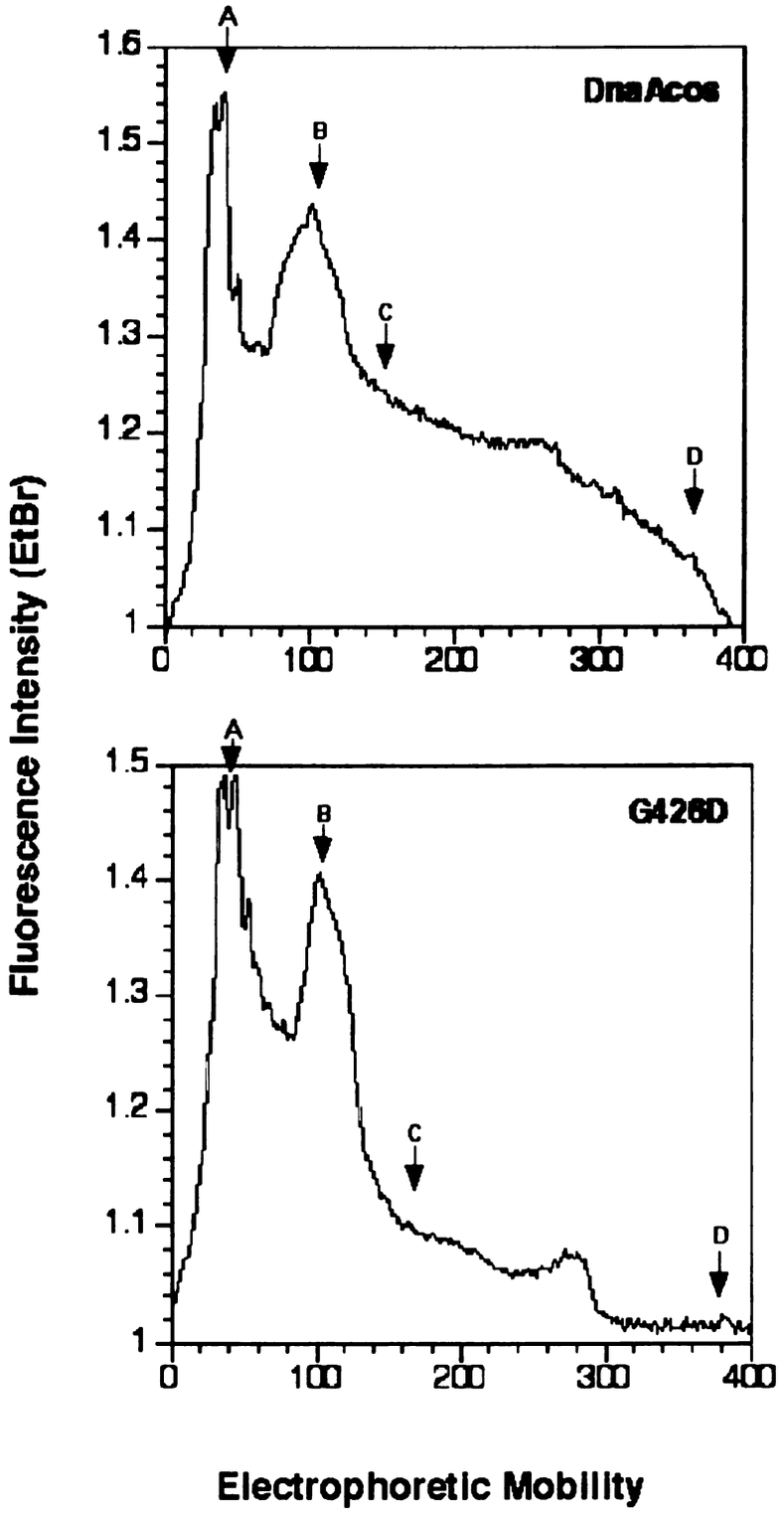
DnaAcos
G426D
A
B
C
D
Fluorescence Intensity (EtBr)
Electrophoretic Mobility

measured by colony formation, is preserved on induced *dnaA*$^+$ expression (Table 3), we infer that the longer time period for colony growth is sufficient to repair these damaged forks. (Please see "The replication restart pathway" in the "Discussion" also.)

It is well-established that blocked replication forks can regress to form Holliday junctions by the annealing of nascent DNA strands (reviewed in Michel, Flores *et al.*, 2001). RuvAB bound to the junctions can support further branch migration; RuvC cleavage induces double strand breaks (DSBs). Current models on DSB repair in *E. coli* invoke that DSBs are lethal because of the inability to duplicate the DNA beyond the break. In support, linear bacterial DNA accumulates in *recB* mutants due to impaired homologous recombination and recombinational DNA replication (Seigneur, Bidnenko, *et al.*, 1998). Based on the microarray results, it is likely that prematurely initiated replication forks catch up and collide with more slowly moving or paused replication forks in front (replication fork collapse), as well as with replication forks that have regressed and been processed subsequently into DSBs. If this explanation is correct, induced expression of *dnaA*$^+$ should be lethal in strains deficient in recombinational DNA replication. As a test of this model, *dnaA*$^+$ expression was induced in strains carrying *recB* and *priA* mutations and viability was compared to a wild type control (Table 3). To confirm published observations (Atlung and Hansen, 1993; Skarstad, Lobner-Olesen *et al.*, 1989), elevated *dnaA*$^+$ expression did not interfere with viability of the wild type strain. In contrast, viability was greatly reduced when *dnaA*$^+$ expression was in a *recB* (implicating RecBCD) or *priA* mutant. Elevated expression of *dnaA* alleles (*T435M*, and *G426D*;(Sutton and Kaguni, 1997a,b) known to be inactive in DNA replication from *oriC* had a benign effect on viability in these mutant strains, indicating that growth interference is due to wild type *dnaA* function and elevated initiation. (As will be shown below, *G426D* also does not elevate the level of DSBs.) By comparison, induced

Table 4. Double strand breaks accumulate in *recB*⁻ strains due to hyperactive initiation.

Plasmid-Borne Allele	Plasmid	Relevant Genotype	% Linear DNA[a]
dnaA⁺	pDS596	*recB*⁻	31.5±2.1
dnaAcos	pLS120	*recB*⁻	42.3±2.0
G426D	pLS*G426D*	*recB*⁻	16.4±1.4
None	pBR322	*recB*⁻	16.5±0.8
None	None	*recB*⁺	4.1±2.2

[a] Cultures of JJC315 (relevant genotype, *recB*::Tn*10*) or MC1061 (*recB*⁺) bearing the indicated plasmids were grown in LB media at 37°C with shaking to mid-log phase (0.2 O.D. at 595 nm), and arabinose (1% w/v final concentration) was added followed by incubation for 1 hr to induce expression of the corresponding proteins. At the end of this incubation, the culture turbidity among these samples was similar (0.7-0.8 O.D. at 595 nm). Chromosomal DNA was analyzed by PFGE analysis (see "Experimental Procedures"). The amount of linear chromosomal DNA (in percent) in the range from 750 to 40 kilobase pairs was quantitated by ethidium bromide staining intensity and is expressed as a percentage relative to the total amount of DNA in each lane. Three independent experiments were performed to calculate the standard deviation.

expression of *dnaAcos* interfered with viability of the wild type host strain, but growth interference was reduced only by about 100-fold. Our experience with a *dnaAcos* mutant (encoded by the chromosome) of a reduction in viability at 30°C compared to 42°C by 6 x 10^{-5} suggests that the growth interference described in Table 3 is not as dramatic because this activity is partially attenuated at 37°C, even despite overproduction of the mutant protein. Nevertheless, this effect was exacerbated in either a *priA*2::*kan* or a *recB*::Tn*10* mutant. These results support the interpretation that hyperinitiation leads to replication fork collapse instead of arrest as discussed above . Furthermore, the lethal effect of *dnaAcos* or *dnaA*$^+$ in either a *recB* or *priA* mutant appears to be due to the failure to repair DSBs, prompting the following experiments.

RecBCD protein functions in DSB repair by processing duplex DNA ends, directing the binding of RecA to the resultant single-stranded DNA to promote homologous recombination (Anderson and Kowalczykowski, 1997). If the reduced viability due to increased expression of *dnaA*$^+$ or *dnaAcos* (Table 3) is due to the failure to repair DSBs, the level of linear chromosomal DNA under these conditions should be elevated. To test this prediction, pulse field gel electrophoresis (PFGE) was performed. This method has been used extensively to quantitate the level of DSBs that result from mutations in genes involved in DNA metabolism (reviewed in Michel, Flores *et al.*, 2001).

As a control for the method, chromosomal DNA from log phase cultures of a *recB*$^+$ strain and *recB* mutant carrying a plasmid lacking *dnaA* (pBR322) was analyzed (Table 4). We quantitated the amount of linear DNA by measuring fluorescence intensity of ethidium bromide-stained DNA, as opposed to the method of measuring the amount of radioactively labelled DNA in gel slices (Seigneur, Bidnenko *et al.*, 1998) because the equipment for PFGE was borrowed. DNA from 750-40 kilobase pairs was selected for analysis because the fluorescence intensity of *S. cerevisiae* chromosomal DNA markers

within this range was proportional to their sizes. For larger DNAs that were poorly resolved, we were not confident that their fluorescence corresponded to their respective molecular weights. Below 40 kilobase pairs, very little fluorescent material was detected. The amounts of linear DNA in each sample confirm values previously reported for a wild type and *recB* mutant (4% and 16%, respectively; (Michel, Ehrlich *et al.*, 1997), confirming that the method of analysis is appropriate. In a *recB* mutant induced for hyperinitiation by elevated levels of DnaA$^+$ or DnaAcos, the elevated amount of linear chromosomal DNA compared to the empty vector control (2- or 2.6-fold, respectively; Table 4, Figure 3) is indicative of DSBs that arise from replication fork collapse and possibly also from the cleavage of regressed replication forks by RuvC. Compared to the *recB* mutant harboring pBR322, induced expression of a mutant protein bearing an aspartate residue at glycine 426 (G426D) did not affect the amount of linear DNA detected (Table 4, Figure 3). Because the mutant protein is inactive in DNA replication due to its inability to bind to the chromosomal origin (Sutton and Kaguni, 1997a,b) this result confirms that growth interference due to elevated *dnaA*$^+$ or *dnaAcos* expression is due to the failure to repair DSBs.

Discussion

Replication forks do not appear to stall at specific sites. Because accumulated evidence indicates that replication forks arrest under normal growth conditions, we examined whether specific sites in the bacterial genome functioned to impede replication fork movement. An attractive candidate is *datA* at which 370 DnaA monomers are estimated to bind (Kitagawa, Mitsuki *et al.*, 1996; Kitagawa, Ozaki *et al.*, 1998; Ogawa, Yamada *et al.*, 2002). This site is proposed to titrate excess DnaA protein to prevent overinitiation. As additional support that *datA* may hinder fork movement, others have described that DnaA bound to duplex DNA impedes RNA polymerase as it transcribes DNA (Roth and Messer, 1998). Using microarray technology which measures the abundance of essentially every open reading frame of the *E. coli* genome, our results support the conclusion that replication forks are not blocked at specific sites except at *ter* bound by Tus at both an ectopic location and at sites in the terminus region. This conclusion is even under an elevated level of DnaA in which the *datA* locus should be fully occupied by DnaA. Apparently, replication fork movement is insensitive to nucleoprotein complexes other than the Tus-*ter* complex.

Under the stringent response in *B. subtilis*, replication forks arrest at replication checkpoints located about 200 kilobase pairs on either side of *oriC* (Autret, Levine *et al.*, 1999; Levine, Vannier *et al.*, 1991). A more recent study of the replication checkpoint terminator to the left of *oriC* showed that the sequence is bound by the replication terminator protein of *B. subtilis*, but that the function of this sequence *in vivo* is independent of stringent control (Gautam and Bastia, 2001). Although we did not investigate the effect of the stringent response on replication fork arrest, our results suggest that the *E. coli* genome does not contain specific sites that function as replication checkpoints.

Hyperinitiation induces replication fork collapse. We showed that elevated levels of DnaA$^+$ protein do not interfere with viability in a wild type host strain, and that this condition induces hyperinitiation from *oriC* (Table 5, Table 2, Figure 1). When analyzed by microarray, replication forks accumulate in the vicinity of *oriC* (within about 15 map units flanking *oriC*, Figure 1). The dependence on *priA* and *recB* for viability (Table 3) and the increased amount of linear chromosomal DNA in a *recB* mutant (Table 4) indicate that replication forks have collapsed and also have regressed to be processed by RuvC. Whereas we did not assess the proportion of linear DNA that accumulates due to fork regression upon elevated initiation in the *recB* mutant, it is attractive to consider that replication forks formed by hyperinitiation collide from behind with stalled replication forks to cause replication fork collapse. If replication forks do not move at constant speed, it is conceivable that replication fork collapse occurs when a speedy fork catches up to a laggard. As another possibility, replication fork collapse may occur if the sealing of Okazaki fragments is slow. In this model, a newly initiated replication fork collapses when it encounters the discontinuity separating nascent Okazaki fragments synthesized on the lagging strand template (Zieg, Maples *et al.*, 1978). None of these models explains why collapsed replication forks are more abundant near *oriC*.

In a recent report (Grigorian, Lustig *et al.*, 2003), elevated *dnaA*$^+$ expression was described to reduce viability when the host strain carried a mutation in one of a number of genes (*recA*, *recB*, *recC*, *recF*, *ruvA* or *ruvC*). These genes function in homologous DNA recombination and in the repair of double strand breaks. In our study, we present direct evidence that DSBs are produced by hyperinitiation, supporting the model that newly initiated replication forks collide from behind with those from previous initiations. This is an alternate mechanism of replication fork collapse as other models invoke a stalled fork due to a lesion in DNA or inactivity of a component of the replication fork machinery.

Whereas the observations above show that hyperinitiation leads to replication fork collapse, forks occasionally abort or collapse when *E. coli* is propagated under normal growth conditions, requiring the reassembly of the replication fork machinery to complete duplication of the genome and to maintain viability . A provocative notion is that the period of sequestration of *oriC* after initiation serves to allow collapsed replication forks to restart so that a trailing replication fork is unlikely to run into the one ahead. The premise for this idea is based on studies done with yeast. In the DNA replication checkpoint response, Rad53 and Mec1 in *S. cerevisiae* and their orthologues are proposed to protect stalled or slowly moving replication forks from experiencing DSBs and potentially lethal genomic rearrangements until the repair of damaged DNA is finished (Cha and Kleckner, 2002; Sogo, Lopes *et al.*, 2002).

Lethality of dnaAcos. In rich media, induced expression of heterologous genes under *araBAD* promoter control is elevated several hundred-fold (Guzman,Belin *et al.*, 1995). Interestingly, this magnitude of DnaA$^+$ overproduction is not lethal in a wild type host strain (Table 3). By comparison, DnaAcos expression when chromosomally encoded interferes with viability at 30°C . Its phenotype has been attributed to hyperinitiation. Because the *dnaAcos* allele carried in the bacterial chromosome is difficult to manage, often giving rise to spontaneous extragenic suppressors that compensate for its phenotype of growth interference, we placed it under *araBAD* promoter control . On measuring the level of initiation by the ratio of *oriC* to *relE* by quantitative PCR, elevated DnaAcos protein at 30°C yielded an *oriC*-to-*relE* ratio of 10.1 ± 1.1, 2.7-fold greater than that obtained with DnaA$^+$ protein at a comparable level (*oriC*-to-*relE* ratio of 3.7 ± 0.1). These observations strongly suggest that the *dnaAcos* allele fails to respond to a regulatory signal whereas the lesser magnitude of hyperinitiation by DnaA$^+$ suggests that it responds to negative regulation. In the present study, we showed that induced expression of *dnaAcos* interferes with viability (Table 3), and results in an

increased amount of broken chromosomal DNA in the absence of *recB* function (42% linear DNA compared to 16% for the empty vector control, Table 4). These observations support the conclusion that the lethality of *dnaAcos* is due to a level of hyperinitiation beyond that seen for the *dnaA*$^+$ allele, followed by the accumulation of collapsed replication forks that are not efficiently processed by the replication restart pathway.

The replication restart pathway. Under conditions that cause replication fork collapse and double strand breaks in DNA, it has been proposed that the viability of *recA* null mutants is due to degradation of linear chromosomal DNA back to *oriC* where DNA replication can be reinitiated . We examined the effect of DnaA$^+$ overproduction in a *recA* null strain (JJC16, relevant genotype Δ(*recA-srl*)::Tn*10*; (Michel, Ehrlich *et al.*, 1997)), and observed that viability was not affected, in agreement with this mechanism. Despite this, the colonies that arose were smaller than those observed when *dnaA*$^+$ expression was induced in a *recA*$^+$ host. Colonies obtained in the presence of arabinose are smaller than those obtained in the absence of induced expression. This result suggests that the growth of each colony, the product of multiple cell cycles, is retarded due to the time required to repair collapsed replication forks with each round of DNA replication.

Hyperinitiation in eukaryotes. Recently, the structure of the C-terminal two-thirds of a DnaA orthologue from *Aquifex aeolicus* was determined and shown to be very similar to the structure of Cdc6/Orc1 from *Pyrobelicum aerophilum* (Erzberger, Pirrucello *et al.*, 2002). Assuming that the structure of *S. cerevisiae* Cdc6 (and Cdc18 of *S. pombe*) is similar to this archael orthologue, it is remarkable that Cdc6 and DnaA share conserved structures and functions. In *S. cerevisiae*, Cdc6 (and Cdc18 in *S. pombe*) recruits Mcm 2-7 helicase onto chromatin to assemble the pre-replicative complex (Tanaka, Knapp *et al.*, 1997; You, Komamura *et al.*, 1999). DnaA performs a similar

role in recruiting DnaB helicase to *oriC* (Marszalek and Kaguni, 1994; Sutton, Carr *et al.*, 1998).

Liang and Stillman described a *cdc6* allele (*cdc6-3*) that showed an abnormal increase in nuclear DNA content (>2C) in both asynchronously growing cells at nonpermissive temperature, as well as in mutants released from an α factor block at permissive temperature (Liang and Stillman, 1997). These investigators suggested that Cdc6-3 fails to respond to the negative control exerted by mitotic cyclins. It is interesting to consider the similar defects of *cdc6-3* and *dnaAcos*, and that the inviability of the *cdc6-3* mutant is due to the failure to repair collapsed replication forks induced by more frequent initiations.

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Chapter VI

Summary and Perspectives

The genetic and biochemical analysis of numerous *dnaA* alleles has provided important details revealing the biochemical functions of DnaA protein that are 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). It has been suggested that the N-terminal region of DnaA protein is important for both oligomerization and interaction with the replicative helicase DnaB (Sutton, Carr et al. 1998; Seitz, Weigel et al. 2000; Mima, Makise et al. 2002). Specific amino acids have not been identified that are required for oligomerization, and oligomerization has not been demonstrated as a required function for replication from the bacterial origin. Here, we identified three amino acids at the N-terminal region that are required for DnaA protein oligomerization, and this activity was demonstrated to be required for replication from *oriC*. These results suggest that the first 10 amino acids of the N-terminal region are important for oligomer formation, and substitution of only certain amino acids in this region results in a failure of DnaA protein to form higher order complexes. Based on homology modeling of putative DnaA protein monomer interaction, it has been suggested that DnaA protomers may form a hexamer at *oriC* (Erzberger, Pirruccello et al. 2002). We were unable to clearly define a hexamic complex formed by DnaA protein, although oligomeric species corresponding to dimers, trimers and tetramers were observed.

The alleles encoding the N-terminal amino acid substitutions described above can be grouped into a category of *dnaA* alleles that are defective for DNA replication. A few hundred of these alleles have been described, yet only two alleles have been clearly identified that encode a more active form of DnaA protein (Kellenberger-Gujer, Podhajska et al. 1978; Braun, O'Day et al. 1987). The most well characterized is the *dnaAcos* allele. This allele encodes four amino acid substitutions (Q156L+ A184V+H252Y+Y271H). The contribution of these individual amino acid changes to

the increase in replication activity observed with DnaAcos had not been examined. It seemed unlikely that all four missense mutations contributed to the *dnaAcos* phenotype, and we felt that determining the amino acid substitutions responsible for the *dnaAcos* phenotype would reveal the requirements for hyperactive initiation. In this work, we determined that the A184V and Y271H amino acid substitutions were responsible for the phenotype elicited by *dnaAcos*. This work indicated that the major contributing factor is a decrease in ATP binding activity of the mutant protein, and the Y271H substitution appears to stabilize the activity of the A184V bearing protein at elevated temperatures. This result underscores the importance of ATP binding as a regulatory factor that limits the frequency of initiation.

After determining the mutations required for the *dnaAcos* phenotype we decided to identify novel alleles of the *dnaA* gene with similar behavior. These experiments were intended to determine the possible causes of hyperactive initiation. In this study, we identified seven novel hyperactive *dnaA* alleles. These alleles were studied genetically and we determined that they are defective in the regulation of initiation from the bacterial origin of replication, *oriC*. It appeared as though mutant alleles had been isolated that encoded amino acid substitutions affecting several different functional domains of DnaA protein. Homology modeling revealed that several of the amino acid changes were in close proximity to the site for ATP binding. Despite this, amino acid substitutions were isolated in the N-terminus (G79D), and in the helix-turn-helix (HTH) DNA binding (E445K) motif of DnaA protein. These studies indicate that DnaA protein requires at least three biochemical activities to initiate DNA replication with the appropriate frequency.

The mechanism that causes lethality after hyperactive initiation has never been identified. It has been suggested that replication forks stall because a processivity factor for the replicase has become limiting. Although this explanation is possible, it seems

unlikely because overexpression of the beta clamp (required for processive DNA synthesis of DNA polymerase III Holoenzyme) does not suppress *dnaAcos* (data not shown) or restore the chromosome content to wild type cell profiles after DnaA protein overexpression. To determine the pattern of replication fork stalling we used genomic microarrays to analyze the replication fork distribution during normal culture growth and under-conditions inducing hyperactive initiation. This study revealed that hyperactive initiation causes replication fork stacking near *oriC*. This suggested to us that viability might require replication fork restart and homologous recombination proteins to repair, and then restart stalled replication forks. These expectations were verified by determining that DnaA protein overexpression was lethal in strains deficient in homologous recombination and replication fork restart. We verified these results by visualizing linear DNA accumulation in *recB*$^-$ cells (deficient in homologous recombination and linear DNA degradation by exonucleaseV activity contained in the RecBCD enzyme). These results indicate that, when replication forks are released from *oriC* with an increased frequency, the consequence is damage to genome integrity.

The data obtained in these studies raises several important questions that are relevant to our understanding of replication initiation in *E. coli*. Determination of the N-terminal structure of DnaA protein will be important to reveal the contacts that are made between DnaA monomers. Structural modeling of DnaA protein monomers to the p97 hexameric complex (human protein required for assembly of the SNARE complex) suggests that DnaA protein could form a hexamer (Erzberger, Pirruccello et al. 2002). It would also be useful to crystalize DnaA protein bound to ATP to reveal the amount of structural remodeling that occurs. This experiment would confirm that AAA$^+$ proteins require ATP binding for a conformational change required for their activity (Koonin 1992; Koonin and Gorbalenya 1992; Koonin 1993).

We know that DnaA protein requires ATP binding to be active for replication (Sekimizu, Bramhill et al. 1987). Many mutant proteins that are defective for ATP binding are also defective for replication. With the exception of DnaAcos protein this conclusion is correct. DnaAcos protein has feeble ATP binding activity yet it is more active than the wild type protein (Katayama and Kornberg 1994; Katayama and Crooke 1995). How is this possible? The mechanism causing hyperactive initiation is still unknown, and experiments need to directed toward revealing the specific biochemical requirements that result in the regulation of DNA replication.

In the case of hyperactive *cdc6* alleles (*S. cerevisiae*) they cause a constant loading of an MCM2-7 complex containing helicase activity (Liang and Stillman 1997). It is possible that hyperactive DnaA mutant proteins load DnaB more frequently than the wild type protein. Both primase (DnaG) and DNA polymerase III holoenzyme have specificity for DnaB. Thus, once DnaB is loaded the other components of the replication fork should assemble. Some of the mutant DnaA proteins may be impaired in their ability to discriminate between ATP and ADP. In this situation the mutant proteins might remain active in the ADP bound form and are inert to the regulation imposed by the intrinsic ATPase activity of DnaA protein. Another potential mechanism that causes the hyperactive mutant DnaA proteins to remain active, is that their ATPase activity is not stimulated by the beta clamp of DNA polymerase III holoenzyme or Hda protein. Under this model, mutant proteins are not responsive to negative regulation by beta clamp and Hda resulting in the continued binding of ATP leaving the mutant protein in a constitutively active form. Future experiments should be directed to discriminate between these possible models for hyperactive initiation.

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