EFFECTS OF ISOLATED MISSENSE MUTATIONS FROM THE *DNAA5* AND *DNAA46* ALLELES IN INITIATION OF *ESCHERICHIA COLI ORIC* REPLICATION

By

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ABSTRACT

EFFECTS OF ISOLATED MISSENSE MUTATIONS FROM THE *DNAA5* AND *DNAA46* ALLELES IN INITIATION OF *ESCHERICHIA COLI ORIC* REPLICATION

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The temperature sensitive alleles *dnaA5* and *dnaA46* each contain two missense mutations. These have been separated by molecular genetic techniques and the resulting mutant proteins studied in initiation of DNA replication *in vitro*. An alanine–to–valine substitution at amino acid 184 is common to both of the alleles and is responsible for the thermolabile defect and prolonged lag in DNA synthesis of DnaA5 and DnaA46. This protein was also defective in binding ATP as are DnaA5 and DnaA46. Aggregated, wild type DnaA was also severely impaired in ATP binding, suggesting protein aggregation and not the amino acid substitution, per se, is the primary cause of defective ATP binding. The *dnaA5* allele also contains a mutation resulting in substitution of glycine at residue 426 with serine. The single mutant is partially thermolabile in activities of DNA replication as well as binding to *oriC* DNA. DnaA46 and the A184V mutant product bind *oriC* DNA more efficiently at 30 °C than $DnaA^+$ and both retained affinity for *oriC* at elevated temperature. The effects on *oriC* binding of the A184V and G426S mutations balance each other when they are both present in DnaA5. All of the mutant forms of DnaA were able to form complexes with *oriC* but a reduced abundance of certain complexes was observed for proteins harboring the A184V mutation. By contrast, the histidine 252 to tyrosine alteration of the *dnaA46* allele, when present alone, did not affect the biochemical activities of DnaA protein.

To

Mom
Dad
John
Karen
Chris

Acknowledgements

While my path here might be considered nontraditional, I will stick to tradition by acknowledging those people who have helped me in this endeavor. First, of course, is my thesis advisor, Dr. Jon Kaguni, who found me wandering in the lobby of the biochem building one night and decided to offer me a job. This work would not have been possible without his guidance and patience. He provided me a place to develop as a scientist and I provided him with several beers by betting on Michigan - Notre Dame football games. I must also thank my second thesis advisor, Dr. Laurie Kaguni, free spirit, latin scholar, keen wit, for many helpful insights and suggestions along the way. My appreciation also to Dr. Zach Burton, ad hoc member of my thesis committee.

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Table of Contents

List of Tables

Chapter II

List of Figures

Chapter I

Chapter II

List of Abbreviations

bp	base pair
BSA	Bovine serum albumin
DnaA box	the 9 bp consensus sequence recognized by DnaA
dNTP	deoxyribonucleotide
DTT	dithiothreitol
EDTA	ethylenedinitrilo tetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperidineethane sulfonic acid
IPTG	isopropyl β–D–thiogalactopyranoside
kDa	kilodalton
PVA	polyvinyl alcohol
SDS	sodium dodecyl sulfate
SSB	single stranded DNA binding protein
Tris	Tris(hydroxymethyl)aminomethane

Chapter I

Literature Review

Introduction

Study of the mechanisms of DNA replication is of significance because of the fundamental nature of this process to all life. With the initial description of the structure of DNA, a possible mode by which the genetic material can direct its own duplication through a semiconservative process became apparent (Watson and Crick, 1953). The semiconservative nature of DNA replication was soon demonstrated (Messelson and Stahl, 1958). Intense study of the mechanism and regulation of DNA replication has been continuous ever since.

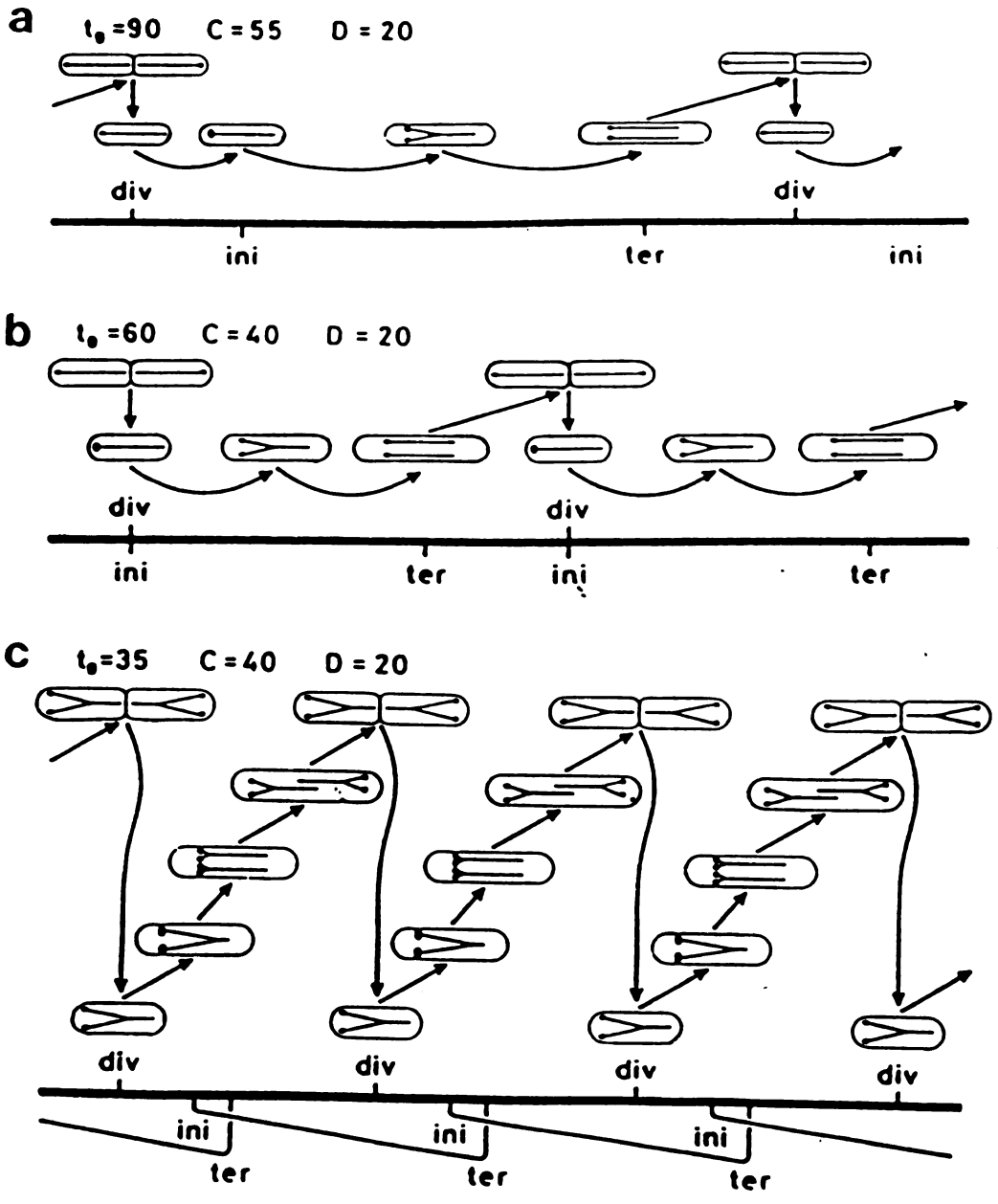
The *Escherichia coli* chromosome is a circular, duplex DNA molecule of 4,700 kilobase pairs (reviewed in McMacken, et al., 1987; von Meyenburg and Hansen, 1987). Replication of the genome of *E. coli* begins at a unique site on the chromosome and replication forks proceed bidirectionally around the circular DNA to the site where replication terminates. Daughter molecules segregate to complete the cycle of chromosome duplication.

Principles of Regulation

Early in the study of DNA replication in *E. coli*, it was recognized that replication is a very regular event (Messelson and Stahl, 1958). Soon after it was understood that the regulated step in synthesis of DNA is at the initiation step (Maaloe and Kjeldgaard, 1966). Jacob and coworkers proposed a model for the control of replication, called the replicon model, which has two components (Jacob, et al., 1963). The first is the replicator which is the site of regulation and where replication begins, now known to be *oriC*. The second is the initiator, which acts on the replicator to initiate replication in a regulated manner. The DnaA protein is the best candidate for the initiator in this model. The concept of initiation mass (Donachie, 1968) is central to all models attempting to describe regulation of initiation. Initiation mass is defined as the ratio of cell mass to the number of origins present in the cell at the time of initiation. If initiation of replication is tightly regulated and coordinated to the cell cycle then it is predicted the initiation mass remains constant. Anything which disrupts the regulation of initiation will alter the initiation mass.

Examination of different periods in the cell cycle (Cooper and Helmstetter, 1968; Helmstetter, 1968) revealed that the period between initiation and termination, the C time which represents the complete duplication of the chromosome, is constant at all growth rates and is 40 minutes long (Figure 1). More recent analysis has shown that at very slow

Figure 1. Schematic representation of the relationship between cell division and chromosome replication cycle (von Meyenburg and Hansen, 1987). C, the time between initiation (ini) and termination (ter) of replication, may be extended at very slow growth rates but is constant and equal to 40 min at more rapid growth rates. D, the time between termination and cell division (div), is 20 min at all growth rates. The time between initiations varies according to the doubling time (t_D) of the cell (**a** t_D=90 min; **b** t_D=60 min; **c** t_D=35 min). If t_D is less than C + D (e.g. in **c**) then the cells must initiate a new round of initiation before previous rounds are completed. Initiations are synchronous on all origins present in the cell.


a
t_0=90 C=55 D=20
div
div
ini
ter
ini
b
t_0=60 C=40 D=20
div
div
ini
ter
ini
ter
c
t_0=35 C=40 D=20
div
div
div
div
ini
ter
ini
ter
ini
ter

growth rates this time can be extended (Churchward and Bremer, 1977; Kubitschek, 1974; Kubitschek and Newman, 1978), but it is never shorter than 40 minutes. The time between termination and subsequent division, D time, was also a constant. This time is 20 minutes, resulting in a minimum of 60 minutes between the time replication is initiated and the appearance of two new daughter cells from a particular round of replication. However,*E. coli* is capable of doubling time as short as 15–20 minutes, which means it must also initiate a new round of replication at this same time interval. In rapidly growing cultures, new round of replication are started before ongoing rounds are completed, resulting in chromosome structures in varying stages of duplication (Figure 1).

Flow cytometry has been used to determine the number of genome equivalents of DNA in a cell in which new initiation of DNA replication and cell division have been inhibited (Boye, et al., 1988; Skarstad, et al., 1986; Skarstad, et al., 1983; Skarstad, et al., 1985). It indirectly measures the number of origins which were present when the drugs were administered to the cell. In samples of normal cultures, the vast majority of the cells are found to have 2^n (e.g. 2, 4 or 8) genome equivalents. This finding indicates that in rapidly growing cells with multiple origins present, initiation occurs simultaneously at all origins. This property is referred to as synchronous initiation and it is further evidence of the high level of regulation of chromosomal replication. Mutants which affect the synchronous initiation of replication have been identified in a subclass of *dnaA* mutants (Skarstad, et al., 1988) that have a

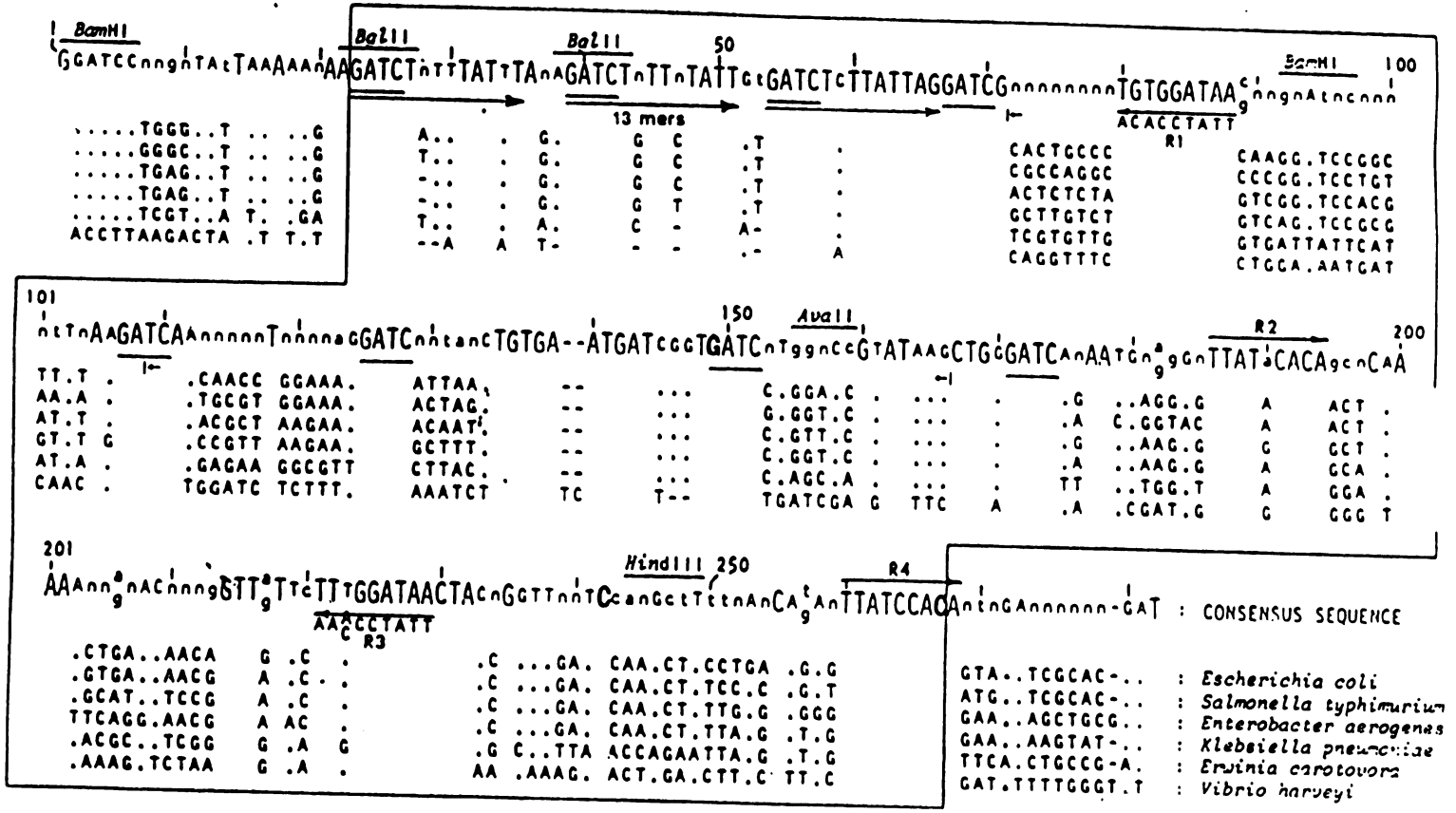
mutation near the ATP binding site. Other cellular factors potentially involved in this process have been identified by mutants which result in an asynchronous phenotype. These include *dam* (Dam methylase) (Boye and Lobner-Olesen, 1990), *seqA* (sequestration) (Lu, et al., 1994), *him* (IHF), *fis* (FIS protein) (Boye, et al., 1993) and *gyrB* (gyrase B subunit) (von Freiesleben and Rasmussen, 1991). The roles these proteins may play in regulation of *oriC* function are discussed below.

The origin of replication

Structural features

The origin of replication, *oriC*, was identified and localized to 83.5 minutes on the *E. coli* chromosome based on examination of gene dosage in replicating cells (Bird, et al., 1972; Masters and Broda, 1971). Later, the origin region was cloned by its ability to confer autonomous replication in *E. coli* to a DNA fragment carrying a drug resistance gene but no functional origin (Hiraga, 1976; Yasuda and Hirota, 1977). Progressive deletions identified the minimal functional origin as 245 bp (Oka, et al., 1980). Sequence information from the *E. coli* origin and the origins of other enteric bacteria was obtained (Cleary, et al., 1982; Zyskind, et al., 1983; Zyskind, et al., 1981). The aligned sequences showed a high degree of homology with several clusters of identity distributed throughout, suggesting sites of functional importance (Figure 2). These clusters are separated by regions of nonconserved sequence but of fixed

Figure 2. Consensus sequence of the minimal origin of the bacterial chromosome (Zyskind, et al., 1983). The consensus sequence is derived from six bacterial origins indicated. The alignment of the sequences is such that the least number of changes are introduced into the consensus sequence. In the consensus sequence, a large capital letter means that the same nucleotide is found in all six origins; a small capital letter means the nucleotide is present in five of the six sequences; a lowercase letter is used when that nucleotide is present in three or four of the six origins but only two different nucleotides are found at that site; and where three or four of the four possible nucleotides, or two different nucleotides plus a deletion, are found at a site, the letter n is used. The minimal origin of *E. coli* is enclosed within the box and the numbering of the nucleotide positions is the used for *E. coli*. Bold large capital letters located at positions 149, 242, and 267, indicate where single-base substitutions produce an *oriC*$^-$ phenotype in *E. coli*. GATC (dam methylation) sites are underlined in the consensus sequence. The four dnaA boxes are indicated as R1, R2, R3 and R4; and their orientations indicated by arrows. The three 13mer repeats are indicated by arrows in the upper left.


BamHI
Bgl II
Bgl II
50
BamHI
100
13 mers
R1
101
150
AvaII
R2
200
201
HindIII
250
R4
R3
CONSENSUS SEQUENCE
Escherichia coli
Salmonella typhimurium
Enterobacter aerogenes
Klebsiella pneumoniae
Erwinia carotovora
Vibrio harveyi

length suggesting spacing or phasing of the conserved sites on the DNA duplex is probably important (Asada, et al., 1982; Hirota, et al., 1981). Among the conserved sites are four DnaA boxes which have been designated R1, R2, R3 and R4; they are arranged in alternating orientations with R1 near the three well conserved AT-rich 13mers at the 5′ end of *oriC*. DnaA protein has been shown to bind specifically to the recognition sequences in *oriC* in a highly cooperative manner (Fuller, et al., 1984). The 13mer region of *oriC* may also be bound by DnaA in the process of being converted to the open complex (below) (Yung and Kornberg, 1989). Studies in which the DnaA boxes were mutagenized showed that single base changes in any one box did not affect the activity of *oriC* (Holz, et al., 1992; Messer, et al., 1991). Only when changes were introduced into two boxes simultaneously was a phenotype observed. When the spacing between boxes R3 and R4 or R2 and R3 was altered, *oriC* was completely inactivated (Messer, et al., 1992; Woelker and Messer, 1993) unless the change was plus or minus 10 base pairs (one helical turn). This suggests that the orientation of the binding sites relative to the helical axis is important probably due to DnaA:DnaA interactions of the proteins bound at these sites.

The sequence GATC present in the 13mers and repeated eight more times in *oriC* (Zyskind, et al., 1981) is the recognition sequence for Dam methyltransferase which methylates the adenine base in the sequence. Other sites for interaction with cellular factors include a Fis binding site located between R2 and R3 (Gille, et al., 1991) as well as an IHF site just 3′ to box R1

(Hwang and Kornberg, 1992a; Polaczek, 1990). Fis (Factor for Inversion Stimulation) and IHF (Integration Host Factor) are DNA binding proteins originally identified as assisting in recombinational events by altering the conformation (bending) of DNA (Koch and Kahmann, 1986; Yang and Nash, 1989). These proteins probably assist DnaA in formation of an open complex at *oriC* (Filutowicz, et al., 1992; Gille, et al., 1991; Hwang and Kornberg, 1992a). Disruption of the binding sites for either of these proteins inactivates *oriC* (cited in Messer and Weigel, 1994)). However mutants can survive but are altered in *oriC* functions showing an asynchronous phenotype (Boye, et al., 1993; Kano and Imamoto, 1990).

Transcription around oriC

Activity of *oriC* function requires a transcriptional event, not involved in protein synthesis, prior to initiation (Lark, 1972; Messer, 1972). It is thought that this event is required for transcriptional activation of *oriC*. A complex pattern of transcription is observed around *oriC* (Stuitje, et al., 1986; Theisen, et al., 1993). The origin is flanked on the right (3') side by the *mioC* and *asnC* genes, on the left side of *oriC* is the *gidA* gene. A DnaA box in the promoter for *mioC*, in the intergenic region between *mioC* and *asnC*, represses transcription from the promoter (Schauzu, et al., 1987; Wang and Kaguni, 1987) and is also responsible for transcriptional termination of the *asnC* transcripts (Gielow, et al., 1988; Schaefer and Messer, 1988). Transcripts from *mioC* generally extend leftward through *oriC* or are terminated within *oriC*

(Junker, et al., 1986; Rokeach and Zyskind, 1986). The leftward promoter of *gidA* has a positive effect on *oriC* activity (Ogawa and Okazaki, 1991). Evidence the *mioC* transcript is involved in regulating the activity of *oriC* is seen in the fact that *mioC* is regulated by DnaA and its expression is also growth–rate–regulated under stringent control (Chiaramello and Zyskind, 1989). The actual mechanism of transcriptional activation is not known but it may have to do with increasing or decreasing superhelical densities of the 13mer region as transcripts either move toward or away from this region (Liu and Wang, 1987). Another suggestion that topology of the origin region can affect its activity is the observation that a deletion of *topA*, the which encodes topoisomerase I can suppress a *dnaA* mutant (Louarn, et al., 1984). Replication in the suppressed strain is still dependent on *oriC* and the *dnaA* allele. The alteration in the supercoiling of the origin may allow function with reduced amounts of, or residually active DnaA.

Membrane attachment and dam *methylation*

A model for regulating the activity of *oriC* by cyclic attachment and detachment from the membrane (Norris, 1990) is based on a number of observations. Conservation of the large number of dam methylation sites in the origin (Zyskind, et al., 1983), and the observation that *dam* mutants fail to initiate replication synchronously (Boye and Lobner-Olesen, 1990) suggest a role in the functioning of *oriC*. Dam sites in *oriC* remain hemi–methylated for a much longer period after a round of replication than sites elsewhere on

the chromosome (Szyf, et al., 1982). Delay in methylation of the dam sites in *oriC* is due to sequestration of the origin in the membrane (Campbell and Kleckner, 1990). Hemi-methylated *oriC* DNA is capable of specifically binding to membrane fractions *in vivo* and *in vitro* whereas fully or unmethylated DNA cannot (Ogden, et al., 1988). The sequestration of the origin in the membrane also prevents it from being reinitiated immediately if there is an excess of initiation capacity. Cyclic detachment and reattachment to the membrane may regulate the activity of *oriC* as part of a control system (Messer and Noyer-Weidner, 1988). The recently identified *seqA* gene is a membrane protein which preferentially binds hemi-methylated DNA, and mutants of this gene result in asynchronous initiation as predicted if it is responsible for sequestering *oriC* (Lu, et al., 1994).

Replication from *oriC in vitro*

oriC *plasmids/minichromosomes*

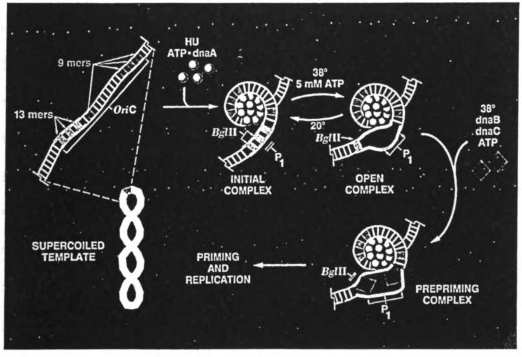
The origin of replication from *E. coli* was cloned into a plasmid by its ability to confer autonomous replication on a DNA fragment carrying a drug resistance marker (Hiraga, 1976). These minichromosomes are compatible with the *E. coli* genome and do not impair growth even when present at high copy numbers (von Meyenburg, et al., 1979). Minichromosomes require the same factors for replication of the genome. Replication proceeds bidirectionally (Meijer and Messer, 1980) from the cloned origin and they are replicated synchronously with the chromosome (Koppes and

von Meyenburg, 1987). These observations indicate that the mechanisms of replication and regulating initiation of these *oriC* plasmids are not significantly different than that of *oriC* on the chromosome, which makes them ideal tools for more detailed study of the biochemical mechanisms of initiation and replication of DNA.

In vitro *replication of* oriC *plasmids*

Replication was first accomplished *in vitro* with a crude enzyme fraction (Fuller, et al., 1981). Several criteria indicated that the replication observed was representative of replication *in vivo*: i) the activity was absolutely dependent on exogenously added template with a functional *oriC*, ii) replication initiated in the origin and proceeded bidirectionally through a theta structure (Kaguni, et al., 1982), iii) all proteins known to be essential for replication were required including DnaA, DnaB, DnaC, SSB, DNA gyrase and RNA polymerase. Fractionation of the required proteins from crude extracts resulted in the reconstitution with purified proteins of a system capable of replicating DNA specifically from *oriC* (Fuller, et al., 1981; Kaguni and Kornberg, 1984). A model has emerged of the biochemical processes involved in initiation of DNA replication from *oriC* (Figure 3). DnaA protein binds cooperatively to the origin through association with the four DnaA boxes and forms a large nucleoprotein structure called the initial complex (Fuller and Kornberg, 1983); electron microscopic studies of these

Figure 3. Hypothetical model for the initiation of chromosome replication in *E. coli*. The stages in the proposed model of initiation are 1) DnaA protein binding to *oriC* at the four DnaA boxes forming a nucleoprotein complex; 2) duplex unwinding of the AT–rich 13mer region; 3) direction of DnaB–DnaC complex to the opening; 4) further opening of the duplex by the DnaB helicase; 5) priming and elongation events. Additional factors which assist in the initial complex and open complex formation include proteins HU, Fis and IHF. Single strand binding protein (SSB) coats the unwound strand of DNA to stabilize them.


HU
ATP•dnaA
9 mers
13 mers
OriC
38°
5 mM ATP
20°
BglII
P1
INITIAL COMPLEX
OPEN COMPLEX
38°
dnaB
dnaC
ATP
SUPERCOILED TEMPLATE
PRIMING AND REPLICATION
PREPRIMING COMPLEX

complexes suggest about 20–40 DnaA monomers bound per origin (Funnell, et al., 1987). This complex does not require the ATP–bound form of DnaA, the ADP–bound or nucleotide–free form can also produce the same structure at the origin. Elevated temperature (38 °C) and high levels of ATP (5 mM) result in the destabilization and local melting of the AT–rich 13mer repeat region; this is the open complex (Bramhill and Kornberg, 1988a). The accessory proteins, HU, Fis and IHF enhance formation of the open complex (Skarstad, et al., 1990). This step requires that at least some of the DnaA be bound to ATP (Sekimizu, et al., 1987). Since ATP hydrolysis is not a part of open complex formation, the requirement for the ATP–bound form of DnaA is probably to stabilize a particular conformation of the protein. The requirement for the high levels of ATP is also not understood. The negative regulator IciA can bind to the 13mers to prevent the formation of the open complex (Hwang and Kornberg, 1992b; Hwang, et al., 1992). DnaB helicase, delivered to the open complex in association with DnaC and ATP, then results in an extension of the unwound region (Gille and Messer, 1991). There is evidence to indicate that DnaB is directed to the forming replication fork by direct interaction with DnaA (Marszalek and Kaguni, 1994). DnaC is not maintained in complex with the other proteins, its function appears to be solely the delivery of DnaB to the complex. If not dissociated from DnaB, it potently inhibits the helicase activity of DnaB (Wahle, et al., 1989a; Wahle, et al., 1989b). With the formation of the prepriming complex, SSB is needed to stabilize the growing single strand regions of DNA. DnaB proceeds to

unwind the template in both directions. Priming and DNA replication follow the establishment of replication forks composed of all necessary components including primase, SSB, DNA polymerase III holoenzyme, gyrase and others (McHenry, 1985).

DnaA Protein

Physical and biochemical properties

DnaA, the initiator protein, is essential for initiation of DNA replication from *oriC* both *in vivo* and *in vitro*. It is a single polypeptide of 467 amino acids, is very basic and has a molecular weight of 52.5 kDa calculated from the deduced amino acid sequence (Hansen, et al., 1982; Yuasa and Sakakibara, 1980). *dnaA* homologues from a number of species from widely separated phylogenetic groups have been identified and examined (reviewed in (Messer and Weigel, 1994)). Alignment of the amino acid sequences revealed a short N–terminal region and a much larger C–terminal region that are highly conserved. These are separated by a region of little homology (Figure 4). A well conserved motif characteristic of ATP binding proteins (P–loop) is present (Saraste, et al., 1990), and, more recently, similarities in this region to the ATPases of the NtrC transcriptional regulator superfamily have been identified (Koonin, 1993). Thus far, no specific functional domains of DnaA have been identified.

DnaA is a sequence–specific DNA binding protein which binds preferentially to the 9 bp sequence 5′–TTAT(C/A)CA(C/A)A–3′, termed the

DnaA box (Fuller, et al., 1984; Holz, et al., 1992; Woelker and Messer, 1993), although a number of variations of this consensus are found which are functional in the cell. The protein binds to adenine nucleotides with high affinity. Its K_D for ATP is 0.03 μM and for ADP is 0.1 μM (Sekimizu, et al., 1987). The binding is believed to occur at a consensus ATP binding site or 'P–Loop' found at amino acids 174–179. It has not been demonstrated directly whether or not this site is involved in the high affinity ATP binding activity. However, mutants with an amino acid substitution near this site were apparently defective in ATP binding (Hupp and Kaguni, 1993c; Hwang and Kaguni, 1988a). Bound ATP is very slowly hydrolyzed to ADP by a weak DNA dependant ATPase activity (Sekimizu, et al., 1987). The turnover of ADP and ATP is normally extremely slow. Cardiolipin and other acidic phospholipids can bind to DnaA. This interaction of cardiolipin with DnaA has been shown to affect the displacement of ADP, allowing the protein to bind again to ATP but only when the cardiolipin was inactivated after displacement of the bound ADP. Otherwise it also prevented the protein from binding ATP (Sekimizu and Kornberg, 1988). It has been suggested that the turnover of the various nucleotide–bound forms is important in regulation of the activity of DnaA since only the ATP–bound form of DnaA is active in initiation of DNA replication from *oriC*.

DnaA function in transcription

Several genes have been shown to be regulated by DnaA. Binding of DnaA to DnaA boxes in promoters resulting in repression of transcription has been demonstrated for the *rpoH* and *mioC* (Lother, et al., 1985; Schauzu, et al., 1987; Wang and Kaguni, 1989). DnaA also negatively affects expression of *ftsQ/ftsA* (Masters, et al., 1989), *guaB* (Tesfa-Selase and Drabble, 1992) and by transcriptional termination of *asnC* in the intergenic region between *asnC* and *mioC* (Gielow, et al., 1988; Schaefer and Messer, 1988). Binding by DnaA protein to boxes in the promoter of *nrd* in conjunction with Fis protein binding, activates expression of this operon (Augustin, et al., 1994).

Perhaps the most important function of DnaA related to transcription is its ability to regulate its own expression (Atlung, et al., 1985). Based on the fact that certain temperature sensitive mutants can accumulate initiation potential in excess of that seen in wild-type strains at nonpermissive temperatures (Hansen and Rasmussen, 1977), it was suggested that *dnaA* is autoregulated. Several subsequent studies have shown that DnaA is able autoregulate expression by binding to the DnaA box between the two *dnaA* promoters (Polaczek and Wright, 1990). Comparative S1 mapping showed that oversupply of DnaA by expression from an inducible promoter on a multicopy plasmid represses expression of *dnaA* (Kucherer, et al., 1986). In a *dnaA* mutant strain, expression of *dnaA* is increased at the nonpermissive temperature. Addition of multiple copies of the DnaA binding sequence on a plasmid resulted in derepression presumably by titrating the DnaA protein

(Hansen, et al., 1987). *In vitro* studies demonstrated the correlation between DnaA binding to the promoter and repression of transcription (Wang and Kaguni, 1987). The autoregulation of *dnaA* maintains its cellular abundance at between 800–2100 copies per cell when various strains were examined, but when these values were corrected for cell mass the abundance was very similar (Sekimizu, et al., 1988).

Regulation of chromosome replication by DnaA

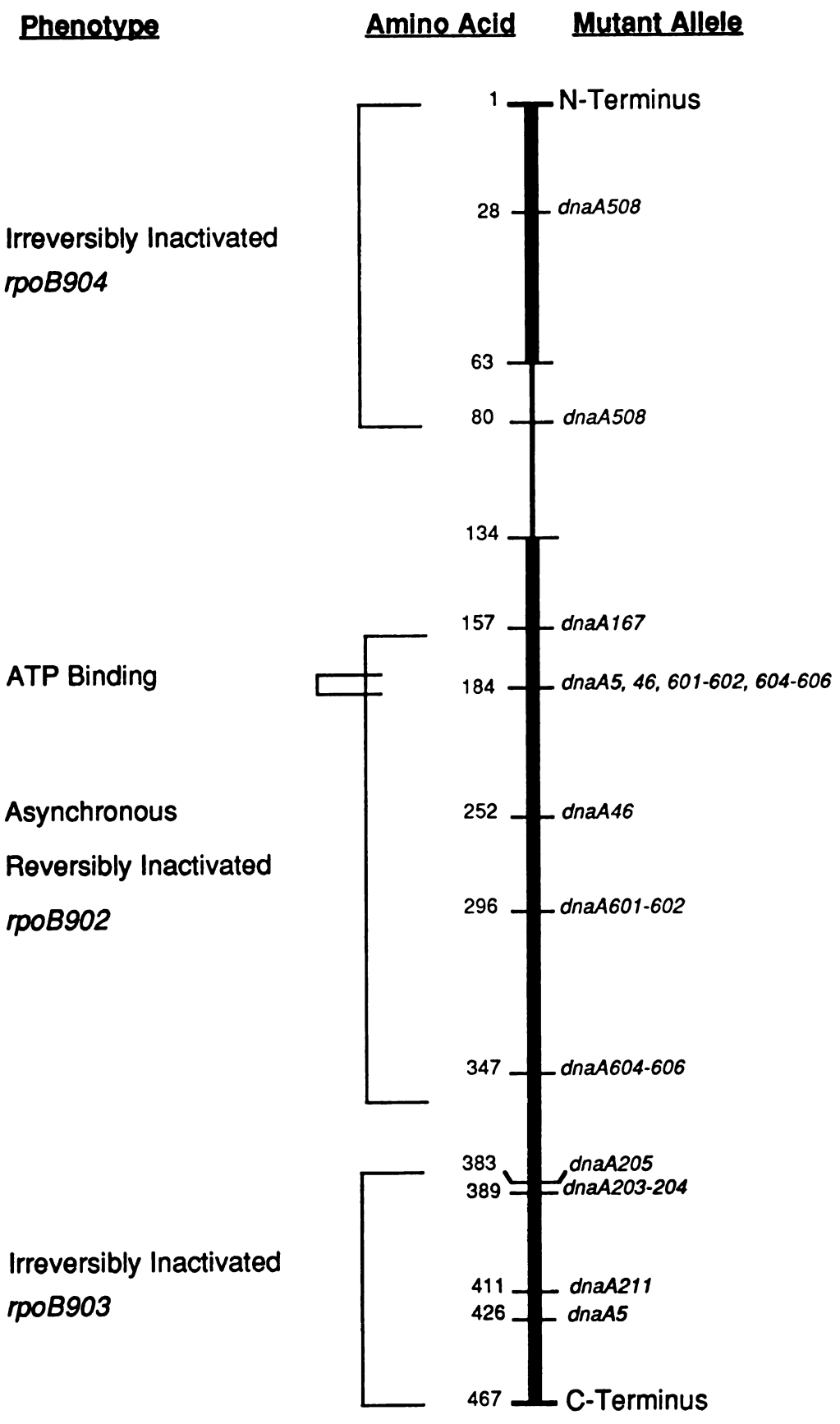
DnaA protein is essential for initiation of replication from the *E. coli* origin. It acts very early in the process of initiation and appears to be dispensable after replication forks have been assembled. This is indicated by the fact that *dnaA* mutants have a 'slow stop' phenotype for DNA replication (Abe and Tomizawa, 1971; Hirota, et al., 1968). Upon shift from the permissive to nonpermissive temperature, ongoing rounds of replication are completed but new initiations are blocked. A possible role in regulating the timing of initiation is suggested by several observations. A subclass of *dnaA* mutants are reversible and can accumulate potential to initiate replication at their nonpermissive temperature (Eberle and Forrest, 1982; Evans and Eberle, 1975). Upon downshift to the permissive temperature and in the absence of de novo protein synthesis, which is normally required before each round of initiation, these Ts proteins are capable of triggering initiation. An intragenic suppressor of the *dnaA46* allele, *dnaAcos*, results in a cold sensitive phenotype due to excess initiation events at low temperatures

(Kellenberger–Gujer, et al., 1978). If DnaA is limiting for initiation, then an increase in the abundance of DnaA should increase the rate of initiations. Initial reports indicated that oversupply of DnaA from an inducible promoter did not lead to a significant increase in the DNA content of the cell (Bremer and Churchward, 1985). More careful examination revealed that after rapid induction of *dnaA* from a strong promoter, a corresponding burst of initiations occurs from *oriC*. However, the replication forks stall shortly after leaving the origin (Atlung and Hansen, 1993), suggesting there may some other limiting factor or secondary control mechanism. Controlled expression of the *dnaA* gene results in a decrease in the initiation mass of the cell and replication is initiated earlier in the cell cycle (Skarstad, et al., 1989) Conversely, the initiation mass is increased in some *dnaA* mutants at permissive temperatures (Frey, et al., 1981). Additional evidence that DnaA is involved in timing initiation comes from studies of mutant alleles of *dnaA*. As discussed above, initiation from multiple origins in rapidly growing *E. coli* occurs synchronously. A subclass of *dnaA* mutants that contain a mutation near the proposed ATP binding site (Figure 4) are defective in synchronously initiating replication from multiple origins (Skarstad, et al., 1988).

Mutant DnaA proteins

dnaA was first identified from mutants unable to synthesize DNA at elevated temperatures, and the block was determined to be specifically at the initiation stage (Hirota, et al., 1970). *dnaA* mutants have been used to study

Figure 4. Map of mutations and correlated phenotypes in *dnaA*. Positions of known missense alleles of *dnaA* and the altered amino acid are indicated. The conserved N– and C–terminal regions, indicated by the heavier line, are separated by a nonconserved internal domain (amino acids 63–134). Phenotypes are grouped into three regions. The alleles of *rpoB* listed show allele specific suppression of the *dnaA*(Ts) mutants. Reversible vs. irreversible inactivation and asynchronous initiation phenotypes (described in text) are associated in groups. "ATP binding" indicates both the position of the consensus ATP binding domain (amino acids 170–179) and mutants which are defective in ATP binding (*dnaA5* and *dnaA46*). Information is reviewed in (Hansen, et al., 1992).


Phenotype
Amino Acid
Mutant Allele
1
N-Terminus
28
dnaA508
63
80
dnaA508
134
157
dnaA167
184
dnaA5, 46, 601-602, 604-606
252
dnaA46
296
dnaA601-602
347
dnaA604-606
383
dnaA205
389
dnaA203-204
411
dnaA211
426
dnaA5
467
C-Terminus
Irreversibly Inactivated
rpoB904
ATP Binding
Asynchronous
Reversibly Inactivated
rpoB902
Irreversibly Inactivated
rpoB903

the process of initiation and the role DnaA protein normally plays in cell. Several mutants have been identified and examined physiologically and genetically. Two have been studied in detail biochemically. Figure 4 shows 11 point (missense) mutants of the *dnaA* gene and the phenotypes associated with them (reviewed in Hansen, et al., 1992). The phenotypes of the mutants fall into clusters. The group of mutants with a substitution at amino acid 184, near the ATP binding site (*dnaA5, 46, 601/602, 604/606*) all possess a second mutation elsewhere in the gene suggesting that this mutation is extremely deleterious to *dnaA* function and that secondary, compensatory mutations are required. This group of mutants is more severely impaired in maintaining synchronous initiation from multiple origins (Skarstad, et al., 1988). Their activity is reversible upon temperature downshift, indicating they can transition between active and inactive states (Hansen, et al., 1984).

Temperature sensitive mutants of *dnaA* can be suppressed by different mechanisms. Two mechanism which bypass the requirement for DnaA at *oriC* are stable DNA replication(Kogoma and Lark, 1975) and integrative suppression(Nishimura, et al., 1971). Stable DNA replication results from inactivation of the *rnhA* allele (Torrey, et al., 1984). The encoded RNaseH degrades RNA in RNA:DNA duplexes. In the absence of this activity, it is thought that transcripts in certain regions of the chromosome are stabilized and function as primers for DNA replication (de Massy, et al., 1984). In integrative suppression, an alternative replicon integrated into the chromosome directs DNA replication. Replicons which can drive

chromosome replication include R1, P2, F, P1 and R100 (Bird, et al., 1976; Felton and Wright, 1979). The latter three replicons cannot suppress a *dnaA* null mutant, indicating that some function of DnaA is still required (Kogoma and Kline, 1987). Inactivation of *topA*, the gene for topoisomerase I, has been shown to suppress the temperature sensitive phenotype of *dnaA46* (Louarn, et al., 1984). The thermotolerant growth requires the presence of *oriC* and the mutant *dnaA* gene, which again indicates that the mutant protein retains some function at the nonpermissive temperature. Inactivation of *topA* results in an increased negative superhelicity of the chromosome. How this suppresses *dnaA46* is not known but it is suggested that the altered structure of the chromosome either allows increased expression of *dnaA* or reduces the amount required to initiate replication from *oriC*. Secondary mutations in *rpoB*, the gene encoding the β subunit of RNA polymerase, suppress the temperature sensitivity of *dnaA* mutants in an allele–specific manner (Atlung, 1984), suggesting an interaction between the two proteins. The extragenic suppressor *dasC* was shown to be allelic with *trxA*, the gene for thioredoxin (Hupp and Kaguni, 1988). The mechanism by which it suppresses *dnaA* mutants is not known.

Two related mutants, *dnaA5* and *dnaA46* have been cloned in an expression vector and the mutant proteins characterized *in vitro* (Hupp and Kaguni, 1993c; Hwang and Kaguni, 1988b). These two alleles are part of the group which carries two missense mutations, one at amino acid 184, very near the putative ATP binding site which is common to both, and second

mutations at position 426 in *dnaA5* or at amino acid 252 for *dnaA46* (Figure 4). They were thermolabile in initiation of DNA replication in an assay using a crude enzyme extract and completely inactive in replication assays with purified proteins. This suggested that some other factor(s) was required for their function. The heat shock proteins DnaK and GrpE were identified as being required for activation of these DnaA proteins (Hupp and Kaguni, 1993b; Hwang and Kaguni, 1991). Heat shock proteins are involved in many cellular processes including protecting proteins from thermal stress, proper folding of nascent polypeptides, and maintaining proteins in partially unfolded states for transport across membranes (Yamamori and Yura, 1982). How these two proteins accomplish this activation is not understood on a molecular level but it is likely to involve a conformational alteration in DnaA. The thermolabile defect in DnaA5 and DnaA46 was shown to be in interaction with these heat shock factors. Whether or not these proteins normally interact with wild type DnaA is not clear. $DnaA^+$ clearly does not require them *in vitro* (Kaguni and Kornberg, 1984) and null mutants of *dnaK* are viable (Bukau and Walker, 1989), but $DnaA^+$ has been shown to interact with DnaK *in vitro* (Malki, et al., 1991). Heat shock proteins are involved in many cellular processes and are therefore difficult to examine phenotypically, but two mutants of *dnaK* have been isolated which have initiation specific defects (Ohki and Smith, 1989; Sakakibara, 1988). Ability to suppress *dnaA* mutants by oversupply of DnaK *in vivo* has not been reported; however, expression of the heat shock proteins GroEL and GroES from a plasmid is

capable of suppressing the temperature sensitive phenotype of *dnaA46* (Fayet, et al., 1986).

Besides their thermolabile phenotype in DNA replication, these two proteins do not bind ATP, presumably because of the mutation near the ATP binding site. DnaA5 was also shown to form an altered complex with *oriC* by DNase footprinting and was unable to form an open complex at *oriC* (Hupp and Kaguni, 1993c; Hwang and Kaguni, 1988a).

The focus of this work is the examination *in vitro* of the functions of the three individual missense mutations from *dnaA5* and *dnaA46* in comparison to DnaA$^+$ and the mutants harboring two substitutions. Correlation of these data to previous *in vitro* and physiological studies of the mutants will improve our understanding of the role of DnaA in initiation and regulation of DNA replication.

Chapter II

Effects of Isolated Missense Mutations from the *dnaA5* and *dnaA46* Alleles in Initiation of *Escherichia coli oriC* Replication

Introduction

Initiation of DNA replication in *Escherichia coli* is highly coordinated to the cell cycle. In rapidly growing cultures, initiation of subsequent rounds of replication occurs before ongoing rounds are completed (Helmstetter, 1968). This is necessary because the time required for complete duplication and segregation of daughter chromosomes, which is invariant, is longer than the time required for cell division (Cooper and Helmstetter, 1968). Once DNA replication has been initiated, it normally proceeds to completion without apparent regulation. Regulation of DNA synthesis occurs at the initiation step; the time between successive initiations is varied according to the growth rate of the cell (Maaloe and Kjeldgaard, 1966). The molecular basis of the regulation mechanism is currently not understood. Genetic methods have identified several cellular factors involved in replication Among these is the *dnaA* gene product (Hirota, et al., 1968; Kohiyama, 1968). DnaA protein is an essential gene product as demonstrated by the fact that only conditional mutants are allowed. This protein acts very early in the replication process but appears to be dispensable for the later stages of elongation or termination, suggesting its function in DNA replication is required only for initiation (Schaus, et al., 1981).

DnaA protein binds to nucleotides with high affinity, its K_D for ATP is 0.03 μM and 0.1 μM for ADP (Sekimizu, et al., 1987). While both the ATP– and ADP–bound forms of DnaA can bind to *oriC*, only the ATP–bound form is active for DNA replication. DnaA protein binds cooperatively to *oriC* at the four DnaA boxes present in the origin (Fuller, et al., 1984) and forms a large nucleoprotein complex consisting of 20-40 monomers of DnaA (Funnell, et al., 1987). In the presence of 5 mM ATP and HU protein, binding leads to unwinding of the AT–rich 13mer region of *oriC* (Bramhill and Kornberg, 1988a). Only the ATP–bound form is active in unwinding. The requirement for high levels of ATP has led to the suggestion the DnaA contains a second, low affinity binding site for ATP. Subsequent to strand separation, other factors in the preinitiation complex are directed to the origin (Bramhill and Kornberg, 1988b) , possibly through interaction with DnaA (Marszalek and Kaguni, 1994). After establishment of a competent replication fork, DnaA is apparently no longer required for subsequent steps of DNA replication.

DnaA protein is the only identified factor that functions only in the initiation stage of DNA replication. As it acts very early in this process, it is a reasonable target for regulation. Physiological and genetic analysis of *dnaA* mutants have suggested that it plays a role in the regulation of initiation of chromosomal replication. Some *dnaA*(Ts) mutants are defective in synchronously initiating replication from multiple origins in rapidly growing cultures even at temperatures permissive for cell viability (Skarstad, et al., 1988). Increased initiation mass is also observed in some *dnaA*(Ts) strains

(Frey, et al., 1981). Conversely, oversupply of DnaA by placing the gene under control of a strong inducible promoter results in a decrease in the initiation mass (Skarstad, et al., 1989). It is apparent that DnaA plays a key role in determining the timing and frequency of initiation events.

The *dnaA5* and *dnaA46* alleles have been extensively studied and shown to be defective in the timing of DNA replication (Skarstad, et al., 1988). These mutants each contain two point mutations that result in amino acid substitutions (Hansen, et al., 1992). One of the mutations is found in both alleles and is present in two other alleles, *dnaA601(602)* and *dnaA604(606)*. These also contain two missense mutations. The common mutation results in an alanine–to–valine substitution at amino acid 184, which is very close to the conserved, putative ATP binding site (Saraste, et al., 1990). In *dnaA5*, the second mutation at glycine 426 encodes serine instead. The unique mutation in *dnaA46* results in replacement of histidine 252 with tyrosine. Both unique mutations of *dnaA5* and *dnaA46* are also in highly conserved residues (for review, see Messer and Weigel, 1994). Although these regions of the protein have not been correlated to specific functions, the C–terminal domain, including amino acid 426, has been suggested to be involved in DNA binding (cited in Messer and Weigel, 1994). This chapter describes the separation of the three missense mutations and examination of the biochemical defects of the resulting proteins *in vitro*. The objective was to determine the contribution of each of the mutations to previously described biochemical defects of DnaA5 and DnaA46 protein. Comparison of the activities of the

single versus the double mutants may also provide information regarding possible interaction between the mutations in altering the activity of DnaA.

Experimental Procedures

Materials

Reagents were obtained from the following sources: polyvinyl alcohol (type II), phosphocreatine, heparin, ribonucleotides, isopropyl β-D-thiogalactopyranoside (IPTG), guanidine hydrochloride, chloramphenicol, and ampicillin, Sigma; deoxyribonucleotides and Sepharose 4-B, Pharmacia-PL Biochemicals; HEPES, Tris and dithiothreitol (DTT), Calbiochem-Behring; [α-^{32}P] ATP (3000 Ci/mmol), [α-^{32}P] dGTP (3000 Ci/mmol), and [α-^{32}P] dTTP (3000 Ci/mmol), New England Nuclear Corp.; [methyl-^{3}H] dTTP, ICN Radiochemicals; acrylamide, and N,N-methylene bis-acrylamide, Boehringer Mannheim; agarose (analytical grade), BioRad; low melting temperature agarose, Fisher; hydroxylamine hydrochloride, Aldrich; and Triton X-100 (scintillation grade), Research Products International Corp.. Tryptone and yeast extract were from Difco. LB media was prepared as described (Sambrook, et al., 1989).

Enzymes and Proteins

Restriction endonucleases were from the following sources: *Bam* HI and *Rsr* II, Boehringer Mannheim; *Bsm* I, *Nde* I and *Sma* I, New England

BioLabs; *Eco* RI, *Sph* I and *Xho* I, Gibco BRL; *Hin*d III, United States Biochemical Corp. Sequenase, version 2.0, was from United States Biochemical Corp. Replinase (Taq DNA polymerase) was from Dupont, NEN; the large fragment of DNA polymerase I, T4 DNA ligase, and egg white lysozyme were from Boehringer Mannheim. Bovine serum albumin (Fraction V), and creatine phosphokinase (type I, from rabbit muscle), Sigma Chemical Corp.

Buffers

Buffer A contained 25 mM HEPES–KOH pH 7.6, 15% glycerol, 0.1 mM EDTA, 2 mM DTT. Buffer B is 120 mM $KHPO_4$ pH 6.8, 15% glycerol, 0.1 mM EDTA, 2 mM DTT. Buffer C is 50 mM HEPES–KOH pH 7.6, 20% glycerol, 1 mM EDTA, 2 mM DTT. Buffer D is 50 mM HEPES–KOH pH 7.6, 20% glycerol, 10 mM magnesium acetate, 0.2 M $(NH_4)_2SO_4$, 0.1 mM EDTA, 2 mM DTT. Buffer E is 50 mM Tris–HCl pH 8.3, 15% glycerol, 0.1 mM EDTA, 2 mM DTT. Buffer G is 25 mM HEPES–KOH pH 7.6, 50% glycerol, 0.1 mM EDTA, 2 mM DTT.

Bacterial strains and DNAs

The bacterial strains and plasmid DNAs used in this study are listed in Table 1. Primers for polymerase chain reaction (PCR), JK–6 and JK–7 (30mers) were synthesized by the Macromolecular Structure, Sequencing and Synthesis Facility, Michigan State University. JK–7 overlaps the translation start codon

Table 1. Bacterial Strains and Plasmids

Plasmid	Relevant Features	Source or Reference
pdnaA/dnaN	Complete promoter and coding sequences of *dnaA* and *dnaN* cloned in pMOB45.	(Burgers, et al., 1981)
pDS596	*dnaA+* cloned in pING1 expression vector.	(Hwang and Kaguni, 1988b)
pDS105	*dnaA5* cloned in pING1.	(Hwang and Kaguni, 1988b)
pDS215	*dnaA46* cloned in pING1.	(Hwang and Kaguni, 1988b)
pET11a	Expression vector with T7 RNA polymerase promoter and translation signal upstream of multiple cloning site.	Novagen
pET11AW	*dnaA+* in pET11a.	This study. *dnaA* fragment generated by PCR.
pKC596	*dnaA+* in pET11a.	This study. Majority of *dnaA* in pET11AW replaced by homologous sequence from cloned DNA.
pKC105	*dnaA5* in pET11a.	This study.
pKC215	*dnaA46* in pET11a.	This study.
pKC–A184V	*dnaA–A184V* in pET11a.	This study.
pKC–G426S	*dnaA–G426S* in pET11a.	This study.
pKC–H252Y	*dnaA–H252Y* in pET11a.	This study.

Table 1 (continued). Bacterial Strains and Plasmids

Strain	Genotype	Comments and Reference
XL1-Blue	*supE*44*hsdR*17*recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*⁻ F'[*pro*AB⁺ *lacI*^{q} *lacZ*ΔM15 Tn10(*tet*^{r})]	Recombination deficient strain used for cloning (Bullock et al., 1987)
HMS174	*recA*1 *hsdR rif*^{r}	Recombination deficient strain used for cloning. (Novagen)
HMS174(DE3)	*recA*1 *hsdR rif*^{r} (λcI*ts*857 *ind*1 *Sam*7 *nin*5 *lac*UV5–T7 gene 1)	Strain for high level expression of genes cloned under control of T7 RNA polymerase (Novagen)
BL21(DE3)(pLysS)	*hsdS gal* (λcI*ts*857 *ind*1 *Sam*7 *nin*5 *lac*UV5–T7 gene 1)	Alternative strain for expression of cloned genes. T7 lysozyme provided by pLysS down regulates activity of T7 RNA polymerase in the absence of expression. (Novagen)
BL21(DE3) *dnaA204*(pLysS)	Isogenic with BL21(DE3) except *dnaA204 tnaA*::Tn*10*	Temperature sensitive derivative of pET expression strain used for assay of complementation *in vivo*.

of *dnaA* and contains four base changes from the natural sequence in this region to generate an *Nde* I restriction site. The base substitutions introduced change the start codon for *dnaA* from GTG to ATG but do not alter the amino acid sequence. The downstream primer, JK–6, anneals approximately 300 bp past the translation stop codon for *dnaA*. It contains one base alteration from the natural sequence creating a *Bam* HI restriction site.

DNA Manipulations

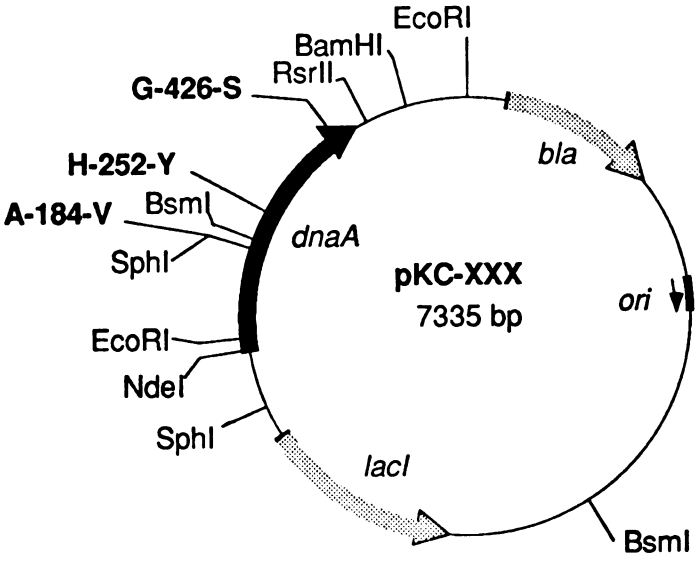
Subcloning of dnaA *into a pET expression vector*

Plasmid pdnaA/dnaN was used as template for PCR to generate a clonable fragment containing the coding sequence of the *dnaA* gene with appropriate restriction enzyme sites. Reactions (100 µl in 1X Replinase Buffer) contained 50 ng pdnaA/dnaN linearized with *Hin*d III; primers JK–6 and JK–7, 20 pmol each; dATP, dGTP, dCTP and dTTP, 50 µM each; and Replinase (Taq) DNA polymerase, two units. DNA amplification was carried out in a Perkin Elmer Cetus Thermal Cycler. Products from PCR were cleaved with restriction enzymes *Nde* I and *Bam* HI. The fragment containing the *dnaA* gene was purified from an agarose gel. The T7 expression vector, pET11a was similarly cleaved with *Nde* I and *Bam* HI and gel purified. These fragments were ligated and the ligation products used to transform HMS174; transformants were selected on LB plates containing 50 µg/ml ampicillin. The proper plasmid structure of selected clones was

confirmed by restriction digestion and agarose gel electrophoresis. The recombinant plasmid was designated pET11AW (Figure 1).

The mutant alleles *dnaA5* and *dnaA46* were subcloned by replacement of homologous DNA in pET11AW with DNA from plasmids bearing the mutant genes; wild type *dnaA* was also treated in this way to replace the majority of the coding sequence with DNA from a cloned (plasmid) source as opposed to DNA originating from PCR. pET11AW was cleaved with restriction endonuclease *Rsr* II, followed by limited digestion with *Eco* RI to remove the majority of the *dnaA* coding sequence. The desired fragment, from the *Rsr* II to the *Eco* RI site within *dnaA* containing all of the original vector sequence and coding sequence for the 21 N-terminal amino acids of *dnaA* (Figure 1), was separated from other cleavage products on a 0.7% agarose gel. DNA fragments corresponding to the sequence removed from pET11AW were obtained by digestion of pDS596 (*dnaA*$^+$), pDS105 (*dnaA5*), and pDS215 (*dnaA46*) (Hwang and Kaguni, 1988b) (Table 1) with *Eco* RI and *Rsr* II. Purified vector and coding sequence fragments were ligated and used to transform XL1-Blue, followed by selection on LB containing 50 μg/ml ampicillin. Plasmids containing *dnaA*$^+$ (pKC596), *dnaA5* (pKC105) and *dnaA46* (pKC215) were confirmed by restriction digestion and agarose gel analysis.

Figure 1. Physical map of pKC plasmids carrying alleles of *dnaA*. A fragment containing the *dnaA* gene with *Nde* I and *Bam* HI ends engineered by PCR was inserted into the multiple cloning site of the vector pET11a. The ***Nde* I** site includes the ATG translation start codon. Immediately upstream (not indicated) of the target gene is a consensus Shine Delgarno ribosome binding site, binding site for the $lacI^q$ repressor and T7 RNA polymerase promoter. Restriction enzyme cleavage sites relevant to the subcloning of the ***dnaA*** alleles (Experimental Procedures) are indicated. The relative positions of the three missense mutations in *dnaA* are shown with the corresponding amino acid position and substitution. Other loci are *bla* , ß-lactamase gene conferring resistance to ampicillin; *lacI*, the $lacI^q$ repressor protein which binds downstream of the promoter to downregulate expression in the absence of induction; *ori*, pBR322 origin of replication. Plasmids and their corresponding alleles are **pKC596**, $dnaA^+$; **pKC105**, *dnaA5* (A184V + G426S); **pKC215**, *dnaA46* (A184V + H252Y); **pKC–A184V**, *dnaA–A184V*; **pKC–H252Y**, *dnaA–H252Y;* **pKC–G426S**, *dnaA–G426S*.


EcoRI
BamHI
RsrII
G-426-S
H-252-Y
A-184-V
BsmI
SphI
bla
dnaA
pKC-XXX
7335 bp
ori
EcoRI
NdeI
SphI
lacI
BsmI

Separation of the individual mutations in dnaA5 *and* dnaA46.

Plasmid DNAs pKC596, pKC105 and pKC215 were cleaved with restriction endonuclease *Bsm* I. There is one cleavage site for this enzyme within the coding sequence of *dnaA* just downstream from the mutation resulting in the amino acid change at position 184. This enzyme also cleaves once in the vector. Cleavage by *Bsm* I generates two DNA fragments, containing the coding sequence for the N-terminal portion of *dnaA*, with or without the A184V mutation depending on the starting material; the other fragment contains the C-terminal coding portion of *dnaA* composed of either the wild type sequence or the unique mutations from *dnaA5* (G426S) or *dnaA46* (H252Y). The six DNA fragments resulting from cleavage of the three plasmids were separated by agarose gel electrophoresis and fragments recovered from the gel. Fragments containing the individual point mutations were combined with the opposite fragment from the wild type plasmid. The fragments were ligated and transformed into XL1-Blue; transformants were selected on LB agar containing 100 μg/ml ampicillin at 30 °C. Individual colonies were selected and grown in LB supplemented with ampicillin to further screen the recombinant plasmids.

Purification of DnaA protein

Purification of DnaA proteins was carried out using a combination of published procedures with minor modification (Hwang and Kaguni, 1988b; Sekimizu, et al., 1988). Recombinant plasmids carrying the different alleles of

dnaA were transformed into either HMS174(DE3) or BL21(DE3)(pLysS) for expression of the target protein. Cultures were grown at 30 °C in a New Brunswick environmental shaker in two liters of LB + 50 μg/ml ampicillin, and 25 μg/ml chloramphenicol if a pLysS strain was being used. Cell growth was monitored by measuring the OD at 595 nm. When the OD_{595} was between 0.6 and 0.8, expression was induced. IPTG was added to a final concentration of 0.4 mM; expression of protein was for $2^1/_2$ hours at 30 °C. The cultures were chilled by placing the flasks in ice–water baths, and cells collected by centrifugation in a Sorvall GS–3 rotor at 5000 rpm for 10 minutes at 4 °C. The cleared supernatant was poured off and the cell pellet was resuspended in 20 ml of 50 mM Tris–HCl, pH 8.0 and 10% sucrose. The resuspended cells were frozen in liquid nitrogen and stored at –70 °C.

Cells were thawed on ice and lysed by addition of NaCl to 0.25 M, spermidine–HCl to 5 mM, EDTA to 20 mM, DTT to 5 mM, and lysozyme to 0.3 mg/ml. Samples were incubated on ice for 30 minutes, then frozen in liquid nitrogen. All subsequent operations were carried out at 0–4 °C unless otherwise indicated. Lysed cells were thawed, centrifuged in a Sorvall SS–34 rotor at 17,000 rpm for 30 minutes and the supernatant recovered. Protein was precipitated by addition of solid ammonium sulfate to 50% saturation (0.35 g/ml), collected by centrifugation in an SS–34 rotor at 15,000 rpm for 30 minutes and resuspended in Buffer A. The samples were diluted with Buffer A until the conductivity was equivalent to Buffer A + 0.1 M KCl. Protein was adsorbed batchwise by gentle mixing for two hours to heparin

Sepharose–4B resin which had been equilibrated with Buffer A + 50 mM KCl. One ml of packed resin was added for each 10–15 mg of protein. After transferring the slurry to a column, the resin was washed extensively (20 bed volumes) with Buffer A + 150 mM KCl. Protein was eluted with a linear gradient of 150 mM–1.0 M KCl in Buffer A in a total of eight column volumes. DnaA protein–containing fractions, determined by replication assays or by SDS–PAGE, were frozen in liquid nitrogen and stored at –70 °C.

Further purification of the mutant proteins varied depending on the particular mutant. This was necessary because use of methods successful for purification of $DnaA^{+}$ protein resulted in inactive preparations or insufficient amounts. Fractions from heparin Sepharose chromatography of DnaA–G426S were used without further treatment. Fractions which were found by gel filtration to be predominantly aggregated wild type protein were used as the source of $DnaA^{agg}$. DnaA5 and DnaA–A184V were concentrated by ammonium sulfate precipitation (50%) and resuspended with $^{1}/_{20}$ their original volume of Buffer G. DnaA46 protein was treated as described (Hwang and Kaguni, 1988b). Fractions were combined and dialyzed against two changes of Buffer E + 100 mM KCl until the conductivity of the sample was equivalent to Buffer E + 130 mM KCL. Precipitated material was collected and resuspended in $^{1}/_{10}$ the original volume of Buffer B + 0.5 M $(NH_4)_2SO_4$. In order to preserve activity, samples were aliquoted into 10 µl volumes, frozen and stored in liquid nitrogen. Aliquots taken from liquid nitrogen were used once and not saved. The activity of these mutant proteins is

extremely labile and after a limited number of freeze/thaw cycles the protein becomes inactive.

$DnaA^+$ and DnaA–H252Y were treated with guanidine hydrochloride (Sekimizu, et al., 1988). Fractions from heparin Sepharose chromatography were dialyzed against Buffer C for 12-16 hours, the precipitated protein was collected and the pellet backwashed with Buffer C containing 0.6 M $(NH_4)_2SO_4$ and 10 mM magnesium acetate. Pellets were then resuspended in Buffer C + 0.6 M $(NH_4)_2SO_4$, 10 mM magnesium acetate, and 4.0 M guanidine hydrochloride in one tenth the original sample volume. Guanidine–treated samples were subjected to gel filtration chromatography by FPLC on a Superose–12 (HR 10/30) column (Pharmacia) in Buffer D.

The purity of the various samples of DnaA protein, judged by silver stained SDS–PAGE, ranged from 60 to greater than 95%.

DNA replication assays

In vitro replication assays dependent on DnaA protein and a crude enzyme fraction (Fraction II) were performed as previously described (Fuller, et al., 1981). Reactions (25 µl) contained HEPES–KOH pH 7.8, 40 mM; ATP, 2 mM; GTP, CTP and UTP, 0.5 mM; dATP, dGTP, dCTP and [^{3}H] dTTP (15–25 cpm/pmol), each at 100 µM; magnesium acetate, 11 mM; phosphocreatine, 40 mM; creatine phosphokinase, 0.1 mg/ml; supercoiled M13*oriC*2LB5 (245 bp *oriC* insert in M13ΔE101), 200 ng (600 pmol in nucleotide); and Fraction II from WM433 (relevant genotype *dnaA204*), 150–200 µg. Reaction mixtures

were assembled at 0 °C and incubated at 30 °C unless otherwise indicated. The time of incubation was adjusted as appropriate for each mutant and are listed in the legend for Figure 5. Reactions were stopped and DNA precipitated by addition of 1.0 ml 10% (wt/vol) trichloroacetic acid and 0.1 M sodium pyrophosphate. Total nucleotide incorporation into acid insoluble form was determined by filtration on glass fiber filters (Whatman GF/C) and liquid scintillation counting. One unit of activity is defined as one pmol of nucleotide incorporation per minute at 30 °C.

ATP binding assays

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

DNA binding assays

oriC fragment retention assays to test the ability of the mutant DnaA proteins to bind to DNA were performed essentially as described (Wang and

Kaguni, 1987). Reactions (20 µl) contained HEPES–KOH, 40 mM pH 7.8; magnesium acetate, 5 mM; DTT, 2 mM; KCl, 100 mM; ATP, 0.5µM; *Hinf* I–digested pBR322 as competitor, 50 ng (17.5 fmol as plasmid); and 20 fmol of purified *Sma* I–*Xho* I fragment (from pBS*oriC*) containing *oriC* radioactively labeled by end filling with [α–^{32}P] dTTP and DNA polymerase I (large fragment). Reaction mixtures were assembled and preincubated at either 30 °C or 40 °C for two minutes prior to addition of DnaA protein. After addition of DnaA, reactions were incubated for 10 minutes at the indicated temperature. The reactions were filtered through nitrocellulose (Millipore, HAWP, 0.45 µm, 13 mm diameter) and washed with 250 µl of the reaction buffer without ATP. Retention of *oriC* was quantitated by liquid scintillation counting.

Alternatively, gel mobility shift assays (Parada and Marians, 1991) were performed to examine complexes formed by the mutant DnaA proteins with *oriC* DNA. Twenty–five fmol of the same labeled fragment as above was used in reactions (10 µl) containing HEPES–KOH pH 8.0, 40 mM; magnesium acetate, 5 mM, EDTA, 2 mM; DTT, 8 mM; Triton X–100, 0.4% (vol/vol); glycerol, 20% (vol/vol); and BSA, 10 mg/ml. DnaA protein was added and incubated at room temperature for five minutes. Samples were loaded on a 4% acrylamide (60:1) gel in 45 mM Tris–borate buffer, and electrophoresed at 80 V for four hours. The dried gels were autoradiographed using Hyperfilm MP (Amersham) and a Cronex Quanta III intensifying screen at –70 °C overnight.

Protein Determinations

Total protein determinations were performed using the dye binding method (Bradford, 1976), with BSA as a standard. Concentrations of the various DnaA proteins were determined by densitometric scanning of fractions after separation by SDS–PAGE in comparison to a highly purified sample of $DnaA^{+}$ as a standard. Silver stained gels were scanned on a Molecular Dynamics computerized scanning densitometer, and the image analyzed using the ImageQuant software package (ver. 3.1) also from Molecular Dynamics.

Results

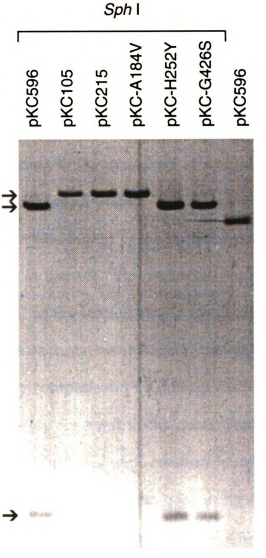
Confirmation of mutations in the various dnaA *alleles*

Separation of the missense mutations of the *dnaA5* and *dnaA46* alleles was confirmed by a combination of three methods; sensitivity to restriction endonuclease *Sph* I, heteroduplex cleavage analysis and DNA sequencing.

The *dnaA* gene has one recognition sequence for the restriction enzyme *Sph* I in its coding sequence, and one site is present in the vector pET11a. Cleavage of the various pKC plasmids should yield two fragments of 6489 and 846 bp. (Figure 1). The nucleotide substitution resulting in the mutation at amino acid 184 disrupts the *Sph* I site in *dnaA*. Cleavage by *Sph* I of pKC596 (*dnaA*$^+$), pKC–H252Y and pKC–G426S generated two fragments of the appropriate size (Figure 2). Digestion of those plasmids harboring an allele with the common mutation, pKC105 (*dnaA5*), pKC215 (*dnaA46*) and pKC–A184V, resulted in a single cleavage product corresponding to full–length, linear plasmid, confirming the presence of the mutation at amino acid 184.

Heteroduplex mapping identifies single base substitutions in large sections of DNA. Heteroduplexes were formed with an *Nde* I–*Rsr* II fragment of wild type *dnaA* (Figure 1) labeled either on the sense or antisense strand

Figure 2. Digestion of *dnaA* plasmids with *Sph* I. Plasmids digested with restriction enzyme *Sph* I are indicated. The lane marked **pKC596** (far right) is the untreated plasmid.


Sph I
pKC596
pKC105
pKC215
pKC-A184V
pKC-H252Y
pKC-G426S
pKC596

and unlabeled fragment from the other alleles of *dnaA*. Mispaired bases are chemically modified by base specific chemicals and the strand subsequently cleaved by piperidine. Hydroxylamine hydrochloride chemically modifies mispaired cytidine residues (Fraenkel-Conrat and Singer, 1972). All of the missense mutations in *dnaA5* and *dnaA46* are C⇨T transitions, two on the sense strand (A184V and H252Y) and one (G426S) on the antisense strand (Hansen, et al., 1992). In samples with the sense strand of $dnaA^+$ labeled, two cleavage products of the expected size were seen for the wild type/*dnaA46* heteroduplex, corresponding to the mutations at amino acids 184 and 252 (Figure 3A). The wild type/*dnaA–A184V* heteroduplex resulted in a single cleavage product. Its size was similar to the product generated by the corresponding mutation in *dnaA46*. Heteroduplex mapping with the antisense strand of $dnaA^+$ as the labeled strand resulted in a cleavage product expected from the missense mutation that gives rise to the G426S substitution in *dnaA5* (Figure 3B). By comparison to similarly treated homoduplexes, no other significant bands were observed in the heteroduplexes.

Treatment of heteroduplexes with osmium tetroxide, which modifies mispaired thymine residues, did not detect mispairing (data not shown).

DNA sequencing confirmed the expected C⇨T base change of the mutation in *dnaA–H252Y* (Figure 4).

Figure 3. Heteroduplex analysis of *dnaA* mutants. Heteroduplexes were formed with an end–labeled *dnaA*$^+$ restriction fragment and unlabeled fragments of the various *dnaA* alleles as indicated. **(A)** Heteroduplex reactions with the sense strand of *dnaA*$^+$as the labeled strand. **(B)** Heteroduplex reactions with the antisense strand al the labeled strand. The lanes marked **Untreated** are *dnaA*$^+$ duplex samples not treated with hydroxylamine or piperidine and indicate the position of uncleaved fragment.

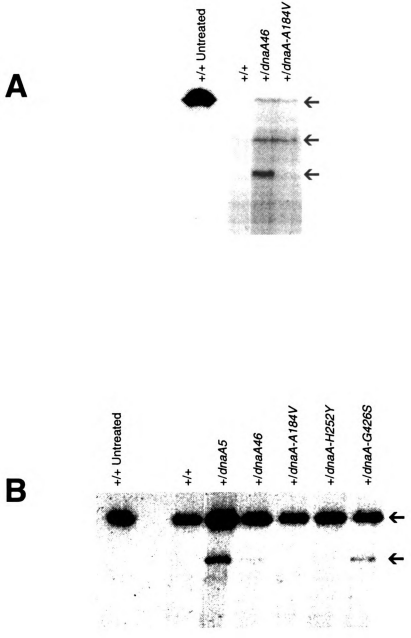
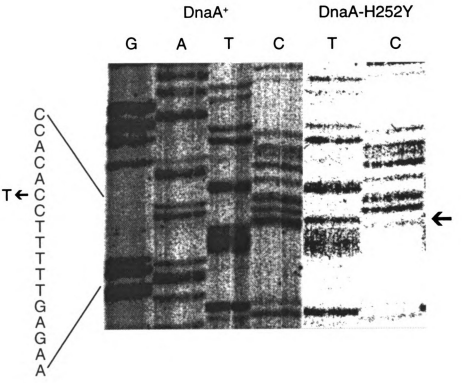

A
+/+ Untreated
+/+
+/dnaA46
+/dnaA-A184V
B
+/+ Untreated
+/+
+/dnaA5
+/dnaA46
+/dnaA-A184V
+/dnaA-H252Y
+/dnaA-G426S

Figure 4. DNA sequence of *dnaA–H252Y* mutant. Plasmids pKC596 (*dnaA*$^+$) or pKC–H252Y were used as templates for sequencing reactions by the Sanger dideoxy method (Sambrook, et al., 1989). Primer JK–21 (15–mer, 5'–ATAACCCGTTGTTCC–3') corresponds to nucleotides 494–508 of the coding sequence of *dnaA*. The sequence in the region surrounding the substitution is shown on the left with the expected C⇨T change at nucleotide 754 of the coding sequence. The nucleotide substitution in the sequence of *dnaA–H252Y* is indicated on the right (arrow).


DnaA+
DnaA-H252Y
G
A
T
C
T
C
T←
CCACACCTTTTTTGAGAA

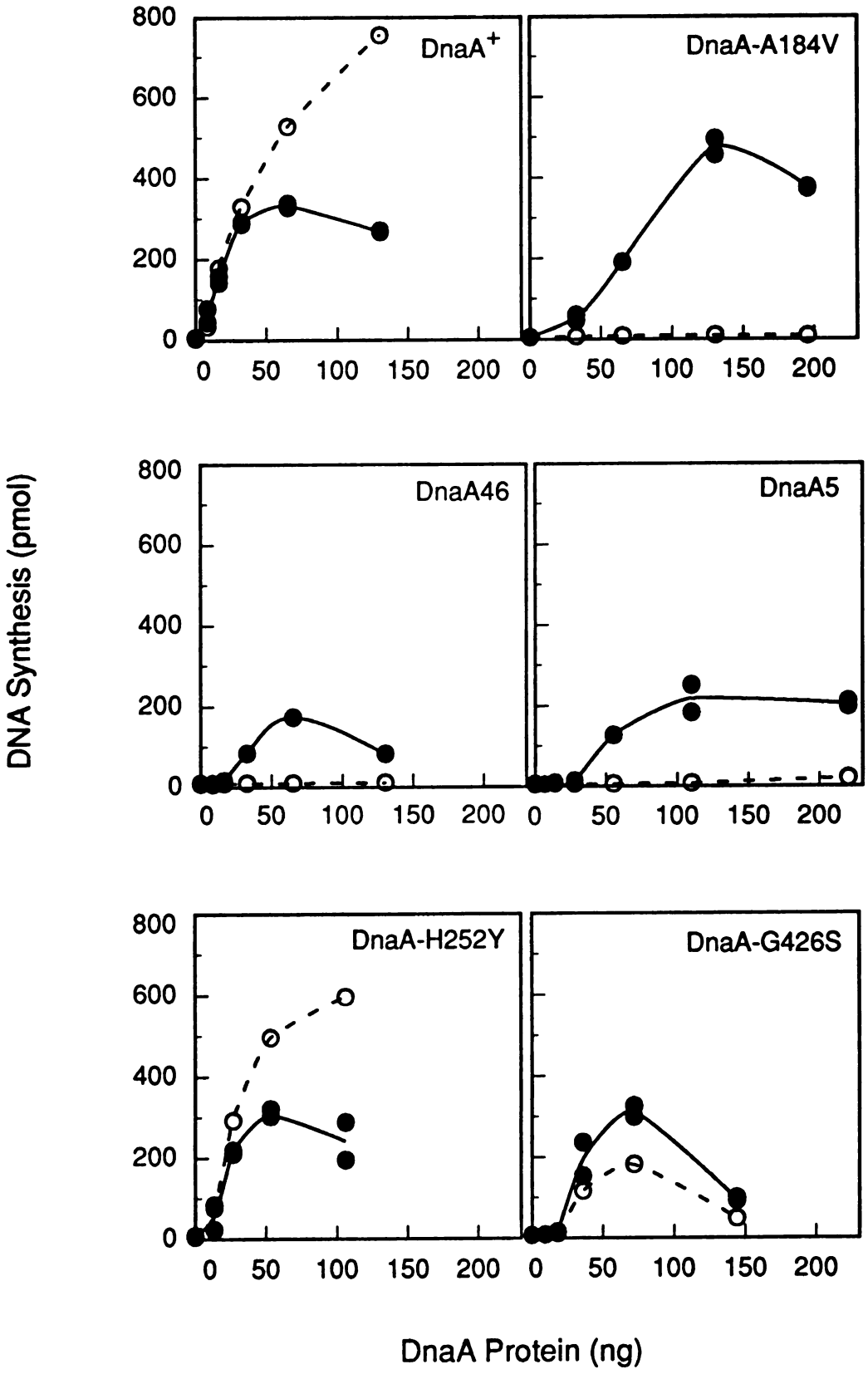
The thermolabile defect of dnaA5 *and* dnaA46 *is due to the mutation at amino acid 184*

DnaA protein is active in initiation of DNA replication in vitro. Replication activity can be measured in either a crude enzyme preparation from a *dnaA204* (Ts) strain or reconstituted with purified enzymes (Fuller, et al., 1981; Kaguni, et al., 1985). Both replication systems require supercoiled plasmid DNA containing either the complete (463 bp) or minimal (245 bp) replication origin from *E. coli*. DnaA5 and DnaA46 proteins were active in the crude enzyme replication assay at a temperature permissive for activity *in vivo*. At 40 °C, the non–permissive temperature, they were inactive in the initiation of DNA synthesis, consistent with the phenotype of these mutants.

The isolated single mutants were also examined in this assay (Figure 5). The mutant A184V was thermolabile for DNA replication. By contrast, DnaA–H252Y displayed no thermolability. Its activity was indistinguishable from that of wild type DnaA. The mutant DnaA–G426S showed partially temperature sensitive activity. Its activity was reduced approximately 50% at 40 °C relative to its activity at 30 °C. This defect is probably masked by the more severe temperature-sensitivity of the A184V mutation, and probably does not contribute significantly to the thermolabile phenotype of *dnaA5*.

Effects of the individual mutations on the activity of DnaA at the permissive temperature were subtle. DnaA–H252Y did not differ significantly in replication activity from wild type DnaA protein. For DnaA–G426S, there

Figure 5. Temperature sensitivity of mutant DnaA proteins in *in vitro* replication. The various DnaA proteins were titrated in DNA replication assays at 30 °C (●) or 40 °C (○). Times of incubation were: DnaA$^+$ and DnaA–H252Y, 20 min; DnaA–G426S, 30 min; DnaA5, DnaA46 and DnaA–A184V, 40 min.


DnaA+
DnaA-A184V
DnaA46
DnaA5
DnaA-H252Y
DnaA-G426S
DNA Synthesis (pmol)
DnaA Protein (ng)
0
200
400
600
800
0
50
100
150
200

was a more severe inhibition of activity at higher concentrations compared to DnaA$^+$. By contrast, DnaA–A184V protein was more active at this higher concentration.

Complementation of dnaA204*(Ts) by the mutant proteins*

Activity of the mutants *in vivo* was assayed by their ability to complement a strain which harbors the *dnaA204* allele (Table 2). The *dnaA204* allele confers a temperature–sensitive phenotype. *dnaA5*, *dnaA46* and *dnaA–A184V* were unable to complement the temperature–sensitive phenotype of this strain, consistent with the thermolability observed in *in vitro* replication assays. There was a slight increase in colony forming ability observed with *dnaA5* but it is not clear from this assay if this represents a significant difference in its phenotype relative to *dnaA46* or *dnaA–A184V*. The *dnaA–H252Y* allele supported growth at 42 °C almost as efficiently as *dnaA*$^+$, consistent with the observation that its replication activity was not thermolabile *in vitro*. Interestingly, *dnaA–G426S*, when supplied on a plasmid, resulted in an intermediate efficiency of colony formation. *In vitro*, this protein showed partially temperature–sensitive replication activity. It should be noted that the *in vivo* activity of these proteins at 30 °C can not be assessed with this assay since the chromosomal *dnaA204* allele is active at this temperature. Also, the level of plasmid–encoded protein was not determined and may have varied between the different mutants. None of the

Table 2. *in vivo* complementation of *dnaA204* by the alleles of *dnaA*

Plasmid Borne Allele	Plating Efficiency (CFU 42°/CFU 30°)
none	1.6×10^{-5}
dnaA^{+}	0.94
dnaA46	0.7×10^{-5}
dnaA5	1.3×10^{-4}
dnaA-H252Y	0.79
dnaA-G426S	1.5×10^{-2}
dnaA-A184V	2.5×10^{-5}

Strain BL21(DE3)*dnaA204*(pLysS) was transformed with plasmids harboring the various alleles of *dnaA* as well as the vector pET11a. Transformants were selected on LB plates containing 50 µg/ml ampicillin and 25 µg/ml chloramphenicol at 30° C. Plasmid minipreps were performed to verify the presence of the appropriate plasmid. Overnight cultures grown in LB media containing ampicillin and chloramphenicol were diluted in LB media and spread in duplicate on LB plates with the same antibiotics. Relative plating efficiency at 42 °C versus 30 °C was determined after overnight incubation. (My appreciation to Joe Lipar for assistance in collecting this data.)

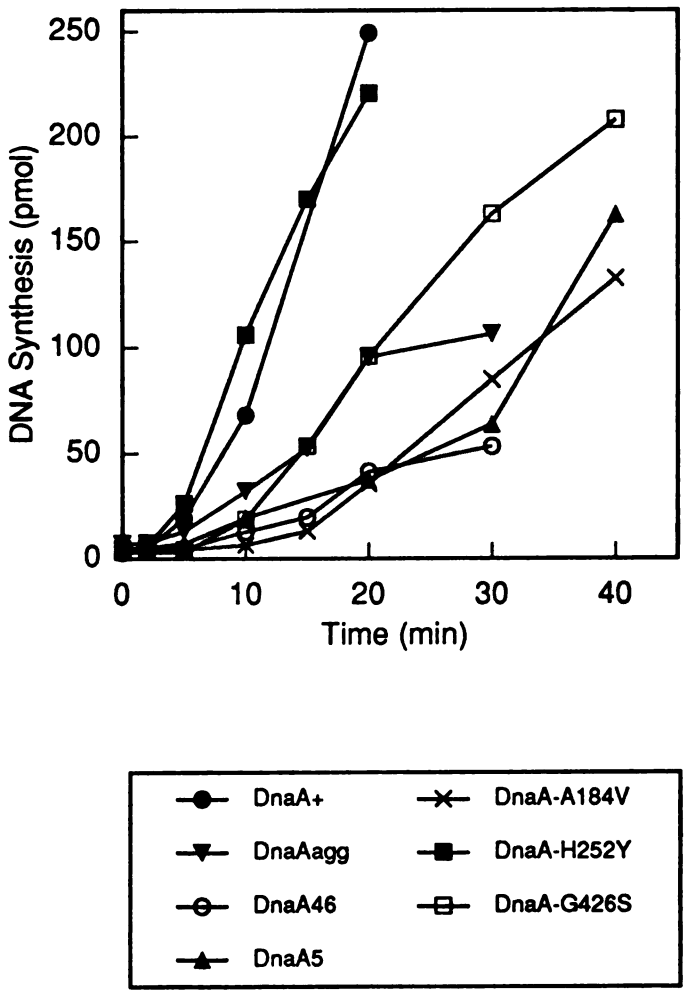
plasmid–encoded proteins had an observable effect on growth at the permissive temperature.

The prolonged lag associated with DnaA5 and DnaA46 proteins is due to the mutation at amino acid 184

In vitro replication assays dependent on DnaA are characterized by lag period of 2–5 minutes prior to incorporation of deoxynucleotides (Fuller, et al., 1981). Previous biochemical characterizations of DnaA5 and DnaA46 protein have shown that the lag period associated with these proteins is much longer (12–18 minutes). The extended lag period is the result of a required activation step in this assay for DnaA5 and DnaA46 which involves the heat shock proteins DnaK and GrpE. This interaction takes place prior to interaction with *oriC* (Hupp and Kaguni, 1993b; Hwang and Kaguni, 1988a).

A time course of DNA synthesis was performed with the various mutant proteins (Figure 6). Reactions containing 50–65 ng of the DnaA proteins were incubated at 30 °C, and stopped at times indicated. As previously observed, wild type DnaA had a short lag period of approximately 2 minutes before incorporation of dNTPs. DnaA5 and DnaA46 had much longer lag periods of 10–15 minutes. The lag period of DnaA–A184V was similar to that of DnaA5 and DnaA46. DnaA–H252Y had a lag period similar to that of $DnaA^+$. The lag period for DnaA–G426S, while longer than that of wild type was shorter than that of DnaA–A184V. The A184V and G426S mutations did not appear to have an additive effect on the lag period of

Figure 6. Time course for DNA synthesis by mutant DnaA proteins. DNA replication assays were assembled as described in Experimental Procedures with constant amounts of the specified protein (50–65 ng). The reactions were incubated at 30 °C, stopped at the indicated times and DNA synthesis was quantitated (Experimental Procedures).

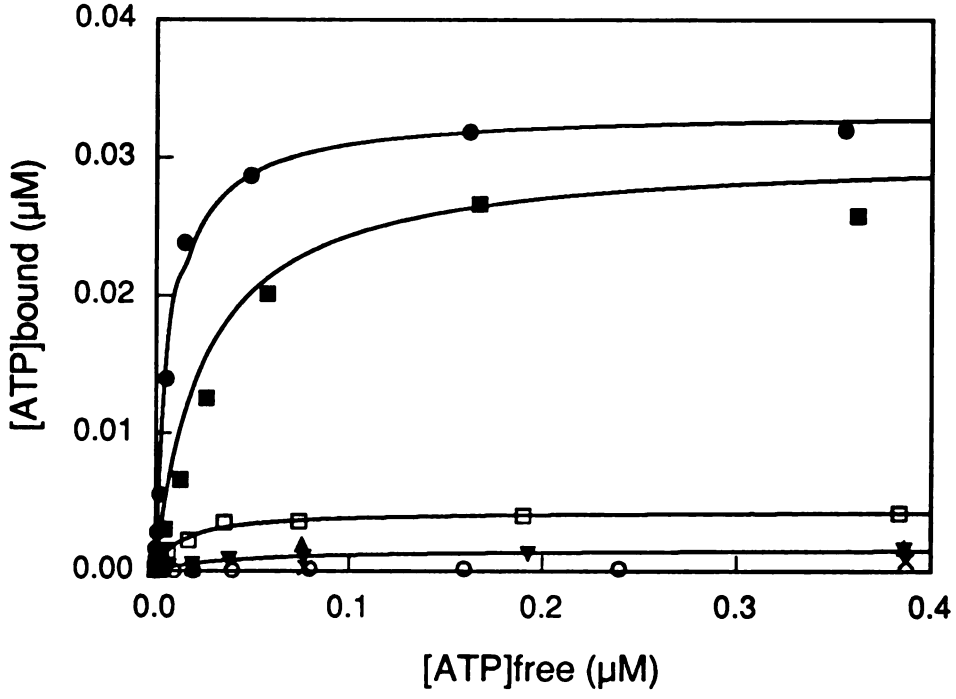

250
200
150
100
50
0
DNA Synthesis (pmol)
0
10
20
30
40
Time (min)
DnaA+
DnaAagg
DnaA46
DnaA5
DnaA-A184V
DnaA-H252Y
DnaA-G426S

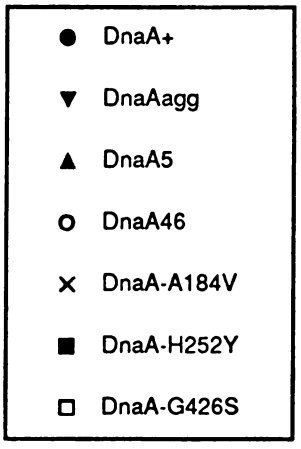
DnaA5. The extended lag of DnaA5 was similar to the lag period of the single mutant A184V. This may indicate that the lag periods represent independent processes and the shorter lag period resulting from mutation at amino acid 426 is masked by the longer process associated with the mutation at amino acid 184. Aggregated DnaA$^+$ was also examined and the lag period of 5–10 minutes seen was longer than for monomeric DnaA protein.

The ATP binding defect appears to be a function of the A184V mutation

DnaA protein binds ATP with high affinity (K_D=0.03μM), and bound ATP is required for replication activity (Sekimizu, et al., 1987). DnaA5 and DnaA46 proteins fail to bind ATP. This defect had previously been attributed to the amino acid substitution at amino acid 184 (Hupp and Kaguni, 1993c; Hwang and Kaguni, 1988a), common to both, since it is very close to a consensus ATP binding motif (P–loop). Using a filter binding assay, DnaA$^+$ was observed to bind with high affinity (Figure 7). The calculated ratio of ATP bound per monomer of DnaA was 0.42. Less than stoichiometric binding of ATP by DnaA$^+$ has consistently been reported in the literature (Hwang and Kaguni, 1988a; Sekimizu, et al., 1987) with values ranging from 0.1–0.3 ATP bound per DnaA monomer. DnaA–H252Y bound ATP with an affinity similar to DnaA$^+$and with a ratio of 0.38 ATP bound per monomer. DnaA5, DnaA46 and DnaA–A184V failed to bind ATP at any appreciable level. This would tend to confirm that the ATP binding defect is attributable to the

Figure 7. ATP binding by mutant DnaA proteins. Increasing amounts of [α–^{32}P] ATP were incubated with a fixed amount of DnaA protein (2 pmol). ATP binding was determined by retention on nitrocellulose filters (Experimental Procedures). Curves were drawn, the dissociation constant (K_D) and stoichiometry of binding (n) were determined by fitting the data to the binding equation : $[ATP]_{bound} = n \times K_{eq} \times [E_t] \times [ATP]_{free}/(1 + K_{eq} \times [ATP]_{free})$; $[E_t]$ = concentration of DnaA protein (0.08μM as monomer), $K_D = 1/K_{eq}$. K_D and n values calculated are DnaA$^+$, 0.01 μM, 0.42; DnaA–H252Y, 0.025 μM, 0.38; DnaA–G426S, 0.014 μM, 0.055; DnaA^{agg}, 0.04 μM, 0.02.


0.04
0.03
0.02
0.01
0.00
[ATP]bound (μM)
0.0
0.1
0.2
0.3
0.4
[ATP]free (μM)

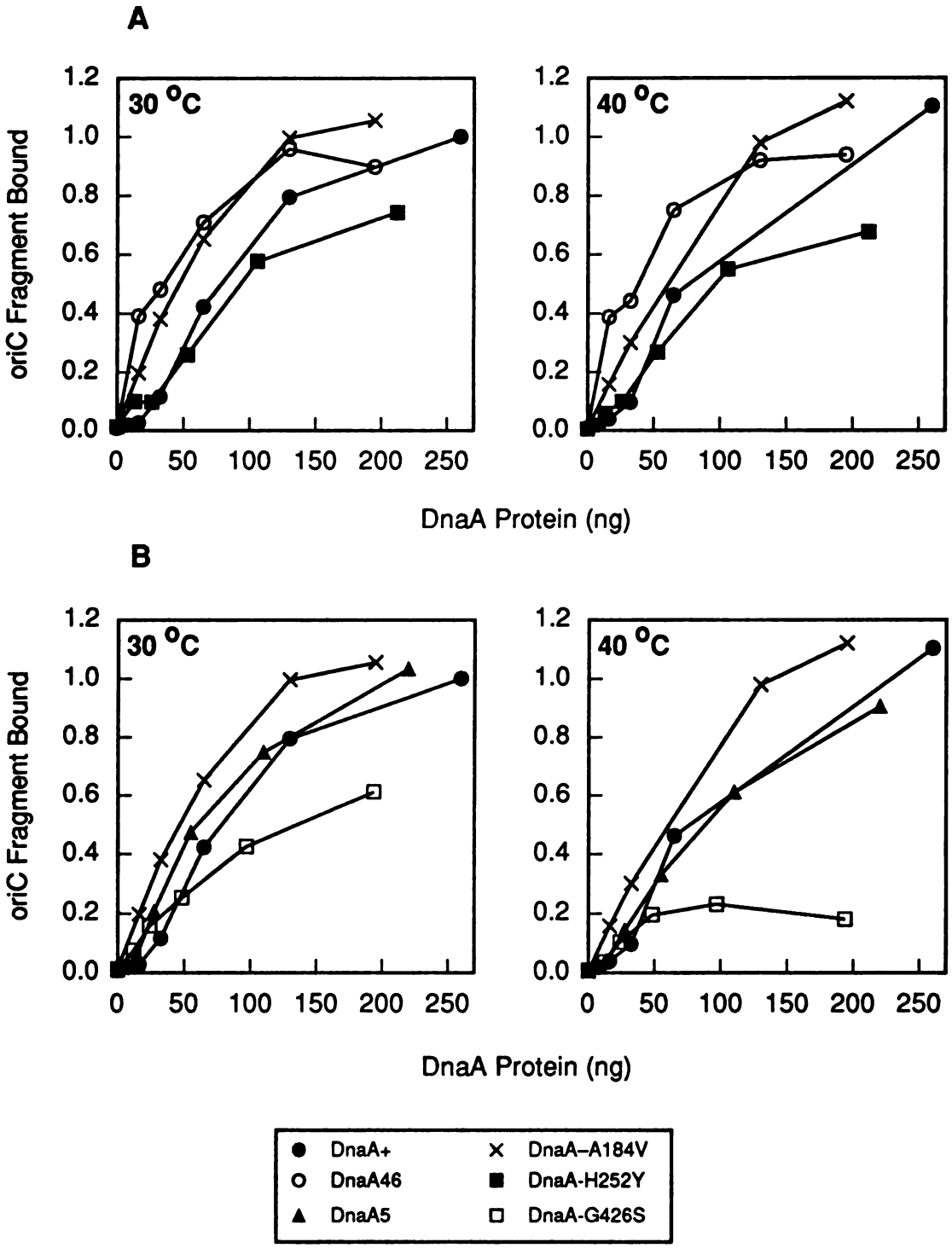

DnaA+
DnaAagg
DnaA5
DnaA46
DnaA-A184V
DnaA-H252Y
DnaA-G426S

mutation at amino acid 184. However, assay of $DnaA^{agg}$ showed it to have an extremely low, but detectable ATP binding activity. ATP binding by DnaA–G426S was only slightly better than the aggregate. The $DnaA^{+}$ (monomer) used in this study was derived directly from the $DnaA^{agg}$ sample by guanidine hydrochloride treatment followed by gel filtration chromatography (Experimental Procedures). This method did not result in active protein except for H252Y. The very low extent of ATP binding by the aggregated DnaA protein suggests that the physical state of the protein has a significant impact on its ability to bind ATP. In addition to $DnaA^{+}$, DnaA–H252Y was subjected to guanidine treatment to obtain monomer. High affinity ATP binding by DnaA–H252Y was observed before or after guanidine treatment, and an increase in the ratio of ATP bound after treatment was observed (data not shown). Extended incubation of $DnaA^{agg}$ at 0 °C or 25 °C did not result in increased binding of ATP by $DnaA^{agg}$ (data not shown).

The mutant proteins retain affinity for oriC *DNA*

DnaA protein has been shown to bind cooperatively to *oriC* at the four DnaA boxes present (Fuller, et al., 1984). *In vitro,* binding to a supercoiled *oriC* plasmid has been shown to result in local unwinding of the DNA (Bramhill and Kornberg, 1988a). This unwinding is postulated to be required for binding of other prepriming components. The ability of the various mutant proteins to recognize and bind to *oriC* was tested using a linear DNA

Figure 8. Fragment retention assay for *oriC* binding by mutant DnaA proteins. ^{32}P–labeled, 461 bp *Sma* I–*Xho* I fragment from pBS*oriC* was incubated with the indicated amounts of DnaA proteins at 30 °C or 40 °C. The amount of fragment retained on filters was quantitated by liquid scintillation counting. Binding is expressed relative to the amount of fragment bound by the highest level of DnaA$^+$ at 30 °C (=1.0). **(A).** Binding by DnaA46 and corresponding single mutants (A184V & H252Y) relative to wild–type DnaA. **(B).** Binding by DnaA5 and its corresponding single mutants (A184V & G426S). Binding by DnaA$^+$ and DnaA–A184V from (A) are shown again for comparison.


A
30 °C
40 °C
oriC Fragment Bound
DnaA Protein (ng)
B
30 °C
40 °C
oriC Fragment Bound
DnaA Protein (ng)
DnaA+
DnaA46
DnaA5
DnaA-A184V
DnaA-H252Y
DnaA-G426S

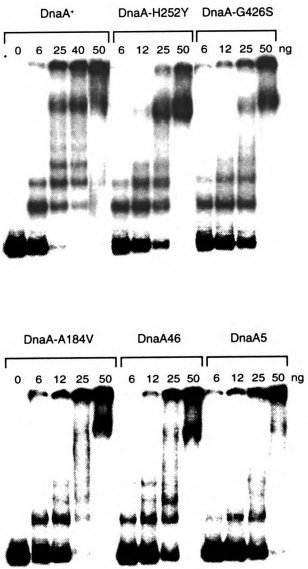
fragment containing *oriC* by a filter binding assay. All of the forms of DnaA were able to bind to the origin at either 30 °C or 40 °C (Figure 8). The cooperative nature of binding by $DnaA^+$ is suggested by the sigmoidal shape of the curve. DnaA46 and DnaA–A184V, while retaining affinity for *oriC* DNA, appeared altered in their binding (Figure 8A). Binding of *oriC* did not appear to be cooperative and was more efficient than $DnaA^+$ at lower concentrations of protein. DnaA–H252Y protein bound to *oriC* in a sigmoidal manner similar to wild type. Apparently, this mutation does not influence the DNA binding activity of DnaA46 since its binding was similar to that of DnaA–A184V. Binding to *oriC* by DnaA46 and DnaA–H252Y was unaffected at 40 °C relative to 30 °C, whereas DnaA–A184V showed a slight reduction. It was still able to bind to *oriC* at least as well as the wild type at the elevated temperature. That the DNA binding activity of DnaA46 appears similar to DnaA–A184V suggests that this common mutation affects DNA binding. The DnaA–H252Y mutant protein appears similar to wild type in DNA binding suggesting that this residue is not important in this activity.

DnaA5 and the corresponding single mutation, DnaA–G426S, were also measured for *oriC* binding activity (Figure 8B). Binding by DnaA5 to *oriC* was slightly temperature sensitive as was previously reported (Hupp and Kaguni, 1993c). DnaA–G426S protein was reduced in its ability to bind to *oriC* at 40 °C compared to 30 °C. Relative to wild type DnaA, the binding to *oriC* was somewhat reduced at 30 °C. Interestingly *oriC* binding by DnaA5 protein was intermediate between the activity observed for the two single mutant

proteins suggesting that their effects counteract each other when both are present. Whether the activity of DnaA5 results from an interaction between the two mutations or represents an average of independent effects cannot be determined from this experiment.

Fragment retention or filter binding assays only measure the total amount of DNA bound by protein but reveal little about the nature of the protein–DNA complexes. DNA gel shift assays were performed to examine the complexes formed between the mutant proteins and a restriction fragment containing *oriC* (Figure 9). Addition of increasing amounts of $DnaA^+$ protein resulted in the appearance of a number of complexes which are resolved by native gel electrophoresis. These complexes represent the ordered binding of DnaA to the four DnaA boxes present in *oriC* (C. E. Margulies and J. M. Kaguni, unpublished results). Examination of the mutant proteins revealed that DnaA–H252Y and DnaA–G426S formed complexes nearly identical to $DnaA^+$. The other three proteins, DnaA5, DnaA46 and DnaA–A184V also formed at least some of the same complexes with *oriC* as wild type DnaA. However, there was a reduction in the abundance of intermediate complexes formed. This could be the result of the A184V mutation present in these proteins. Alternatively, it may be related to the physical state of the purified protein. No new or anomalous complexes were observed between the mutant DnaA proteins and *oriC* suggesting that gross alterations in the association of these proteins with *oriC* were not occurring.

Figure 9. Gel shift assay of *oriC* binding by mutant DnaA proteins. The indicated amounts of DnaA protein were incubated with DNA fragment (described in the legend for Fig. 8) containing *oriC*. Separated complexes were visualized by autoradiography. The uppermost band corresponding to the position of the well are of complexes which did not enter the gel.


DnaA+
DnaA-H252Y
DnaA-G426S
0 6 25 40 50 6 12 25 50 6 12 25 50 ng
DnaA-A184V
DnaA46
DnaA5
0 6 12 25 50 6 12 25 50 6 12 25 50 ng

Discussion

In order to understand better the contribution of the individual missense mutations in *dnaA5* and *dnaA46* to their observed biochemical defects, the missense mutations were separated from each other and the resulting single mutant proteins were studied. The mutation at amino acid 184 (alanine ⇨ valine) is common to both of these alleles and was found to be responsible for the thermolabile defect and the prolonged lag in initiation of chromosomal replication. Correlation of these two biochemical defects with a single mutation would be predicted because both of the defects were previously shown to be related. The prolonged lag period is due to a required activation of the mutant proteins by the heat shock proteins DnaK and GrpE (Hupp and Kaguni, 1993a; Hwang and Kaguni, 1991) and it is this interaction which was thermolabile (Hupp and Kaguni, 1993b; Hwang and Kaguni, 1988a). Aggregated $DnaA^{+}$ protein also showed a lag in *in vitro* DNA synthesis that was shorter than that for those proteins with the A184V mutation. Since there was only a very short lag for monomeric $DnaA^{+}$, these results suggest that the activation process involves disaggregation of the mutant proteins. This is supported by reports that $DnaA^{agg}$, like DnaA5 and DnaA46 is unable to function in *in vitro* replication systems using purified proteins without prior activation by DnaK protein (Hwang, et al., 1990). The DnaK activation does result in conversion of DnaA to a monomeric form.

The mutation at amino acid 184 results in a less efficient and thermolabile interaction with heat shock proteins. The short lag period observed for DnaA–G426S is probably also the result of protein aggregation like that of $DnaA^{agg}$ and not related to its defect in binding to *oriC*, since it has been shown previously that the activation of DnaA5 protein, which harbors the 426 mutation, can occur prior to its interaction with *oriC* (Hupp and Kaguni, 1993b).

The ATP binding defect previously reported for DnaA5 and DnaA46 (Hupp and Kaguni, 1993c; Hwang and Kaguni, 1988a) had been attributed to the A184V mutation since it is very close to a consensus ATP binding (P–loop) motif. No ATP binding was observed with DnaA–A184V, whereas detectable binding by the other two single mutant proteins was observed. This supports the hypothesis that the mutation at amino acid 184 is directly responsible for the inability of DnaA5 and DnaA46 to bind ATP. Comparison of the ATP binding activities of $DnaA^{+}$ and $DnaA^{agg}$, however, suggests that physical state of the purified protein greatly influences the ability of DnaA to bind ATP. Aggregated $DnaA^{+}$ protein is known to be associated with phospholipids, among them cardiolipin (Hwang, et al., 1990). Acidic phospholipids have been implicated in regulating the activity of DnaA and cardiolipin has been shown to be able to prevent ATP binding by DnaA under certain conditions (Sekimizu and Kornberg, 1988) or alternatively to allow the exchange of ADP and ATP on DnaA. Presumably the guanidine hydrochloride treatment in the purification of $DnaA^{+}$ and DnaA–H252Y

removes bound phospholipids but this has not been examined directly. DnaA–G426S, which like DnaA^{agg}, has not been treated with guanidine, binds ATP only slightly better than DnaA^{agg} and significantly less well than DnaA^{+}. In those proteins where binding can be detected, the K_D values determined are similar to that previously reported for DnaA of 0.03 μM (Sekimizu, et al., 1987) but they vary tremendously in the calculated n value. n is a measure of the number of ATP molecules bound per molecule of DnaA protein, but the n value may also be interpreted as a representing the fraction of DnaA molecules in the sample capable of binding ATP. This fraction may vary as a function of aggregation, the presence of bound phospholipids, or the contribution of both of these factors. These results further suggest that the processes of activation, disaggregation and ATP binding are related. A number of reports in the literature would tend to support this view. Nucleotide binding by DnaA stabilizes the monomeric form of the protein (Yung, et al., 1990). Cardiolipin can, under varying conditions, inhibit nucleotide binding by DnaA or facilitate the turnover from the inactive, ADP–bound DnaA to active ATP–bound DnaA (Sekimizu and Kornberg, 1988). Aggregated DnaA protein, which is inactive in *in vitro* replication using purified proteins, is complexed with phospholipids. The aggregated protein can be activated for initiation of DNA replication by DnaK protein or by phospholipase A_2, an enzyme which degrades phospholipids (Hwang, et al., 1990).

It is still a possibility that the mutation at amino acid 184 does result in a defect in ATP binding not dependent on aggregation. DnaA5 was unable to form the characteristic open complex at *oriC* (Hupp and Kaguni, 1993c). Formation of this complex is dependent on the ATP-bound form of DnaA (Sekimizu, et al., 1987) so the deficiency of DnaA5 was attributed to its lack of ATP binding. DnaA5 which had been activated for replication by DnaK and GrpE was still unable to form this complex (cited in Hupp and Kaguni, 1993a), which may indicate it is still unable to bind ATP. Disaggregation of $DnaA^{agg}$ resulting in ability to bind ATP may not be an appropriate model for activation of DnaA5 and DnaA46. $DnaA^{agg}$ is activated for replication in reconstituted systems by DnaK alone (Hwang, et al., 1990), whereas DnaA5 and DnaA46 absolutely require GrpE in addition to DnaK (Hupp and Kaguni, 1993a).

Binding of DnaA protein to a DNA fragment containing the *oriC* sequence, measured by nitrocellulose filter binding assay, was altered by two of the missense mutations, A184V and G426S. DnaA-A184V and DnaA46 bound *oriC* more efficiently at low protein concentrations but the cooperative nature of the DNA binding appeared reduced. A previous report that DnaA46 failed to bind *in vitro* to DNA containing the consensus DnaA binding sequence was apparently erroneous (Hwang and Kaguni, 1988a). The activity of these mutant proteins is very labile when purified (numerous personal observations) and this may have been a factor in the previous observations. The ability of DnaA46 to bind to *oriC* does however contradict

numerous other reports that DnaA46 cannot repress transcription from the *dnaA* promoter at the nonpermissive temperature (Kucherer, et al., 1986). Inability to repress transcription has been taken to indicate that the mutant protein does not bind to DNA at elevated temperature. A number of factors may explain this apparent discrepancy, most obvious is that the binding to two different DNA fragments is being measured; *oriC* contains four DnaA boxes whereas the *dnaA* promoter contains only one. Another difference is the assay used. In the fragment retention assay the mutant protein is exposed to the elevated temperature for only a short time. In *in vivo* transcriptional repression assays, the protein is held at the nonpermissive temperature for a longer period. The presence of other cellular factors in the *in vivo* assay may also negatively effect the ability of the protein to bind at elevated temperatures. DnaA–H252Y was not altered in binding to *oriC* relative to $DnaA^+$ and the mutation did not influence the DNA binding activity of the DnaA46 protein. The altered nature of the *oriC* binding activity of DnaA46 was apparently due to the A184V mutation alone. The mutation G426S gave rise to a partially temperature sensitive defect in binding to DNA and it is likely the cause of the observed partially thermolabile replication activity. Although no direct evidence has been reported in the literature, there are suggestions that the C–terminal domain of DnaA is involved in DNA binding (cited in Messer and Weigel, 1994; Skarstad and Boye, 1994). The clear defect in DNA binding by DnaA–G426S is the first biochemical evidence documenting the involvement of this region of DnaA in DNA binding.

Further investigation of the interaction of the mutant DnaA proteins with *oriC* was done using a gel shift assay. The similarity of complex formation observed for DnaA$^+$ and DnaA–G426S indicates that the defect resulting in the temperature sensitive binding of DnaA–G426S did not apparently alter the structure of the DnaA:*oriC* complex, at least at 25 °C, the assay temperature for the gel shift. The reduced abundance of the intermediate complexes seen with mutants harboring A184V may be due directly to the mutation, or secondarily to some altered conformation of the protein resulting from the mutation. DnaA5 protein appeared to be the most affected in formation of these intermediate complexes. Previous examination of the interaction of DnaA5 with *oriC* by DNase I footprinting (Hupp and Kaguni, 1993c) revealed that the protein was altered in its association with *oriC*, protecting broader and less well defined regions within *oriC*. Altered patterns in the gel shift assay support the previous report that DnaA5 is somehow altered in its association with *oriC*. Since both of the amino acid substitutions in DnaA5 were shown to affect interaction with *oriC*, it is not clear if one of them or interaction of the two is responsible for the altered footprint.

An alanine–to–valine substitution at amino acid 184 has been identified in two other mutants of *dnaA* besides *dnaA5* and *dnaA46*. Like them, these other mutants also have a second missense mutation (Hansen, et al., 1992). Though no direct evidence exists, this curious finding has been used to suggest that a mutation at this amino acid is so deleterious it can only

be maintained if there is a secondary mutation to compensate. The *oriC* binding activity of DnaA5, measured in the fragment retention assay, was affected by both of the mutations present. Increased binding to *oriC* caused by the A184V mutation was balanced by a decrease in binding due to the G426S mutation. The net result was activity more closely approximating wild type DnaA. The *oriC* binding activity of DnaA46 was unaltered relative to DnaA–A184V however, which would suggest that this altered activity does not represent a lethal effect. Other than the DNA binding activity, the only other difference between the DnaA–A184V and the double mutants observed in this study was in the DNA replication. The increased activity at higher levels of protein observed for DnaA–A184V and the more pronounced inhibition seen with DnaA–G426S appeared to offset each other when present jointly in DnaA5. The same reduction in activity of DnaA46 relative to DnaA–A184V was seen, and was the only difference observed between DnaA–A184V and DnaA46. In all of the biochemical activities examined thus far, DnaA–H252Y was indistinguishable from $DnaA^+$, so there is no apparent explanation for having this effect in DnaA46. Since amino acid 252 is so highly conserved (identical in 14 out 15 species, reviewed in Messer and Weigel, 1994) as are 184 and 426, it is surprising that a mutation at this position did not alter the activity of the protein in at least some small way. A phenotype resulting from this mutation is of course still possible since the entire range of DnaA protein function has not been examined, or an effect may only result through interaction with the A184V mutation.

Chapter III

Summary and Perspectives

Three missense mutations that are found in the *dnaA5* and *dnaA46* alleles were separated in order to correlate biochemical defects to the individual mutations. Recombinant DNA techniques were used to separate the mutations and the T7 RNA polymerase expression system (Novagen) used to overproduce the various forms of DnaA for study.

The alanine–to–valine substitution at amino acid 184 that is common to both of the mutants was responsible for the thermolabile defect and prolonged lag prior to initiation of DNA replication *in vitro*. A partial temperature sensitive defect was also observed when glycine 426 is substituted with serine, the mutation unique to DnaA5. The ability of the various mutant or wild type *dnaA* alleles to complement the temperature sensitive phenotype of *dnaA204 in vivo* correlated with the results obtained *in vitro*. Examination of the mutant alleles in this genetic background however precludes assessing their function *in vivo* at the permissive temperature since the *dnaA204* strain is viable at 30 °C. Reintroducing the alleles of *dnaA* with the isolated mutations onto the chromosome in an otherwise normal genetic background will allow for a more careful examination of their phenotypes *in vivo*. Physiological studies of the single mutants would also provide more insight into their effects on DnaA protein function in initiation of replication. Of particular interest would be flow

cytometry experiments to examine the synchrony of initiation with each of the alleles. It will also be possible to address directly whether or not the A184V mutation is lethal when present alone and if the second mutations compensate for this.

ATP binding by mutants carrying A184V was not detected. This is expected since the mutation is very close to the putative ATP binding site. Aggregated, wild type DnaA, however, was also unable to bind ATP at any significant level, suggesting an alternate explanation for the inability of these mutants to bind ATP. Determination of the ability of these mutants to bind ATP is of central importance to understanding the role ATP binding plays in regulating the activity of DnaA. The effects of DnaK/GrpE activation of DnaA5, DnaA46 and DnaA–A184V on the physical state of these proteins can be examined by centrifugation or gel filtration studies. Whether activation of the mutant proteins for replication also confers the ability to bind ATP may be addressed by crosslinking and SDS–PAGE analysis to separate the crosslinked DnaA protein from DnaK. DnaK also binds ATP.

DNA binding activity to *oriC* DNA was found to be altered by two of the mutations examined, A184V and G426S. DnaA–A184V and DnaA46 bound to *oriC* more efficiently at lower protein concentrations and the binding was not thermolabile. DnaA–G426S was partially temperature sensitive for binding to *oriC* and likely explains the thermolability observed in the DNA replication assay. This defect did not result in detectable alterations of the DnaA:*oriC* interaction in a gel shift assay. Alteration of

oriC:DnaA interaction with these mutants had already been suggested by genetic and biochemical studies. Deletion of *topA*, encoding topoisomerase I can suppress the temperature sensitive phenotype of *dnaA46* (Louarn, et al., 1984). Loss of topoisomerase I activity results in a greater degree of negative superhelicity in the chromosome. Altered supercoiling may compensate for a defect in the protein:DNA interaction. DNase I protection patterns at *oriC* were altered with DnaA5 relative to $DnaA^+$. Further investigation of the defects observed in DNA binding with these mutants by footprinting, determination of affinity on supercoiled versus relaxed templates and ability to promote strand unwinding will better clarify the nature of the defect(s) in DNA binding.

The histidine–to–tyrosine change at amino acid 252 in DnaA46 did not alter the activity of the protein relative $DnaA^+$ in any of the assays used in this study. This is quite surprising given that this amino acid is conserved in 14 out of 15 homologues of *dnaA* which have been sequenced thus far (reviewed in Messer and Weigel, 1994). It also did not have a significant influence on the activity of DnaA46. Except for a subtle difference in activity in the DNA replication assay, no other difference was observed between DnaA46 or the single mutant DnaA–A184V. An alternate function of this region is suggested by the observation the codon for amino acid 252 is part of a DnaA box present in the reading frame of *dnaA*. A transcriptional termination event has been shown to occur in this region of *dnaA* but it is

unclear if it is dependent on DnaA protein or this binding sequence (Wende, et al., 1991).

Identification of specific biochemical defects associated with a mutation and correlation to physiological alterations are common and useful methods to understand of the normal functioning of proteins. Genetic studies strongly suggest that the mutation at amino acid 184 is responsible for the asynchronous initiation phenotype observed in *dnaA5* and *dnaA46* strains (reviewed in (Hansen, et al., 1992). There are multiple biochemical defects observed for DnaA–A184V, including an increased tendency for self aggregation, a possible defect in ATP binding and an alteration in *oriC* interaction. All of these have been proposed as being involved in the regulatory function of DnaA protein.

Biochemical characterization of other *dnaA* mutants will complement knowledge gained from the study of DnaA5, DnaA46 and the corresponding single mutant proteins. Such studies will be easier due to improved expression of *dnaA* in the pET system allowing more rapid purification of new mutant proteins.

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