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Genetic and Biochemical Analysis of its
Replication Activity

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Mark D. Sutton

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**Mutants of the *E. coli dnaA* Gene: Genetic and Biochemical Analysis
of its Replication Activity**

By

Mark David Sutton

A DISSERTATION

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

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Abstract

Mutants of the *E. coli dnaA* Gene: Genetic and Biochemical Analysis of its Replication Activity

By

Mark David Sutton

The *Escherichia coli* DnaA protein is a sequence-specific DNA binding protein required for initiation of chromosomal replication. Numerous plasmids and bacteriophage including pSC101, R6K, F factor, and P1 also require DnaA protein for replication. Although the *dnaA* gene was identified three decades ago, only a handful of mutant alleles have been described. Furthermore, very little is known regarding the domain structure of DnaA protein at the functional or structural level. To identify functional domains of DnaA, novel mutants of the *dnaA* gene were isolated. These were obtained using two independent approaches, both of which relied on the pSC101 origin which is dependent on the *dnaA* gene for replication. Genetic and biochemical characterization of these novel alleles suggest that the DnaA protein contains four functionally distinct domains. Detailed characterization of one class of missense mutations that mapped to the C-terminal 85 residues indicated that this region is required for DNA binding.

To

**Laurie,
my wife and
best friend**

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List of Abbreviations

bp	base pair(s)
BSA	bovine serum albumin
Dam	deoxyadenosine methyltransferase
DnaA box	asymmetric 9-mer consensus sequence bound by DnaA
DTT	dithiothreitol
EDTA	(ethylenedinitrilo)tetraacetic acid
Fis	Factor for inversion stimulation
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IHF	Integration host factor
kDa	kilodalton
RNAP	RNA polymerase
SDS	sodium dodecyl sulfate
Tris	Tris(hydroxymethyl)aminomethane

Chapter I

Literature Review

Introduction

As accurate duplication of the genetic material is a prerequisite for cell division in all organisms, the study of the mechanisms and regulation of DNA replication is of considerable significance. DNA replication and its regulation have been studied extensively since the discovery that DNA was the genetic material (140) with the process best understood in the enteric bacterium *Escherichia coli*.

The *E. coli* chromosome is a covalently closed, circular, duplex molecule of 4,700 kilobase pairs. Replication initiates at a single, unique site termed the origin of chromosome replication, or *oriC*, and continues in a bidirectional fashion until terminating at a site called *terC* located directly opposite *oriC* (74). Following duplication of the bacterial chromosome, cell division proceeds wherein each daughter cell receives a complete copy of the chromosome (31).

Principles of Replication

Less than a decade following the discovery that DNA was the genetic material (10), a model for its structure was proposed (140). Soon after, Jacob, Brenner, and Cuzin proposed a model for its duplication that is still accepted to this day (63). In this model, an initiator, that is encoded by the replicon, acts at a unique site termed the replicator to promote duplication of the replicon. To date, the best candidates for the replicator and initiator are the loci defined as *oriC* and *dnaA*, respectively.

Soon after Jacob and coworkers proposed their model, it was recognized that DNA replication must be both a periodic and tightly controlled event. The most obvious mode of regulation, that of regulation of initiation, was soon demonstrated (84).

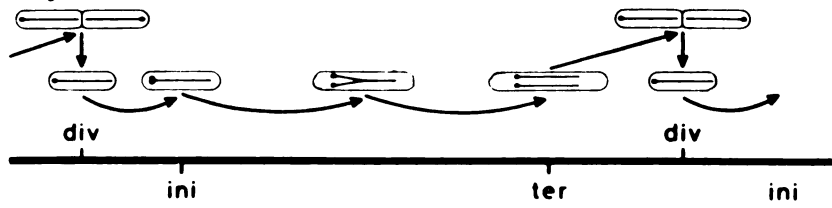
Central to any model attempting to explain initiation of replication is the concept of initiation mass (30). The initiation mass is defined as the ratio of cell mass to chromosome number at the time of initiation and is thought to be constant irrespective of growth rate. Any factor that perturbs the initiation mass is expected to affect the time of initiation of replication as well.

The Bacterial Cell Cycle

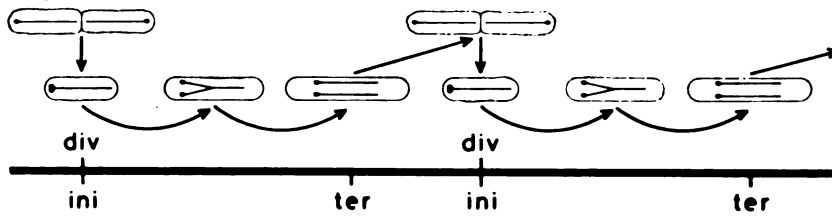
That the initiation mass must be an important aspect of DNA replication led to the examination of different periods of the bacterial cell cycle (27, 51). This examination revealed that the time between DNA replication and cell division, termed *D*, is a constant period of 20 minutes (75) (Figure 1). Furthermore, complete duplication of the bacterial chromosome, termed *C*, requires a minimum of 40 minutes. Although a lag may occur between

Figure 1. Schematic representation of the relationship between the bacterial cell cycle and cell division (135). The bacterial cell cycle consists of two distinct periods, C and D. C, the period between initiation (ini) and termination (ter) of replication, may vary at slow growth rates but is a constant equal to 40 min at rapid growth rates. D, the period between termination of replication and cell division (div), is a constant equal to 20 min and is independent of the growth rate. The time between subsequent initiation events varies as a function of the doubling time (t_D) of the cell (**a** $t_D > C + D$, **b** $t_D = C + D$, **c** $t_D < C + D$). If t_D is less than $C + D$ (as is the case in **c**) then the cell must initiate a new round of replication prior to termination of the ongoing round. When this occurs, initiation of all origins within the cell occurs simultaneously, or synchronously.

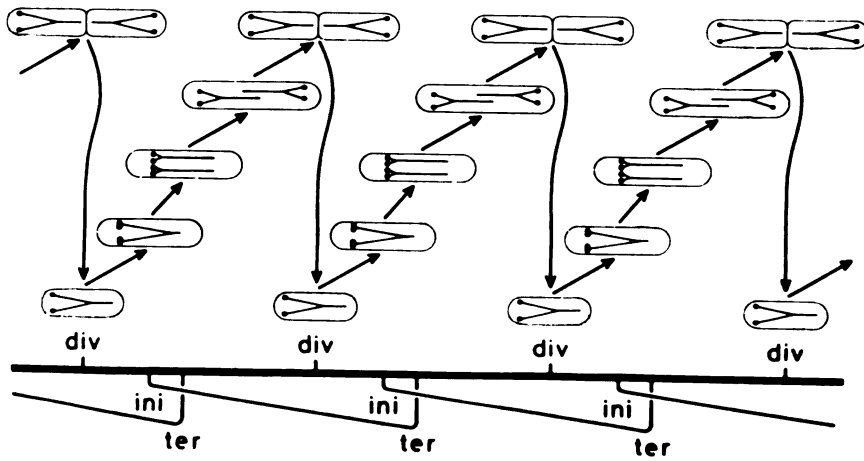
a $t_0 = 90$ $C = 55$ $D = 20$



b $t_0 = 60$ $C = 40$ $D = 20$



c $t_0 = 35$ $C = 40$ $D = 20$



duplication of the genome and the next initiation event, it appeared that at least 60 minutes was required for cell division. That *E. coli* can divide at time intervals as short as 20 minutes appeared to contradict these findings. However, this apparent dilemma was resolved by the finding that initiation events occur prior to the completion of ongoing rounds of replication.

A recent method corroborating this observation is flow cytometry that measures DNA mass relative to cell size. This may then be extrapolated into the number of chromosome equivalents per cell. This method indicated that rapidly growing *E. coli* cells contain more than one chromosome equivalent per cell, consistent with the proposal that initiation of replication may occur prior to the completion of ongoing rounds of replication, thereby permitting *E. coli* to divide at time intervals shorter than that required for duplication of the bacterial genome (122).

The *Escherichia coli* *dnaA* Gene

The *Escherichia coli* *dnaA* gene was identified nearly three decades ago as an essential gene (52, 72). Mutations mapping to the *dnaA* locus exhibit a temperature sensitive phenotype for growth (reviewed in reference 92). That *dnaA*(Ts) mutations exhibit a slow-stop phenotype following an upshift to the nonpermissive temperature (48, 72) is consistent with the requirement of the *dnaA* gene product for initiation of replication. This slow-stop phenotype is due to completion of ongoing rounds of replication without new initiation due to inactivation of DnaA by a temperature upshift.

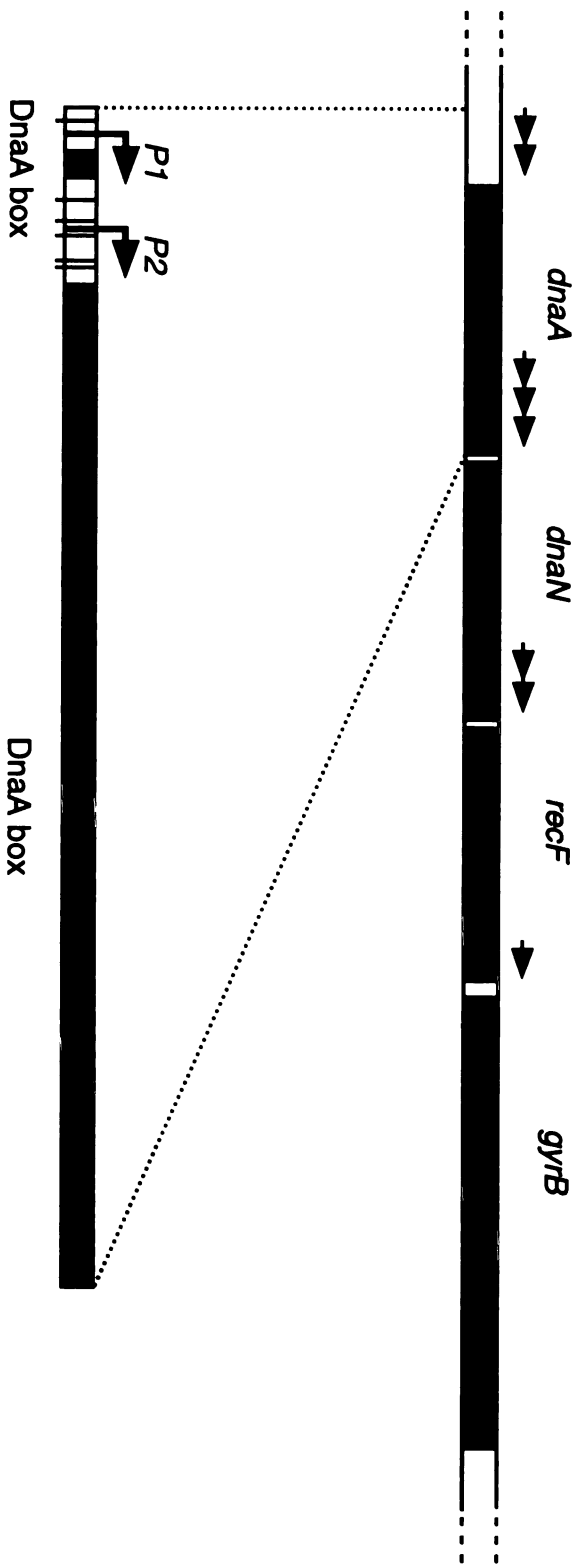
The *E. coli* *dnaA* gene was cloned by complementation of the temperature-sensitive phenotype of a *dnaA* mutant. It maps 42 kb to the left of the chromosomal origin at 83.5 minutes on the revised map of the *E. coli*

genome (11). It is the first in an operon that also contains *dnaN*, *recF*, and *gyrB* (49, 106) (Figure 2). *dnaN* encodes the β subunit of the replicative DNA polymerase, DNA polymerase III holoenzyme, *recF* encodes a protein involved in recombination-directed DNA repair following damage by ultraviolet light, and *gyrB* encodes the B subunit of DNA gyrase, a heterotetrameric type II topoisomerase. Although these genes appear to form an operon, *dnaN*, *recF*, and *gyrB* are also transcribed from promoters located upstream from each gene (3, 4, 102, 104, 110).

Nucleotide sequence analysis of *dnaA* revealed an open reading frame of 467 residues with a calculated molecular mass of 52.5 kDa and deduced pI of approximately 9.6 (43). Transcription is from two promoters, *dnaAP1* and *dnaAP2*. S1 nuclease protection analysis indicated that *dnaAP2* is the stronger of the two promoters resulting in approximately 70-80% of total transcripts (25, 76). Binding of DnaA protein to a 9-mer sequence termed a DnaA box (see below) in between *dnaAP1* and *dnaAP2* results in repression of transcription from both promoters (138) (see "*DnaA Protein as a Transcription Factor*").

A greater than expected number of deoxyadenosine methyltransferase (Dam) sites were observed in the *dnaA* promoter region (46, 124) (see Figure 2). Immediately following DNA replication, the *dnaA* promoter region is naturally hemimethylated. Whereas transcription from *dnaAP2* is essentially blocked in the absence of Dam methylation, transcription from *dnaAP1* appears to be independent of Dam methylation (17, 76). That the *dnaA* promoter region does not become fully methylated for up to 1/3 of a generation, while other sites on the chromosome become fully methylated after only 1-2 minutes, has led to the proposal that the promoter region is sequestered when hemimethylated by one or more membrane associated proteins (20). This sequestration is thought

Figure 2. Schematic representation of the *dnaA* operon. Genes and promoters are indicated by shaded boxes and filled triangles, respectively. In the blow-up of the *dnaA* gene (bottom) symbols are: shaded rectangle, *dnaA* coding sequence; bent arrows, *dnaA* promoters; thin ticks in the *dnaA* promoter region, Dam methylation sites; filled boxes, sequences bound by DnaA protein (DnaA box motifs).



to inhibit transcription of *dnaA* from *dnaAP2* and has been proposed to serve some regulatory function.

DnaA Protein

Functional Characteristics of DnaA Protein

DnaA protein, the product of the *dnaA* gene, is essential for the initiation of replication of the bacterial chromosome (38, 52, 72) as well as numerous plasmids and bacteriophage including pSC101 (32, 34, 50), R6K (143), F factor (44, 69), and P1 (44, 142). Shortly after the *dnaA* gene was cloned from *E. coli* (49), *dnaA* homologs from other members of the family *Enterobacteriaceae* were identified (reviewed in reference 92). In all, 15 homologs have been described. Based on their deduced amino acid sequence, a remarkable degree of conservation is observed (Figure 3).

Biochemical characterization of DnaA protein revealed numerous functional activities. DnaA is a sequence-specific DNA binding protein that recognizes an asymmetric 9-mer consensus matching the sequence 5'-TTAT^C/_ACA^C/_AC-3', or very close matches (36). However, the 50-fold range in K_D depending upon the sequences flanking this 9-mer indicates the importance of these flanking sequences on binding affinity (113).

DnaA protein is also a nucleotide binding protein. It binds avidly to ATP and ADP (K_D of 30 nM and 100 nM, respectively) in a mutually exclusive manner (116). Bound ATP is hydrolyzed slowly to ADP in the presence of DNA in a sequence-nonspecific manner. Although nucleotide binding does not appear to affect the affinity of DnaA for the DnaA box (15), the ATP-bound form is significantly more active than either the ADP-bound or non-nucleotide-bound forms for replication of an *oriC* minichromosome *in vitro* (145). However, the

Figure 3. Comparison of the amino acid sequences of fifteen different *dnaA* homologs (92). The deduced amino acid sequence of the *E. coli* DnaA protein is indicated relative to the consensus sequence obtained by comparison of the deduced amino acid sequences of fifteen (*E. coli*, *S. typhimurium*, *S. marcescens*, *P. mirabilis*, *B. aphidicola*, *P. putida*, *B. subtilis*, *S. coelicolor*, *M. luteus*, *C. crescentus*, *R. meliloti*, *Synechocystis sp.*, *B. burgdorferi*, *S. citri*, and *M. capricolum*) different *dnaA* homologs. For the consensus sequence, a dot (·) represents a residue conserved (either identical residue or conservative replacement) in less than 9, a letter represents a residue conserved in 9 or more, and an underlined letter represents a residue conserved in 12 or more of the 15 homologs. See reference 92 for a description of the method used for the sequence comparison.

1 60
E. coli MSLSLWQQCLARLQDELPATEFSMWIRPLQAELSDNTLALYAPNRFVLDWVRDKYLNNIN
consensus MSL.LW.Q.LA.L..EL....F..WIR.LQ.EL...TL.L.APN.FVLDWV..KYL..I.

61 120
E. coli GLLTSFCGRIAPQLRFEVGTKPVTQTPQAAVTSNVAAPAQVAQTQPORAAPSTRSGWDNV
consensus .LL..F.....L.F.V.....W...

121 180
E. coli PAPAEPITYRSNVNVKHTFDNFVEGKSNQLARAAARQVADNPGGAYNPLFLYGGTGLGKTH
consensus S.VN.K.TFDNFVEG.SN.LA.AAAR.VADNPG.AYNPLFLYGG.GLGKTH

181 240
E. coli LLHAVGNGIMARKPNAKVVMHSERFVQDMVKALQNNAIIEFKRYRSVDALLIDDIQFF
consensus LLHAVGN..M...PNAKVVM.SERFV.DMV.ALQ.N.IIEFK.YYRSVD.LLIDDIQFF

241 300
E. coli ANKERSQEEFFHTFNALLEGNQIILTSDRYPKEINGVEDRLKSRFGWGLTVAIEPPELE
consensus A.KE..OEEFHTFNALLE...QIILTSDRYPKEI.GVEDRLKSRF.WGL.VAIEPPELE

301 360
E. coli TRVAILMKKADENDIRLPGEVAFFIAKRLRSNVRELEGALNRVIANANFTGRAITIDFVR
consensus TRVAIL.KKADE..I.LP.EV.FFIA.RL.SNVRELEGALNRVIA.A.F....ITIDFV.

361 420
E. coli EALRDLLALQEKLVTIDNIQKTVAEYKIKVADLLSKRRSRSVARPRQMAMALAKELTNH
consensus E.LRDLL...E.LVTIDNIQK.VAEYKIKV.DLLSKRRSRSVARPRQAM.L.KELTNH

421 467
E. coli SLPEIGDAFGGRDHTTVLHACRKIEQLREESHDIKEDFSNLIRTLS
consensus SLPEIGD.FGGRDHTTVLHACRKIE.LR.E..DIK.DE..LIR.L..

ADP-bound form may augment limiting levels of the ATP-bound form, indicating that it is able to function at certain stages of initiation, and is active *in vitro* at a concentration approximately four-fold higher than the ATP-bound form.

It has also been reported that DnaA protein binds cAMP (K_D of 1 μ M) at a site distinct from the ATP and ADP binding site (54). The binding of cAMP appears to induce the release of bound ADP but not ATP and is postulated to aid in regeneration of the active form of DnaA. Furthermore, the cAMP-bound form had an increased affinity for *oriC* relative to the ATP-, ADP-, and non-nucleotide-bound forms.

Physical Characteristics of DnaA Protein

DnaA protein is isolated in two physical states: a monomeric and aggregated form (59). Although the aggregated form is poorly characterized, it is known to be a large complex. The monomer is active in *in vitro* replication of an *oriC* minichromosome; the aggregate is not, suggesting that its physical state (i.e. ratio of monomer to aggregate) may in some way determine the net level of DnaA activity.

The aggregate may be activated for *in vitro* replication by treatment with protein denaturants such as guanidine (118) or the heat shock protein DnaK (59). Both treatments appear to activate the aggregate by its conversion to the monomeric form.

Limited biochemical characterization of the aggregate indicated that it was associated with acidic phospholipids (146). It was this observation that led to the finding that the aggregated form could be monomerized also by treatment with phospholipase A_2 (59), presumably by cleavage of the phospholipids to result in the destabilization of the aggregated complex.

That ATP can stabilize the monomeric form of DnaA and prevent its conversion into the aggregate (28) is consistent with the ATP-bound form of DnaA being the form preferred for replication *in vitro*.

Role of DnaA in Replication of Plasmids and Bacteriophage

That a strain containing a *dnaA* null mutation was unable to maintain F factor (44, 69), R6K (143), pSC101 (32, 34, 50), or P1 (44, 142) demonstrated that these replicons are dependent upon *dnaA* function. Consistent with this observation is the similarity in structural organization of their origins (2, 26, 44, 143). All contain one or more DnaA box motifs in addition to an adjacent AT-rich region.

Although the AT-rich region of *oriC* can be unwound by DnaA protein (15), its role in the unwinding of plasmid origins dependent on it for replication, with the exception of bacteriophage P1, is largely unknown. The AT-rich region of the P1 origin can be unwound by DnaA (95). That this unwinding is facilitated by the phage-encoded initiator, RepA, suggests that both proteins are involved in the unwinding process *in vivo*.

DnaA protein is dispensable yet stimulatory for replication of R1 (44, 89, 101) and ColE1-like plasmids (1, 34). The *in vivo* role of DnaA protein in replication may be both versatile and widespread.

Mutations of dnaA

As described above (see "*The Escherichia coli dnaA Gene*"), the *dnaA* gene was first identified by a conditional allele mapping to the *dnaA* locus (52, 72). This mutant exhibited a slow-stop phenotype (48, 72) indicative of a defect at the point of initiation of replication. Since its discovery, a limited collection of *dnaA* alleles have been reported (reviewed in reference 92), all of which exhibit

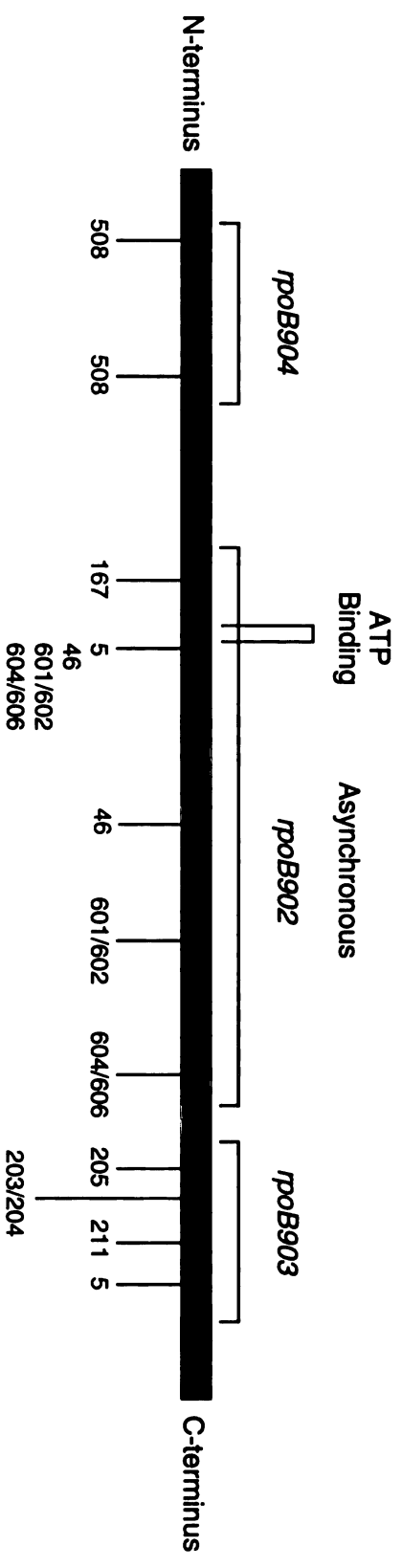
a conditional phenotype (Figure 4). Both the small number and the fact that five of the 14 reported alleles share a common substitution of valine for alanine at position 184 indicates that the current collection is neither representative of all functional domains nor sufficiently large for detailed structure-function studies. However, some insight into *dnaA* function has been gained from the genetic and biochemical characterization of these alleles.

First, the thermolabile phenotype of *dnaA* mutants may be suppressed in an allele-specific fashion by mutations in *rpoB* (7) (Figure 4). *rpoB* encodes the β -subunit of RNA polymerase (RNAP), an essential enzyme under certain conditions for replication of *oriC* minichromosomes *in vitro* (65, 96). Although this suggests a physical interaction between DnaA protein and RNAP, no evidence for such an interaction has as yet been reported.

Arguing against a DnaA-RNAP interaction is the observation that an *rpoC* mutation results in an increased chromosome copy number (103, 105). *rpoC* encodes the β' subunit of RNAP. That this *rpoC* mutation results also in an increase in expression of *dnaA* is presumably related to the observed increase in chromosome copy number (i.e. via a gene dosage effect). Some *rpoB* mutations appear to result also in an increase in chromosome copy number suggesting that a similar mechanism may account for the allele-specific suppression of *dnaA*(Ts) alleles by mutant *rpoB* alleles. Indeed, the thermolabile phenotype of some *dnaA*(Ts) mutations may be suppressed by an increase in their gene dosage (or level of expression) (47).

In addition to *rpoB*, the thermolabile phenotype of *dnaA*(Ts) mutants may be suppressed by extragenic suppressors mapping to *trxA* (55), *mhA1* (130), *seqA* (83, 133), or *topA* (81). Furthermore, mutations of *dnaA* have been identified which can suppress the thermolabile phenotypes of *dnaR* (phosphoribosylpyrophosphate [PRPP] synthase) (109) or *dnaX* (encoding

Figure 4. Summary of *dnaA* mutations and corresponding phenotypes. DnaA protein is represented by the shaded rectangle. The P-loop motif encompassing residues 172-179 and proposed to be the high affinity ATP binding site is indicated. The approximate positions of known amino acid substitutions for missense mutations of *dnaA* are also shown (below). Phenotypes are grouped into three different classes (above) based on allele-specific suppression of their thermolabile phenotypes by the different *rpoB* alleles (see text). The mutants that are most affected in synchrony of initiation map to the middle region of the protein.



the γ and τ subunits of DNA polymerase III holoenzyme) (42) mutants. It has been speculated that these second site suppressors represent genes whose products interact physically with DnaA in the replication process.

Biochemical characterization of thermolabile DnaA mutants (DnaA5 and DnaA46) has also been performed (56, 57, 58, 60, 61). DnaA5 and DnaA46 protein are activated for *in vitro* *oriC*-dependent replication by the combined action of the heat shock proteins DnaK and GrpE. The thermolabile phenotype of *dnaA46* may be suppressed *in vivo* by overexpression of *groELS* (68), suggesting that these heat shock proteins and chaperonins may play a role in initiation of replication of the bacterial chromosome.

The requirement of DnaK for *oriC*-dependent replication *in vitro* with mutant forms of DnaA is consistent with the finding that *dnaK* mutants are defective for replication at the point of initiation (108). Replication activity of DnaA5 and DnaA46 protein is temperature sensitive and more labile than wild type DnaA *in vitro* (58, 61), leading to the speculation that the role of DnaK is to maintain DnaA (particularly mutant forms) in an active conformation. Consistent with this is the fact that DnaK is not required for *oriC*-dependent replication *in vitro* using the replication system consisting of reconstituted purified proteins unless mutant forms of DnaA are used (56, 57, 65).

Finally, physiological characterization of *dnaA* mutants indicated that those sharing a common substitution of alanine-to-valine at position 184 (near a P-loop motif proposed to function in nucleotide binding) exhibited an asynchrony in initiation of replication (123). Asynchrony refers to the inability of a cell containing multiple origins to initiate replication at all origins simultaneously. In a wild type bacterium, all origins are initiated simultaneously, or synchronously, resulting in a 2^n (where $n=1, 2$, or 3) number of chromosome equivalents (i.e. 2, 4 or 8) (121). Conversely, cells that initiate

asynchronously have a variable number of chromosome equivalents (i.e. 2, 3, 4, and 5).

That the DnaA5 and DnaA46 proteins, both of which contain the alanine-to-valine substitution, are defective for ATP binding *in vitro* (58, 60), and are asynchronous for initiation *in vivo* (123) has prompted speculation that the asynchrony is due to the common ATP binding defect. The ATP-bound form is active in *in vitro* replication from *oriC* (145) and appears to be important for proper timing of initiation of replication *in vivo*.

DnaA Protein as a Transcription Factor

The role of DnaA as a transcriptional regulator is well documented (reviewed in reference 92). DnaA protein has been demonstrated either to activate or repress transcription of genes that contain one or more DnaA box motifs in either their promoter region or coding sequence. The best studied of these is the *dnaA* gene itself. A DnaA box is in the promoter region located in between *dnaAP1* and *dnaAP2*. A second DnaA box is in the coding region (see Figure 2). Binding of DnaA protein to the DnaA box in the promoter region results in transcriptional repression from both *dnaA* promoters as measured by S1 nuclease protection (76, 138). Furthermore, transcription from *dnaAP1* was repressed *in vivo* when *dnaAP2* was deleted and the DnaA box was present but not when both *dnaAP2* and the DnaA box were deleted (16). This was determined using translational fusions consisting of various portions of the *dnaA* promoter region joined in-frame to the *lacZ* structural gene, and measuring β -galactosidase activity as a function of *dnaA* induction. The significance of the DnaA box in the coding region of the *dnaA* gene is not yet known, although it does not appear to affect transcription (91).

Other genes whose transcription is repressed by DnaA include *rpoH* (139), *uvrB* (132), *ftsQ/ftsA* (90), the *gua* operon (128), and the *trp* operon (8). Although DnaA protein binds to a DnaA box within the *polA* promoter, the effect of binding on *polA* transcription has not been demonstrated (137). Transcriptional repression is also observed for a number of promoters within or nearby *oriC* including P_{oriL} (5), *mioC* (79, 129), and *asnC* (40, 112) (see “*Transcription in and Around oriC*”).

Transcription of P_{oriR1} , a promoter in *oriC*, is stimulated by DnaA protein (5). It also stimulates transcription of the *nrd* operon encoding both subunits for ribonucleotide reductase (9) and *glpD* (aerobic glycerol-3-phosphate dehydrogenase) (cited in reference 92). Stimulation of transcription of the *nrd* operon by DnaA protein also requires Fis (Factor for Inversion stimulation) protein.

The *Escherichia coli* Origin of Replication, *oriC*

Physical Characteristics of oriC

The *E. coli* origin of chromosome replication, *oriC*, was first cloned by virtue of its ability to confer autonomous replication upon a nonreplicating DNA fragment containing a selectable antibiotic resistance cassette (144). The minimal *oriC* sequence, and element within it were then identified by a combination of both deletion (100) and random mutagenesis (99). These regions are well conserved in homologs of *oriC* in the family *Enterobacteriaceae* (Figure 5).

Structurally, the *E. coli* origin of replication contains a number of sequence elements required for origin function, including four DnaA box motifs (R1-R4) (36), three AT-rich 13-mer sequences (L, M, and R) (15), 11

Figure 5. Consensus sequence of the minimal chromosomal origin (147). This consensus sequence is derived from six different bacterial organisms and was aligned such that the least number of changes are introduced into the consensus sequence. In the consensus, a large capital letter indicates that that nucleotide is found in all six origins; a small capital letter indicates that it is found in five of the six origins; a lowercase letter indicates that it is found in three or four of the six origins and only two different nucleotides are found at that position; n indicates that three or four of the four possible nucleotides, or that two different nucleotides and a deletion are present at that position. Bold, large capital letters located at positions 149, 242, and 267, indicate where single-base substitutions produce an *oriC*⁻ phenotype. The minimal origin of *E. coli* is bracketed and numbers refer to positions in the *E. coli* sequence. The three AT-rich 13mers repeats in the 5'-end of the origin are indicated by arrows. Dam methylation (GATC) sites are underlined. Positions and orientations of the four DnaA boxes are indicated by arrows and R1, R2, R3, and R4.

[illegible]

deoxyadenosine methyltransferase (Dam) sites (148), two promoters (5), and binding sites each for Fis protein (33, 41, 107) and IHF (Integration Host Factor) (62, 107).

The observation that mutations mapping to *oriC* abolished replication activity when assayed *in vivo* using a minichromosome but exhibited no mutant phenotype when present in the chromosome (53) suggests that their respective mechanisms of replication may differ. Based on this finding, the minimal *oriC* as currently defined may be incomplete. Consistent with this is the lack of incompatibility observed between the chromosomal *oriC* and *oriC* minichromosomes, even when the minichromosomes are present at high copy numbers (144). Indeed, plasmids containing the *Bacillus subtilis* origin exhibit incompatibility with the *B. subtilis* chromosome (93, 94).

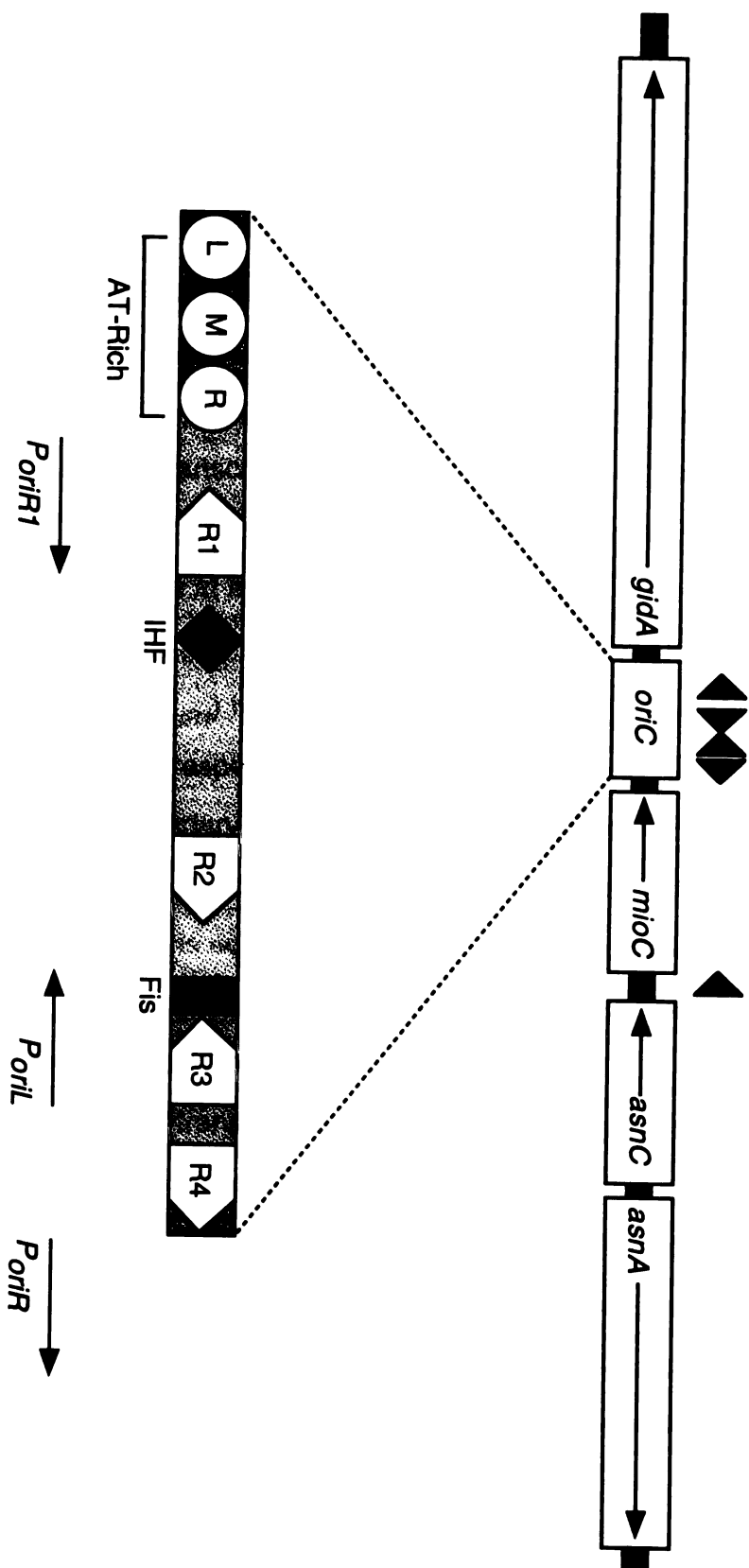
However, that minichromosomes initiate replication in a cell-cycle-specific manner (77), replicate bidirectionally (64), and show a similar dependence in replication on proteins known to be essential for replication of the chromosome (65) argues that much, if not all of the essential sequences are present.

In summary, *oriC* defines a locus known to be essential for initiation of replication of the bacterial chromosome and represents the best studied and most understood of all chromosomal origins. Whether additional sequences are required for regulation or accurate initiation of chromosomal replication is a subject under continued investigation.

Transcription in and Around oriC

oriC is flanked by a number of promoters, many of which exhibit a striking degree of regulation by DnaA protein (Figure 6). Located to the right of *oriC*, *mioC* (modulation of initiation at *oriC*) encodes a 16 kDa gene product of

Figure 6. Promoters in and around *oriC*. Genes and DnaA box motifs (►; 5'–TTATCCACA–3') in the vicinity of *oriC*, the chromosomal origin, are indicated (above). Relevant structural features of *oriC* including the promoters within *oriC* (see text) are shown in the blow-up (below). Symbols are: open circles, AT-rich 13mers (L, M, and R); pentagons, DnaA boxes (R1-R4). Binding sites for IHF and Fis protein are also indicated.



unknown function (127). The *mioC* promoter contains a single DnaA box and its leftward transcription through *oriC* is repressed by DnaA at the point of transcriptional initiation (115, 138). The *asnC* gene encodes a transcriptional activator of *asnA*, one of the two asparagine synthetases of *E. coli* (73). Extension of *asnC* transcripts past *mioC* and into *oriC* is regulated by DnaA at the boundary between *asnC* and *mioC* by transcriptional termination (40, 112). The *gidA* gene (glucose inhibition of division) is located to the left of *oriC*. The *gidA* promoter directs transcription leftward away from *oriC* and has a positive effect on *oriC* function by its ability to elevate the copy number of an *oriC* plasmid when contained at its proper position (6, 97).

In addition to the promoters discussed above that flank *oriC*, two promoters (P_{oriL} and P_{oriR1}) have been identified within it that direct transcription leftward and rightward, respectively (5, 115). An additional promoter, P_{oriR} , located at the right boundary but outside of *oriC* directs transcription rightward, away from *oriC*. Whereas transcription from P_{oriL} is repressed by DnaA, transcription from P_{oriR1} is activated. However, transcriptional activation of P_{oriR1} is dependent upon its location in the bacterial chromosome as similar activation is not observed using *oriC* minichromosomes.

In the process of transcription, RNA polymerase generates positive supercoils ahead of the transcription complex and negative supercoils behind (78). DnaA represses (or terminates) transcription from promoters that enter into *oriC* and stimulates transcription from promoters that lead away from *oriC*, suggesting that the role of RNAP in initiation of replication may be through facilitation of unwinding in the *oriC* region by an increase in its negative superhelical density.

To test the effect of transcription on the function of *oriC*, T7 promoters were inserted at various distances from *oriC* and origin function was measured

as a function of T7 RNAP (12, 119). This analysis indicated that transcription dependent on T7 RNAP was able to enhance *oriC* function when the promoters were within 1.4 kb of *oriC*. It was speculated that the observed activation was through alteration of the DNA structure in the vicinity of *oriC*, thereby facilitating unwinding of the AT-rich region by DnaA protein.

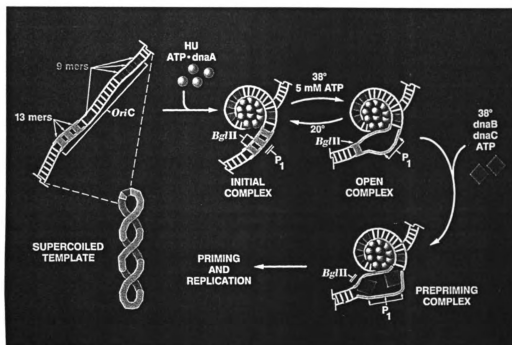
***in vitro* Replication of *oriC* Minichromosomes**

The cloning of *oriC* permitted the development of *in vitro* replication systems specific for *oriC*. The first such system relied on use of a crude, soluble protein fraction obtained from a *dnaA*(Ts) mutant (37). This system was completely dependent upon both exogenously added template DNA containing a functional *oriC* and DnaA protein (38).

Fractionation of the crude, soluble protein fraction permitted the identification of those proteins essential for *in vitro* replication from *oriC*. These include DnaA, DnaB, DnaC, primase, RNAP, gyrase, topoisomerase I, RNase HI, single stranded DNA binding protein, and DNA polymerase III Holoenzyme. This led to the development of a replication system reconstituted with these purified proteins (65).

Making use of these *in vitro* replication systems, a model for the biochemical mechanism of initiation of replication from *oriC* has emerged (Figure 7). DnaA protein binds to *oriC* in a cooperative manner through specific recognition of the four DnaA boxes present within the origin resulting in the formation of a large and highly ordered structure referred to as the initial complex (117). Characterization of this initial complex by electron microscopy suggests that it contains 20-30 DnaA monomers per *oriC* (36).

Figure 7. Proposed model for initiation of chromosomal replication in *E. coli* (74). The four stages of DnaA-dependent initiation of DNA replication in *E. coli* are (1) cooperative binding of the ATP-bound form of DnaA protein to four DnaA boxes contained within *oriC* resulting in a large nucleoprotein structure referred to as the initial complex; (2) DnaA-dependent unwinding of the three, tandemly repeated AT-rich 13mer sequences in the left-most end of *oriC* resulting in conversion of the initial complex to the open complex; (3) loading of DnaB, the replicative helicase, into the unwound region followed by dissociation of DnaC protein from the DnaB-DnaC complex creating a structure termed the prepriming complex; (4) further unwinding of the DNA strands by DnaB helicase allowing subsequent priming and bidirectional replication. Both HU protein and IHF have been shown to aid DnaA protein in unwinding the AT-rich region. 5 mM ATP and elevated temperature (38°C) is required for conversion of the initial complex to the open complex.



Although the nucleotide-free form of DnaA protein is able to form the initial complex with an efficiency similar to that observed for the ADP- and ATP-bound forms, only the ATP-bound form is capable of converting the initial complex to the open complex (15, 145). In the open complex, the AT-rich left most region of *oriC* becomes unwound. This unwinding also requires elevated temperature (38°C) and high levels of ATP (5 mM). Furthermore, histone-like proteins such as HU and IHF aid DnaA in the conversion of the initial complex to the open complex (62).

Subsequent entry of DnaB, the replicative helicase, proposed to occur through direct physical contact between DnaA protein in the open complex and the DnaB-DnaC complex, followed by the release of DnaC results in formation of the prepriming complex (39, 88). The sole function of DnaC appears to be the inhibition of the ATPase activity of DnaB helicase prior to its loading into the open complex (141). That DnaA is dispensable following the loading of DnaB helicase was confirmed by isolation of the prepriming complex by gel filtration chromatography in the presence of potassium glutamate (145). Under these conditions, the prepriming complex lacked detectable levels of DnaA and was competent for replication.

Establishment of a multi-subunit heterodimeric replication fork complex consisting of all remaining necessary replication factors fulfills the final requirement for the priming and bidirectional synthesis of daughter strands (74).

Regulation of Replication

Although regulation of replication has been demonstrated to occur at the point of initiation (84), the biochemical mechanism of this regulation is as yet unknown. As the initiation mass, the ratio of chromosomal origins to cell mass,

is thought to be a constant and independent of the growth rate (30), it has been postulated that the accumulation of a key factor signals the time of initiation. DnaA protein may act to regulate the frequency of initiation.

Two separate groups have determined the intracellular concentration of DnaA protein as a function of growth rate. Whereas one reported it to be a constant irrespective of the growth rate (80), the other found that it varied as a function of the growth rate (24). The reason for this discrepancy is unknown. Consequently, two different models have emerged to attempt to explain regulation of replication.

In the model proposed by Zyskind and co-workers (86), initiation of replication is regulated by the level of activity of DnaA protein (i.e. the ATP-bound form), not its concentration. This was based on their finding that the intracellular DnaA protein concentration varied as a function of the growth rate, suggesting that its intracellular concentration was not the key factor determining the initiation mass.

In the model of Atlung and co-workers (45), the total concentration of free intracellular DnaA protein is proposed to regulate initiation of replication. As they detect no change in the level of DnaA protein as a function of growth rate, they propose that it must be the concentration of DnaA that is important. They suggest that DnaA protein has a lower affinity for sites within *oriC* relative to sites outside of *oriC*, and that only at high levels of DnaA are these low affinity sites bound thereby permitting initiation of replication.

A number of observations support the latter model. First, *in vivo* footprinting of *oriC* plasmids indicated that all but one of the four DnaA boxes (R3) of *oriC* were occupied for the majority of the cell cycle (111), leading to the proposal that binding of DnaA to box R3 is the rate-limiting step for initiation. Second, binding of DnaA protein to the four DnaA boxes within *oriC* is highly

ordered (87). Consistent with the *in vivo* footprinting results (111), R3 is the last of the four boxes to be occupied. Finally, binding constants for DnaA protein to various individual DnaA box motifs were determined (113). In this analysis, DnaA was unable to bind specifically to box R3.

Other observations support the former model in which the net level of DnaA activity regulates the frequency of initiation. Two potential candidates that may negatively regulate the activity of DnaA protein have been identified. These are SeqA (83, 133) and an uncharacterized soluble factor of ~150 kDa (66).

The *dam* gene of *E. coli* encodes DNA adenine methyltransferase that methylates adenine in GATC sequences. Eleven sites are in *oriC*. The *seqA* gene, encoding a 22 kDa protein of 181 amino acids (18, 83), was identified by the ability of an *E. coli* strain containing a *dam13::Tn9* allele and a mutation in *seqA* to be transformed by a fully methylated *oriC* minichromosome (83). In contrast, a *seqA*⁺, *dam*⁻ mutant is transformed inefficiently because, after one round of replication, the hemimethylated daughter molecules that are formed are not replicated further (20). It has been proposed that SeqA is a membrane associated protein that binds to and sequesters hemimethylated *oriC* to prevent subsequent initiation events (83). Biochemical studies that support this proposal include the finding that SeqA preferentially binds to *oriC* when hemimethylated relative to the fully methylated form (18, 125). A *seqA* null allele suppresses the thermolabile phenotype of *dnaA*(Ts) mutations and an increase in its gene dosage and suppresses the cold sensitive phenotype of the hyperactive *dnaAcos* mutation, suggesting that SeqA may also regulate the level of activity of DnaA protein (83).

dnaAcos is an intragenic suppressor of *dnaA46* (35). Although *dnaAcos* does not exhibit a thermolabile phenotype, it is hyperactive at 30°C resulting in

a cold sensitive phenotype. Biochemical characterization of DnaAcos protein revealed that in contrast to wild type DnaA protein that was inactivated by a soluble factor of ~150 kDa, DnaAcos was not (66, 67). This inactivation was dependent upon DNA (in a sequence-independent manner) and hydrolyzable ribonucleoside triphosphates. Based on this, it has been proposed that the ~150 kDa factor inactivates DnaA protein by stimulating its intrinsic DNA-dependent ATPase activity to convert the ATP-bound form to the less active ADP-bound form by a mechanism analogous to inactivation of Ras GTPase by GTPase-activating protein (GAP) (19).

Replication in the Absence of *dnaA* Function

Stable DNA Replication

Conditions exist under which replication initiates from sites other than *oriC* in a DnaA-independent fashion. One, referred to as **constitutive stable DNA replication**, cSDR (130), results from inactivation of *mhaA* that encodes RNase H. RNase H degrades RNA in RNA-DNA hybrids and appears to confer specificity in initiation to *oriC* by destruction of potential primers for replication at other sites (71, 98). On inactivation of *mhaA*, initiation of replication appears to occur at random sites referred to collectively as *oriK* (29). cSDR is completely dependent upon *recA* function. Whereas replication from *oriC* is dependent upon concomitant transcription and translation (136), cSDR is independent of concomitant translation (134).

A second type of SDR has been reported to occur following induction of the SOS response. This type of SDR, termed **inducible stable DNA replication**, or iSDR, may drive replication for several hours (70). iSDR initiates from two origins referred to collectively as *oriM* (85). One is located within *oriC* and the

other near *terC*. iSDR is dependent upon derepression of the *lexA* regulon and is independent of concomitant transcription and translation.

Integrative Suppression

Integrative suppression is the second condition wherein replication of the chromosome is no longer dependent upon *oriC*. It occurs by integration of an autonomous replicon that serves as a site for initiation of chromosomal replication in the absence of *oriC* function (14, 21, 82). If replication from this origin is independent of *dnaA* function, the *dnaA* gene becomes dispensable as well (13, 44).

Although insertion of F plasmid (131), R6K (126), or bacteriophage P1 (22, 23) into the bacterial chromosome is able to suppress the thermolabile phenotype of *dnaA*(Ts) mutants, only plasmid R1 (13, 44) and bacteriophage P2 (114) have been demonstrated to suppress a *dnaA* null or amber mutation. This observation suggests that F, R6K, and P1 are dependent on *dnaA* function but may not require the full complement of activities of DnaA protein as does *oriC*.

In addition to the use of R1 suppressed, *dnaA* null mutants to identify DnaA-dependent replicons, they have been used to study *dnaA* function in cell division (13). Cell division proceeds normally in the absence of *dnaA* function, suggesting that DnaA does not play a significant role in the process of cell division.

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Chapter II

Novel Alleles of the *Escherichia coli* *dnaA* gene That Are Defective in Replication of pSC101 but Not of *oriC*

Abstract

Five novel mutations of the *Escherichia coli dnaA* gene were isolated that were temperature sensitive in maintenance of pSC101, a plasmid that is dependent on this gene for replication. Nucleotide sequence analysis revealed that four of the five mutations arose from single base substitutions whereas the fifth contained three base substitutions, two of which were silent. Whereas all were temperature sensitive *in vivo* for pSC101 maintenance, genetic and biochemical characterization indicated that only two were defective in replication from the chromosomal origin, *oriC*. As previously characterized mutations are defective in replication for both pSC101 and *oriC*, the *dnaA* mutations specifically defective in pSC101 maintenance represent a novel class. We speculate that one or more of these pSC101-specific mutations are defective in interaction with pSC101 RepA protein that is also required for initiation of plasmid DNA replication.

Introduction

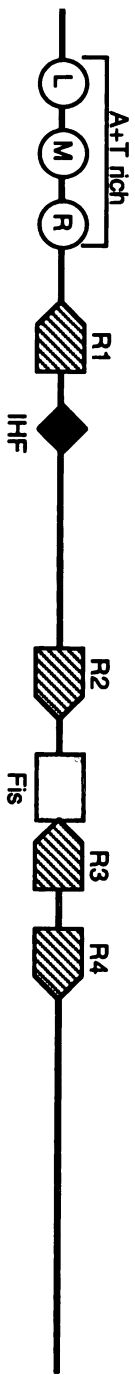
The *dnaA* gene of *Escherichia coli* is essential for initiation of replication of the bacterial chromosome (23, 32, 37). A number of plasmids and bacteriophage also require this gene for DNA replication as indicated by the inability of F (28, 36), R6K (55) and P1 (28, 54) to be maintained in a *dnaA* null mutant. Replication of pSC101 is also dependent on the *dnaA* gene (18, 20, 31). However, it is not capable of integrative suppression of a temperature sensitive *dnaA* allele, in contrast to F (43), R6K (46), and P1 (12, 13). These findings suggest that many of the same functions of DnaA protein are required for replication of pSC101 and for the bacterial chromosome.

Biochemical characterization of DnaA protein indicates that it is multifunctional. It is a sequence-specific DNA binding protein that recognizes 9-mer sequences (DnaA boxes) present in *oriC*, the chromosomal origin, as well as in replication origins of plasmids and bacteriophage that require this protein for replication (21). At *oriC*, DnaA protein, bound to ATP and stimulated by integration host factor (IHF) or HU protein, is proposed to induce a structural alteration resulting in strand separation of the AT-rich region (7). As binding of DnaA protein to *oriC* is mutually exclusive and independent of IHF binding, stimulation of unwinding by IHF is apparently at a step subsequent to DNA binding by DnaA protein (34). By contrast, at the pSC101 origin, enhancement of DnaA protein binding by either IHF, or pSC101-encoded RepA protein is proposed to occur through formation of a looped structure (47).

The differences in mechanisms of initiation at *oriC* relative to the pSC101 origin likely relates to differences in their structural organization (Figure 1). Whereas both strong and weak DnaA protein binding sites in the pSC101 origin are similarly oriented (47), the four DnaA boxes in *oriC* are arranged with

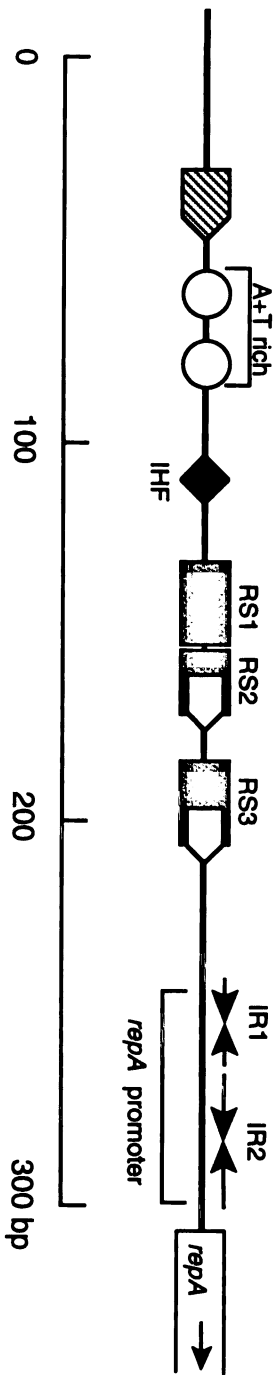
Figure 1. Structural organization of *oriC* and the pSC101 replication origin. DnaA protein binding sites (DnaA boxes) are represented by pentagons with the indicated orientations. DnaA boxes containing at most one mismatch from the consensus are crosshatched whereas those with more than 4 or 5 mismatches are not. AT-rich 13-mer repeats (L, M, and R) are represented by open circles. RepA binding sites (50, 52) are represented by shaded boxes (direct repeats RS1-RS3) or arrows (inverted repeats IR1 and IR2). Also indicated are IHF (19, 48) and Fis (25) binding sites.

oriC



51

pSC101 *oriV*



opposing polarities (21). Each replicon has an IHF binding site between DnaA boxes and 13-mer motifs (6, 19, 48). Iterons bound by RepA protein are important for enhancement of DnaA protein binding, leading to the speculation that RepA protein with IHF interact directly with DnaA protein in the initiation process (47).

Although the *dnaA* gene was first identified nearly 3 decades ago (32, 37), the collection of conditionally defective mutations is limited to 16 alleles (8, 17, 26, 30, reviewed in reference 40) (see Figure 2A). In addition, the amino acid sequence has been deduced for 15 homologs of the *dnaA* gene from bacteria in the family *Enterobacteriaceae* (reviewed in reference 40). Their comparative analysis, revealing a remarkable degree of amino acid sequence conservation, suggests that conserved residues contribute to the structure or function of DnaA protein. If so, mutations that affect its functions are expected in conserved residues of this essential gene.

We embarked on the isolation and characterization of novel mutations of the *dnaA* gene by a genetic assay that measures pSC101 maintenance. As *oriC* and pSC101 appear to have different mechanisms of initiation, we felt it possible that mutations of *dnaA* defective in replication of pSC101 but not *oriC* may be identified. Some of the mutations described here are specifically defective for pSC101 maintenance. The substituted residues may affect a proposed interaction with pSC101 RepA protein.

Materials and Methods

Bacterial strains and plasmids. The bacterial strains and plasmid DNAs used in this study are described in Table 1. P1 transduction was performed essentially as described by Miller (41) using P1 *vir*. pACYC*dnaA* was constructed by ligation of the 1.9 kb *Cla* I-*Xho* I fragment (see Figure 2A) containing the *dnaA* gene and its promoter from *pdnaA/dnaN* (10) to the 3.6 kb *Cla* I-*Sa* I pACYC184 vector fragment (11). The *dnaA*⁺ gene of pACYC*dnaA* was replaced by mutant sequences using the unique *Eco* NI and *Rsr* II restriction sites (encompassing the *dnaA* coding region but without the *dnaA* promoters) located internal to the *Cla* I-*Xho* I restriction sites (see Figure 2A). Bacteriological methods were performed essentially as described by Miller (41). Bacterial transformation was either by the calcium chloride method or electroporation using a Bio-Rad Gene Pulser according to the manufacturer's recommendation. Bacterial strains and their plasmid-containing derivatives were routinely grown in LB medium supplemented, as indicated, with 50 µg/ml ampicillin, 40 µg/ml kanamycin and 10 µg/ml tetracycline. M9 minimal medium supplemented with 0.5% casamino acids, 0.2% glucose, 1 µg/ml thiamine and the indicated antibiotic(s) was used as noted.

***in vitro* mutagenesis of the *dnaA* gene.** 30 µg of pDS596 DNA (33) was incubated for 60 min at 65°C in 1 ml of 1 M hydroxylamine-HCl (Sigma) and 0.1 M sodium phosphate pH 6.5 (41). The sample was then dialyzed against 200 volumes of 10 mM Tris-HCl pH 8.0 and 1 mM EDTA for 2 h, ethanol precipitated, washed with 70% ethanol and resuspended in the above buffer. Hydroxylamine treatment reduced the transformation efficiency of the plasmid DNA to 1% of the untreated DNA. An *Eco* NI-*Xho* I restriction fragment containing the complete *dnaA* coding region from

TABLE 1. Bacterial strains and plasmids^a

Strain	Genotype ^b	Source or construction
MC1061	<i>araD139 Δ(ara-leu)7696 ΔlacX74 galU galK hsdR2 (r_K-m_K+) mcrB1 rpsL (F⁻)</i>	Lab stock
EH3894	<i>asnB32 relA1 spoT1 thi-1 fuc-1 lysA ilv-192 zia::pKN500 (pKN500=mini-R1) ΔdnaA mad-1^c tnaA::Tn10 (F⁻)</i>	M. Yarmolinsky (28)
MS3895	EH3894 but lacking Tn10	Selection for Tc ^s of EH3894 on Bochner media (4)
MS3896	MS3895: <i>mad-2^c</i>	Selection for growth of MS3895 at 42°C
KL16-99	<i>recA1</i> HfrKL16	L. Snyder
MS3897	MS3896: <i>recA1 lysA⁺ fuc⁺</i>	(MS3896) X (KL16-99), select Lys ⁺ and screen for RecA ⁻ by UV ^s
TH433	<i>leu-19 pro-19 trp-25 his-47 thyA59 arg-58 met-55 deoB23 lac-11 gal-11 strA56 sul-1 hsdS_{K12} dnaA204 tnaA::Tn10 (F⁻)</i>	This lab
MS204	MS3896: <i>dnaA204 tnaA::Tn10</i>	(MS3896) X P1(TH433), select Tc ^r , screen for Ts pSC101 maintenance
MS205	MS204 but lacking Tn10	Selection for Tc ^s of MS204 on Bochner media (4)
MS206	MS205: <i>recA1 lysA⁺ fuc⁺</i>	(MS205) X (KL16-99), select Lys ⁺ and screen for RecA ⁻ by UV ^s

TABLE 1 (continued).

Plasmid	Characteristics	Source
pING1	Ap ^r ; pBR322 origin, expression vector, <i>Salmonella typhimurium</i> <i>araB</i> promoter and <i>araC</i>	D. Ray (35)
pDS105	<i>dnaA5</i> cloned into pING1	This lab (33)
pDS215	<i>dnaA46</i> cloned into pING1	This lab (33)
pDS319	<i>dnaA204</i> cloned into pING1	This lab (33)
pDS596	<i>dnaA</i> ⁺ cloned into pING1	This lab (33)
pDS596 [*]	pDS596 containing an hydroxylamine-treated <i>Eco</i> NI- <i>Xho</i> I fragment of the <i>dnaA</i> gene	This work
pA31T	A31T cloned into pING1	This work
pV198M	V198M cloned into pING1	This work
pG287S	G287S cloned into pING1	This work
pT301I	T310I cloned into pING1	This work
pL447W	L447W cloned into pING1	This work
pACYC184	Cm ^r , Tc ^r ; p15A origin, cloning vector	Lab stock (11)
pACYC <i>dnaA</i>	Cm ^r ; <i>dnaA</i> ⁺ cloned into pACYC184	This work
pACYC <i>dnaA46</i>	Cm ^r ; <i>dnaA46</i> cloned into pACYC184	This work
pACYCA31T	Cm ^r ; A31T cloned into pACYC184	This work
pACYCG287S	Cm ^r ; G287S cloned into pACYC184	This work
pACYCT301I	Cm ^r ; T301I cloned into pACYC184	This work
pACYCL447W	Cm ^r ; L447W cloned into pACYC184	This work
pSC101	Tc ^r ; DnaA-dependent low-copy-number replicon	S. Cohen (14)
pKN505	Apr; pBR322 origin, expresses R1 <i>copA</i> activity	K. Nordström (24)

^a Abbreviations are Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline; UV^s, ultraviolet sensitive; Ts, temperature sensitive; P1, P1 lysate; X, genetic cross.

^b See the report of Bachmann (3) for genetic symbols.

^c *mad-1* is a mutation that confers cold-resistant growth on minimal medium and temperature-sensitive growth on rich medium to a strain that contains a *dnaA* null mutation and is integratively suppressed by a mini-R1 plasmid (*zia::pKN500*) (28). It is unlinked by P1 transduction to both the integrated mini-R1 and *dnaA* null mutation and is thought to be a large chromosomal inversion (28). For consistency, we have

TABLE 1 (continued).

assigned the *mad-2* designation to derivatives of $\Delta dnaA$, *zia*::pKN500, *mad-1* strains that spontaneously acquired temperature resistant growth on both rich and minimal medium. We speculate that *mad-2* is also a chromosomal inversion resulting in a more optimal placement of *zia*::pKN500 relative to *terC* (39). Replication of both the *mad-1* and *mad-2* mutations remains dependent on the integrated mini-R1 plasmid (*zia*::pKN500) as demonstrated by the cold-sensitive phenotype of the strains following their transformation to ampicillin resistance with pOU420, a plasmid encoding an R1 *copA*(Ts) allele (28, data not shown).

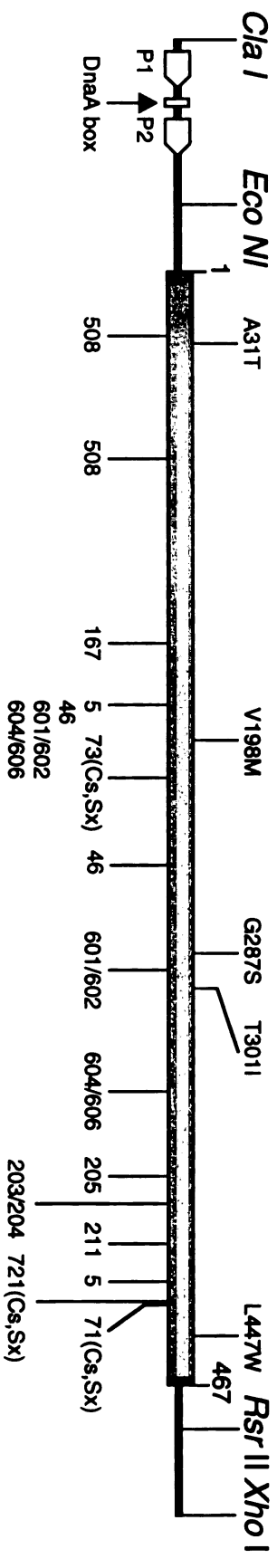
hydroxylamine-treated pDS596 was gel-purified (Qiaex DNA extraction kit, Qiagen), and ligated to gel-purified pING1 vector fragment from nonmutagenized pDS596 to form pDS596* (Figure 2B). This construction places the *dnaA* gene without most of its promoter region distal to the *araB* promoter that is regulated by the plasmid-encoded AraC protein.

Genetic assay to identify mutations of *dnaA* defective in pSC101 maintenance. A genetic screen was devised to identify plasmid-borne mutations of *dnaA* unable to complement the conditional phenotype of the *dnaA204*(Ts) allele for pSC101 maintenance. MS206 containing pSC101 was transformed with the mutagenized *dnaA*-containing plasmid, pDS596*, by selection for ampicillin resistance at 30°C on LB medium containing kanamycin and tetracycline (Figure 3). Transformants were then grown in LB medium containing the above antibiotics at 30°C in 96-well microtiter plates and diluted to a cell density of approximately 10^4 colony forming units/ml. Aliquots (1-2 μ l) were replica plated in duplicate onto supplemented M9 medium with or without tetracycline and incubated at 30°C or 41.5°C to screen for the inability of transformants to maintain pSC101 at the elevated temperature. The phenotype of plasmid-borne mutations was confirmed by retransformation into MS206 (pSC101) followed by incubation at 30°C and 41.5°C on supplemented M9 medium. The vector only (pING1), or it containing the *dnaA5*(Ts) (pDS105), *dnaA46*(Ts) (pDS215), *dnaA204*(Ts) (pDS319) and *dnaA*⁺ alleles (pDS596) served as controls.

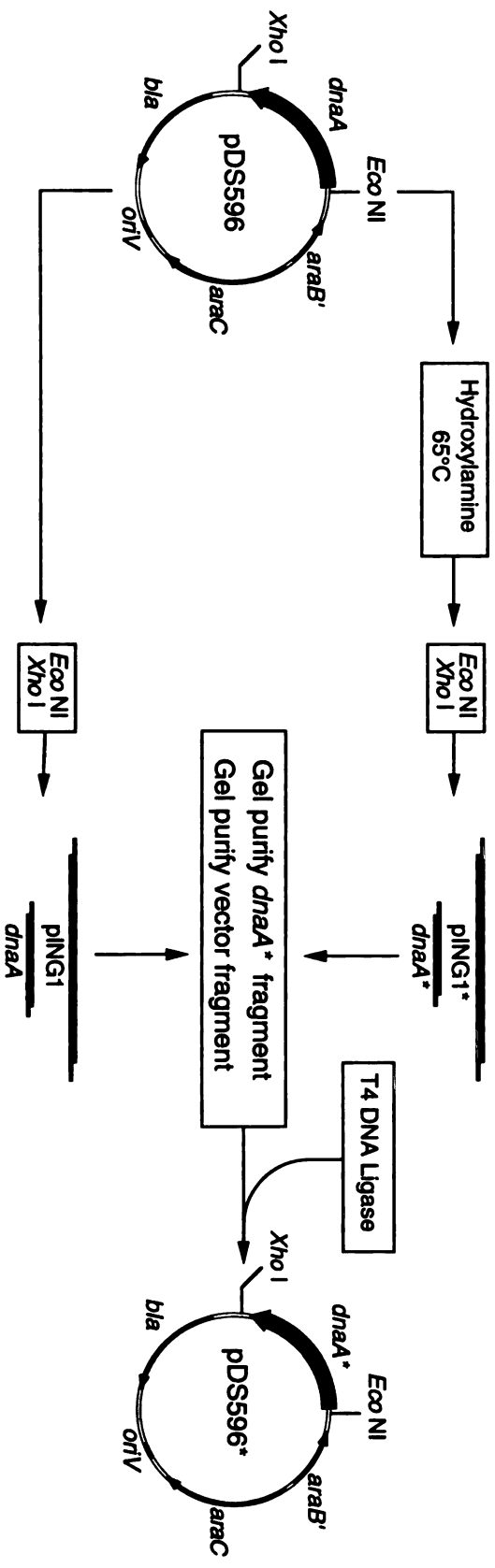
Chemical cleavage of mismatched bases. The chemical mismatch cleavage procedure was performed on heteroduplex DNA essentially as described (15). As hydroxylamine modifies cytosine mismatches, and osmium tetroxide modifies thymine mismatches (16, 42), analysis of both strands provides mapping information for all possible substitutions. The sizes of

Figure 2. (A) Map of the *dnaA* gene. Relevant restriction enzyme sites, promoters (pentagons), the DnaA box, positions of mutations for existing *dnaA* alleles (listed below) (8, 17, 26, 30, reviewed in reference 40) as well as those described here (listed above) are indicated. Extragenic suppressors of *dnaX2016*(Ts) that reside in the *dnaA* gene are denoted by “Cs, Sx” (26). Positions are not indicated for intragenic suppressor mutations (*dnaA46cos*, *dnaA508cos*) of the *dnaA46* and *dnaA508* alleles. Numbers refer to the first and last amino acid residues of DnaA protein. **(B) pDS596* construction.** pDS596* was constructed (see Materials and Methods) by insertion into the vector pING1 of an *Eco*NI-*Xho*I restriction fragment containing the hydroxylamine-treated *dnaA* coding region. The recombinant plasmid carries the mutagenized *dnaA* gene without most of the promoter region downstream from the *araB* promoter that is regulated by AraC protein, also encoded by the plasmid vector.

A



B



cleavage products detected by autoradiography were determined relative to molecular weight markers. By chemical cleavage, the nature of putative base changes was deduced by the base preference of hydroxylamine (C-specific) or osmium tetroxide (T-specific), and the template or coding strand that was cleaved. By taking into account the position of the labelled nucleotide relative to the coding sequence of the *dnaA* gene, the approximate location of the mispaired nucleotides was determined.

DNA sequence analysis. Based on the results from chemical cleavage, relevant regions for each of the five mutations were sequenced by the chain-terminating enzymatic method. Oligonucleotide primers JK-7, 5'-GTGGAGTCCCATATGTCACTTTCGCTTTGG-3'; JK-21, 5'-ATAACCCGTTGTTCC-3'; JK-22, 5'-GACCACCTAACGGACCGCTC-3'; JK-26, 5'-GAGCGCTTTGTTTCAGGACAT-3', synthesized by an in-house facility with an Applied Biosystems model 394, were used with Sequenase (U.S. Biochemicals) essentially as described by the manufacturer with plasmid DNAs purified with the Qiagen mini- or midi-column method. Samples were electrophoresed in a 6% DNA sequencing gel and autoradiographed. The oligonucleotide primers used for sequencing are homologous to the following nucleotide positions: JK-7, residues -12 to 18 of the coding strand and contains mismatches at positions -3, -2, -1 and 1; JK-21, 494 to 508 of the coding strand; JK-22, 1,459 to 1,478 of the template strand; JK-26, 610 to 629 of the coding strand.

Quantitative immunoblot analysis of DnaA protein. Cultures were grown in supplemented M9 medium (41), unless otherwise indicated, containing the appropriate antibiotics at 30°C. At mid-log growth (OD_{595nm} of 0.5-0.7), cells from 1.0 OD_{595nm} of culture were collected by centrifugation and resuspended in 50 mM Tris pH 7.6 and 20% sucrose (w/v). An equal volume of

50% glycerol, 0.2 M Tris-HCl pH 7.6, 1% SDS, 0.1% bromophenol blue, and 40 mM DTT was then added. The final concentration of each sample was equivalent to 10^{-2} OD_{595nm}/μl. Samples were frozen in liquid nitrogen and stored at -70°C prior to electrophoresis in 10% SDS polyacrylamide gels, then subjected to immunoblot analysis (51). Detection was with a monoclonal antibody (M43 that recognizes residues 133-141) to DnaA protein incubated in 10 mM Tris pH 7.4, 154 mM NaCl, 0.05% Tween 20, and 2% nonfat milk. ¹²⁵I-labelled Goat anti-Mouse IgG (ICN) was used as the second antibody. Quantitation of immunoreactive species was with a Packard Instant Imager, or with a Kodak Bio Image Visage 110, as indicated.

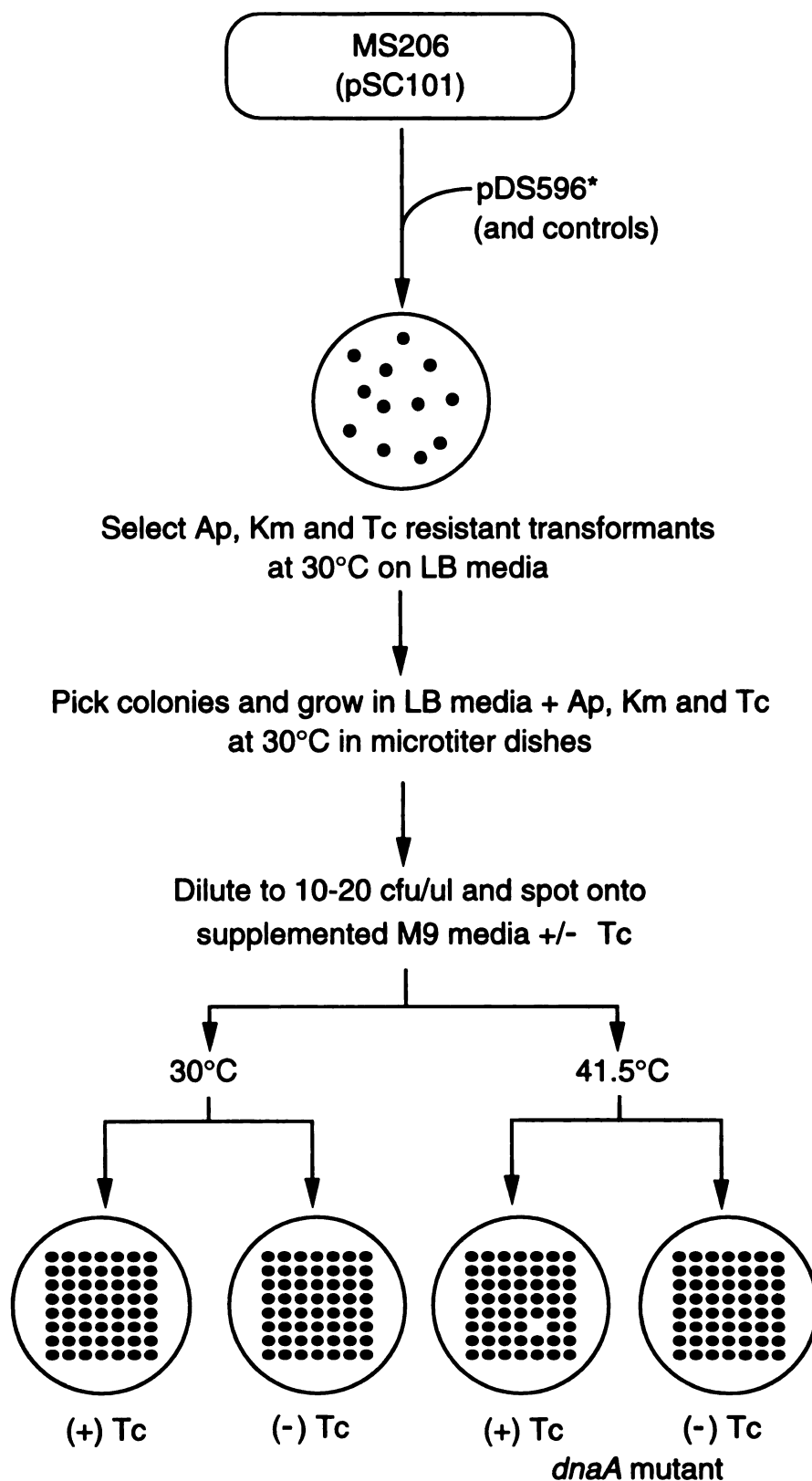
Assay of *in vitro* *oriC* replication. Reactions (25 μl) were performed with a crude protein fraction deficient in DnaA protein activity prepared from WM433 (22). Purified DnaA⁺ protein was used as a control. Lysate supernatants (Fraction I) were prepared using a gentle lysis procedure as described (33) of cell pellets obtained from 1 L cultures of MS3897 harboring the mutant alleles in pING1. Induced expression of respective gene products was by addition of L-arabinose (Sigma) to 0.7%, followed by growth for 3 hr at 30°C.

Results

Isolation of five novel *dnaA* mutations. To obtain novel mutations of the *dnaA* gene, a genetic assay was developed (Figure 3). This method measured the inability of a plasmid-borne mutation to complement the conditional defect of the *dnaA204(Ts)* allele for pSC101 maintenance. To measure pSC101 maintenance at an elevated temperature, the host strain (MS206) was also integratively suppressed by a mini-R1 plasmid. Whereas this strain was viable up to 41.5°C due to integrative suppression, an isogenic strain lacking the mini-R1 was viable only up to 38°C (data not shown). When the integratively suppressed strain was transformed by pSC101, this plasmid was not maintained at the elevated temperature unless it was co-resident with another containing the *dnaA*⁺ gene (pDS596) (Table 2) whose expression is under control of the *araB* promoter but was not induced (see Figure 2B). Sufficient levels of DnaA protein were expressed from this construct in the absence of arabinose induction to permit the establishment and maintenance of pSC101. Other controls were performed with this strain containing the latter plasmid but bearing other alleles (*dnaA5*, *dnaA46*, and *dnaA204*) temperature sensitive for replication from *oriC* (reviewed in reference 40). These *dnaA* alleles were also temperature sensitive for pSC101 maintenance (Table 2), confirming that pSC101 maintenance is *dnaA*-dependent (18, 20, 31).

By this method in combination with hydroxylamine mutagenesis of the *dnaA* gene (Figure 2 and 3), five mutations defective for pSC101 maintenance at high temperature (41.5 °C) were identified from a collection of 1,790 transformants (Table 2). At the elevated temperature, G287S (with a G-to-S mutation at position 287), T301I and L447W displayed small or minute colonies relative to 30°C, or when compared to the *dnaA*⁺ transformant (pDS596). A31T

Figure 3. Genetic assay to identify *E. coli dnaA* mutants defective in pSC101 maintenance at 41.5°C. See Materials and Methods for a description. Abbreviations are Ap, ampicillin; Km, kanamycin; Tc, tetracycline.



and V198M each showed a modest defect, having a reduction in plating efficiency to 0.11 and 0.09, respectively, at the elevated temperature (Table 2).

Nucleotide sequence determination of mutant *dnaA* alleles.

The respective mutation(s) of each allele was mapped by a chemical modification method (data not shown) (15), and the appropriate regions for each sequenced (Table 3). A single nucleotide substitution for four of the five alleles was identified. L447W was found to contain 3 nucleotide substitutions, two of which are silent. In all, each mutant allele was found to contain a single deduced amino acid substitution. All mutations were consistent with hydroxylamine mutagenesis (C→T transitions) (16, 42) except for the transversion (T→G) of L447W that we presume arose spontaneously.

Compared to the *dnaA* alleles previously described (reviewed in reference 40), these are novel (Figure 2A). The amino acids, V-198 (valine at position 198), T-301, and L-447 are highly conserved among 15 *dnaA* homologs (Table 3). A-31 and G-287 are less well conserved among all homologs but are fairly well conserved among Gram negative bacteria. As hydroxylamine treatment should produce nonsense mutations (9), the failure to obtain these or to reisolate previously identified mutations may be due to the small number of transformants screened (1,790). Indeed, *dnaA5*, *dnaA46* and *dnaA204*, were temperature sensitive for pSC101 maintenance as measured by this genetic screen (see Table 2). Furthermore, the residual activity of the *dnaA204* allele at 42°C (29) may have restricted the types of mutations detected as those able to function in concert with the *dnaA204* gene product would not have been obtained.

Replication activity of the novel *dnaA* alleles in a Δ *dnaA* strain. To assess the phenotypes of the novel mutations without the possible contribution of a chromosomal *dnaA* allele, plasmids bearing the mutant alleles

TABLE 2. Inability of plasmid-borne *dnaA* mutations to confer temperature resistant growth to *E. coli* MS206 (pSC101)

MS206 (pSC101) transformant ^a	<i>dnaA</i> allele	Efficiency of plating ^b
pING1	None	8.1 X 10 ⁻⁶
pDS596	<i>dnaA</i> ⁺	1.4
pDS105	<i>dnaA5</i>	5.1 X 10 ⁻⁵
pDS215	<i>dnaA46</i>	2.1 X 10 ⁻⁵
pDS319	<i>dnaA204</i>	5.6 X 10 ⁻⁵
pA31T	A31T	0.11
pV198M	V198M	9.1 X 10 ⁻²
pG287S	G287S	1.3 ^c
pT301I	T301I	0.82 ^c
pL447W	L447W	1.3 ^d

^a MS206 (pSC101) transformants contain the indicated *dnaA* alleles in pING1 and expressed from the *araB* promoter.

^b Transformants were grown at 30°C in LB medium containing kanamycin, ampicillin and tetracycline before plating. Plating efficiency is expressed as the number of colony forming units observed at 41.5°C relative to 30°C on supplemented M9 medium containing the above antibiotics. Efficiency of plating was determined in the absence of arabinose induction. On medium without tetracycline, the colony morphology of MS206 (pSC101) with the different plasmid-borne mutations was similar to the *dnaA*⁺ transformant. By contrast to these results with synthetic medium, pSC101 maintenance was not temperature-sensitive on LB medium with these plasmid-encoded *dnaA* mutations, or with temperature sensitive alleles (*dnaA5*, *dnaA46* and *dnaA204*).

^c Small cfu observed at 41.5°C.

^d Tiny cfu observed at 41.5°C.

TABLE 3. Nucleotide and deduced amino acid alterations of five *dnaA* alleles^a

<i>dnaA</i> allele	Nucleotide position ^b	Nucleotide substitution ^c	Amino acid position	Deduced amino acid substitution (sequence conservation) ^d
A31T	91	G→A	31	Ala→Thr (6/15)
V198M	592	G→A	198	Val→Met (13/15)
G287S	859	G→A	287	Gly→Ser (6/15)
T301I	902	C→T	301	Thr→Ile (12/15)
L447W	1335	G→A	445	Glu→Glu
	1338	G→A	446	Gln→Gln
	1340	T→G	447	Leu→Trp (12/15)

^a Chemical modification of the template and coding strand to map the approximate positions of mutations (data not shown) was performed as described in Materials and Methods. As controls, the *dnaA*⁺ and *dnaA46* alleles were analyzed. The (cytosine) substitutions of the *dnaA46* allele at residues 551 and 754 (30) were confirmed by this method. No mispairs in either strand of the *dnaA*⁺ gene were detected. Appropriate regions of each mutant allele based on the chemical modification mapping results were sequenced by the chain-terminating enzymatic method.

^b Numbers refer to those in *dnaA* coding region with position 1 corresponding to the first nucleotide of the coding sequence (27, 44).

^c Nucleotide substitutions are expressed as those in the coding strand.

^d Sequence conservation is indicated by the frequency of residues conserved at respective positions among the 15 *dnaA* homologs (reviewed in reference 40).

were transformed into an integratively-suppressed strain lacking the *dnaA* gene (MS3897). Except for the *dnaA* null mutation, this strain is isogenic to that used to isolate the novel mutations. Plasmids bearing each of the novel alleles were taken up by this strain as efficiently as controls of the vector (pING1), or it bearing the wild type (pDS596) or *dnaA46*(Ts) allele (pDS215) (data not shown). The *dnaA* null strain carrying each of the novel mutations was then transformed by pSC101. At 30°C, their transformation efficiencies were comparable to controls of the *dnaA*⁺ or *dnaA46* alleles in pING1 (data not shown). At 41.5°C, the transformation efficiency with plasmids carrying the novel mutations or the *dnaA46* allele was greatly reduced relative to *dnaA*⁺ (data not shown). The temperature sensitive phenotype for pSC101 maintenance was confirmed by plating representative transformants at 30°C and 41.5°C on supplemented M9 medium (Table 4).

At the elevated temperature, an increased plating efficiency was observed with these plasmid-borne mutations co-resident with the chromosomal *dnaA204* allele relative to the *dnaA* null allele in the isogenic strain (Table 2 compared to Table 4). This may be due to contribution of the *dnaA204* allele that maintains residual activity at 42°C (29).

An elevated level of DnaA protein is required for *oriC* replication compared with that required for pSC101 replication. We discovered that the *dnaA*⁺-containing plasmid, pDS596, was unable to maintain an *oriC* plasmid (data not shown), whereas pSC101 was maintained under comparable conditions (Table 4, line 1). This was in the absence of arabinose induction in the *dnaA* null strain (MS3897).

By contrast, the *dnaA*⁺ gene expressed from its own promoter contained in pACYC184 was sufficient to maintain an *oriC* plasmid (data not shown), or to support chromosomal replication from *oriC* upon inhibition of integrative

TABLE 4. Plasmid-borne *dnaA* mutations are temperature sensitive for pSC101 maintenance

MS3897 transformant ^a	<i>dnaA</i> allele	Efficiency of plating ^b
pDS596	<i>dnaA</i> ⁺	0.91
pDS215	<i>dnaA46</i>	9.0 X 10 ⁻⁵
pA31T	A31T	2.8 X 10 ⁻³
pV198M	V198M	1.2 X 10 ⁻³
pG287S	G287S	1.9 X 10 ⁻²
pT301I	T301I	8.1 X 10 ⁻⁴
pL447W	L447W	1.1 X 10 ⁻⁴

^a MS3897 transformants contain the indicated *dnaA* alleles in pING1 and expressed from the *araB* promoter.

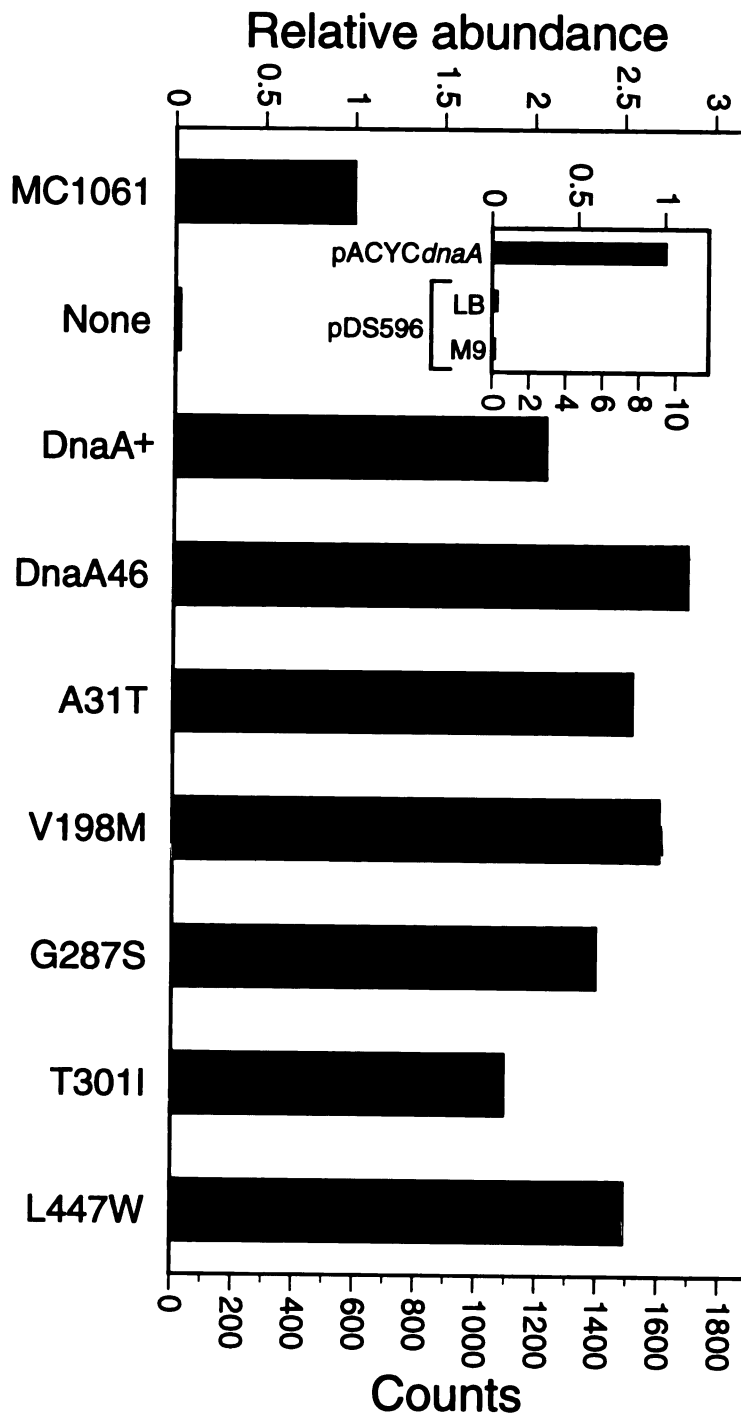
^b Transformants were grown at 30°C in LB medium containing kanamycin, ampicillin and tetracycline before plating. Efficiency of plating is expressed as the ratio of the number of colony forming units observed at 41.5°C relative to 30°C on supplemented M9 medium containing the above antibiotics. Plating efficiency was determined in the absence of arabinose induction.

suppression (see Table 5, line 1 and below). These results suggest that expression of DnaA protein, when under *araB* promoter control and not induced, is limiting for *oriC* replication. Quantitative immunoblot analysis supported this interpretation (Figure 4). The level of DnaA⁺ protein expressed from pACYC*dnaA* contained in the null strain was about 40-fold higher than the uninduced level expressed from pDS596 (Figure 4, inset). In the latter plasmid, the level of expression was not dramatically affected by growth medium. The level of DnaA⁺ protein expressed from pDS596, barely detectable by autoradiography, concurs with the observation of Abeles *et al.* (1). Together, these results indicate that the level of DnaA protein required for pSC101 replication *in vivo* is less than that required for replication of an *oriC* plasmid or the bacterial chromosome.

In the *dnaA* null strain, DnaA⁺ and DnaA46 protein encoded in pACYC184 were expressed about 2.1-fold and 2.9-fold higher, respectively, relative to the chromosomally-encoded level of MC1061 (Figure 4). The calculated cellular abundance of chromosomally encoded DnaA protein (1.9×10^3 molecules per 10^{-9} O.D._{595nm}, see Figure 4 legend) is slightly greater than published estimates ($1\text{--}1.5 \times 10^3$ molecules per 10^{-9} O.D._{595nm}) (45). In this experiment, DnaA protein was not detected in the null strain lacking a plasmid or harboring pACYC184, confirming its genotype.

Replicon-specific defect of the novel *dnaA* alleles. The method to obtain novel mutations screened for the inability of a plasmid-encoded allele to maintain pSC101 in a host strain harboring the *dnaA204*(Ts) allele at the nonpermissive temperature (Figure 3). Consequently, it is possible that the mutations obtained were not defective in replication from *oriC*. To address this issue, chromosomal replication from *oriC* was measured by inhibition of replication from the integrated R1 origin in the *dnaA* null strain (MS3897).

Figure 4. Quantitative immunoblot analysis of DnaA protein. The level of DnaA protein encoded by the various recombinant plasmids in the *dnaA* null strain (MS3897) was determined as described in Materials and Methods. “None” refers to the MS3897 (pACYC184) transformant. The levels were quantified with a Packard Instant Imager. For each sample, the counts detected during a 100 min exposure minus background, indicated by the right ordinate, were normalized relative to the chromosomally encoded level of the wild type strain MC1061 (indicated by the left ordinate). In the inset, scanning densitometry of an autoradiogram (not preflashed) was required due to the low levels of expression that could not be detected with the Instant Imager. Optical densities of DnaA protein encoded by pDS596 grown in either LB medium (0.24 units), or M9 minimal medium (0.15 units) were normalized relative to the level encoded by pACYC*dnaA* (9.43 units). Relative to known amounts of purified DnaA protein analyzed in parallel, the cellular abundance of DnaA protein encoded by MC1061 was determined.



Inhibition was achieved by introduction of a plasmid containing the R1 *copA* gene (pKN505) that encodes an antisense RNA to the transcript for R1 RepA protein (24). Formation of the RNA duplex interferes with expression of RepA protein required for R1 replication (49).

The level of DnaA protein, when encoded by pACYC184 and expressed from its own promoter, was sufficient to support *oriC* replication (data not shown, Table 5). To examine the *dnaA* mutations for *oriC* replication activity, derivatives of pACYC184 containing these alleles were constructed. They were introduced into the *dnaA* null strain followed by the R1 *copA*-containing plasmid (pKN505) to inhibit integrative suppression (Table 5). In contrast to the others, the A31T, G287S and L447W alleles supported replication from *oriC* as effectively as the wild type control (pACYC*dnaA*). This observation suggests that the defect of the A31T, G287S and L447W alleles is specific for pSC101 replication. Transformants harboring the *copA*-containing plasmid (pKN505), and either the plasmid-borne *dnaA46* or T301I allele were obtained at 30°C but were extremely unstable, preventing an assessment of their replicon specificity. This observation is not understood but is unlikely to be due to insufficient levels of DnaA protein (Figure 4). DnaA46 and T301I proteins were expressed at levels comparable to the other mutant proteins.

As a control, pSC101 maintenance was examined in the integratively-suppressed null strain (Table 5). G287S, T301I and L447W were temperature sensitive for maintenance of pSC101. By contrast, A31T and V198M were not defective for pSC101 maintenance at the elevated temperature.

Suppression of the temperature sensitive phenotype for pSC101 maintenance by elevated expression. All of the mutant *dnaA* alleles, except G287S which was unchanged, showed an increase in plating

TABLE 5. A subclass of *dnaA* mutations is specifically defective in pSC101 maintenance

MS3897 transformant ^a	<i>dnaA</i> allele	Efficiency of plating ^c	
		pSC101	pKN505
pACYC <i>dnaA</i>	<i>dnaA</i> ⁺	1.17	1.16
pACYC <i>dnaA46</i>	<i>dnaA46</i>	5.9 X 10 ⁻³	NA ^c
pACYCA31T	A31T	1.01	0.89
pACYCV198M	V198M	1.30	6.3 X 10 ⁻²
pACYCG287S	G287S	1.6 X 10 ⁻²	0.91
pACYCT301I	T301I	7.8 X 10 ⁻²	NA
pACYCL447W	L447W	4.2 X 10 ⁻²	1.03

^a MS3897 transformants contain the indicated *dnaA* alleles in pACYC184 and expressed from the *dnaA* promoter.

^b Transformants of pSC101 were grown at 30°C in LB medium containing kanamycin, chloramphenicol and tetracycline before plating. Transformants of pKN505 were grown on supplemented M9 agar medium, picked and diluted in M9 salts before plating. Efficiency of plating is expressed as the number of colony forming units observed at 41.5°C relative to 30°C on supplemented M9 medium containing kanamycin, chloramphenicol and either tetracycline (for pSC101) or ampicillin (for pKN505).

^c Efficiency of plating is not available ("NA") due to the instability of the transformant containing both the plasmid-encoded *dnaA* allele and pKN505.

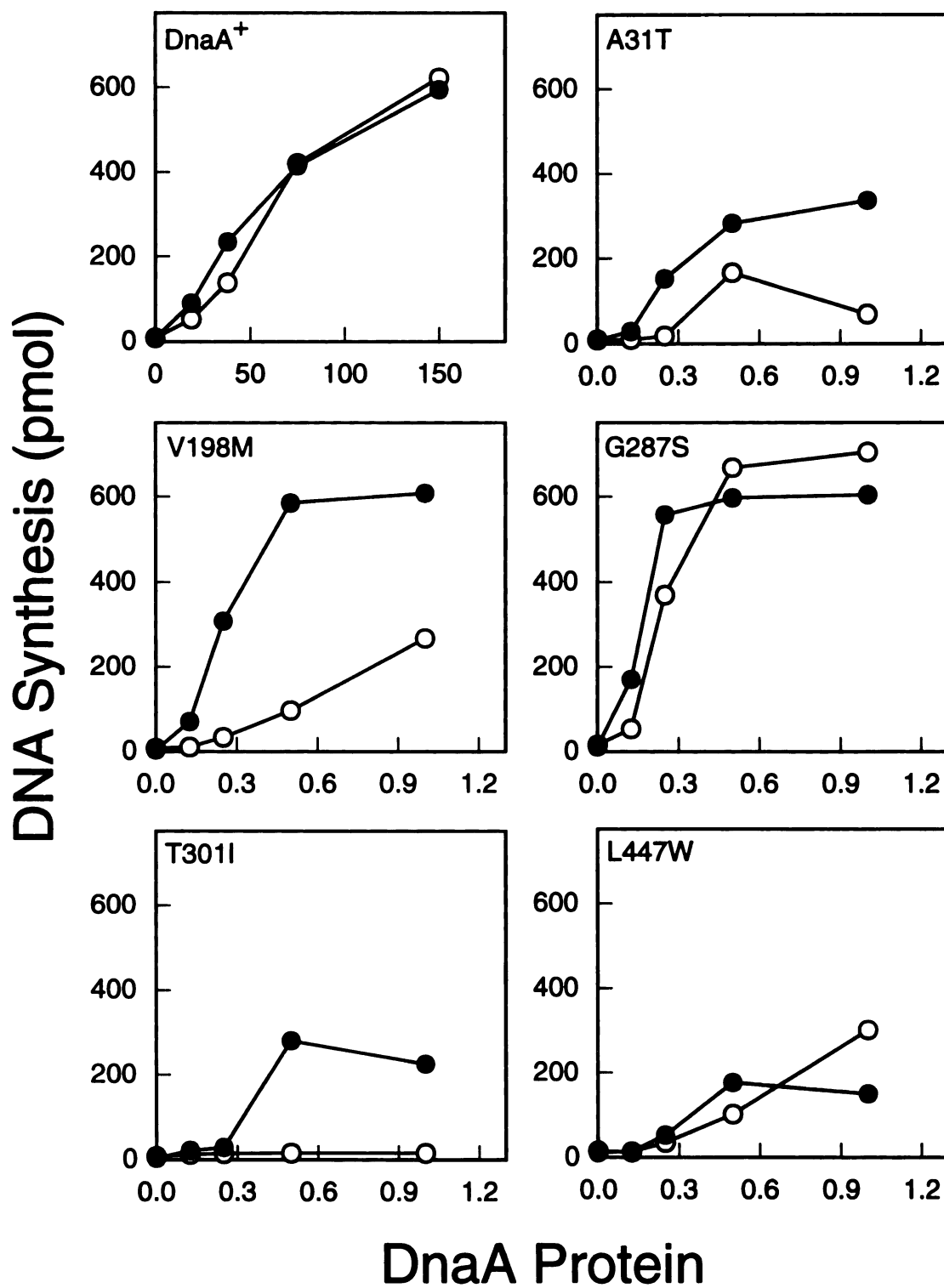
efficiency of two to three orders of magnitude at the elevated temperature when contained in pACYC184 relative to pING1 (compare Table 4 and 5). The A31T and V198M alleles were not temperature sensitive for pSC101 maintenance when contained in pACYC184. Although we have not measured the cellular abundance of these or the other novel mutants when contained in pING1, we believe that their elevated expression when in pACYC184 suppresses, sometimes partially, the temperature-sensitive phenotype for pSC101 maintenance. This interpretation is based on two observations. First, immunoblot analysis showed that DnaA⁺ protein was approximately 40-fold elevated in the pACYC184 construct relative to pING1 (Figure 4, inset). Second, the viability of many *dnaA*(Ts) mutants increases when the alleles were present on both the chromosome and a pBR322 derivative (30), presumably by a gene dosage effect.

Also in this analysis (Figure 4), the levels of the mutant proteins encoded in pACYC184 were found to be between 1.9- and 2.7-fold higher than the chromosomally-encoded *dnaA*⁺ control (MC1061), and comparable or slightly elevated compared to the control of DnaA⁺ protein encoded by the comparable plasmid (pACYC*dnaA*). The latter difference may possibly relate to differences in protein stability. Alternatively, the difference in expression levels of the pACYC184 constructs may relate to their relative abilities to autoregulate their own expression. The *dnaA* promoter region containing a DnaA box is autoregulated by the binding of DnaA protein (2, 28, 38, 53).

We do not know whether A31T or V198M, when expressed at the chromosomal level, will be temperature sensitive for pSC101 maintenance nor whether A31T will be temperature sensitive for replication from *oriC*. This issue may be addressed by substituting the chromosomal copy with each allele, or by modulating their expression from the *araB* promoter to the chromosomal level.

A31T, V198M and T301I are thermolabile in *in vitro* replication of an *oriC* plasmid. To corroborate the genetic studies, *in vitro* replication activity of the mutant DnaA proteins was measured at both 30°C and 42°C (Figure 5). The V198M and T301I mutant proteins were thermolabile *in vitro* for *oriC* plasmid replication. Whereas G287S protein appeared comparable in replication activity at both temperatures to the wild type protein, A31T protein was moderately thermolabile. The replication activity of L447W protein was not thermolabile. However, the extent of nucleotide incorporation with A31T and L447W protein was lower than that observed for both the wild type and G287S proteins. The crude protein fractions used in this assay contained comparable levels of mutant or wild type protein as judged by immunoblot analysis (data not shown). Whether a contaminating factor in the samples may have affected the results obtained can be addressed when these proteins are purified. The marginal effect of temperature on the replication activity of A31T, G287S and L447W proteins is in agreement with the *in vivo* phenotypes of these mutants for chromosomal replication (Table 5), suggesting that these alleles are specifically defective for pSC101 maintenance.

Figure 5. *in vitro* *oriC* replication activity of novel DnaA mutant proteins. Replication assays were performed as described in Materials and Methods at 30°C (●) or 42°C (○). Crude lysate supernatants containing the indicated mutant DnaA proteins used in these *in vitro* replication assays were comparable in total protein concentration determined by the dye-binding method (5), and DnaA protein abundance as judged by immunoblot analysis (data not shown). DnaA protein is expressed either in ng (purified DnaA⁺ protein) or μl of Fraction I (mutants). By immunoblot analysis, 1 μl of the respective Fraction I corresponds to ~150 ng of DnaA protein.



Discussion

***dnaA* mutations specifically defective in pSC101 maintenance.**

The method to identify mutations of the *E. coli dnaA* gene relied on use of an integratively suppressed strain (MS206) containing the *dnaA204*(Ts) allele. Due to the integrated mini-R1, the *dnaA* gene and *oriC* are not essential for bacterial growth (28). Incubation at a temperature nonpermissive for the *dnaA204* allele provided a means to assess the phenotype of the plasmid-encoded *dnaA* gene for maintenance of pSC101 (Figure 3). Whereas bacterial growth in the absence of tetracycline was observed at 30°C and 41.5°C (unpublished results), pSC101 was not maintained at the elevated temperature in the absence of a functional *dnaA* gene product (Table 2). With this approach, five mutations unable to complement the conditional phenotype of the *dnaA204* allele for pSC101 maintenance were obtained. Additionally, these mutations were temperature sensitive for pSC101 maintenance in a strain lacking the *dnaA* gene (Table 4). Compared to the 16 other missense mutations of the *dnaA* gene (8, 17, 26, 30, reviewed in reference 40), these are novel (see Figure 2A). Furthermore, V198M, T301I and L447W have substitutions in amino acid residues that are highly conserved in *dnaA* homologs of the *enterobacteriaceae* family (Table 3).

Of the five mutations, A31T, G287S, and L447W were not temperature sensitive for *oriC* replication under conditions in which replication from the integrated R1 origin was inhibited (Table 5). Biochemical studies corroborated these genetic results (Figure 5). Assays using lysate supernatants enriched with the respective mutant proteins indicated that G287S and L447W, and to a lesser degree A31T, were also temperature resistant in *in vitro* replication of an *oriC* plasmid. Collectively (Table 6), these results suggest that the G287S and

TABLE 6. Summary of mutant *dnaA* phenotypes^a

<i>dnaA</i> allele	pSC101 maintenance		<i>oriC</i> replication	
	Low level of DnaA ^b	High level of DnaA ^c	<i>in vivo</i> (High level of DnaA ^c)	<i>in vitro</i>
A31T	Ts	+	+	+/-Ts
V198M	Ts	+	Ts	Ts
G287S	Ts	Ts	+	+
T301I	Ts	Ts	NA	Ts
L447W	Ts	Ts	+	+

^a This Table is based on results presented in Figures 4 and 5 and Tables 4 and 5. Abbreviations are Ts, temperature sensitive; +, temperature insensitive; +/-, moderately temperature sensitive; NA, data not available.

^b "Low level of DnaA" refers to the level of expression observed when the mutant alleles were contained in pING1 and under control of the *araB* promoter in the noninduced state (see Figure 4, inset).

^c "High level of DnaA" refers to the level of expression observed when the mutant alleles were contained in pACYC184 and expressed from the *dnaA* promoter (see Figure 4).

L447W mutations, and possibly A31T are specifically defective in replication of pSC101.

We do not understand the instability of the *dnaA* null strain bearing both the *copA* plasmid and either plasmid-borne *dnaA46* or T301I allele (Table 5). That these alleles were plasmid-borne and not chromosomally encoded may be of relevance. Suppressors of *dnaX2016*(Ts) that reside in the *dnaA* gene are dominant to plasmid-borne *dnaA*⁺ when chromosomally located, but recessive in the converse construction (26). A similar difference in activity depending upon mutant allele location (chromosome versus plasmid) has been reported for those *dnaA*(Ts) allele containing an alanine-to-valine substitution at residue 184 (30). These alleles, expressed from the *dnaA* promoter, exhibit a cold-sensitive phenotype when plasmid-encoded and in a strain containing a chromosomal *dnaA*⁺ allele. However, cold sensitivity was not observed when the thermolabile allele was present on the chromosome with the wild type allele residing in a plasmid.

pSC101 maintenance requires a lower level of DnaA protein than the level needed for replication from *oriC*. With the *dnaA*⁺ gene in pDS596, our inability to establish an *oriC* plasmid in a *dnaA* null mutant (unpublished results) contrasts with the establishment and maintenance of pSC101 under comparable conditions (Table 4). With the *dnaA*⁺ gene in pACYC184 in the *dnaA* null strain, we were able to establish an *oriC* plasmid (unpublished results), as well as to sustain chromosomal replication from *oriC* on inhibition of replication from the integrated R1 origin (Table 5). This result, suggesting that the level of DnaA protein required for pSC101 replication is less than that required for replication of an *oriC* plasmid or the bacterial chromosome, was confirmed by quantitative immunoblotting (Figure 4).

DnaA protein may interact with RepA protein of pSC101. Among the plasmids of *E. coli*, many require DnaA protein for replication. Of these, F and P1 are capable of integrative suppression of strains containing the *dnaA46*(Ts) allele (12, 13, 43), but not a *dnaA* null allele (28, 36). By comparison, the strict dependence of pSC101 on DnaA protein for maintenance (18, 20, 31) was interpreted to suggest that it requires many of the same functions of *dnaA* that are required for initiation from *oriC*. The mutations G287S, L447W, and possibly A31T, obtained in this study that appear to have a specific defect in pSC101 replication are particularly interesting for they suggest a unique role of DnaA protein in pSC101 replication. One or more may be defective in the proposed interaction of DnaA protein with pSC101-encoded RepA protein that facilitates the binding of DnaA protein to the pSC101 replication origin (47). Further examination of these mutant proteins in binding to the pSC101 replication origin, and the development of an *in vitro* system to study pSC101 replication should provide a greater understanding of the interplay between these proteins, and if the amino acids that have been substituted normally interact with pSC101 RepA protein.

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Chapter III

Novel Alleles of the *Escherichia coli* *dnaA* Gene

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Abstract

The *Escherichia coli dnaA* gene is required for replication of the bacterial chromosome. In order to identify residues critical for its replication activity, a selection to obtain novel mutations was developed. The method relied on lytic growth of lambda from an inserted pSC101 replication origin. Replication from the lambda origin was inhibited by *ci* repressor protein encoded by a lysogen of the host. Replication from the pSC101 origin that resulted in lytic growth was dependent on active DnaA protein that was provided by a plasmid. The host strain lacked the chromosomal *dnaA* gene. With this approach, a large collection of missense, nonsense, and a few internal deletion mutations were obtained. Nucleotide sequence analysis of the missense mutations indicated that 28 of 50 were unique. Of these, one is identical to the *dnaA205* allele whereas the remainder represent novel mutations. Examination of the locations of mutation and replication activity indicate that only those mutations mapping to the C-terminus were inactive for pSC101 replication.

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Introduction

The *Escherichia coli dnaA* gene was identified nearly three decades ago (24, 30). It is required for initiation of replication of the bacterial chromosome (18, 24, 30) as well as a number of plasmids and bacteriophage including F (21, 29), pSC101 (15, 16, 23), and P1 (10, 11). This requirement correlates with its activity as a sequence-specific DNA binding protein and the observation that sequences termed DnaA boxes that are in these replicons are bound by DnaA protein (17). At *oriC* and aided by HU protein or integration host factor, DnaA protein unwinds an AT-rich region within *oriC* (4, 5, 28). As DnaA protein and the replicative helicase, DnaB, interact physically (31), a model has been proposed whereby DnaA protein then loads DnaB in the vicinity of *oriC*, presumably in the single stranded AT-rich region, to establish both replication forks.

In an effort to identify functional domains of DnaA protein, mutant alleles have been characterized genetically (reviewed in references 32 and 38) and biochemically (25, 27, reviewed in references 32 and 38). Although the biochemical characterization of DnaA protein has revealed that it contains numerous activities including sequence-specific DNA binding (17) and ATP binding (37), little has been revealed regarding its domain structure. A P-loop motif (GX₄GKT, where X is any amino acid) mapping to positions 172-179 and found in many nucleotide binding proteins has been implicated in ATP binding (25, 27). The DNA binding activity appears to reside in the C-terminal 94 residues (36).

Characterization of a large collection of *dnaA* alleles has the potential of identifying functional domains of DnaA protein. However, the current collection of mutant alleles is small and their mutations not widely distributed (6, 14, 19,

reviewed in reference 32), rendering them inadequate for in-depth structure-function studies. To identify a large number of residues of DnaA protein critical for its replication activity, a novel selection for *dnaA* alleles defective in pSC101 replication was developed. *E. coli* strains harboring lambda as a lysogen express *cl* repressor to maintain it as a prophage (35). The activity of the prophage-encoded *cl* repressor also inhibits subsequent infection by lambda by repressing lytic functions, including the expression of phage-encoded genes required for lambda replication. However, if the infecting lambda contains in its genome an alternative replication origin, lytic growth ensues because of the inability of *cl* repressor to inhibit replication from this origin (7). We relied on these features to develop a method to select novel mutants of the *E. coli dnaA* gene. The alternative replicon was the pSC101 origin that depends on DnaA protein for its replication (15, 16, 23). The inability of mutant *dnaA* alleles to replicate the lambda derivative containing the pSC101 origin provided a powerful method for their selection. Characterization of these novel mutant *dnaA* alleles revealed three regions that affect the replication activity of DnaA protein.

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Materials and Methods

Bacteriological methods. Bacterial strains, plasmids and bacteriophage used in this study are described in Table 1. Bacteriological methods were performed essentially as described by Miller (33). Bacterial transformation was either by the calcium chloride method or by electroporation using a Bio-Rad Gene Pulser according to the manufacturer's recommendation. Bacterial strains and their plasmid-containing derivatives were routinely grown in LB medium supplemented, where indicated, with 100 µg/ml ampicillin, 40 µg/ml chloramphenicol, 40 µg/ml kanamycin, and 10 µg/ml tetracycline. M9 minimal medium supplemented with the indicated antibiotics and either 7 mM MgSO₄, 0.5% casamino acids, 0.2% maltose, and 1 µg/ml thiamine (for work with phage lambda) or 0.5% casamino acids, 0.2% glucose, and 1 µg/ml thiamine (to measure the phenotypes of *dnaA* alleles) was used as noted. Lambda lysogens and cross-streaking of bacteria and phage lambda were done essentially as described by Davis *et al.* (13).

Recombinant DNA methods. λpCM128, λpCM129, and λ*imm*⁴³⁴ were constructed as described in the legend to Figure 1.

***in vitro* chemical mutagenesis of plasmid DNA.** pACYC*dnaA* DNA (30 µg) was incubated for 60 min at 65°C in 1 ml of 1 M hydroxylamine-HCl (Sigma) and 0.1 M sodium phosphate pH 6.5 (33) then ethanol precipitated 2 times to remove hydroxylamine. Hydroxylamine treatment reduced the transformation efficiency of the plasmid DNA to 10% that of the untreated DNA.

Genetic selection for mutations of *dnaA* defective for replication from the pSC101 origin. A selection method was developed to obtain mutations of the *Escherichia coli dnaA* gene defective for pSC101 replication (Figure 3). After transformation of MS3898 with hydroxylamine-mutagenized pACYC*dnaA* by selection for chloramphenicol resistant growth at

TABLE 1. Bacterial strains, plasmids, and bacteriophage^a

Strain, plasmid, or bacteriophage	Genotype ^b or characteristics	Source or construction
Strains		
HMS174	<i>recA</i> (<i>r_{K-12}</i> ⁻ <i>m_{K-12}</i> ⁺) <i>Rif^r</i> (<i>F</i> ⁻)	Novagen, Inc.
MS175	HMS174 but lysogenized with <i>λimm</i> ⁴³⁴	This work
MS3897	<i>asnB32 relA1 spoT1 thi-1 ilv-192 zia::pKN500</i> (<i>pKN500</i> =mini-R1) <i>ΔdnaA mad-2 recA1</i> (<i>F</i> ⁻)	This lab
MS3898	MS3897 but lysogenized with <i>λimm</i> ⁴³⁴	This work
Plasmids		
pACYC184	<i>Cm^r</i> , <i>Tc^r</i> ; p15A origin, cloning vector	Lab stock (9)
pACYC <i>dnaA</i>	<i>dnaA</i> ⁺ cloned into pACYC184	This lab
pACYC <i>dnaA46</i>	<i>dnaA46</i> cloned into pACYC184	This lab
pSC101	<i>Tc^r</i> ; DnaA-dependent low-copy-number replicon	S. Cohen (12)
pCM128	<i>Ap^r</i> ; copy-number-up mutant of pSC101	S. Cohen (41)
Phage^c		
<i>λcl857</i>	<i>clts857 imm</i> ^λ	L. Snyder
<i>λclts857Sam7</i>	<i>clts857 Sam7 imm</i> ^λ	Lab stock
<i>λgt10</i>	phage lambda cloning vector, <i>srlλ3° srlλ4°</i> <i>srlλ5° imm</i> ⁴³⁴ <i>λb527</i> (<i>Att</i> ⁻)	David Dewitt (26)
<i>λimm</i> ⁴³⁴	<i>λgt10</i> but without <i>λsrl3°</i> and <i>λb527</i> (<i>Att</i> ⁺)	This work
<i>λpCM128</i>	<i>Ap^r</i> ; pCM128 cloned into the <i>Eco</i> RI site of <i>λgt10</i> , pSC101 origin replicates leftward	This work
<i>λpCM129</i>	<i>Ap^r</i> ; pCM128 cloned into the <i>Eco</i> RI site of <i>λgt10</i> , pSC101 origin replicates rightward	This work

^a Abbreviations are Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline.

^b See the report of Bachmann (1) for genetic symbols.

^c See Figure 1 for a description of the construction of *λimm*⁴³⁴, *λpCM128*, and *λpCM129*.

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37°C, colonies were replica plated in duplicate using sterile velveteen onto supplemented M9 medium (as described above) seeded with 10^{11} λ pCM128 infective particles. After overnight incubation at either 30° and 42°C, isolates were colony purified on the above medium at the same temperature that they were originally selected for. Immunity to λ pCM128 infection was confirmed by cross-streaking. That immunity was due to a specific plasmid-borne mutation was confirmed by retransformation of MS3898 with the isolated plasmid followed by cross-streaking with λ pCM128. λ *cl857* and λ gt10 were used as controls to confirm that all clones were sensitive to lambda infection (λ *cl857* sensitive) but expressed phage 434 immunity (λ gt10 immune). MS3898 harboring pACYC184 (see Table 1), pACYC*dnaA*, or pACYC*dnaA46* were included in parallel as controls. Colonies (5) from the control plate of MS3898 harboring pACYC*dnaA* that were immune to λ pCM128 infection were assumed to be spontaneous mutations of *dnaA* and were pursued in parallel.

Immunoblot analysis of DnaA protein. Preparation of whole cell lysates and immunoblot analysis was performed as described (40) except that cultures were grown in LB medium supplemented with kanamycin and chloramphenicol to late log phase. Detection of horseradish peroxidase coupled to goat-anti mouse Immunoglobulin G (Bio-Rad) was by chemiluminescence (ECL, Amersham).

Nucleotide sequence analysis of mutant *dnaA* alleles.

Nucleotide sequence determination was either by the chain-terminating enzymatic method with Sequenase (U. S. Biochemicals) and [α^{35} S]-dATP (Dupont, NEN) or an on-campus sequencing facility equipped with an Applied Biosystems Model 343A DNA Sequencing System. For the latter, at least seven unique primers were used that provided overlapping sequence information for the majority of the gene (Figure 4). Sequence compilations were performed

using either Seq-Ed (Applied Biosystems) or Sequencher (version 2.1.1, Gene Codes Corporation). Mutations were identified by comparison of the compiled sequences to the *dnaA*⁺ gene sequence in GeneBank (accession no A24944).

Template DNAs were prepared either by the Qiagen mini- or midi-column procedure or by PCR amplification of the *dnaA* gene using the oligonucleotide primers JK-22 and JK-23 and Deep Vent DNA polymerase (New England Biolabs) with a Perkin Elmer Cetus DNA Thermal Cycler.

Oligonucleotide primers were synthesized by an in-house facility with an Applied Biosystems Model 394 and are complementary to the indicated positions relative to the coding region (with position 1 corresponding to the first nucleotide of the coding region; see Figure 4): JK-7, 5'-GTGGAGTCCCATATGTCACTTTCGCTTTGG-3' (-12 to 18 of the template strand and contains mismatches at positions -3, -2, -1 and 1); JK-22, 5'-GACCACCTAACGGACCGCTC-3' (1,459 to 1,478 of the coding strand); JK-23, 5'-AAGCCAATTTTTGTCTATGG-3' (-320 to -301 of the template strand); JK-25, 5'-CACGTTTGATAACTTCGTTG-3' (408 to 427 of the template strand); JK-26, 5'-GAGCGCTTTGTTTCAGGACAT-3' (610 to 629 of the template strand); JK-27, 5'-CGTTGAGGATCGTTTGAAAT-3' (831 to 850 of the template strand); JK-28, 5'-CATTGCCAATGCCAACTTTA-3' (1,029 to 1,048 of the template strand); JK-31, 5'-CGTTATCCCAACCTGAGCGC-3' (339 to 358 of the coding strand); JK-32, 5'-GGTGAGTTTTACCCAGACCC-3' (519 to 538 of the coding strand); JK-33, 5'-GAGATCGTTCTTTATTAGCA-3' (720 to 739 of the coding strand); JK-34, 5'-CGTTTTCGTCGGCCTTTTTTC-3' (921 to 940 of the coding strand); JK-35, 5'-TCCGCCACCGTCTTCTGAAT-3' (1,135 to 1,154 of the coding strand).

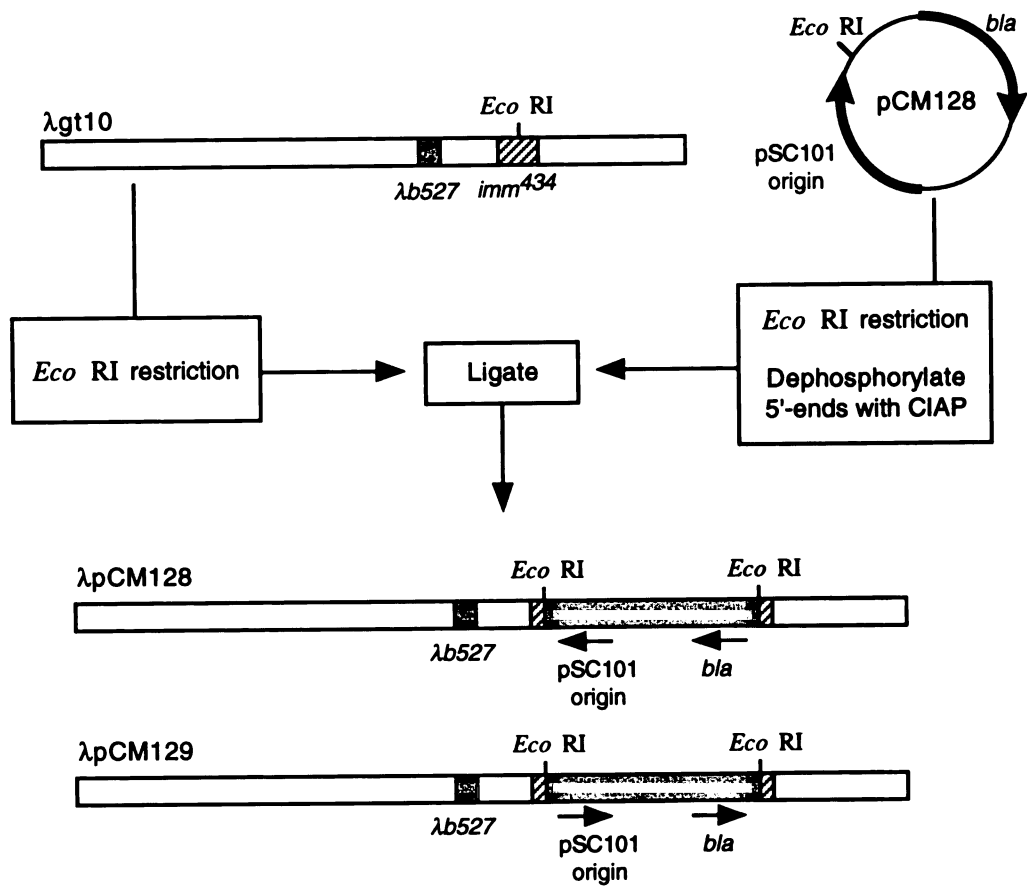
Results

λ gt10 recombinants containing an alternative replication origin form plaques on a host strain immune to infection by λ gt10. Infection by bacteriophage lambda of an *Escherichia coli* host containing lambda as a prophage is repressed by virtue of the prophage-encoded *ci* repressor (35). *ci* repressor inhibits lytic propagation of lambda by repressing expression of genes required for lambda replication. However, lytic growth is observed if the infecting lambda genome contains an alternative replication origin (7). Recombinants of λ gt10 were constructed that contained the pSC101 origin in both orientations inserted at the unique *Eco* RI site (Figure 1A). The ability of these bacteriophage to plate on a control *E. coli* strain, or its isogenic partner was measured (Table 2). λ gt10 containing the pSC101 origin in either orientation plated with an efficiency of approximately 0.8 on the lysogen expressing phage 434 repressor relative to the nonlysogen. By comparison, nonrecombinant λ gt10 was unable to form plaques on the lysogenic strain expressing phage 434 repressor, but did on the nonlysogen.

The λ gt10 derivatives that were active in lytic growth on a lysogen expressing phage 434 repressor utilized a copy-number-up mutant of pSC101 (λ pCM128 and λ pCM129; see Figure 1A). As suggested by others (7), we assume that lytic growth of the lambda derivative requires titration of the prophage-encoded *ci* repressor to a level where it is no longer inhibitory to lambda propagation. The wild type pSC101 origin or *oriC* inserted in either orientation into a lambda vector were unable to promote cell lysis (data not shown). This observation correlated with the reduced copy numbers of these plasmids relative to pCM128 (41, data not shown).

Figure 1. (A) Construction of λ gt10 derivatives containing the pSC101 origin. λ pCM128 and λ pCM129 are λ gt10 derivatives that were constructed by insertion of *Eco* RI linearized pCM128, a pSC101 derivative with a copy-number-up *repA* mutation (41), into the unique *Eco* RI site of λ gt10. For this construction, pCM128 was treated with calf intestinal alkaline phosphatase prior to ligation *in vitro* with T4 DNA ligase. As insertion into the *Eco* RI site of λ gt10 inactivates the *cl⁴³⁴* gene, λ gt10 recombinants were identified as clear plaques (26), then confirmed by DNA restriction analysis. **(B) Construction of a lambda derivative expressing phage 434 repressor and capable of lysogeny.** λ *imm⁴³⁴* was constructed *in vitro* by joining of the left arm of λ clts857Sam7 and the right arm of λ gt10 at the unique *Xho* I site. Recombinant phage that were S⁺ and *imm⁴³⁴* were selected by transformation of *E. coli* HMS174 (relevant marker, Sup^o). The phenotype of recombinants was confirmed by screening for immunity to λ gt10 and sensitivity to λ *cl857* by cross-streaking colony purified lysogens originally picked from the center of plaques.

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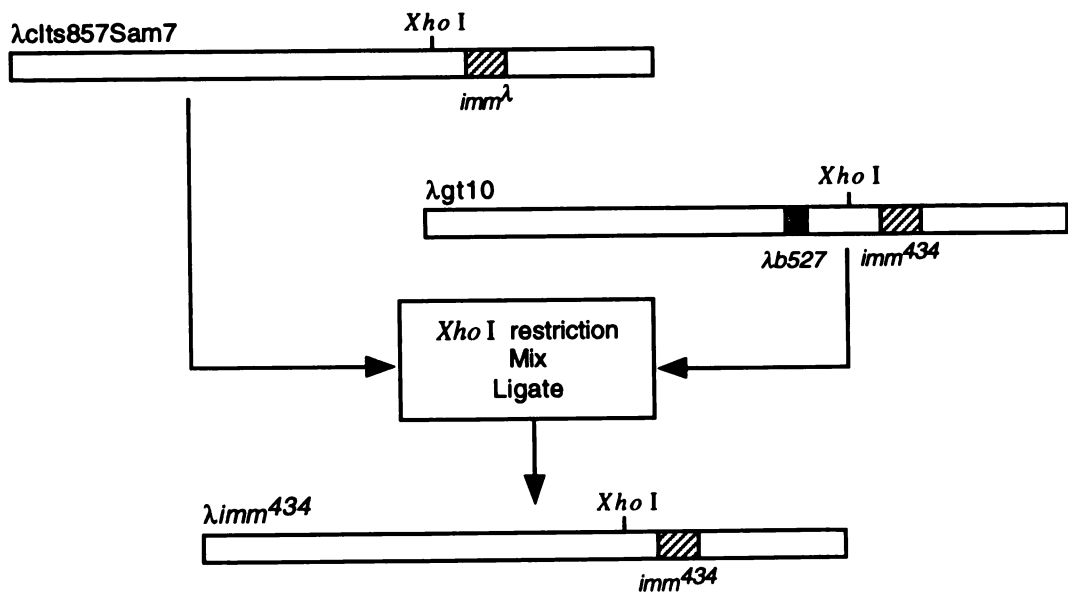


TABLE 2. λ gt10 derivatives containing the pSC101 replication origin are able to plate on a nonlysogenic and lysogenic host quantitatively

Host strain ^a	Phage ^b	Average phage titer ^c	Relative phage titer ^d
HMS174	λ gt10	2.4×10^9	—— ^f
MS175	λ gt10	ND ^e	
HMS174	λ pCM128	1.6×10^9	0.81
MS175	λ pCM128	1.3×10^9	
HMS174	λ pCM129	8.6×10^9	0.78
MS175	λ pCM129	6.7×10^9	

^a HMS174 and MS175 are isogenic except for the λ imm⁴³⁴ prophage contained in MS175 that expresses the same immunity region as λ gt10, λ pCM128, and λ pCM129 (see Table 1).

^b Lambda derivatives are described in Table 1 and their structures are shown in Figure 1.

^c Expressed as the number of infective particles per ml of phage lysate.

^d Expressed as the phage titer calculated on MS175 relative to that calculated on HMS174.

^e ND, none detected.

^f Relative phage titer could not be calculated due to the inability of λ gt10 to form plaques on MS175.

Replication of lambda bearing the pSC101 origin in a host expressing phage 434 repressor is *dnaA*-dependent. pSC101 replication is dependent on the *dnaA* gene (15, 16, 23). To demonstrate that replication of the lambda derivative containing the replication origin of pSC101 was dependent on the *dnaA* gene, growth of the bacteriophage was tested by spotting 10^7 infective particles onto a *dnaA* null host expressing phage 434 repressor and harboring different plasmid-encoded *dnaA* alleles (Figure 2). Lytic growth was observed on this strain harboring the *dnaA*⁺-containing plasmid at 30° or 42°C. Lytic growth was also observed on the strain bearing the plasmid-encoded *dnaA46* allele at 30°C, but not at 42°C. *dnaA46* is inactivated for pSC101 replication at 42°C (16, 23). The negative control of this strain containing the vector only did not support lytic growth at either temperature.

Other controls were performed as well. λ c/857 propagated on this host at both temperatures and independent of *dnaA* function. In contrast, growth was not observed with λ gt10 (that contains the immunity region of phage 434). These results indicate that the host strain was sensitive to lambda infection and expressed phage 434 repressor. Furthermore, they indicate that growth of the λ gt10 derivative containing the pSC101 origin on this host strain is dependent on the *dnaA* gene and the inserted pSC101 origin.

A selection method to obtain novel mutants of the *E. coli dnaA* gene. To identify residues of DnaA essential for its replication activity, a genetic selection for mutant alleles defective for replication from the pSC101 origin was developed (Figure 3). As described above, a lambda derivative containing the pSC101 replication origin with a copy-number-up *repA* mutation permitted the identification of *dnaA* mutations either inactive or conditionally defective for pSC101 replication (see Figure 3). Replica plating transformants

Figure 2. Lytic growth of λ pCM128 is *dnaA*-dependent. The *dnaA* null strain expressing phage 434 repressor (MS3898) and containing the indicated plasmid-borne *dnaA* allele was streaked then spot tested with 10^7 infective particles of each indicated lambda derivative (see Table 1). Plates were incubated at 30° (A) or 42°C (B) for 16 h. SM refers to lambda diluent that was used for dilution of the phage and included as a negative control.

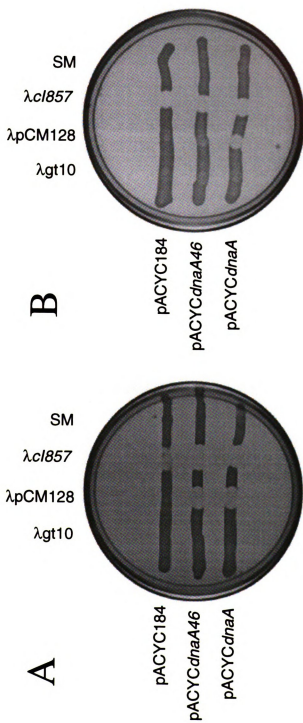
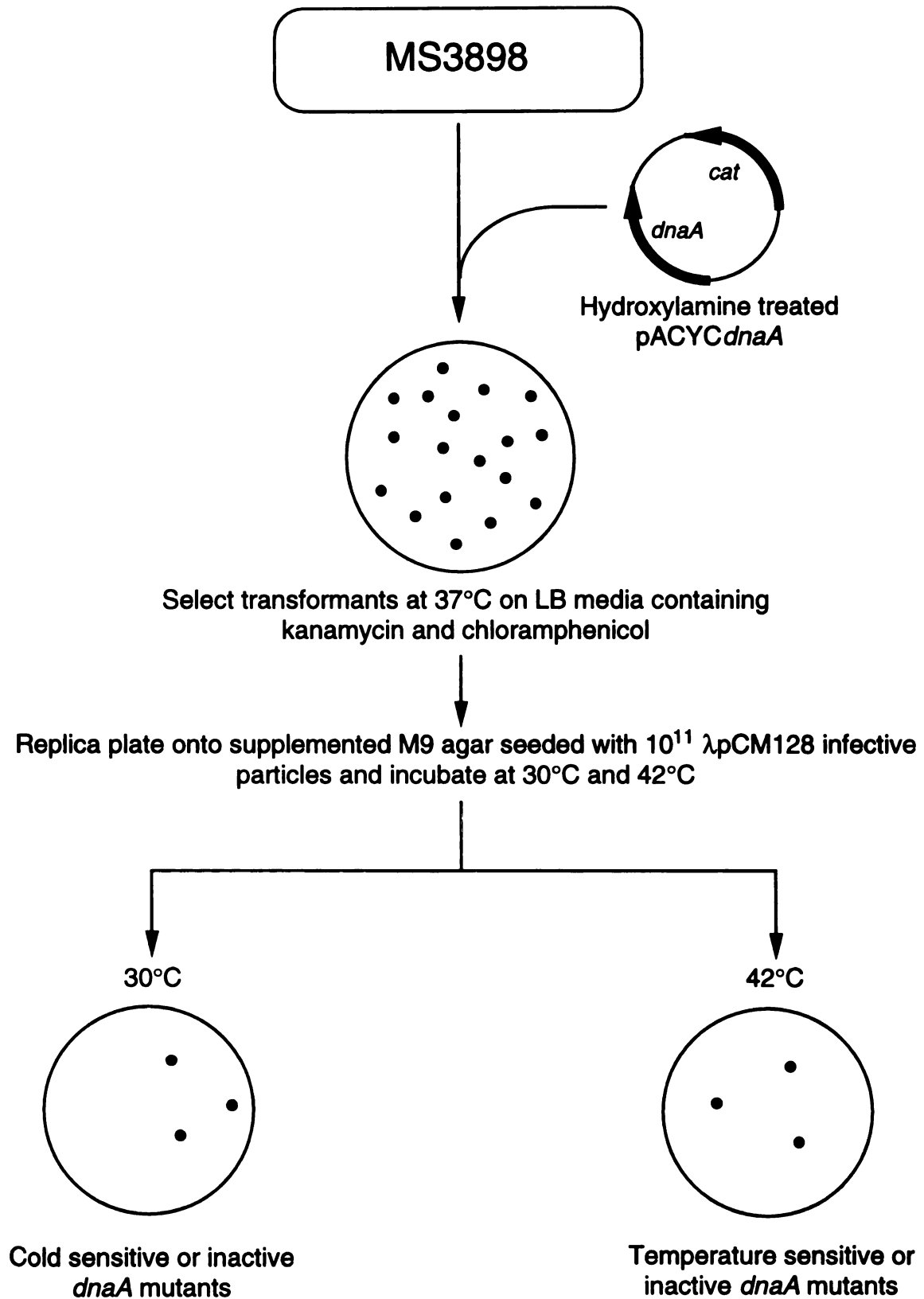


Figure 3. Genetic selection for mutations of *dnaA* defective in pSC101 replication. See Materials and Methods for a description.



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of a *dnaA* null strain expressing phage 434 repressor and harboring a chemically mutagenized, plasmid-borne *dnaA* gene provided a powerful selection for mutations of *dnaA*.

Using this approach, 289 mutants immune to the lambda derivative were obtained from a collection of 8,921 transformants. Five of these 289 were from the control plate of MS3898 bearing the nonmutagenized *dnaA*⁺-containing plasmid. These were presumed to be spontaneous mutations of *dnaA*. To confirm the mutant phenotype of these 289 isolates, plasmid DNA corresponding to each putative *dnaA* mutant was isolated and retransformed into the *dnaA* null strain used for their original isolation. By cross-streaking, 266 of the 289 were either temperature sensitive or inactive for lytic growth of the lambda derivative, confirming the plasmid-dependence of the mutant phenotype.

Qualitative immunoblot analysis of mutant *dnaA* alleles.

Immunoblot analysis of whole cell lysates was performed to identify and discard those plasmid-borne mutations that resulted in a reduced level of DnaA protein due to a reduced copy number of the plasmid, production of an unstable polypeptide, or insufficient expression, perhaps due to a mutation in the *dnaA* promoter region (data not shown). As controls, transformants of the vector, pACYC184, or it containing the *dnaA*⁺ or *dnaA46* allele were included. No product was detected for the pACYC184 transformant, confirming the *dnaA* null mutation of the host strain.

Immunoreactive polypeptides that comigrated at the position of purified DnaA protein were observed for the *dnaA*⁺ and *dnaA46* transformants. Immunoreactive polypeptides were detected for 129 of the 266 plasmid-borne mutations. Of these, 50 appeared identical in size to DnaA protein and were presumed to be missense mutants. This interpretation was confirmed by DNA

sequence analysis (see below). 76 encoded proteins with greater electrophoretic mobilities than DnaA protein. We presumed that these encoded nonsense mutations. DNA sequence analysis of representative isolates identified seven unique nonsense mutations (data not shown). In addition, a few mutations containing internal, in-frame deletions of coding sequence were identified (data not shown). The nonsense mutations and those containing an internal, in-frame deletions will be described elsewhere (see Chapter IV).

As no immunoreactive species was detected for the remaining 137 plasmid-borne mutations, we assume that they contained mutations either in the promoter region to diminish expression or elsewhere that resulted in unstable gene products. A third possibility is that they contain a mutation that reduced the copy number of the plasmid to lower the amount of DnaA protein to undetectable levels. These mutants were not characterized further.

Nucleotide sequence analysis of mutant *dnaA* alleles. The group of 50 missense mutations were sequenced as described in Materials and Methods (see Figure 4). 28 of these 50 were unique (Table 3). 27 of the 28 were novel with one of the 28 being identical to the *dnaA205* allele identified previously (3). The five mutations identified on the control plate of the *dnaA* null strain harboring the non-mutagenized *dnaA*⁺-containing plasmid were confirmed and found to represent two different alleles (A412P and 412[ΩMA]413; see footnote a to Table 3). In all, the 28 novel alleles identified 31 different residues that when altered affect the replication activity of DnaA protein. Many result in substitutions of residues that are extremely well conserved among *dnaA* homologs (reviewed in reference 32) (Table 3). Four alleles contain mutations that result in substitutions at the same positions as in alleles previously identified (*dnaA508*, P-28 (proline at position 28); *dnaA5*, 46, 601/602, 604/606, A-184; *dnaA205*, V-383; *dnaA5*, G-426) (22). However,

Figure 4. Scheme for nucleotide sequence analysis of *dnaA* alleles. The top portion represents the *Cla*I-*Xho*I *dnaA* gene fragment contained in pACYC184 (40). The bold line represents pACYC184 sequence. Nucleotide sequence determinations were performed as described in Materials and Methods. At least seven of the 14 indicated primers were used to sequence each allele. The seven primers used in most cases are indicated within dashed boxes that correspond to the portion of the gene sequenced for each allele. Others were used to resolve ambiguities or to confirm mutations. Arrows represent the approximate location and extent of sequence information obtained, on average, for each primer. In some cases, if a clone with a phenotype similar to an allele already sequenced in its entirety was found to contain mutation(s) identical to the latter allele after partial nucleotide sequence analysis, the remaining portion was not sequenced and the alleles were assumed to be identical. Upon completion of the nucleotide sequence analysis (and consistent with their almost wild-type phenotype with respect to lytic growth of λ pCM128), no mutations were detected in three of the presumed 50 missense mutations (data not shown). It is possible that these three may have contained mutations that, for some reason, were unstable, thereby preventing their detection. A similar phenomenon has been reported for a mutation residing in *dnaA* that suppresses the thermolabile phenotype of the *dnaX2016*(Ts) allele (19).

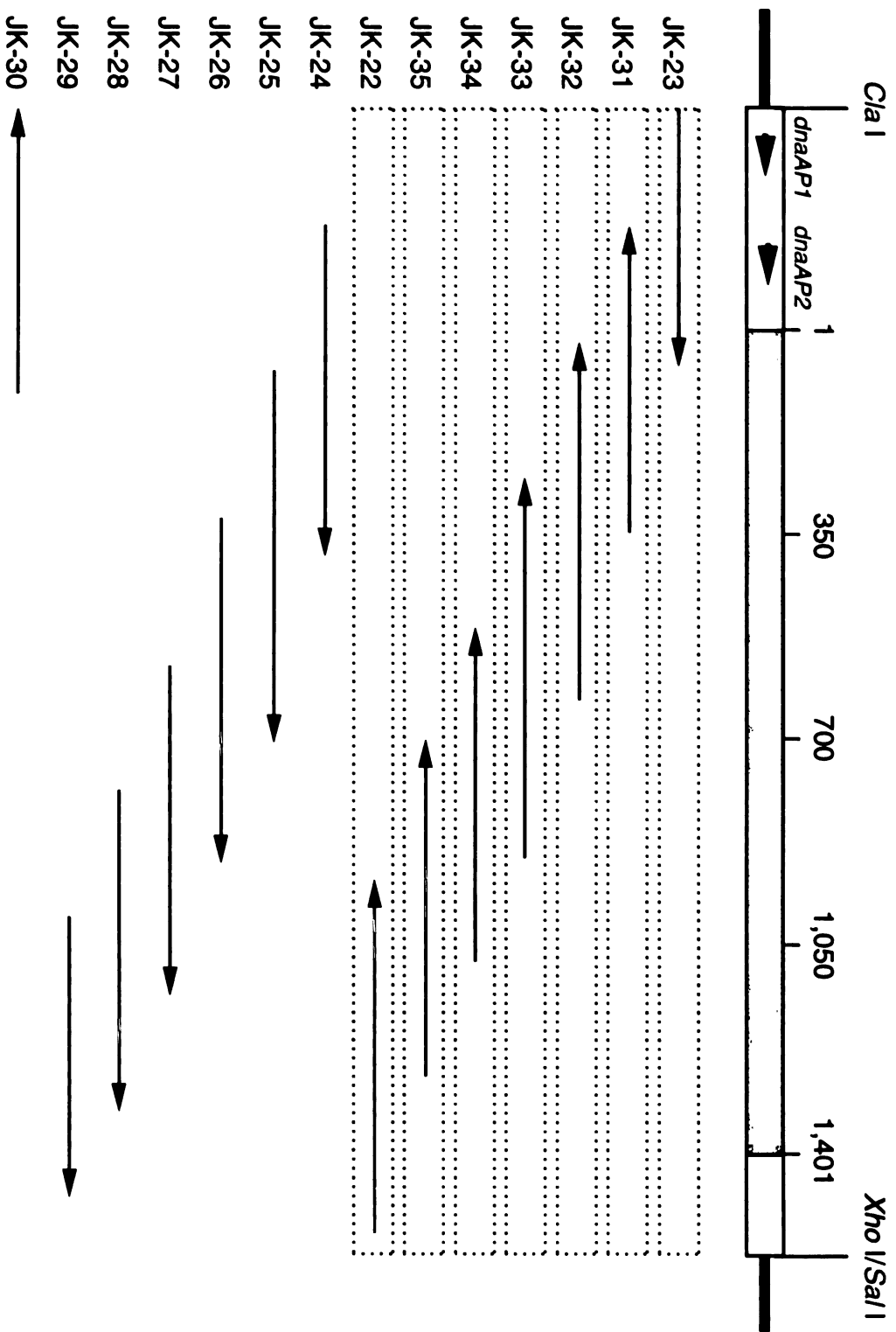


TABLE 3. Nucleotide and deduced amino acid alterations of novel *dnaA* missense mutations

Mutant <i>dnaA</i> -containing plasmid ^a	Nucleotide position ^b	Nucleotide substitution ^c	Amino acid position	Deduced amino acid substitution (degree of conservation) ^d	Number of times identified ^e
pACHA-9	68	G→A	23	Ser→Asn (5/15)	1
	976	A→T	326	Ala→Thr (14/15)	
	1,397	C→T	466	Ser→Leu (5/15)	
pACHA-32	77	T→A	26	Ile→Lys (9/15)	1
	1,354	C→T	452	His→Tyr (5/15)	
pACHA-39	26	G→A	9	Cys→Tyr (6/15)	1
	191	C→T	64	Thr→Ile (1/15)	
	1,110	G→A	370	Silent (Gln)	
	1,149	G→A	383	Silent (Val)	
pACHA-40	3	G→A	1	Met→Ala (15/15)	3
pACHA-43	1,318	G→A	440	Ala→Thr (12/15)	2
pACHA-45	26	G→A	9	Cys→Tyr (6/15)	5
pACHA-49	1,304	C→T	435	Thr→Met (14/15)	3
pACHA-50	74	G→C	25	Trp→Ser (12/15)	1
pACHA-54	1,220	G→A	407	Arg→His (15/15)	1
pACHA-70	1,309	G→A	437	Val→Met (15/15)	3
pACHA-72	61	G→A	21	Glu→Lys (6/15)	2
	1,099	C→T	367	Silent (Leu)	
	1,362	C→T	454	Silent (Leu)	
pACHA-79	1,277	G→A	426	Gly→Asp (15/15)	2
pACHA-83	1,319	C→T	440	Ala→Val (12/15)	3
pACHA-94	530	G→A	177	Gly→Asp (15/15)	1
	742	G→A	248	Glu→Lys (14/15)	
	745	G→A	249	Glu→Lys (13/15)	
pACHA-101	524	G→A	175	Gly→Asp (15/15)	1
pACHA-105	550	G→A	184	Ala→Thr (14/15)	2
	1,113	A→G	371	Silent (Glu)	
	1,224	G→A	408	Silent (Gln)	
pACHA-106	26	G→A	9	Cys→Tyr (6/15)	1
	878	C→T	293	Ala→Val (7/15)	
pACHA-110	1,147	G→A	383	Val→Met (11/15)	1
pACHA-117	128	C→T	43	Pro→Leu (11/15)	1
	529	G→A	177	Gly→Ser (15/15)	

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TABLE 3 (continued).

Mutant <i>dnaA</i> - containing plasmid ^a	Nucleotide position ^b	Nucleotide substitution ^c	Amino acid position	Deduced amino acid substitution (degree of conservation) ^d	Number of times identified ^e
pACHA-130	1,282	G→A	428	Ala→Thr (5/15)	1
	1,318	G→A	440	Ala→Thr (12/15)	
pACHA-143	28	C→T	10	Leu→Phe (9/15)	1
	154	C→T	52	Arg→Trp (5/15)	
pACHA-146	530	G→A	177	Gly→Asp (15/15)	1
	1,149	G→A	383	Silent (Val)	
	1,164	G→A	488	Silent (Lys)	
pACHA-152	82	C→T	28	Pro→Leu (6/15)	1
	83	C→T			
pACHA-162	625	G→A	209	Asp→Asn (8/15)	1
	1,304	C→T	435	Thr→Met (14/15)	
pACHA-170	1,304	C→T	435	Thr→Met (14/15)	1
	1,307	C→T	436	Thr→Met (15/15)	
pACHA-200	48	G→A	16	Silent (Glu)	1
	61	G→A	21	Glu→Lys (6/15)	
	430	G→A	144	Gly→Asn (14/15)	
	431	G→A			
	526	C→T	176	Silent (Leu)	
pACM-2	1,234	G→C	412	Ala→Pro (6/15)	1
pACM-7	1,237- 1,242	Ω(ATGGCG) ^f	413- 414	412(Ω Met-Ala)413	4

^a Some MS3898 (pACYC*dnaA*) transformants were immune to λpCM128 infection (data not shown). These were pursued under the assumption that they may represent spontaneous mutations of *dnaA*. pACHA refers to mutations of *dnaA* identified by hydroxylamine treatment of pACYC*dnaA*. pACM refers to mutations of *dnaA* identified from non-hydroxylamine treated pACYC*dnaA* control transformants.

^b Numbers refer to the *dnaA* coding region, with position one corresponding to the first nucleotide of the coding sequence (20).

^c Nucleotide substitutions are expressed as those in the coding strand.

^d Sequence conservation is indicated by the frequency of residues conserved among the 15 *dnaA* homologs (reviewed in reference 32).

^e The number of times that each allele was identified is indicated. No mutations were detected in three of the presumed 50 missense mutations (data not shown).

^f Ω(ATGGCG) indicated an insertion of the noted nucleotides at the indicated location.

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substitutions at positions A-184 and G-426 are with different amino acid residues than in previously isolated alleles. For *dnaA508*, a secondary mutation not present in the allele described here substitutes threonine at position 80 with isoleucine.

Among the 50 alleles characterized at the nucleotide level, all but six mutations were consistent with hydroxylamine treatment (see Table 3) that produces C→T transitions (34, 13). We assume that these six mutations arose spontaneously. Indeed, two of the six (A412P and 412[ΩMA]413) were identified on the plate corresponding to the control of the host strain harboring the *dnaA*⁺-containing plasmid that was not mutagenized (see footnote a to Table 3).

Replication phenotypes of novel *dnaA* alleles are either inactive or temperature sensitive. We were interested in determining if the replication phenotypes among the missense mutations might be distinguishable. To do this with the number of mutations in hand, about 10⁵ cells of the *dnaA* null strain expressing phage 434 repressor and containing the different plasmid-borne *dnaA* alleles were spotted with 10², 10³, and 10⁴ infective particles of λpCM128 to test the efficiency whereby each allele supported lytic growth of the lambda derivative from the pSC101 origin (Table 4). Lytic growth was observed with all phage dilutions spotted onto the transformant harboring the plasmid-borne *dnaA*⁺ allele at both 30° and 42°C. By comparison, lytic growth on the transformant bearing the *dnaA46* allele was observed at 30°C, the permissive temperature for *dnaA46*, but not at 42°C. The negative control of the transformant harboring the vector pACYC184 did not support lytic growth at either temperature.

Of the 28 missense mutations, two were almost wild-type for *dnaA*-function, 21 were temperature sensitive, and five were inactive. The latter class

TABLE 4. Summary of replication phenotypes of novel *dnaA* mutations

<i>dnaA</i> -containing plasmid ^a	Deduced amino acid substitution(s) ^b	λpCM128 spotting ^c						Replication phenotype ^d
		30°C			42°C			
		10 ⁴	10 ³	10 ²	10 ⁴	10 ³	10 ²	
pACYC <i>dnaA</i>	None	+	+	+	+	+	+	Wild type
pACYC <i>dnaA46</i>	A184V-H252Y	+	+	+	+/-	-	-	Ts
pACYC184	————	-	-	-	-	-	-	Inactive
pACHA-9	S23N-A326T-S466L	+	+	+	+	+	+/-	~Wild type
pACHA-32	I26K-H452Y	+	+	+	+	+	+/-	~Wild type
pACHA-39	C9Y-T64I	+	-	-	-	-	-	Ts
pACHA-40	M1V	+	+	-	+	-	-	Ts
pACHA-43	A440T	+	+	+	+	+	-	Ts
pACHA-45	C9Y	+	+	-	-	-	-	Ts
pACHA-49	T435M	+	-	-	-	-	-	Ts
pACHA-50	W25S	+	-	-	-	-	-	Ts
pACHA-54	R407H	-	-	-	-	-	-	Inactive
pACHA-70	V437M	+	+	+	-	-	-	Ts
pACHA-72	E21K	+	+	-	-	-	-	Ts
pACHA-79	G426D	-	-	-	-	-	-	Inactive
pACHA-83	A440V	+	-	-	-	-	-	Ts
pACHA-94	G177D-E248K- E249K	+	+	+	+	-	-	Ts
pACHA-101	G175D	+	+	+	+	+	-	Ts
pACHA-105	A184T	+	+	+	+	+	-	Ts
pACHA-106	C9Y-A293V	+	+	-	-	-	-	Ts
pACHA-110	V383M	+	+	+	-	-	-	Ts
pACHA-117	P43L-G177S	+	+	-	-	-	-	Ts
pACHA-130	A428T-A440T	+	-	-	-	-	-	Ts
pACHA-143	L10F-R52W	+	+	+	-	-	-	Ts
pACHA-146	G177D	+	+	+	+	-	-	Ts
pACHA-152	P28L	+	+	+	+	+	-	Ts
pACHA-162	D209N-T435M	+	-	-	-	-	-	Ts

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TABLE 4 (continued).

<i>dnaA</i> -containing plasmid ^a	Deduced amino acid substitution(s) ^b	λpCM128 spotting ^c						Replication phenotype ^d
		30°C			42°C			
		10 ⁴	10 ³	10 ²	10 ⁴	10 ³	10 ²	
pACHA-170	T435M-T436M	–	–	–	–	–	–	Inactive
pACHA-200	E21K-G144N	+	+	–	–	–	–	Ts
pACM-2	A412P	–	–	–	–	–	–	Inactive
pACM-7	412(ΩM-A)413 ^e	–	–	–	–	–	–	Inactive

^a See footnote a to Table 3.

^b Deduced from results presented in Table 3 and expressed as the amino acid present in wild type DnaA, its position, and the deduced substitution (i.e. A184V represents an A-to-V mutation at position 184).

^c The ability of the individual *dnaA* mutations to propagate λ pCM128 from the pSC101 origin was measured by spotting dilutions of λ pCM128 (10⁴-10² infective particles) onto ~10⁵ cells of MS3898 bearing the respective plasmid-borne allele. Growth of λ pCM128 on the above MS3898 transformants at 30° and 42°C on supplemented M9 medium was compared with MS3898 harboring the vector only (pACYC184), or it containing the *dnaA*⁺ (pACYC*dnaA*) or *dnaA*46 (pACYC*dnaA*46) alleles. Abbreviations are +, bacterial clearing (λ pCM128 sensitivity); –, bacterial growth (λ pCM128 immunity); +/- partial clearing (moderate λ pCM128 sensitivity).

^d Deduced from the λ pCM128 spotting. Ts, temperature sensitive.

^e 412(Ω MA)413 is a Met, Ala insertion between residues 412 and 413.

contained substitutions in the C-terminal region within residues 407 through 436 (see Table 4). The inability of these five mutant alleles to support replication of pSC101 was confirmed by a transformation assay (data not shown). Whereas transformants of the *dnaA* null strain harboring each of these alleles were transformed efficiently with pBR322 at either 30° or 42°C, they could not be transformed with pSC101. As a control, this strain bearing the plasmid-encoded *dnaA*⁺ allele was transformed by pSC101. If this strain harbored a plasmid encoding the *dnaA46* allele instead, pSC101 transformants were obtained at 30°C, but not at 42°C.

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Discussion

Identification and nucleotide sequence of novel mutant *Escherichia coli dnaA* alleles. To obtain a large collection of novel *Escherichia coli dnaA* alleles to identify residues critical for its activity in replication, we developed a method to select mutants defective for pSC101 replication. Relying on the ability of lambda lysogens to exclude superinfection (35), we constructed a lambda derivative containing the immunity region of phage 434 and a pSC101 origin with a copy-number-up mutation. Lytic growth of this lambda derivative on a lysogen expressing phage 434 repressor was dependent on the pSC101 origin and the *dnaA* gene.

With this lambda derivative to infect a *dnaA* null strain expressing phage 434 repressor, and a plasmid bearing the *dnaA* gene mutagenized with hydroxylamine, *dnaA* mutants were selected that were inactive or conditionally defective. These failed to support lytic growth and remained viable in the presence of the lambda derivative. By this approach, we identified 28 unique missense mutations of the *E. coli dnaA* gene. Twenty seven of the 28 are novel. In all, the 28 alleles identified 31 different residues of DnaA that when substituted affect its ability to support pSC101 replication. Many result in substitutions at positions that are highly conserved among *dnaA* homologs (Table 3).

That no cold sensitive *dnaA* mutations were identified may be due to the fact that the host strain used for selection of *dnaA* mutations contained an intact *oriC* locus. It is possible that a cold sensitive mutant would over initiate replication from *oriC*, thereby resulting in cell death as is the case with the *dnaAcos* mutant (6). Alternatively, the possibility that DnaA protein may facilitate replication from the integrated R1 origin (8, 2, 21) may also have

affected the types of mutations obtained. A third possibility is that cold sensitive *dnaA* alleles may require multiple mutations as in the case of *dnaAcos* (6) and that the *in vitro* mutagenesis method used was insufficient for generation of multiple mutations.

Missense mutations residing in the C-terminus of DnaA abolish replication activity. The ability of the various plasmid-borne mutations to support lytic growth of the lambda derivative from the pSC101 replication origin was measured by a spot test. Of the 28 missense mutations, 21 were thermolabile, five were inactive, and two were similar to wild type *dnaA* for lytic growth of the lambda derivative. Interestingly, all of the mutations that abolished replication activity mapped to residues 407 through 436 (Table 4). That these alleles were inactive for replication of pSC101 was substantiated by our inability to transform a *dnaA* null strain harboring the respective plasmid-borne alleles to tetracycline resistance with the wild-type pSC101 plasmid (data not shown). As the C-terminal region of DnaA is involved in DNA binding (36, Chapter V), we speculate that these mutations affect the DNA binding activity of DnaA protein.

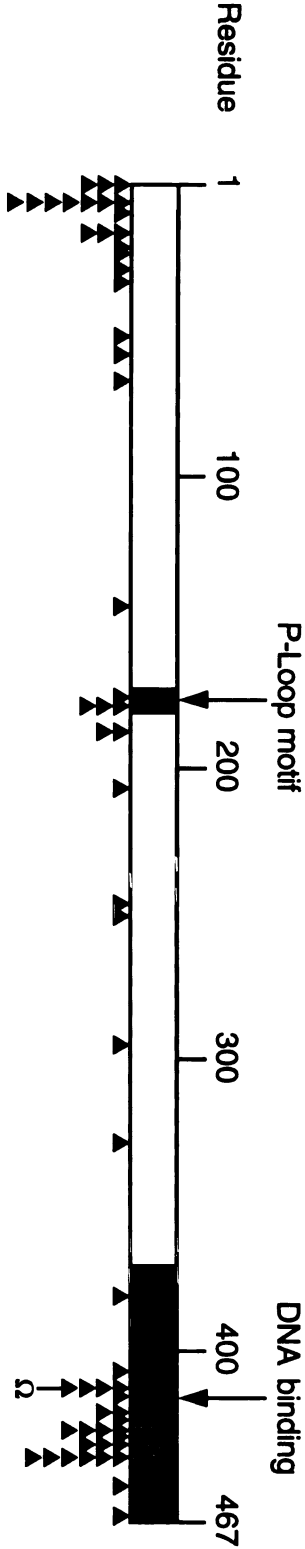
The 21 alleles that were thermolabile for pSC101 replication varied both in their degree of temperature sensitivity and overall activity (Table 4). Furthermore, some of the mutants encoding more than one amino acid substitution were comparable in phenotype to single mutants containing an identical mutation. This finding suggests that the substitutions of Q144N, D209N, E248K-E249K, and A293V that are found with other substitutions that are unique for some alleles may have little effect on replication activity. Finally, it is of particular interest that two alleles with different substitutions at position 440 exhibit such diverse replication phenotypes. Whereas A440V had only minimal replication activity, A440T was nearly wild-type in its ability to support

lytic growth of the lambda derivative (Table 4).

DnaA contains three distinct domains. The missense mutations were clustered in three discrete regions of the *dnaA* gene (Figure 5), suggesting the presence of three distinct domains of DnaA protein essential for replication of pSC101. The C-terminal 94 residues have been determined to be both essential and sufficient for specific DNA binding (36). Although a function for the N-terminus of DnaA is unknown, the large number of mutations mapping to this region indicates its essential role.

The central region of DnaA protein contains a P-loop motif (GX₄GKT, where X represents any amino acid) mapping to positions 172-179 and is thought to comprise a portion of the high affinity ATP binding site. Consistent with this assumption, an alanine-to-valine substitution at position 184 near this P-loop motif has been shown to affect the ability of DnaA to bind ATP (27, 25). Furthermore, mutant alleles containing an alanine-to-valine mutation at position 184 initiate replication from *oriC* asynchronously whereas those containing mutations mapping elsewhere in the gene do not (39). Although each of the alleles containing the A184V mutation contains a second mutation as well, it has been speculated that the A184V mutation is responsible for the asynchrony in initiation and that the ATP-bound form of DnaA is essential for synchronous initiation of multiple origins in rapidly growing cells (38). As the ability of the A184V mutation, without additional secondary mutations, to initiate replication synchronously from multiple origins is not yet known, it will be interesting to see if the novel alleles described here containing mutations mapping within or near the P-loop motif exhibit asynchrony in initiation. Whether the

Figure 5. Summary of positions of mutations of novel *dnaA* alleles. Relative positions of mutations resulting in the novel *dnaA* alleles described here are indicated. Each blackened triangle (▲) represents the approximate location of a single mutation. The mutations indicated between residues 200 and 350 are not unique and contain one or more additional mutations that may account for its mutant phenotype (see Table 3). Ω indicates the position of the Met, Ala insertion mutation between residues 412 and 413. Amino acid residues and proposed functional domains of DnaA protein (reviewed in references 32 and 39) are indicated.



mutations mapping to this region of the protein affect ATP binding activity may be addressed following their purification.

Finally, the genetic and biochemical characterization of the mutant alleles described here will provide significant insight into functional domains of DnaA and aid in the development of a more refined model for the function of DnaA in initiation of DNA replication in *Escherichia coli*.

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Chapter IV

Genetic and Biochemical Characterization of Novel Alleles of the *Escherichia coli dnaA* Gene: Identification of Four Functionally Distinct Domains of DnaA Protein

Abstract

The *Escherichia coli* DnaA protein is a sequence-specific DNA binding protein that promotes the initiation of replication of the bacterial chromosome, pSC101, and other plasmids. We recently described 28 missense mutations of the *E. coli dnaA* gene, identified by their inability to replicate a recombinant phage lambda derivative from the inserted pSC101 origin (see Chapter III). Here, we describe the genetic and biochemical characterization of these missense *dnaA* alleles as well as the identification and characterization of seven nonsense mutations and one in-frame deletion mutation. Our findings suggest that the *E. coli* DnaA protein contains four functionally distinct domains. Mutations that affect residues in the P-loop motif, proposed to function in ATP binding, identify one domain. The second domain maps to the C-terminus and is involved in DNA binding. The function of the third domain that maps near the N-terminus is unknown. Two mutant alleles encoding different truncated gene products retained the ability to promote replication from the pSC101 origin but not *oriC*, identifying a fourth domain dispensable for replication of pSC101 but essential for *oriC*.

Introduction

DnaA protein is a sequence-specific DNA binding protein that is essential for initiation of replication from the *Escherichia coli* chromosomal origin, *oriC* (14). DnaA recognizes and binds to a 9-mer sequence, termed the DnaA box, that is found four times in the *E. coli* replication origin (28), as well as in the replication origins of plasmids (12, 13, 19, 17, 24) and bacteriophage (17, 44) which require this protein for replication. Once bound at *oriC*, DnaA is proposed to induce a structural alteration in the AT-rich region of *oriC*, consisting of three tandem repeats of a 13-mer AT-rich sequence, leading to their unwinding and formation of the prepriming complex (3, 38).

Little is known to correlate the structure of DnaA protein to its various biochemical activities. The isolation of extragenic suppressors of conditional *dnaA* mutations has revealed a number of gene products proposed to interact either functionally or physically with DnaA protein (1, 15, 20, 33). However, with the exception of DnaB helicase (26), biochemical evidence in support of these physical interactions is lacking.

DnaA protein binds ATP and ADP with high affinity (K_D of 0.03 μ M and 0.10 μ M, respectively) (37). Examination of the amino acid sequence identifies a P-loop motif (GX₄GKT from residues 172-179, where X is any amino acid) found in many nucleotide binding proteins (35). An alanine-to-valine substitution at residue 184 near this P-loop motif has been shown to reduce ATP binding affinity (21, 22), supporting the model that this region functions in ATP binding.

DnaA protein interacts physically with DnaB protein (26). This interaction is inhibited by a monoclonal antibody that binds to an epitope within the N-terminal 147 residues of DnaA protein. These observations suggest that

residues within this region are involved in binding to DnaB. In another study, the region of DnaA protein involved in DNA binding was localized to the C-terminal 94 residues. This was determined by fusing portions of DnaA protein to β -galactosidase then measuring DNA binding activity (32).

Here, we report the genetic and biochemical characterization of the mutations described in Chapter III as well as the identification and characterization of seven nonsense mutations and one in-frame deletion mutation. Examination of the mutant phenotypes relative to the positions of the mutations suggests the presence of four functionally distinct domains of DnaA protein. One class resides near a P-loop motif that appears to be involved in ATP binding. The second affects DNA binding activity. The third group is postulated to affect the interaction between DnaA protein and pSC101 RepA protein. The fourth class maps to a region near the N-terminus. The function of this region is unknown. Comparison of these findings with the predicted secondary structure of DnaA protein obtained using the PHD method (31) revealed that many substituted amino acids fall in regions of predicted secondary structure, and affect positions that are highly conserved.

Materials and Methods

Bacteriological methods. The bacterial strains, plasmid DNAs, and phage lambda used in this study are described in Table 1. Bacteriological methods were performed essentially as described by Miller (27). Bacterial transformation was by the calcium chloride method. Lambda lysogens were constructed as described by Davis *et al.* (11). Bacterial strains and their plasmid-containing derivatives were routinely grown in LB medium supplemented, as indicated, with 100 µg/ml ampicillin, 40 µg/ml chloramphenicol, 40 µg/ml kanamycin, and 10 µg/ml tetracycline. M9 minimal medium supplemented with 0.5% casamino acids, 0.2% dextrose, 1 µg/ml thiamine, and the indicated antibiotics was used as noted.

Recombinant DNA methods. pACYC*dnaA* and pACYC*dnaA46* are pACYC184 recombinants (41) that contain the *dnaA*⁺ and *dnaA46*(Ts) alleles, respectively. pMDS421 is a chloramphenicol sensitive derivative of pOU420 (17) constructed by *Eco* RI restriction of pOU420, end-filling of the linear DNA with the large fragment of DNA polymerase I and dATP and dTTP, and its religation with T4 DNA ligase (Boehringer Mannheim) to obtain a four base pair insertion at the *Eco* RI site within the *cat* gene resulting in its inactivation.

Selection for mutations of *dnaA* defective for replication from the pSC101 origin. Mutations of *dnaA* encoding truncated gene products were identified, and their respective replication phenotypes measured, utilizing the phagemid assay described previously (see Chapter III). Briefly, this assay measures the ability of a plasmid-encoded *dnaA* allele to direct replication of a phage lambda vector that contains a pSC101 origin (λpCM128). Such a recombinant phage lambda containing a secondary origin of replication is referred to as a lambda phagemid (8). As the host strain contains a prophage

TABLE 1. Bacterial strains and plasmids^a

Strain or plasmid	Genotype ^b or characteristics	Source or construction
Strains		
MC1061	<i>araD139</i> Δ (<i>ara-leu</i>)7696 Δ <i>lacX74 galU galk</i> <i>hsdR2</i> (r_K -12 ⁻ m_K -12 ⁺) <i>mcrB1 rpsL</i> (F ⁻)	Laboratory stock
MS1062	MC1061 but <i>zia</i> ::pKN500	This work
MS3896	<i>asnB32 relA1 spoT1 thi-1 lysA fuc-1 ilv-192</i> <i>zia</i> ::pKN500 (pKN500=mini-R1) Δ <i>dnaA mad-2</i> (F ⁻)	This laboratory
MS3898	MS3896: <i>recA1 lysA⁺ fuc⁺ λ434</i>	This laboratory
MS207	MS3898 but <i>dnaA204</i> (Ts)	This laboratory
SH210	<i>fhuA22 garB10</i> Δ (<i>argF-lacU</i>)169 <i>zai-736</i> ::Tn10 <i>phoA8 ompF627 fadL701 relA1 glpR2 glpD3</i> <i>pit-10 spoT1 phoM510 mcrB</i> Hfr (PO2A of Cavalli Hfr)	CGSC # 7096 (36)
MS3899	MS3896 but Δ (<i>argF-lacU</i>)169 <i>zai</i> ::Tn10	(MS3896) X (SH210), select Tc ^r , screen for Lac ⁻
MS3900	MS3899 but λ RB1 ^c	This work
MS3907	MS3898 but <i>dnaA⁺</i>	This work
Plasmids		
pACYC184	Cm ^r , Tc ^r ; p15A origin, cloning vector	Laboratory stock (9)
pACYC <i>dnaA</i>	<i>dnaA⁺</i> cloned into pACYC184	This laboratory (41)
pACYC <i>dnaA46</i>	<i>dnaA46</i> (Ts) cloned into pACYC184	This laboratory (41)
pOU420	Ap ^r , Cm ^r ; pBR325 origin, contains an R1 <i>copA</i> (Ts) allele	M. Yamolinsky (17)
pMDS421	Ap ^r ; Cm ^s derivative of pOU420	This work
pSC101	Tc ^r ; DnaA-dependent low-copy-number replicon	S. Cohen (10)
pCM128	Ap ^r ; copy-number-up mutant of pSC101	S. Cohen (42)

^a Abbreviations are Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline; Ts, thermolabile; X, bacterial mating; CGSC, *E. coli* genetic stock center.

^b See the report of Bachmann (2) for genetic symbols.

^c λ RB1 is a λ NF1955 derivative that contains a *dnaA'*-*lacZ* translational fusion (7).

that expresses the same immunity region as λ pCM128, normal lambda propagation of the phasemid is repressed by virtue of the prophage-encoded *ci* repressor protein (29). However, replication from the pSC101 origin, which is DnaA-dependent, has the ability to produce a λ pCM128 copy number sufficiently high to titrate out the prophage encoded repressor protein, thereby promoting cell lysis.

Nucleotide sequence analysis of mutant *dnaA* alleles. The approximate size of each mutant protein was determined by immunoblot analysis of whole cell lysates of a *dnaA* null strain harboring the respective plasmid-encoded *dnaA* mutations. Relevant regions for each of the seven presumed nonsense alleles were then sequenced by the chain-terminating enzymatic method using Sequenase (U.S. Biochemicals) essentially as described by the manufacturer. Plasmid DNAs were purified using the Qiagen mini- or midi-column method. The approximate location of the coding sequence deletion from the pACHA-4 mutation was determined by endonuclease restriction analysis of the plasmid DNA relative to pACYC*dnaA* and markers of known size. The appropriate region was then sequenced as described above. Oligonucleotide primers JK-21, 5'-ATAACCCGTTGTTCC-3'; JK-22, 5'-GACCACCTAACGGACCGCTC-3'; JK-26, 5'-GAGCGCTTTGTTTCAGGACAT-3'; JK-27, 5'-CGTTGAGGATCGTTTGAAAT-3'; JK-32, 5'-GGTGAGTTTTACCCAGACCC-3', synthesized by an in-house facility with an Applied Biosystems model 394, were used for sequencing and are homologous to the following nucleotide positions of *dnaA* (with position 1 corresponding to the first nucleotide of the coding sequence (16)): JK-21, 494 to 508 of the coding strand; JK-22, 1,459 to 1,478 of the template strand; JK-26, 610 to 629 of the coding strand; JK-27, 831 to 850 of the coding strand;

JK-32, 522 to 541 of the template strand.

β -galactosidase assays. Cultures were grown in supplemented M9 medium at 30° and 42°C. When growth approached mid-log phase, as determined by OD₆₀₀, cells were lysed by sonication on ice using a Heat Systems-Ultrasonics, Inc. Sonicator Model W-225 equipped with a microtip. Debris was removed from the lysates by centrifugation in a microcentrifuge at 4°C prior to performing β -galactosidase assays. 0.5 ml aliquots of each cleared lysate were assayed for β -galactosidase activity essentially as described by Miller (27). Aliquots were also assayed for protein concentration by the dye binding method of Bradford (6). β -galactosidase activity was calculated using the following formula: $([213] \times [A_{420}] / ([\text{protein concentration}] \times [\text{volume}] \times [\text{time}])),$ where 213 is the extinction coefficient for 2-Nitrophenyl- β -D-galactopyranoside (ONPG), the protein concentration is in mg/ml, the volume assayed is in ml, and the time of incubation is in minutes. Units of β -galactosidase activity are then expressed as nmol O-nitrophenyl (ONP)/min-mg protein.

Quantitative immunoblot analysis of mutant DnaA proteins. Whole cell lysates and quantitative immunoblot analysis were performed as described previously (41), except that cultures were grown in LB medium at 30°C to late log phase and MS3898 was used as the host strain in place of MS3897 (see Table 1).

S1 nuclease protection assays. S1 nuclease protection assays were performed as described previously (43). As a probe, the 1.4 kb *Pvu* II-*Eco* RI fragment from pACYC*dnaA* was used. This DNA fragment contains pACYC184 sequence (coordinates 515 to 1,517 (see reference 9)), the complete *dnaA* promoter region, and the first 61 nucleotides of the *dnaA* coding region. Preparation of this fragment was as follows: The 3.6 kb *Eco* RI

fragment from pACYC*dnaA* was dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim), 5' end-labelled with [$\gamma^{32}\text{P}$]ATP (Dupont, NEN) and T4 polynucleotide kinase (New England BioLabs), and digested with *Pvu* II. The 1.4 kb fragment was used without further purification.

Determination of relative pCM128 abundance. The abundance of pCM128 relative to the respective *dnaA*-containing pACYC184 derivatives was determined using a modification of the method described by Biek and Cohen (5). Briefly, double transformants of MS3898 harboring both the pACYC184 *dnaA*-containing derivative (as noted in Table 9) and pCM128 were grown at 30°C in LB medium containing kanamycin, chloramphenicol, and ampicillin. These were subcultured into supplemented M9 medium containing kanamycin and chloramphenicol, but lacking ampicillin which is required for selection of pCM128, followed by growth at 30° or 42°C for 4 h. Plasmid DNAs were prepared by the alkaline lysis miniprep method (34) and the *Ava* I (New England BioLabs) linearized plasmid DNAs were electrophoresed in a 0.7% agarose gel. Both DNAs contain a single *Ava* I site and differ in size by approximately 2 kb. The relative abundance of each DNA was determined by scanning densitometry relative to a standard curve using a Bio-Rad Gel Doc 1000 equipped with the Molecular Analyst software package.

Results

Identification of nonsense and deletion mutations of the *Escherichia coli dnaA* gene. Using a selection method for mutations of the *E. coli dnaA* gene, we identified nonsense and deletion mutations. These were originally identified as encoding smaller polypeptides by immunoblot analysis of whole cell lysates of a *dnaA* null strain harboring the plasmid-encoded *dnaA* alleles (data not shown). For the nonsense mutations, electrophoretic analysis of plasmid DNAs revealed these to be similar in both size and structure to the wild type pACYC*dnaA* parent whereas the deletion mutants appeared slightly smaller (data not shown). In addition, the amber suppressors *supE* and *supF* permitted expression of full length polypeptides for the presumed nonsense mutations (data not shown). As many of the nonsense mutations appeared to encode polypeptides judged to be similar in size (data not shown), a representative group was selected. None of the presumed nonsense mutations expressed a detectable level of the full length polypeptide (Figure 1).

The region of each mutant allele expected to contain the nonsense mutation based on the estimated size of the truncated polypeptide was sequenced by the chain-terminating enzymatic method as described in Materials and Methods. Nucleotide sequence analysis confirmed the presence of a nonsense mutation near the expected position for each allele sequenced (Table 2).

The regions encompassing the deletion of sequence for those mutant plasmids that were smaller in size than the pACYC*dnaA* parent plasmid were determined by DNA restriction endonuclease analysis (data not shown). This analysis indicated that of the three deletion mutations that expressed a detectable gene product, one appeared similar to a nonsense mutation

Figure 1. Western blot analysis of truncated forms of DnaA protein. Western blot analysis of whole cell lysates of a *dnaA* null strain harboring the respective *dnaA* alleles under control of the *dnaA* promoter region was performed as described in Materials and Methods.

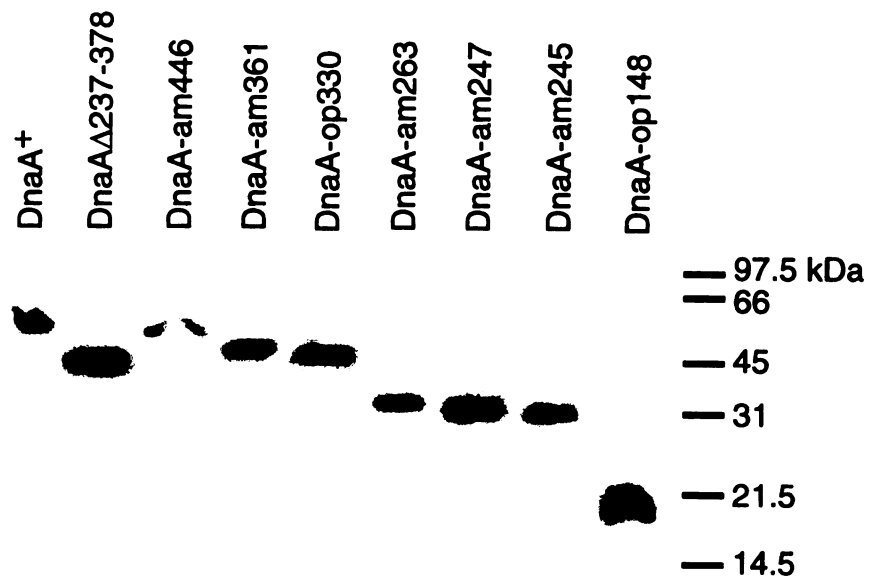


TABLE 2. Summary of nucleotide sequence analysis of nonsense and in-frame *dnaA* alleles

<i>dnaA</i> -containing plasmid	Position of mutation ^a	Nucleotide alteration ^b	Deduced mutation(s) ^c
pACHA-4	709-1,134	In-frame deletion	$\Delta(237-378)$
pACHA-6	988	CGA →TGA	330→opal
pACHA-22	442	CAA →TAA	148→ochre
pACHA-86	787	CAG →TAG	263→amber
pACHA-97	739	CAG →TAG	247→amber
pACHA-119	1,336	CAG →TAG	446→amber
pACHA-121	1,081	GAG →TAG	361→amber
pACHA-178	733	CGA →TGA	245→opal

^a The nucleotide position of each mutation is indicated with position one corresponding to the first nucleotide of the coding sequence (18).

^b Altered nucleotides resulting in the nonsense codon are in bold.

^c Mutations are expressed as the amino acid position in the wild type sequence and the type of nonsense mutation (amber, am; opal, op; ochre, oc) resulting from the base substitution. The allele containing an in-frame deletion has been named *dnaA* $\Delta 237-378$. Nonsense alleles have been named with the designation of the nonsense codon and its position in the coding sequence(*dnaA-op330*).

described above (based on polypeptide size and the finding that C-terminal sequences were deleted; data not shown) and was not pursued further. The remaining two appeared similar to each other (data not shown). The region encompassing the deletion of coding sequence for these two was sequenced as described in Materials. Both contained an identical in-frame deletion of coding sequence corresponding to amino acid residues 237-378 (Table 2).

Mutations mapping within or near the P-loop motif exhibit a cold-sensitive heteropolyloid phenotype. In an effort to identify mutations of *dnaA* that exhibited a dominant negative phenotype, all 36 plasmid-encoded alleles (28 missense, seven nonsense, and one deletion) were transformed into a prototrophic strain (W3110). Chloramphenicol resistant transformants were selected at both 30° and 42°C. By this analysis, three of the 36 were cold sensitive for transformation (data not shown). None were thermolabile for transformation. Based on nucleotide sequence analysis, all three contained substitutions of highly conserved residues mapping within or near the P-loop motif thought to function in nucleotide binding (37).

A similar cold-sensitive heteropolyloid phenotype has been described for other *dnaA* alleles containing the alanine-to-valine substitution at position 184 (18). In light of this, a more careful analysis of the transformation efficiency of all plasmid-borne *dnaA* mutations containing an amino acid substitution mapping within or near the P-loop motif was performed (Table 3). This analysis used two isogenic strains; MC1061 and MS1062 (see Table 1). The latter strain contains a mini-R1 plasmid inserted into its chromosome that serves as a secondary site for initiation of replication in the absence of *dnaA* or *oriC* function (17). In this case, it serves as a control strain inasmuch as it may suppress the otherwise lethal phenotype of a dominant negative *dnaA* mutation (data not shown; 23).

TABLE 3. Substitutions mapping to the P-loop motif exhibit a cold-sensitive heteropolyploid phenotype

<i>dnaA</i> -containing plasmid	Deduced amino acid substitution(s)	Relative transformation efficiency ^a			
		24 hours		48 hours	
		MC1061	MS1062	MC1061	MS1062
pACYC <i>dnaA</i>	None	1.0	0.9	1.0	0.9
pACYC <i>dnaA46</i>	A184V H252Y	2.0 X 10 ⁻³ ^b	0.7 ^c	1.0	0.7
pACYC184	—	0.8	1.0	0.8	1.0
pACHA-94	G177D E248K E249K	3.0 X 10 ⁻³ ^b	1.0 ^d	3.9 X 10 ⁻³ ^b	1.0
pACHA-101	G175D	1.0	1.0	1.0	1.0
pACHA-105	A184T	1.7 X 10 ⁻²	1.3 X 10 ⁻²	1.7 X 10 ⁻² ^b	3.9 X 10 ⁻¹
pACHA-117	P43L G177S	0.9	0.9	0.9	0.9
pACHA-146	G177D	2.8 X 10 ⁻² ^b	1.0 ^d	0.9	1.0

^a Expressed as the transformation efficiency observed at 30° relative to 42°C after 16 h of growth at 42°C and either 24 h or 48 h of growth at 30°C, as noted. Transformation efficiencies at 42°C were on the order of 10⁷-10⁸ per µg of plasmid DNA for each of the eight plasmid DNAs.

^b These values do not include micro colony forming units.

^c Small colony forming units.

^d Tiny colony forming units.

The plasmids containing the *dnaA*⁺, G175D (with a G-to-D mutation at position 175), or P43L-G177S allele each transformed MC1061 with similar efficiencies at 30° relative to 42°C. However, the transformation efficiency of the plasmids containing the *dnaA*46, G177D, G177D-E248K-E249K, or A184T allele was reduced at 30°C relative to both that observed for the *dnaA*⁺ allele at the same temperature, and to their respective transformation efficiencies at the elevated temperature. At 42°C, all transformation efficiencies were similar to that obtained with pACYC184, indicating that none of the plasmid-borne alleles exhibited an inhibitory phenotype at the elevated temperature. We do not understand why the G175D and P43L-G177S alleles did not result in a cold sensitive phenotype in this analysis.

The cold sensitive transformation phenotype of the plasmids containing the *dnaA*46, G177D, or G177D-E248K-E249K allele was suppressed by chromosomal insertion of a mini-R1 replicon. This was demonstrated by the ability of the respective plasmids to transform the R1 integratively suppressed strain (MS1062) at both 30° and 42°C with comparable efficiency. By contrast, the cold sensitive transformation phenotype of the A184T allele was only partially suppressed as indicated by its reduced transformation efficiency at 30° relative to 42°C.

Further investigation of the phenotypes of the plasmids containing the G177D, G177D-E248K-E249K, or A184T allele using MC1061 harboring pBS*oriC* (3), a pBluescript derivative containing *E. coli oriC* sequence, indicated that their cold-sensitive heteropolyploid phenotype was also suppressed by the presence of a multicopy *oriC* plasmid (data not shown). Consistent with the results discussed above regarding the R1 integratively suppressed strain (MS1062), the A184T allele was least efficiently suppressed by the presence of pBS*oriC* while G177D was most efficiently suppressed. This

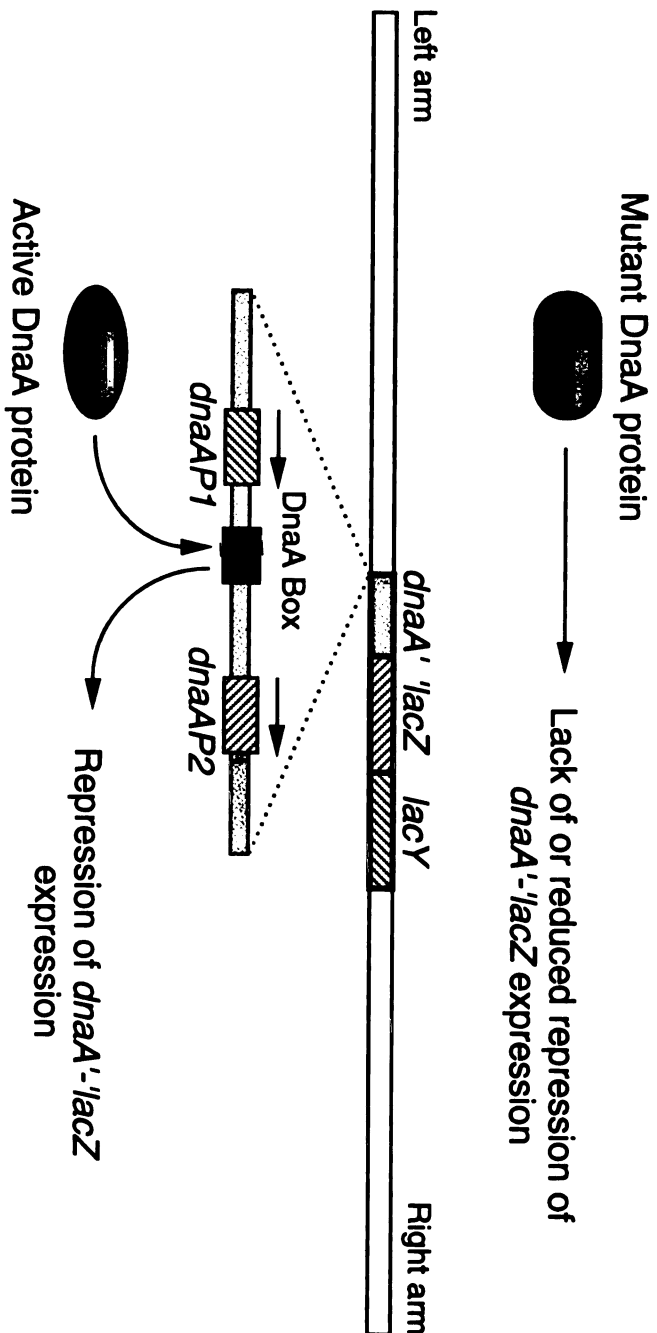
was judged both by growth rate and colony size of the double transformant (data not shown). Together, these observations suggest that the cold-sensitive heteropolyploid phenotype exhibited by these mutations mapping within or near the P-loop motif is *oriC*-specific.

The C-terminus of DnaA is required for repression of *dnaA* gene expression. DnaA protein has been shown to regulate its own expression (7, 25). Autorepression is a function of the binding of DnaA protein to a DnaA box located between the two *dnaA* promoters (43). Although the mechanism of *dnaA* autoregulation is not yet understood at the molecular level, the DNA binding activity of DnaA protein is a major component. With this in mind, we developed an *in vivo* assay to measure the DNA binding activity of DnaA protein (Figure 2). Using a specialized *E. coli* strain (MS3900; see Table 1), we were able to measure the abilities of the plasmid-encoded *dnaA* alleles to repress expression of the *dnaA'*-*lacZ* fusion by quantitation of β -galactosidase activity. Repression was attributed in part to binding of DnaA protein to the DnaA box within the *dnaA* promoter region (43).

MS3900 was transformed to chloramphenicol resistance with all 36 plasmid-encoded mutant *dnaA* alleles (28 missense, one deletion and seven nonsense). Representative isolates were cultured in supplemented M9 medium and β -galactosidase assays were performed as described in Materials and Methods. As the cell size and/or extent of filamentation for the different classes of *dnaA* mutations varied (data not shown), β -galactosidase activity was normalized to total soluble protein.

In this assay, missense mutants mapping to the N-terminus or P-loop motif had only a marginal effect on β -galactosidase activity relative to the controls (vector only and it containing the *dnaA*⁺ or the thermolabile *dnaA46* allele). By contrast, many of the mutants mapping to the C-terminus were

Figure 2. *in vivo* DNA binding assay. The DNA binding activities of the plasmid-borne *dnaA* alleles was measured indirectly using the *dnaA* promoter'-*lacZ* fusion contained in λ RB1 (7). Mutants of DnaA able to bind to the DnaA box in the *dnaA* promoter region repress expression of the *dnaA*'-*lacZ* fusion whereas those unable to bind to the DnaA box have no effect. Expression of the *dnaA*'-*lacZ* fusion was measured by quantitation of total β -galactosidase activity as described in Materials and Methods.



unable to repress the *dnaA*'-'*lacZ* fusion (Table 4). In addition, none of the *dnaA* nonsense mutations analyzed were able to represses expression of the *dnaA*'-'*lacZ* fusion in comparison to pACYC184 (Table 5). Together, these results indicate that the C-terminus of DnaA protein is required for autorepression of *dnaA* expression and suggest that it is required for DNA binding. The inability of the mutation containing an in-frame deletion of residues 237-378 to repress expression of the *dnaA*'-'*lacZ* fusion suggests that it is either defective in DNA binding, or that the region absent is in some way required for autoregulation of *dnaA* gene expression.

Experiments similar to those described above were performed using a plasmid (pDS596) that contained the *dnaA* gene under control of the *araB* promoter (data not shown). Based on quantitative immunoblot analysis, in the absence of *araB* promoter induction, about 2-3% as much DnaA⁺ protein was observed relative to when the *dnaA* gene was expressed from its own promoter (pACYC*dnaA*; see Chapter II). Under these conditions, only subtle repression of β -galactosidase activity was observed, suggesting that the level of expression from *araB* in the noninduced state represented the near minimal level of DnaA⁺ protein required to observe partial repression of *dnaA*'-'*lacZ* expression. Therefore, a likely explanation for the lack of repression observed for the M1V mutation in this assay relates to its low level of abundance (see below and Table 6). We do not yet know why C9Y, P43L-G177S, and G177D were less efficient for repression of expression of the *dnaA*'-'*lacZ* fusion, although it most likely does not relate to their respective levels of abundance as they were present at levels similar to other proteins analyzed (see below and Table 6).

Quantitative immunoblot analysis of mutant DnaA proteins. To corroborate the β -galactosidase assays, quantitative immunoblots of whole cell

TABLE 4. Mutations mapping to the C-terminus of DnaA are defective for regulation of expression of the *dnaA'*-*lacZ* translational fusion *in vivo*^a

MS3900 transformant ^b	Deduced amino acid substitution(s) ^c	Relative units β -galactosidase activity ^d	
		30°C	42°C
pACYC <i>dnaA</i>	None	$\equiv 0$	$\equiv 0$
pACYC <i>dnaA46</i>	A184V-H252Y	23	46
pACYC184	—	$\equiv 100$	$\equiv 100$
pACHA-9	S23N-A326T-S466L	7	-16
pACHA-32	I26K-H452Y	1	-16
pACHA-39	C9Y-T64I	36	52
pACHA-40	M1V	79	68
pACHA-45	C9Y	53	70
pACHA-50	W25S	31	42
pACHA-72	E21K	7	31
pACHA-106	C9Y-A293V	42	71
pACHA-143	L10F-R52W	20	59
pACHA-152	P28L	17	32
pACHA-200	E21K-Q144N	12	42
pACHA-94	G177D-E248K-E249K	39	24
pACHA-101	G175D	25	38
pACHA-105	A184T	9	32
pACHA-117	P43L-G177S	59	78
pACHA-146	G177D	49	40
pACHA-43	A440T	60	60
pACHA-49	T435M	107	106
pACHA-54 ^e	R407H	112	106
pACHA-70	V437M	81	89
pACHA-79 ^e	G426D	112	104
pACHA-83	A440V	97	87
pACHA-110	V383M	51	91
pACHA-130	A428T-A440T	86	106

TABLE 4 (continued).

MS3900 transformant ^b	Deduced amino acid substitution(s) ^c	Relative units β -galactosidase activity ^d	
		30°C	42°C
pACHA-162	D209N-T435M	113	113
pACHA-170 ^e	T435M-T436M	103	104
pACM-2 ^e	A412P	93	122
pACM-7 ^e	412(Ω M-A)413 ^f	104	124

^a β -galactosidase assays were performed as described in Materials and Methods.

^b MS3900 contains a *dnaA* null mutation, a *lac* deletion, and λ RB1 as a prophage. λ RB1 contains a translational fusion of the *dnaA* promoter and *lacZ* structural gene (7). See Table 1 for the complete genotype of MS3900.

^c Deduced amino acid substitutions are based on the nucleotide sequence of these mutations that were presented previously (see Chapter III). The deduced amino acid substitutions in *dnaA46*(Ts) are from Hansen *et al.* (18).

^d β -galactosidase activity was determined as described in Materials and Methods and is presented as the percentage of the activity observed relative to the pACYC*dnaA* and pACYC184 controls with 100% being equal to that for no DnaA present (pACYC184 transformant) and 0% being equal to that for the level of DnaA present when the *dnaA*⁺ allele was plasmid-encoded (pACYC*dnaA* transformant). To normalize the data, units observed with pACYC*dnaA* were subtracted from all samples and the resulting values were divided by the units observed with pACYC184 (minus the units observed with pACYC*dnaA*). Actual units of β -galactosidase activity (nmol ONP/min-mg protein) observed for the controls at 30° and 42°C, respectively, were as follows: pACYC*dnaA*, 82 and 61; pACYC*dnaA46*, 144 and 89; pACYC184, 350 and 122.

^e These alleles are inactive for replication from the pSC101 origin (see Chapter III) and *oriC* (data not shown).

^f 412(Ω M-A)413 is a Met, Ala insertion between residues 412 and 413.

TABLE 5. Truncated *dnaA* mutations are unable to affect expression of a *dnaA*'-'*lacZ* translational fusion *in vivo*^a

MS3900 transformant	Deduced mutation(s) ^b	Relative units β -galactosidase activity ^c	
		30°C	42°C
pACYC <i>dnaA</i>	None	$\equiv 0$	$\equiv 0$
pACYC <i>dnaA46</i>	A184V-H252Y	24	47
pACYC184	—	$\equiv 100$	$\equiv 100$
pACHA-4	$\Delta(237-378)$	98	93
pACHA-6	330→amber	121	104
pACHA-22	148→ocher	84	128
pACHA-86	263→amber	120	112
pACHA-97	247→amber	117	115
pACHA-119	446→amber	124	117
pACHA-121	361→amber	94	95
pACHA-178	245→amber	115	118

^a See the footnotes to Table 4.

^b Based on the nucleotide sequence information presented in Table 2.

^c Relative β -galactosidase activity is expressed as described in the footnote to Table 4. Actual units of β -galactosidase activity (nmol ONP/min-mg protein) for the controls at 30° and 42°C, respectively, were as follows: pACYC*dnaA*, 53 and 56; pACYC*dnaA46*, 82 and 93; pACYC184, 177 and 134.

lysates of a *dnaA* null strain harboring the plasmid-borne *dnaA* alleles was performed. Assuming the various polypeptides were similarly resistant to proteolysis, those defective in DNA binding would be expected to be present at levels higher than those unaffected as they would be unable to autoregulate their own expression.

With the exception of the G177D and G177D-E248K-E249K mutants, only those mapping C-terminal to residue 406 were detected at levels elevated relative to the plasmid-encoded DnaA⁺ and DnaA46 proteins (Table 6). Previously, we reported that DnaA46 protein was present at an abundance approximately 1.4-fold elevated relative to the level observed for the wild type protein when both alleles were plasmid-encoded (see Chapter IV). Although this analysis was done using the same plasmids and an isogenic strain, the variation in results is likely due to the different growth conditions used (see Materials and Methods).

Some of the mutations mapping to the C-terminus were unable to repress expression of the *dnaA*'-'*lacZ* fusion but the mutant proteins were present at levels lower than that observed for DnaA⁺ or DnaA46. Presumably, this is due to proteolytic instability of the mutant proteins. Consistent with this interpretation, these same mutants were proteolytically unstable following their overexpression from an inducible promoter (data not shown; see Chapter V). In general, the results of the quantitative immunoblot analysis are consistent with those of the β -galactosidase assay. Together, they indicate that the C-terminus of DnaA is required for repression of *dnaA* gene expression and suggest that it is required for DNA binding.

Identification of a third promoter directing expression of the *dnaA* gene in pACYC184. In an effort to correlate the results of the β -galactosidase assay with transcription from the two *dnaA* promoters, S1

TABLE 6. Quantitative immunoblot analysis of mutant DnaA proteins

MS3898 transformant	Deduced amino acid substitution(s)	Relative protein level ^a
pACYC <i>dnaA</i>	None	≡1.00
pACYC <i>dnaA46</i>	A184V-H252Y	0.97
pACYC184	—	≡0.00
pACHA-9	S23N-A326T-S466L	0.81
pACHA-32	I26K-H452Y	0.22
pACHA-39	C9Y-T64I	0.19
pACHA-40	M1V	0.04
pACHA-45	C9Y	0.24
pACHA-50	W25S	0.30
pACHA-72	E21K	0.24
pACHA-106	C9Y-A293V	0.24
pACHA-143	L10F-R52W	0.57
pACHA-152	P28L	0.45
pACHA-200	E21K-G144N	0.20
pACHA-94	G177D-E248K-E249K	1.99
pACHA-101	G175D	0.69
pACHA-105	A184T	0.33
pACHA-117	P43L-G177S	0.51
pACHA-146	G177D	1.60

TABLE 6 (continued).

pACHA-43	A440T	1.44
pACHA-49	T435M	3.34
pACHA-54	R407H	3.50
pACHA-70	V437M	0.70
pACHA-79	G426D	1.97
pACHA-83	A440V	1.62
pACHA-110	V383M	0.21
pACHA-130	A428T-A440T	0.64
pACHA-162	D209N-T435M	2.54
pACHA-170	T435M-T436M	2.81
pACM-2	A412P	0.30
pACM-7	412(Ω M-A)413	2.59

^a Relative protein levels were determined by quantitative immunoblot analysis using an ¹²⁵I-labelled antibody as described in Materials and Methods. Immunoblots were quantitated with a Packard Instant Imager. No DnaA was detected in the pACYC184 transformant, confirming its genotype. The counts detected in this sample were therefore defined as background (normalized to 0.00). Protein levels are corrected for cell density (based on OD₅₉₅) and are expressed relative to that observed for the wild type transformant (pACYC*dnaA* that was normalized to 1.00) minus background.

nuclease protection assays were performed (Figure 3). This analysis revealed that the plasmid-encoded *dnaA* alleles were expressed from not only *dnaAP1* and *dnaAP2*, but a third promoter as well. We have designated this third promoter *dnaAP1'*. S1 nuclease mapping of this transcript positioned its 5' end near the start site for the tetracycline resistance gene. Restriction at the *Cla* I site located between the -35 and -10 regions of the promoter for the tetracycline resistance gene in pACYC184 was expected to inactivate transcription from it. Activity from this promoter is presumably due to its reconstruction by the insertion of the *dnaA* gene at the *Cla* I site. A similarity in nucleotide sequence near the -10 region of the *tet* promoter region to that of the hybrid promoter formed is observed (see Figure 3 legend).

Quantitative immunoblot analysis indicated a two-fold higher level of DnaA protein encoded by pACYC184 relative to the chromosomally encoded allele (41). This elevated level of expression may be due to a copy number effect or due to elevated transcription by virtue of the third promoter.

The N-terminus of DnaA protein is essential for replication activity. Although mutations mapping to the N-terminus of DnaA protein neither exhibit a cold-sensitive heteropolyploid phenotype nor were defective for autoregulation, their thermolabile phenotype for pSC101 replication indicates the importance of this region of the protein. Biochemical characterization of select mutants from this class will help to elucidate the function(s) of this domain in replication.

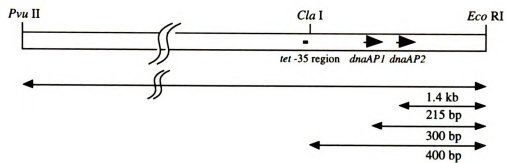
DnaA protein contains two domains that are alternately dispensable for replication from the pSC101 origin. The replication phenotypes of the nonsense and in-frame deletion mutations were measured using the phagemid assay (see Materials and Methods). Two of the eight alleles retained the ability to replicate the lambda derivative from the inserted

Figure 3. Identification of *dnaAP1'* using an S1 nuclease protection assay. (A) Structure of the 1.4 kb DNA fragment used for S1 nuclease protection assays. Assays were performed as described in Materials and Methods. Approximate positions and sizes of fragments identified by S1 nuclease protection shown in part B below are indicated. **(B)** Total RNA was isolated from either the *dnaA*⁺ strain, MS3907, (lane 1) or the *dnaA* null strain, MS3898, harboring pACYC184 (lane 2) or pACYC*dnaA* (lane 3). DNA size standards are indicated (lane 4), as are the positions of protected fragments corresponding to *dnaAP1* (~300 bp), *dnaAP2* (~215 bp), and the hybrid promoter, *dnaAP1'* (~400 bp). The -10 region of the *tet* promoter and the upstream region of the *dnaA* gene are both are AT rich:

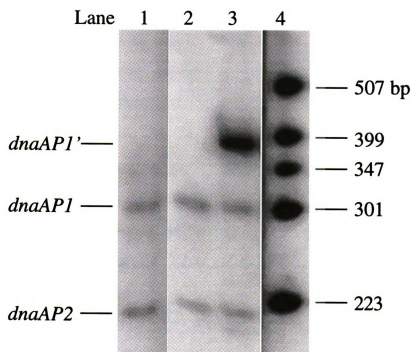
<i>dnaA</i>		CGATTAAAGCCAATTTTGTCTAT
		
<i>tet</i>	TGTTTGACAGCTTATCATCGATAAGCTTTAATGCGGTAGTT	
	-35	-10
		<i>Cla</i> I

The -35 and -10 positions in the *tet* promoter are indicated, as are the *Cla* I site and putative -10 region of the hybrid promoter. Conserved bases are indicated by the blackened dots.

A



B



pSC101 origin; both were thermolabile (Table 7). One was the in-frame deletion of residues 237-378 (*dnaA* Δ 237-378) and the other an amber mutation at position 361 (*dnaA-am361*). Their abilities to maintain pSC101 were investigated further by use of a transformation assay. *dnaA-op245* was included as a control, as were pACYC184 and it containing the *dnaA*⁺ or temperature sensitive *dnaA46* alleles. Isolates of a *dnaA* null strain harboring the plasmid-encoded *dnaA* alleles were transformed with the wild type pSC101 plasmid, the copy-number-up pSC101 mutant, pCM128, or pBR322. The transformation efficiency at 30°C for each plasmid was determined (Table 8). Consistent with the results of the phagemid assay, the *dnaA* null strain bearing *dnaA* Δ 237-378 or *dnaA-am361* was transformed with pSC101 or pCM128 at 30°C as efficiently as were the *dnaA*⁺ and *dnaA46*(Ts) controls. By contrast, we were unable to transform the *dnaA-op245* or pACYC184 controls with either pSC101 or pCM128, but were with pBR322. These observations suggest that the regions defined by the in-frame deletion and the amber mutation at position 361 represent domains of DnaA that are alternately dispensable for pSC101 replication.

The abilities of the various alleles to maintain pSC101 or pCM128 at 42°C relative to 30°C was measured by plating representative isolates of a *dnaA* null strain harboring the *dnaA*-containing plasmid and either pSC101 or pCM128 (Table 8). Whereas the plasmid-encoded *dnaA*⁺ allele was able to maintain pSC101 at both 30° and 42°C with similar efficiency, the *dnaA46*, *dnaA* Δ 237-378, and *dnaA-am361* alleles were temperature sensitive.

The *dnaA*⁺, *dnaA46* and *dnaA* Δ 237-378 alleles were temperature insensitive for pCM128 maintenance while the *dnaA-am361* allele remained thermolabile (Table 8), indicating that the *repA7* mutation contained in pCM128 is able to suppress certain *dnaA* alleles. The inability to transform *dnaA* null

TABLE 7. *dnaA*Δ237-378 and *dnaA-am361* are active for replication of a lambda derivative from the inserted pSC101 origin

<i>dnaA</i> -containing plasmid	Deduced mutation(s) ^a	λpCM128 spotting ^b						Replication phenotype ^c
		30°C			42°C			
		10 ⁴	10 ³	10 ²	10 ⁴	10 ³	10 ²	
pACYC <i>dnaA</i>	None	+	+	+	+	+	+	Wild type
pACYC <i>dnaA46</i>	A184V-H252Y	+	+	+	+/-	-	-	Ts
pACYC184	—	-	-	-	-	-	-	Inactive
pACHA-4	Δ(237-378)	+	+	-	-	-	-	Ts
pACHA-6	330→opal	-	-	-	-	-	-	Inactive
pACHA-22	148→ochre	-	-	-	-	-	-	Inactive
pACHA-86	263→amber	-	-	-	-	-	-	Inactive
pACHA-97	247→amber	-	-	-	-	-	-	Inactive
pACHA-119	446→amber	-	-	-	-	-	-	Inactive
pACHA-121	361→amber	+	+	-	-	-	-	Ts
pACHA-178	245→opal	-	-	-	-	-	-	Inactive

^a See Table 2 for nucleotide sequence information. The nucleotide sequence of the *dnaA46* allele is from Hansen *et al.* (18).

^b λpCM128 spotting consisted of spotting ~10⁵ MS3898 transformants of each of the indicated plasmid-encoded *dnaA* transformants onto supplemented M9 medium in combination with the indicated number of λpCM128 infective particles (10²–10⁴) as described in Materials and Methods. λpCM128 spotting was done in duplicate and plates were incubated at 30° and 42°C. Abbreviations are +, bacterial clearing (λpCM128 sensitivity); -, bacterial growth (λpCM128 immunity); +/-, partial clearing (moderate λpCM128 sensitivity).

^c Deduced from λpCM128 spotting. Ts, thermolabile.

TABLE 8. Truncated *dnaA* mutations are able to maintain pSC101

MS3898 transformant	Mutation(s)	Transformation efficiency ^a		Efficiency of plating ^b	
		pSC101	pCM128	pSC101	pCM128
pACYC <i>dnaA</i>	None	≡1.0	≡1.0	1.0	1.0
pACYC <i>dnaA46</i>	A184V-H252Y	1.1	1.4	6.9 X 10 ⁻⁴	1.1
pACYC184	—	NT ^c	<0.001	NT	NT
pACHA-4	Δ(237-378)	0.8	1.0	1.3 X 10 ⁻³	0.81
pACHA-121	361→amber	1.2	1.3	2.8 X 10 ⁻³	2.2 X 10 ⁻²
pACHA-178	245→opal	≤0.03 ^d	≤0.03 ^d	NT	ND ^e

^a MS3898 harboring each of the indicated plasmid-encoded *dnaA* alleles were transformed with pSC101, pCM128, or pBR322, and kanamycin, chloramphenicol, and tetracycline (for pSC101 and pBR322) or ampicillin (for pCM128 and pBR322) resistant colonies were selected for at 30°C. The transformation efficiency for pSC101 and pCM128 for each *dnaA* allele was normalized relative to the efficiency observed for pBR322, and is expressed relative to the efficiency observed for the *dnaA*⁺ transformant (normalized to 1.0). The transformation efficiency (colony forming units/μg plasmid DNA) of MS3898 (pACYC*dnaA*) for each plasmid DNA was as follows: pSC101, 3.6 X 10⁴; pCM128, 9.3 X 10⁴; pBR322, 2.1 X 10⁵. Transformation efficiencies of the other transformants with pBR322 was on the order of 10⁵ colony forming units/μg plasmid DNA.

^b Representative isolates of MS3898 harboring both the *dnaA*-containing plasmid and pSC101 or pCM128 were grown under selection for both plasmids in LB medium at 30°C, then diluted serially in 1X M9 salts, plated onto supplemented M9 minimal plates containing the appropriate antibiotics, and incubated at 30° and 42°C. Efficiency of plating is expressed as the number of colony forming units observed at 42° relative to the number observed at 30°C.

^c NT, not tested. In the case of the transformation efficiency of the pACYC184 transformant for pSC101, this was not tested because both plasmids confer tetracycline resistance.

^d Double transformants of MS3898 containing pACHA-178 and pSC101 or pCM128 were tiny. Those containing pSC101 would not grow in liquid culture containing kanamycin, chloramphenicol, and tetracycline. Although those containing pCM128 would grow in liquid culture containing kanamycin, chloramphenicol, and ampicillin, no pCM128 was detected by miniprep analysis (see footnote ^e). Reinvestigation of the ability of the R245→opal mutation to support replication of pSC101 or pCM128 using pACHA-213 (not described in this thesis) that contained both an R245→opal and G247→amber mutation was performed (data not shown). We were unable to transform MS3898 (pACHA-213) with either pSC101 (transformation efficiency ≤50 colony forming units/μg DNA) or pCM128 (transformation efficiency ≤50 colony forming units/μg DNA), thus suggesting that the ability of the pACHA-178 mutation to maintain pSC101 and pCM128 was due to read through of the opal mutation. No full length polypeptide was detected for the pACHA-178 mutation. This is not inconsistent with the above conclusion as the level of DnaA protein necessary to maintain such a low level of pCM128 may be undetectable by immunoblot analysis (data and theoretical calculation not shown).

^e Efficiency of plating for MS3898 (pACHA-178)(pCM128) was not determined (ND) because following growth in liquid culture supplemented with kanamycin, chloramphenicol, and with or without ampicillin, less than 1 in 1,000 colony forming units were resistant to ampicillin.

strain harboring pACYC184 with pCM128 is consistent with its *dnaA*-dependence (42).

The differences observed between the replication phenotypes of the mutant alleles in the phagemid assay relative to the transformation assay are not unexpected (compare Tables 7 and 8). Although both measure replication from the pCM128 origin, activity observed in the phagemid assay appears to be proportional to the copy number of the phagemid (8). Titration of the prophage-encoded *cl* repressor is presumed to be more efficient when the phagemid copy number is high. Therefore, we assume the phagemid assay measures an elevated copy number of the λ pCM128 phagemid. However, the transformation assay has few constraints on copy number. A single copy of the *bla* gene is sufficient for resistance to ampicillin (27).

Determination of the relative abundance of pCM128 when maintained by different *dnaA* alleles. The *dnaA*, *dnaA46*, *dnaA Δ 237-378*, and *dnaA-am361* alleles were able to maintain pCM128 for at least 10 generations at 30°C in the absence of its selection (data not shown). However, the plating efficiencies of these alleles for pSC101 and pCM128 maintenance suggested that *dnaA-am361* was less efficient for pSC101 replication than was *dnaA Δ 237-378* (Table 8). To investigate this possibility, we determined the relative abundance of pCM128 in strains harboring each of these plasmid-encoded *dnaA* alleles (Table 9). This analysis indicated that maintenance of pCM128 at 30°C by the *dnaA Δ 237-378* or *dnaA-am361* allele was less efficient than the wild type allele. Furthermore, the level of pCM128 when maintained by *dnaA Δ 237-378* appeared 40% elevated relative to *dnaA-am361*. These observations are consistent with the *dnaA-am361* allele being less efficient than *dnaA Δ 237-378* for pSC101 replication.

The *dnaA46* allele appeared to be less efficient than the wild type allele

for pSC101 replication as well. This was based on the reduced abundance of pCM128 when maintained by *dnaA46* relative to the wild type allele. This finding is consistent with previous reports indicating that *dnaA*(Ts) mutants maintained pSC101 at a reduced copy number relative to an isogenic *dnaA*⁺ host (13).

Both the *dnaA46* and *dnaAΔ237-378* alleles showed a modest reduction in abundance of pCM128 at 42° relative to 30°C (see Table 9), consistent with the finding that these alleles were able to maintain pCM128 at both 30° and 42°C with similar efficiency (Table 8). By contrast, the *dnaA-am361* allele showed a significant reduction in pCM128 abundance at 42° relative to 30°C. This is consistent with the thermolabile phenotype of this allele for pCM128 maintenance (see Table 8).

Truncated *dnaA* mutations are inactive for replication from *oriC*. As *dnaAΔ237-378* and *dnaA-am361* were active for pSC101 replication, we wanted to test their abilities to support replication from *oriC*. The ability of these plasmid-encoded *dnaA* alleles to initiate replication from *oriC* was measured after a temperature downshift from 42° to 30°C of a *dnaA* null strain harboring both the plasmid-encoded *dnaA* alleles and the *copA*(Ts)-containing plasmid, pMDS421. pMDS421 bears an R1 *copA*(Ts) allele that encodes an antisense RNA which inhibits translation of the R1 *repA* mRNA required for R1 replication. The R1 *copA*(Ts) allele is active at 30° but inactive at 42°C. In contrast to the results obtained with pSC101 and pCM128, neither the *dnaAΔ237-378* nor *dnaA-am361* alleles were active for replication from *oriC* (Table 10). As controls, the plasmid-encoded *dnaA*⁺ or *dnaA46* alleles were able to complement the chromosomal *dnaA* null allele at 30°C for replication from *oriC*; pACYC184 was not.

TABLE 9. Relative abundance of pCM128 in various *dnaA* mutants

<i>dnaA</i> allele ^a	Relative abundance of pCM128 ^b	
	30°C	42°C
<i>dnaA</i> ⁺	≡1.00	≡1.00
<i>dnaA46</i>	0.66	0.43
<i>dnaAΔ237-378</i>	0.36	0.33
<i>dnaA-am361</i>	0.26	0.08

^a *dnaA* alleles were plasmid-encoded and harbored by the *dnaA* null strain MS3898.

^b The abundance of pCM128 was determined relative to the indicated *dnaA*-containing plasmid following growth at 30°C to log phase maintaining selection for pCM128, then for 4 h in the absence of selection for pCM128 in supplemented M9 medium at 30° and 42°C as described in Materials and Methods. In each case, values are normalized relative to the *dnaA*⁺ control (pACYC*dnaA* that was normalized to 1.00) at that temperature.

TABLE 10. Truncated *dnaA* mutations are inactive for replication from *oriC*

MS3898 transformant	<i>dnaA</i> allele	Efficiency of plating of pMDS421 transformants ^a
pACYC <i>dnaA</i>	<i>dnaA</i> ⁺	0.54
pACYC <i>dnaA46</i>	<i>dnaA46</i>	0.12
pACYC184	None	5.4 X 10 ⁻⁶
pACHA-4	<i>dnaA</i> Δ237-378	5.5 X 10 ⁻⁶
pACHA-121	<i>dnaA361-am</i>	3.6 X 10 ⁻⁴

^a MS3898 containing the indicated plasmid-encoded *dnaA* alleles (or pACYC184) were transformed with pMDS421 that encodes an R1 *copA*(Ts) allele. Colonies were picked from plates immediately following their transformation, resuspended in 1X M9 salts, diluted serially, and plated onto supplemented M9 medium containing kanamycin, chloramphenicol, and ampicillin. Efficiency of plating is expressed as the number of colony forming units observed at 30°C relative to 42°C.

Discussion

The method described in Chapter III provided a large collection of missense and nonsense mutations that were defective in replication of a lambda derivative containing an inserted pSC101 origin. DNA sequence analysis indicated that the mutations were distributed throughout the coding region of the *dnaA* gene with groupings near the N-terminus, the C-terminus, and the P-loop motif. These mutations were further characterized to correlate a specific functional defect with each individual mutation (Figure 4).

Mutations mapping to the well conserved P-loop motif, proposed to function in nucleotide binding, exhibited a cold sensitive phenotype when plasmid-encoded and harbored by a strain dependent upon the chromosomal *dnaA*⁺ allele and *oriC* for growth (Table 3). DnaA5 and DnaA46 exhibit a similar cold sensitive phenotype (18) and are known to be defective for ATP binding *in vitro* (21, 22). Together, these observations suggest that the cold sensitive phenotype is due to an ATP-binding defect. Biochemical characterization of the novel mutants described here will reveal whether they are affected in ATP binding.

The three alleles described here that exhibit the cold-sensitive heteropolyploid phenotype were active for pSC101 maintenance at 30°, but less so at 42°C. Of the three, A184T appeared most active at the elevated temperature in the phagemid assay (see Chapter III). By contrast, the A184T mutant was less abundant than either DnaA⁺, G177D, or G177D-E248K-E249K as measured by quantitative immunoblot analysis (Table 6). Despite this, it was least tolerated by MC1061 and MS1062 in the transformation assay. Together, these observations suggest that there is a correlation between the level of activity and/or protein abundance and the cold-sensitive heteropolyploid

Figure 4. DnaA protein has four functionally distinct domains. (A) The approximate location of each of the deduced substitutions contained in the 28 missense mutations are indicated by a blackened triangle (▲). Numbers refer to amino acid residues of DnaA protein. **(B)** Examination of the positions of mutation and their mutant phenotypes suggested the presence of four functionally distinct domains of DnaA protein. Delineations of the separate domains are indicated and are based on the genetic characterization and nucleotide sequence of the mutant alleles. Specific end and start points for Domains I and II, respectively, were refined based on the PHD secondary structure prediction (see Figure 5). The proposed function for Domains II, III and IV are indicated. The function of Domain I is unknown as is the role of Domain III in *oriC* replication.

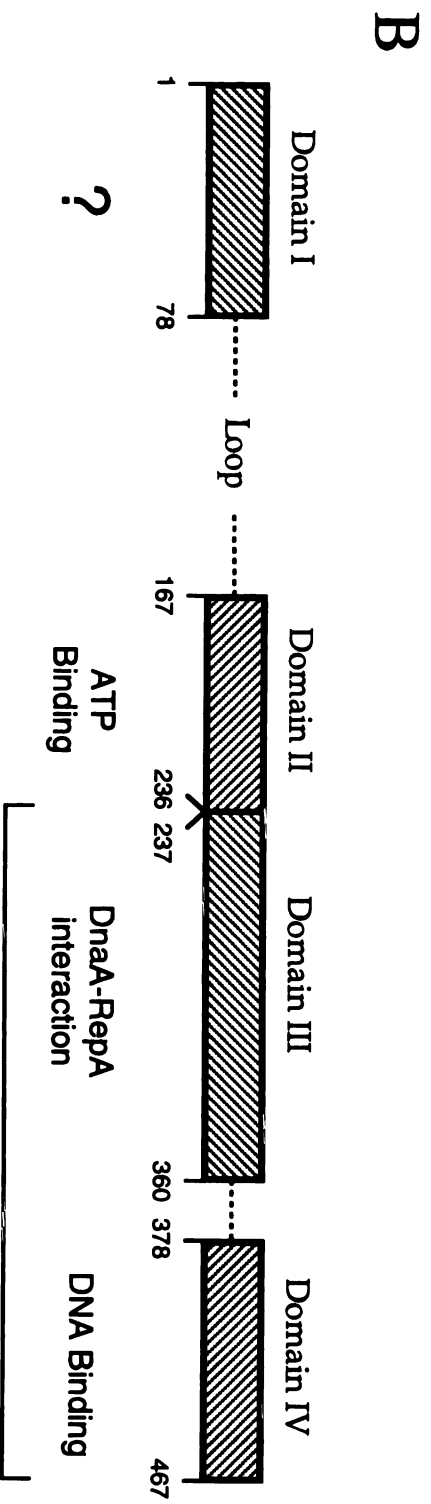
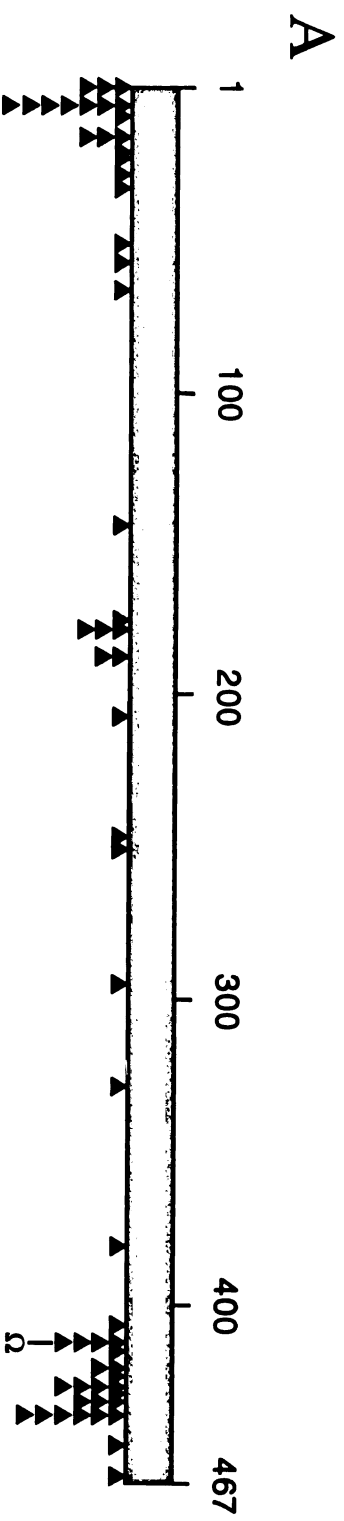
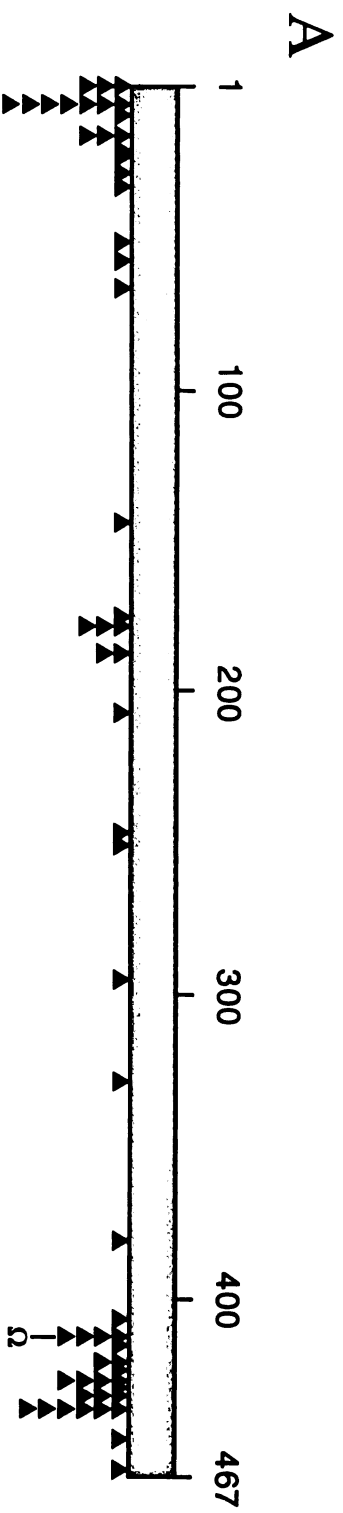


Figure 4. DnaA protein has four functionally distinct domains. (A) The approximate location of each of the deduced substitutions contained in the 28 missense mutations are indicated by a blackened triangle (▲). Numbers refer to amino acid residues of DnaA protein. **(B)** Examination of the positions of mutation and their mutant phenotypes suggested the presence of four functionally distinct domains of DnaA protein. Delineations of the separate domains are indicated and are based on the genetic characterization and nucleotide sequence of the mutant alleles. Specific end and start points for Domains I and II, respectively, were refined based on the PHD secondary structure prediction (see Figure 5). The proposed function for Domains II, III and IV are indicated. The function of Domain I is unknown as is the role of Domain III in *oriC* replication.



B



phenotype.

The P43L-G177S mutant was less abundant than DnaA⁺ and appeared less active in the phagemid assay than either G177D or G177D-E248K-E249K. These observations may account for the lack of cold sensitivity of this allele when harbored by MC1061. The specific substitution at residue 177 (G177S as opposed to G177D), or the secondary mutation (P43L) may also be responsible for the lack of cold sensitivity. Likewise, the G175D mutant may be expressed at too low a level (see Table 6). Whether an elevated level of P43L-G177S or G175D will result in a cold-sensitive phenotype may be addressed by cloning these alleles under control of an inducible promoter and modulating their expression levels accordingly.

The C-terminus of DnaA protein was essential for transcriptional repression from the *dnaA* promoter region (see Tables 4 and 5). Quantitative immunoblot analysis of whole cell lysates of a *dnaA* null strain harboring these alleles on a plasmid confirmed that their inability to repress expression of the *dnaA*'-'*lacZ* fusion was not due to their proteolytic instability or lack of abundance (Table 6). These results, suggesting that the C-terminus of DnaA is required for DNA binding, substantiate those of Roth and Messer. They found that the C-terminal 94 residues of DnaA were sufficient and required for specific DNA binding activity (32).

V383M is identical to *dnaA205* that was identified previously (4, 18). Consistent with its thermolabile phenotype, it was able to repress expression of the *dnaA*'-'*lacZ* fusion at 30° but not at 42°C. That V383M could repress at 30°C suggests that it is able to bind DNA. Its reduced ability to repress, however, indicates that its DNA binding activity may be affected. This finding contrasts with that of Roth and Messer (32) who stated that they could not detect a DNA binding activity for a fusion protein between β -galactosidase and the

C-terminal 94 residues of DnaA205. A similar fusion protein consisting of the C-terminal 94 residues of wild type DnaA protein was active for DNA binding. This discrepancy may be due to either a folding deficiency of the fusion protein, or inactivation on purification. Consistent with the latter proposal, we have noticed that DnaA205 is proteolytically unstable when overexpressed from an inducible promoter (data not shown, see Chapter V). The viability of a *dnaA205(Ts)* mutant at 30°C argues strongly that DnaA205 is able to bind DNA at its permissive temperature, as our results suggest.

None of the mutations mapping to the N-terminus exhibited a phenotype in the assays described. However, their temperature sensitive phenotype for pSC101 replication (see Chapter III) indicates the importance of the N-terminus in replication.

Two truncated forms of DnaA were identified that retained replication activity for pSC101 (Tables 7 and 8) but not for *oriC* (Table 10). One was an in-frame deletion of residues 237-378 and the other an amber mutation at position 361. All other nonsense mutations, including an opal mutation that codes for a stop codon at position 245, were inactive for pSC101 replication. These observations suggest that the domains defined by the in-frame deletion and amber mutation at position 361 identify regions of DnaA protein that are alternately dispensable for pSC101 replication. Furthermore, it suggests that these two domains perform redundant functions. However, that *DnaA Δ 237-378* retained DNA binding activity whereas *DnaA-am361* did not (data not shown, see Chapter V) indicates that these domains perform dissimilar functions. That *dnaA-am361* could maintain pSC101 is consistent with a physical interaction between DnaA protein and the pSC101-encoded initiator, RepA. Such an interaction was proposed based on the ability of RepA protein to facilitate the binding of DnaA protein to the pSC101 origin (40). This proposed interaction

may recruit DnaA protein to the pSC101 origin in the absence of a DNA binding activity. Examination of the phenotypes of *dnaA*Δ237-378 and *dnaA-am361* suggests that residues 237 through 360-to-378 function in a DnaA-RepA interaction and that residues 360-to-378 through 467 constitute the DNA binding domain (see Figure 4).

Although *dnaA-am361* was active for pSC101 replication, the C-terminus of DnaA is not entirely dispensable for replication of pSC101. Five missense mutations mapping in between residues 361-467 were found to be inactive for pSC101 replication (see Chapter III). We propose that these mutants were inactive for pSC101 replication because they were unable to bind specifically to DNA (see Chapter V). Their nonspecific DNA binding activity may be sufficient to sequester them away from the pSC101 origin. Furthermore, we propose that since DnaA-am361 lacks the DNA binding domain, it would be more readily recruited to the pSC101 origin by RepA protein.

Although the missense mutations were widely distributed throughout *dnaA*, those that mapped within residues 237-378 contained one or more additional mutations that may account for the observed mutant phenotype, consistent with this region of DnaA being dispensable for replication of pSC101. In further support of this, two mutations of *dnaA* (G287S and T301I) thermolabile for pSC101 replication that were identified using an alternative approach (see Chapter II) were indistinguishable from the wild type allele in the phagemid assay (data not shown).

To complement the results of the genetic and biochemical characterization of the mutant *dnaA* alleles, a secondary structure prediction of DnaA protein using the PHD method was obtained (31) (Figure 5). Essentially all of the missense mutations were found to map within either an α -helix or β -structure. The most striking result of the secondary structure prediction,

Figure 5. Secondary structure prediction for DnaA protein. The deduced amino acid sequence of the *E. coli* DnaA protein is indicated relative to the consensus sequence obtained by comparison of the deduced amino acid sequences of fifteen (*E. coli*, *S. typhimurium*, *S. marcescens*, *P. mirabilis*, *B. aphidicola*, *P. putida*, *B. subtilis*, *S. coelicolor*, *M. luteus*, *C. crescentus*, *R. meliloti*, *Synechocystis sp.*, *B. burgdorferi*, *S. citri*, and *M. capricolum*) different *dnaA* homologs (27a). For the consensus sequence, a dot (·) represents a residue conserved (either identical residue or conservative replacement) in less than 9, a letter represents a residue conserved in 9 or more, and an underlined letter represents a residue conserved in 12 or more of the 15 homologs. The position of each mutation identified in the 28 *dnaA* alleles and the deduced substitution are also presented. *PHD* refers to the PHD secondary structure prediction for the *E. coli* DnaA protein. Symbols are: -, an unstructured residue, such as one contained in a loop; H, α helical structure; E, β structure.

1 60
E. coli MSLSLWQQCLARLQDELPATEFSMWIRPLQAE~~LS~~DN~~T~~LALYAPNRFVLDWVRDKYLNNIN
consensus MSL.LW.Q.LA.L..EL...F..WIR.LQ.EL...TL.L.APN.FVLDWV..KYL..I.
mutations V YF K N SK L L W
PHD ---HHHHHHHHHHHHHH---HHHHHHHHHHHHHH-----EEEE---HHHHHHHHHHHHHHHH

61 120
E. coli GLLTSFCGRIAPQLRFEVGTKPVTQTQPAAVTSNVAAPAQVAQTQPQRAAPSTRSGWDNV
consensus .LL..F.....L.F.V.....W...
mutations I
PHD HHHHHH-----EEEEEE-----

121 180
E. coli PAPAPTYRSNVNVKHTFDNFVEGKSNQLARAAARQVADNPGGAYNPLFLYGGTGLGKTH
consensus S.VN.K.TFDNFVEG.SN.LA.AAAR.VADNPG.AYNPLFLYGG.GLGKTH
mutations N D D S
PHD -----EE---HHHHHHHHHHHHHH-----EEEE---HHHH

181 240
E. coli LLHAVGNGIMARKPNAKVVMHSE~~RF~~QDMVKALQNN~~AI~~EEFKRYRSVDALLIDDIQFF
consensus LLHAVGN..M...PNAKVVM.SERFV.DMV.ALQ.N.IEEFK.YYRSVD.LLIDDIQFF
mutations T N
PHD HHHHHHHHHHHHH---EEEEEHHHHHHHHHHHHHHHHHHHHHHHHHHHH---EEEE-HHHH

241 300
E. coli ANKERSQEEFFHTFNALLEGNQIILTSDRYPKEINGVEDRLKSRFGWGLTVAIEPPELE
consensus A.KE..QEEFTHTFNALLE...OIILTSDRYPKEI.GVEDRLKSRF.WGL.VAIEPPELE
mutations KK V
PHD H-----HHHHHHHHHHHHHH---EEEEEE-----HHHHHHHHHHHHHH---EEE---HHH

301 360
E. coli TRVAILMKKADENDIRLPGEVAFFIAKRLRSNVRELEGALNRVIANANFTGRAITIDFVR
consensus TRVAIL.KKADE..I.LP.EV.FFIA.RL.SNVRELEGALNRVIA.A.F....ITIDFV.
mutations T
PHD HHHHHHHHHHHHH---HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH---EEHHHH

361 420
E. coli EALRDLLALQEKLV~~T~~IDNIQKTVAEYYKIKVADLLSKRRSRSVARPRQ~~M~~AMALAKELTNH
consensus E.LRDLL...E.LVTIDNIQK.VAEYYKIKV.DLLSKRRSRSVARPRQ~~M~~.L.KELTNH
mutations M H PΩ
PHD HHHHHHHHH---EHHHHHHHHHHHHHH-EEEEHHH---HHHHHHHHHHHHHHHHHHHH---

421 467
E. coli SLPEIGDAFGGRDHTTVLHACRKIEQLREESHDIKEDFSNLIRTLLS
consensus SLPEIGD.FGGRDHTTVLHACRKIE.LR.E..DIK.DF..LIR.L..
mutations D T MMM V Y L
T
PHD --HHHHHH---HHHHHHHHHHHHHHHHHHHH---HHHHHHHHHHHHHH--

however, was the presence of a canonical nucleotide binding domain, or Rossmann fold (30), mapping to residues 168-235. This domain includes the P-loop motif thought to function in nucleotide binding.

Finally, the genetic characterization of these *dnaA* alleles suggests that DnaA protein contains four functionally distinct domains: an N-terminal domain of unknown function, a domain containing the P-loop motif involved in nucleotide binding, a region proposed to interact with pSC101 RepA protein, and a C-terminal domain required for DNA binding (see Figure 4).

More detailed biochemical characterization of select mutant proteins, as well as the design and characterization of more refined deletion mutations will help to more precisely define the function of each of the four proposed domains and will help to provide a more detailed understanding of the mechanism of DnaA-dependent DNA replication.

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Chapter V

Identification of Essential Residues in the DNA Binding Domain of the *Escherichia coli* DnaA Protein

Abstract

The *Escherichia coli* DnaA protein, as a sequence-specific DNA binding protein, promotes the initiation of chromosomal replication by binding to asymmetric 9-mer sequences termed DnaA boxes that are present four times in *oriC*. Characterization of N-terminal, internal, and C-terminal deletion mutants indicated that residues near the C-terminus of DnaA protein are required for DNA binding. Furthermore, genetic and biochemical characterization of eleven missense mutations mapping within the C-terminal 89 residues indicated that they are defective in DNA binding. Detailed biochemical characterization of one mutant protein that contains a threonine-to-methionine substitution at position 435 (T435M) indicated that it retained only nonspecific DNA binding activity, suggesting that the residue involved imparts specificity in binding. Finally, T435M was inactive on its own for *in vitro* replication of an *oriC* plasmid but able to augment limiting levels of wild type DnaA, consistent with the proposal that not all of the DnaA monomers in the initial complex are bound specifically to *oriC*.

Introduction

The *E. coli* origin of chromosome replication, *oriC*, is bound by about 20-30 monomers of DnaA protein to form a large nucleoprotein structure (10). Subsequent unwinding of the AT-rich region at the left end of *oriC* by DnaA that is aided by HU or integration host factor (IHF) (17) allows entry of DnaB helicase resulting in formation of the prepriming complex (13).

Biochemical characterization of DnaA protein has revealed that it is multifunctional possessing activities of binding to adenine nucleotides (29), to acidic phospholipids (30, 36), to DnaB protein (20), and to itself as a self-aggregate (12). Finally, it is a sequence-specific DNA binding protein that binds to an asymmetric 9-mer sequence (5'-TTAT^C/_ACA^C/_AA-3') referred to as the DnaA box (10).

Its activity as a DNA binding protein appears to be critical not only for its role in initiation of chromosome replication, but also for the expression of genes containing DnaA box motifs in promoters or within coding regions. Its binding to these DnaA boxes results in repression (18, 24, 32, 34, 35), activation (2), or termination of transcription (1, 28), respectively (reviewed in reference 21). The best studied of these genes whose transcription is affected by DnaA protein is the *dnaA* gene itself (18, 34). A single DnaA box located in the *dnaA* promoter region is bound by DnaA protein to repress transcription from both *dnaA* promoters.

Recently, we described the identification of a large collection of *E. coli* *dnaA* alleles (see Chapter III). The genetic and biochemical characterization of these alleles suggested that DnaA protein contains four functionally distinct domains (see Chapter IV). An *in vivo* assay to measure DNA binding activity was established that relied on transcriptional repression of a *dnaA* promoter'

'*lacZ* fusion by binding of DnaA protein to the DnaA box in the *dnaA* promoter region. Mutations encoding amino acid substitutions within the C-terminal 89 residues or lacking C-terminal coding sequence were unable to repress expression, suggesting that the C-terminus of DnaA was involved in DNA binding. Consistent with this conclusion was the recent report by Roth and Messer (27) describing that the C-terminal 94 residues of DnaA when fused to β -galactosidase were sufficient for specific binding to a DNA fragment containing *oriC*.

Here, we report that the C-terminal 89 residues of DnaA are required for specific DNA binding. Furthermore, genetic and biochemical characterization of a large set of missense mutations mapping within this region identify residues critical for DNA binding activity. A structure for the DNA binding domain of DnaA is proposed.

Materials and Methods

Bacterial strains. *E. coli* strains were WM433 (4), *leu-19 pro-19 trp-25 his-47 thyA59 arg-58 met-55 deoB23 lac-11 gal-11 strA56 sul-1 hsdS^{K-12} dnaA204(Ts)*; XL1-Blue (7), *recA1 endA1 gyrA96 (Nal^r) thi hsdR17 (r_{K-12}⁻m_{K-12}⁺) supE44 relA1 lac (F'⁺::Tn10 proA⁺B⁺ lac^r Δ(lacZ) M15)*; HMS174(DE3)(pLysS) (9), *recA1 hsdR (r_{K-12}⁻m_{K-12}⁺) Rif^r F⁻*; BLAL21(DE3)(pLysS), *ompT hsdS (r_B⁻m_B⁻) recA56 srl-300::Tn10 F⁻*. BLAL21(DE3) is a *recA56 srl-300::Tn10* derivative of BL21(DE3) (9) kindly provided by Dr. Laurie S. Kaguni.

Recombinant DNA techniques. Plasmid DNAs used in this study are described in Table 1. The deletion mutants containing N-terminal truncations (*dnaAΔ62*, *dnaAΔ129*, and *dnaAΔ219*) were constructed by polymerase chain reaction (PCR)-mediated site-specific mutagenesis using the following oligonucleotide primers (complementary to the indicated positions with position 1 corresponding to the first nucleotide of the coding sequence (15)) synthesized by an in-house facility with an Applied Biosystems Model 394: JK-36, 5'-AATATCAATCATATGCTAACCAGTTTCTGC-3' (178-201 of the template strand); JK-37, 5'-CAGAACCGACCCCATATGTCTAACGTAAACG-3' (371-402 of the template strand); JK-38, 5'-CAAAACAACCCATATGGGAAGAGTTTAAACGC-3' (643-672 of the template strand); JK-6, 5'-CGGAGCGTACCAGGATCCGTTTCACCTTCCA-3' (1,683-1,712 of the coding strand). The oligonucleotides JK-36, JK-37, and JK-38 contained nucleotide sequence that was altered relative to the coding sequence of the *dnaA* gene that generated an *Nde* I restriction endonuclease site (5'-CATATG-3'; indicated above by underlining) and the oligonucleotide JK-6 introduced a *Bam* HI site (5'-GGATCC-3'; indicated above by underlining) at the appropriate locations to allow cloning of the PCR product

TABLE 1. Plasmid DNAs

Plasmid	Characteristics ^a	Source or construction ^b
pET11a	Ap ^r , pBR322 origin, expression vector that places gene of interest under T7 promoter control	Novagen, Inc. (9)
pKC596	<i>dnaA</i> ⁺ cloned into pET11a	This laboratory (8)
pKCΔ62	<i>dnaA</i> Δ62 cloned into pET11a	This work
pKCΔ129	<i>dnaA</i> Δ129 cloned into pET11a	This work
pKCΔ219	<i>dnaA</i> Δ219 cloned into pET11a	This work
pKCΔ220-294	<i>dnaA</i> Δ220-294 cloned into pET11a	This laboratory
pKCΔ237-378	<i>dnaA</i> Δ237-378 cloned into pET11a	This work
pKC43	A440T cloned into pET11a	This work
pKC49	T435M cloned into pET11a	This work
pKC54	R407H cloned into pET11a	This work
pKC70	V437M cloned into pET11a	This work
pKC79	G426D cloned into pET11a	This work
pKC83	A440V cloned into pET11a	This work
pKC110	V383M cloned into pET11a	This work
pKC130	A428T-A440T cloned into pET11a	This work
pKC170	T435M-T436M cloned into pET11a	This work
pKCM2	A412P cloned into pET11a	This work
pKCM7	412(ΩMA)413 cloned into pET11a	This work

^a Nomenclature is as follows: Δ, deletion of amino acid residues; A440T (and like), alanine-to-threonine substitution at position 440; A428T-A440T, dash indicates that both mutations are present in the same mutant (i.e. double mutant); 412(ΩMA)413, Met, Ala insertion between residues 412 and 413.

^b See Materials and Methods for a description of plasmid constructions.

into the *Bam* HI-*Nde* I linearized pET11a vector fragment. PCR reactions contained Vent DNA polymerase (New England Biolabs) as per the manufacturer's recommendation, pKC596 (see Table 1) as the DNA template, and a Perkin Elmer Cetus DNA Thermal Cycler.

pKCΔ220-294 is a pKC596 derivative that lacks a 225 bp fragment corresponding to residues 220-294 of DnaA that was liberated by *Pvu* I endonuclease restriction and was generated by standard *in vitro* molecular biological techniques (J. Lipar, M. D. Sutton, and J. M. Kaguni, data not shown).

*dnaA*Δ237-378 and the *dnaA* missense mutant alleles that were described previously (see Chapters III and IV) were subcloned from pACYC184 into pET11a by *Eco* RI-*Rsr* II restriction of the pACYC184 recombinant containing the mutant *dnaA* allele, purification of the 1,402 bp fragment corresponding to the *dnaA* coding sequence (residues 21-467), and its subsequent ligation to the gel purified 5.9 kb pKC596 vector fragment that was obtained by partial *Eco* RI restriction of the *Rsr* II linearized pKC596 plasmid. Alternatively, some of the mutant *dnaA* alleles were amplified by PCR using the oligonucleotide primers JK-6 and JK-7, (8) restricted with *Nde* I and *Rsr* II, and ligated to the *Nde* I-*Rsr* II pET11a vector fragment.

Overexpression and purification of recombinant proteins.

Overexpression of target genes contained in pET11a recombinants was performed by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (U. S. Biochemicals) to 0.05 mM (final concentration) when cultures reached an OD₅₉₅ of 0.6-0.8 units. Purified forms of monomeric DnaA and T435M were obtained as described (22). Briefly, cells (HMS174(DE3)(pLysS)) induced to express DnaA protein (or T435M) were lysed by a gentle freeze-thaw lysis procedure (11). The soluble fraction was then diluted to a conductivity equivalent to Buffer A (25 mM HEPES-KOH pH 7.6, 15% glycerol, 0.1 mM EDTA, and 0.2 mM DTT)

containing 50 mM KCl and applied to Heparin Sepharose at a ratio of 20 mg protein to 1 ml of packed resin. After loading, the column was washed with 50 column volumes of Buffer A containing 100 mM KCl. Bound proteins were eluted with a linear gradient of 0.1 M to 1 M KCl in Buffer A. Fractions containing DnaA⁺ (or T435M) were identified by SDS-PAGE analysis and pooled. DnaA⁺ (or T435M) was then precipitated by dialysis against Buffer C (50 mM HEPES-KOH pH 7.6, 20% glycerol, 1 mM EDTA, and 2 mM DTT) overnight at 4°C. Precipitated proteins were collected by centrifugation, washed with Buffer C containing 0.6 M (NH₄)₂SO₄, and solubilized in Buffer C containing 0.6 M (NH₄)₂SO₄, 10 mM MgOAc, and 4 M guanidinium hydrochloride at a final protein concentration of approximately 10 mg/ml. Gel permeation chromatography using a Superose 12 column equilibrated with Buffer D (50 mM HEPES-KOH pH 7.6, 15% glycerol, 0.1 mM EDTA, 10 mM MgOAc, 0.2 mM (NH₄)₂SO₄, and 2 mM DTT) was used to remove the guanidinium hydrochloride and obtain the active monomer of DnaA protein (or T435M).

Other mutant forms of DnaA containing amino acid substitutions were purified partially from 100 ml cultures grown at 30°C of BLAL21(DE3)(pLysS) containing the respective mutant *dnaA* alleles in pET11a. As these mutants were more sensitive to proteolysis than were DnaA⁺ or T435M (data not shown), cell lysis was performed in the presence of 4 mM phenylmethyl-sulphonyl fluoride (PMSF). Soluble fractions were chromatographed batchwise with Blue Dextran Agarose. After extensive washing of the bound material with Buffer A containing 100 mM KCl, bound proteins were eluted stepwise with Buffer A containing 1 M KCl. V383M, A412P, and A428T-A440T were found to be extremely unstable. Full length protein for these mutants was obtained only by rapid lysis on ice by sonication in the presence of PMSF (4 mM) followed by

addition of SDS (0.25%) to the soluble fraction.

DnaA Δ 62 and DnaA Δ 129 were purified partially by batchwise chromatography essentially as described above for the mutant forms of DnaA containing amino acid substitutions except that Blue Dextran Agarose chromatography was replaced by Heparin Sepharose. Whereas DnaA Δ 62 and DnaA Δ 129 were present almost exclusively in the soluble fraction (data not shown), DnaA Δ 219 partitioned exclusively to the insoluble fraction (data not shown). DnaA Δ 219 was purified from a 200 ml culture of HMS174(DE3)(pLysS)(pKC Δ 219) that was induced by addition of IPTG (0.05 mM). The insoluble fraction that was obtained following cell lysis as described above was washed once with Buffer A containing 100 mM KCl and 2% Triton X-100, then two times with Buffer A containing 100 mM KCl. The pellet was then solubilized in Buffer A containing 100 mM KCl and 6 M guanidinium hydrochloride and dialyzed against 1,000 volumes of Buffer A containing 0.5 M KCl, 10 mM MgSO₄, and 0.5% Triton X-100 for 16 hours at 4°C. The dialyzed material was then combined with 0.5 volumes of Heparin Sepharose and the mixture was diluted with Buffer A to a conductivity equivalent to Buffer A containing 50 mM KCl. After packing the slurry into a column (0.5 cm diameter) it was washed with 50 column volumes of Buffer A containing 0.5% Triton X-100 and 100 mM KCl, then eluted stepwise with Buffer A containing 0.5% Triton X-100 and 1 M KCl. Fractions containing DnaA Δ 219 were identified by SDS-PAGE analysis.

Protein determinations. Protein concentrations were determined by scanning densitometry of Coomassie-stained polyacrylamide gels using a Bio-Rad Gel Doc 1000 equipped with the Molecular Analyst software package. Alternatively, protein concentrations were determined by quantitative immunoblot analysis using an ¹²⁵I-labelled antibody specific to DnaA protein as

described previously (see Chapter II).

Southwestern blotting. Southwestern blotting was performed based on the method of Bowen *et al.* (5). Samples were electrophoresed and blotted onto Westran membrane (Schleicher and Schuell) as described (31). Membranes were then incubated in binding buffer (20 mM HEPES-KOH pH 7.6, 2.5 mM MgOAc, 15% glycerol, 0.4% Triton X-100, 2 mM EDTA, 4 mM DTT, 5 mg/ml BSA, and 0.5 μ M ATP) at 4°C with gentle rocking for 1 h to allow for protein renaturation. Radiolabelled DNA fragment (either the 464 bp *Sma* I-*Xho* I *oriC* fragment from pBS*oriC* (3) or a 466 bp *Bam* HI restricted PCR-generated fragment corresponding to the C-terminus of DnaA that did not contain a DnaA box motif) was then added at a ratio of 1 to 30 (pmol protein:fmol DNA) and incubated at 4°C for an additional 5 h. 3' end-labelling was performed with the large fragment of DNA polymerase I and the appropriate [α^{32} P]dNTP (Dupont, NEN). Membranes were then washed in binding buffer at 4°C to remove unbound fragment. Quantitation of the amount of radiolabelled DNA fragment retained by each protein sample was by use of a Packard Instant Imager relative to a standard curve of the respective radiolabelled DNA fragment. Bound DNA was stripped from the membrane by incubation in 154 mM NaCl, 10 mM Tris-HCl pH 7.4, and 0.25% SDS. Membranes were then processed as western blots as described previously (31) using a monoclonal antibody specific to DnaA (M43 that recognizes residues 133 to 141) followed by a secondary antibody that was labelled with ^{125} I (ICN Biochemicals). The amount of DNA retained by each protein sample was then normalized for the amount of that protein present on the membrane based on ^{125} I counts relative to a standard curve.

DnaA Δ 62, DnaA Δ 129, and DnaA Δ 219 were retained on Westran membrane as efficiently as was DnaA under the conditions used for

electrotransfer (data not shown). Consequently, the amount of protein present for these samples was assumed to be equivalent to that loaded onto the SDS-PAGE as was the case with wild type DnaA.

DNA binding assays. Fragment retention assays (20 μ l) were performed as described (10) and contained 20 fmol of the indicated 3' end-labelled DNA fragment and 50 ng (17.5 fmol as nucleotide) *Hinf* I digested pBR322 as competitor (unless otherwise stated) in buffer containing 40 mM HEPES-KOH pH 7.8, 5 mM MgCl₂, 50 mM KCl, 2 mM DTT, and 0.5 μ M ATP. DNA fragments used for fragment retention assays included the 464 bp *Sma* I-*Xho* I *oriC* fragment from pBS*oriC* (3), the 1,097 bp *Ban* I ColE1 origin fragment from pBluescript, the 686 bp *Eco* RI-*Spe* I pSC101 origin fragment from pCM128 (33), a 417 bp fragment containing the *dnaA* promoter region obtained by PCR using the oligonucleotide primers JK-23 (5'-AAGCCAATTTTGTCTATGG-3' that is complementary to positions -302 to -321 of the template strand) and the M13 -40 universal primer (U. S. Biochemicals) and pRB1A (6) as the DNA template, and a 466 bp fragment corresponding to the C-terminus of the *dnaA* gene that was used as a nonspecific DNA fragment that was also generated by PCR using the oligonucleotide primers JK-6 (see above for the sequence of JK-6) and JK-29 (5'-GATGGCGCTGGCGAAAGAGC-3' that is complementary to positions 1,230-1,249 of the template strand) and pACYC*dnaA* as the DNA template. Following incubation at 30°C for 10 min with the indicated amount of DnaA or T435M, reactions were filtered through nitrocellulose filters (Millipore HWAP, 0.22 μ M, 13 mm), washed with 250 μ l of the above buffer equilibrated to 30°C, and dried. Bound DNA was quantitated by liquid scintillation counting.

Alternatively, binding to the same 3' end-labelled 464 bp *Sma* I-*Xho* I *oriC* fragment, or to the following 22 bp duplex oligonucleotide corresponding to

box R4 of *oriC* and flanking sequences was measured by gel mobility shift assays (25) (the DnaA box is indicated by bold type):



Reactions (10 μ l) contained the indicated amount of wild type or mutant DnaA protein, 25 fmol of the indicated 3' end-labelled DNA substrate, and 50 ng (17.5 fmol as nucleotide) of *Hinf* I digested pBR322 as competitor in buffer containing 20 mM HEPES-KOH pH 7.6, 2.5 mM MgOAc, 15% glycerol, 0.4% Triton X-100, 2 mM EDTA, 4 mM DTT, 5 mg/ml BSA, and 0.5 μ M ATP. Reactions were electrophoresed through 4% (for reactions containing *oriC*) or 10% (for reactions containing the oligonucleotide) native polyacrylamide gels (60 acrylamide:1 bis-acrylamide) in 90 mM Tris-borate pH 8.3 and 1 mM EDTA, dried, and exposed to Hyperfilm MS (Amersham) at -70°C using a Cronex Quanta III intensifying screen.

Competitive gel mobility shift assay. Competitive gel mobility shift assays were performed as described above using the same *oriC* fragment and the indicated amounts of DnaA or T435M except that in place of *Hinf* I digested pBR322 as competitor, either the same unlabelled *oriC* fragment or the 1,031 bp *Afl* III-Ava II fragment from pBluescript were used as competitor at the indicated concentrations. DNAs were added prior to addition of proteins and reactions were incubated for 10 min at room temperature prior to electrophoresis through 4% native gels. Quantitation of the bound and free radiolabelled *oriC* fragment was by use of a Packard Instant Imager.

ATP binding assays. Reactions (25 μ l) were performed as described (29) and contained 1 pmol (52 ng) of DnaA or T435M and the indicated amount of [γ^{32} P]ATP (Dupont, NEN) in buffer containing 50 mM Tris-HCl pH 8.0, 0.5 mM MgOAc, 15% glycerol, 0.01% Triton X-100, and 5 mM DTT. Incubations were at

0°C for 10 min followed by filtration through nitrocellulose filters (Millipore HWAP, 0.22 µM, 13 mm) that were equilibrated in ATP binding buffer. Filters were washed with 0.5 ml of the same buffer and dried. Bound [$\gamma^{32}\text{P}$]ATP was quantitated by liquid scintillation counting.

Assay of *in vitro* replication. Assays of *in vitro* replication were performed as described previously using a crude protein fraction deficient in DnaA protein (11).

Results

The C-terminal 89 residues of DnaA are required for specific DNA binding. DnaA protein binds to four DnaA boxes within *oriC* to promote initiation of replication, as well as to DnaA boxes within origins of plasmids and bacteriophage that require this protein for their replication (10). To determine the region of DnaA protein required for DNA binding activity, quantitative Southwestern blot analysis was performed with deletion mutants that lacked N-terminal, C-terminal, or internal coding sequences (Figure 1).

The respective ability of DnaA protein to retain a DNA fragment containing *oriC* (464 bp) was compared to a DNA fragment from the C-terminus of the *dnaA* gene (466 bp) lacking a DnaA box motif. This analysis indicated that the *oriC* fragment was retained approximately 1.5-fold more efficiently (Figure 2). Mutant proteins lacking either N-terminal or internal sequences retained the *oriC* fragment with an efficiency similar to DnaA protein, indicating that the N-terminal 378 residues of DnaA are not required for specific DNA binding (Figure 3 and Table 2). Deletion mutants lacking C-terminal sequences were defective in retaining the *oriC* fragment, suggesting that this region is required for DNA binding activity.

To confirm that residues N-terminal to position 379 were dispensable for DNA binding activity, gel mobility shift assays were performed with an *oriC*-containing fragment or a duplex oligonucleotide containing a DnaA box (box R4 of *oriC* and natural flanking sequences) (data not shown). Consistent with the results of Southwestern blotting, the deletion mutants, DnaA Δ 219 (lacking the N-terminal 219 residues), DnaA Δ 220-294, and DnaA Δ 237-378 (see Figure 1), were able to bind to either DNA in a manner comparable to that observed with the wild type protein. Attempts to overexpress the C-terminal 88 residues

Figure 1. Structures of truncated forms of DnaA protein. Structures of the truncated forms of DnaA protein used in this study are indicated. Numbers refer to amino acid residues. DnaA Δ 62, DnaA Δ 129, and DnaA Δ 219 code for a methionine at positions 62, 129, and 219, respectively. DnaA Δ 220-294 and DnaA Δ 237-378 are in-frame deletions. All were constructed *in vitro* as described in Materials and Methods except *dnaA* Δ 237-378 that was identified using a novel selection for mutations of *dnaA* (see Chapter IV).

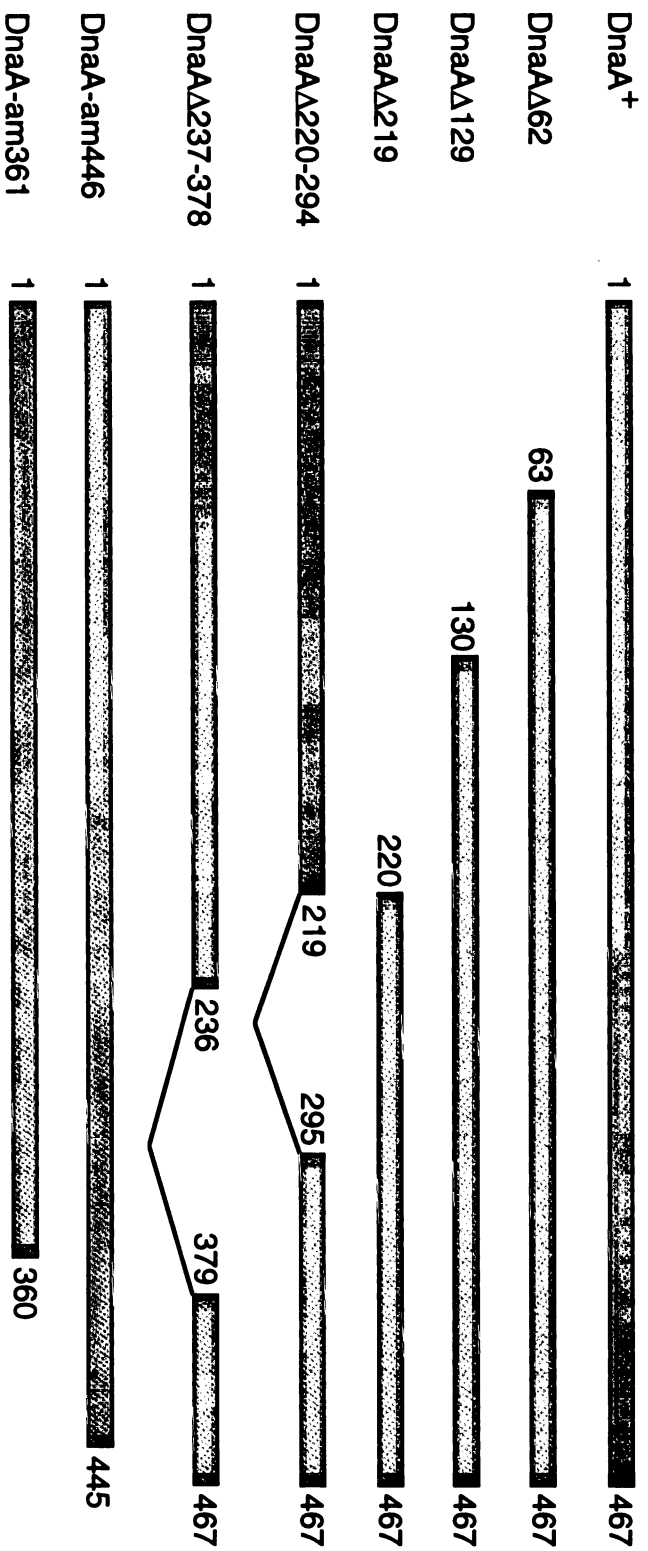


Figure 2. DnaA protein binds preferentially to DNA fragments containing *oriC* by Southwestern blot analysis. Southwestern blot analysis was performed in duplicate using the indicated amounts of DnaA protein as described in Materials and Methods. Samples were run on the same 10% SDS-PAGE. After electrotransfer, Westran membrane was cut in half. One half was probed with a 464 bp radiolabelled DNA fragment containing *oriC* (O), and the other with a 466 bp radiolabelled DNA fragment corresponding to the C-terminus of the *dnaA* gene that lacked a DnaA box motif (□). Both halves were incubated in identical volumes of binding buffer containing equivalent amounts (fmol) of the respective radiolabelled DNA fragments. Quantitation of the amount (fmol) of each radiolabelled DNA fragment retained by DnaA⁺ was with a Packard Instant Imager. To calculate fmol of DNA retained, the specific activity of each DNA fragment was determined simultaneously with quantitation of the Southwestern blot by counting known amounts of each fragment.

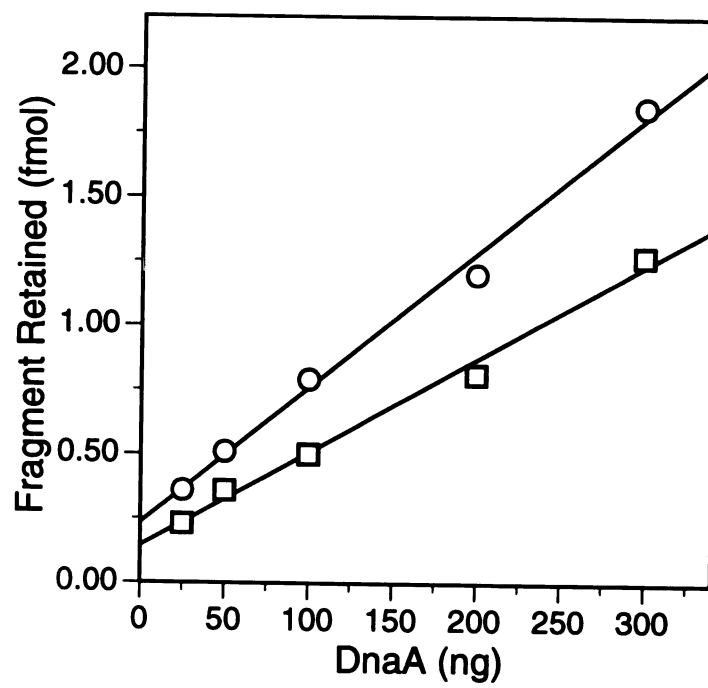


Figure 3. Southwestern blot analysis of truncated forms of DnaA protein. A representative Southwestern blot probed with a radiolabelled DNA fragment containing *oriC* is shown. Truncated forms of DnaA protein used in this analysis were greater than 50% pure as determined by SDS-PAGE (data not shown). Prior to Southwestern blotting, the membrane was stained with Ponceau S to confirm that proteins were transferred to the Westran membrane (data not shown). Similar experiments were performed to obtain the data presented in Tables 2 and 3 except that Ponceau S staining was replaced by quantitative immunoblot analysis to normalize fmol DNA retained to protein concentration as described in Materials and Methods.

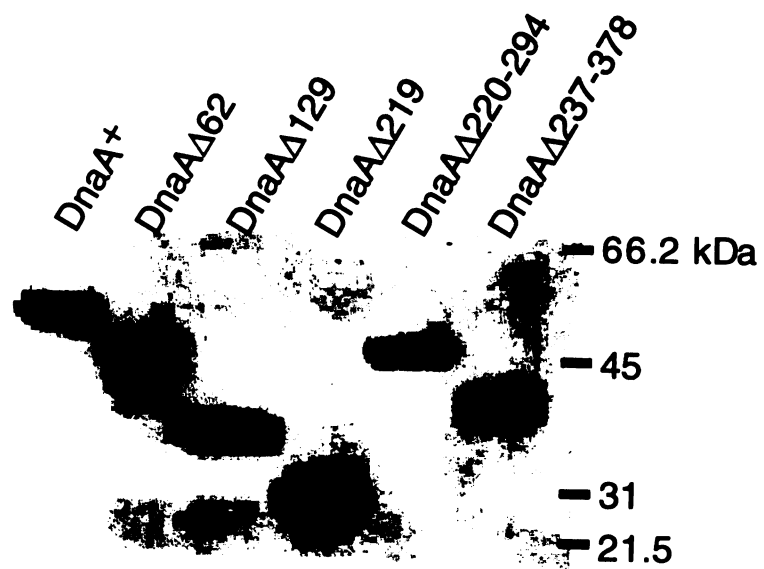


TABLE 2. Quantitative Southwestern blot analysis of truncated forms of DnaA^a

Protein	Amino acids deleted	Relative retention of <i>oriC</i> ^b
DnaA ⁺	None	≡1.00
DnaA-am446	446-467	ND ^c
DnaA-am361	361-467	ND
DnaAΔ62	2-62	1.34 (±0.47)
DnaAΔ129	2-129	1.33 (±0.41)
DnaAΔ219	2-219	1.19 (±0.22)
DnaAΔ220-294	220-294	1.04 (±0.29)
DnaAΔ237-378	237-378	0.92 (±0.14)

^a Binding to *oriC* was measured by Southwestern blot analysis using 200 ng of each protein as described in Materials and Methods.

^b Retention of *oriC* is expressed relative to that observed by DnaA⁺ that was normalized to 1.00. Depending on the experiment, 200 ng of DnaA⁺ retained between 0.9 and 1.8 fmol of the *oriC* fragment. Each value is normalized and represents the average of at least three experiments ± the standard deviation (calculated after normalization).

^c ND, none detected. Binding to *oriC* was not detected by either autoradiography or scanning with a Packard instant imager.

of DnaA protein to measure its DNA binding activity were unsuccessful. The reason may relate to proteolytic instability of the recombinant protein.

Mutants of DnaA protein with substitutions within the C-terminal 89 residues are defective in DNA binding. The above results suggested that residues from position 379 to the C-terminus are sufficient for DNA binding activity. To substantiate this conclusion, eleven mutants containing amino acid substitutions in this region were examined by quantitative Southwestern blotting (Table 3). All were much less efficient at retention of the *oriC* fragment compared to DnaA.

DNA binding activity was also measured in a gel mobility shift assay with a duplex oligonucleotide containing a DnaA box motif (box R4 of *oriC* and natural flanking sequences) and a constant amount of wild type or mutant DnaA protein (Figure 4B). Whereas none of the mutant proteins formed a complex with the oligonucleotide at the level tested, wild type DnaA protein did at levels as low as 2 ng (Figure 4A).

Due to proteolysis of the mutants V383M (with a V-to-M mutation at position 383), A412P, and A428T-A440T on induced expression, we were not able to purify them for biochemical study to corroborate the results of Southwestern analysis. Proteolysis was indicated by the observation that they were overexpressed efficiently then rapidly converted, over time, from a species that migrated to a position similar to purified DnaA protein in an SDS-PAGE (as monitored by Immunoblotting) to a mixture of smaller species. For these mutants, full length protein was recovered only if induced expression was for 45 minutes instead of for 3 hours that was used for the other missense mutants, followed by cell lysis on ice by sonication in the presence of phenylmethylsulfonyl fluoride (PMSF) and addition of SDS (0.25%) to the soluble fraction. With these exceptions, results from these two methods indicate that mutants

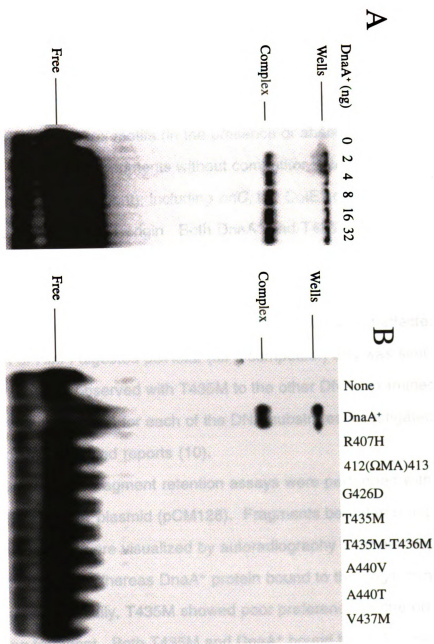
TABLE 3. Mutant proteins with substitutions in the C-terminal region of DnaA protein are defective in binding to *oriC*^a

Protein	Relative retention of <i>oriC</i> ^b
DnaA ⁺	≅1.00
V383M	0.10
R407H	0.18
A412P	0.06
412(ΩMA)413	0.07
G426D	0.12
T435M	0.46
T435M-T436M	0.28
V437M	0.09
A440V	0.08
A440T	0.12
A428T-A440T	0.12

^a Binding to *oriC* was measured by Southwestern blot analysis using 200 ng of each protein as described in Materials and Methods .

^b Retention of *oriC* is expressed relative to that observed by DnaA protein that was normalized to 1.00 (200 ng of DnaA retained 1.8 fmol of the *oriC* fragment).

Figure 4. Mutant forms of DnaA protein are defective in DNA binding. (A) Binding of DnaA⁺ protein to a duplex oligonucleotide (25 fmol) containing nucleotide sequence corresponding to that of box R4 of *oriC* (and natural flanking sequences) by gel mobility shift analysis. The amounts of DnaA protein used are indicated. assays were performed as described in Materials and Methods. **(B)** Mutant forms of DnaA protein (25 ng) were assayed for their ability to bind the same duplex oligonucleotide as above. The amino acid, the substitution, and its position are indicated for each of the mutants (i.e. R407H, arginine-to-histidine substitution at position 407; 412(ΔMA)413, Met, Ala insertion between residues 412 and 413). The positions of wells, complexes, and free oligonucleotide are indicated.



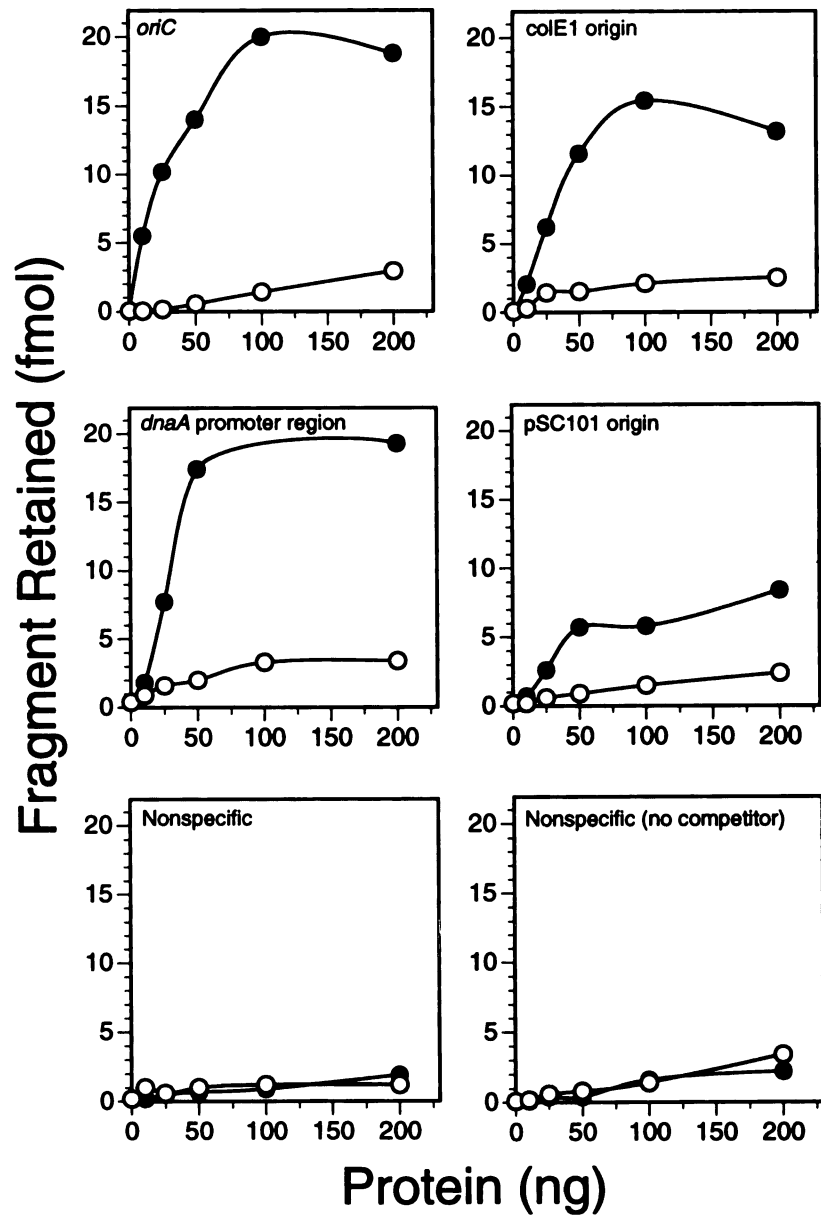
containing substitutions near the C-terminus of DnaA protein are defective in DNA binding.

T435M is defective in specific DNA binding activity. As T435M retained the greatest affinity for *oriC* as measured by Southwestern blotting, its DNA binding activity was investigated in more detail by nitrocellulose fragment retention assays using a variety of DNA substrates (Figure 5). In comparison to DnaA⁺ protein, T435M was inefficient in retention of DNA fragments containing one or more DnaA box motifs (in the presence or absence of competitor DNA; data not shown for experiments without competitor DNA except in the case of the “nonspecific” fragment), including *oriC*, the ColE1 origin, the *dnaA* promoter region, and the pSC101 origin. Both DnaA⁺ and T435M were similarly ineffective in binding to a DNA fragment lacking a DnaA box (this DNA fragment termed “nonspecific” was from the C-terminal coding region of the *dnaA* gene). The extent of retention by either protein to this DNA was not affected by the inclusion of *Hinf* I digested pBR322 (as a competitor) and was similar in extent of binding to that observed with T435M to the other DNAs examined. The relative affinity of DnaA for each of the DNA substrates investigated was similar to previously published reports (10).

Comparable fragment retention assays were performed with a restriction digest of a pSC101 plasmid (pCM128). Fragments bound and not bound by DnaA⁺ or T435M were visualized by autoradiography after electrophoresis (data not shown). Whereas DnaA⁺ protein bound to the origin-containing fragment preferentially, T435M showed poor preference for the origin-containing fragment. Both T435M and DnaA⁺ bound to DNA nonspecifically at protein concentrations of 100 to 200 ng. Collectively, these observations suggest that the DNA binding activity of T435M is largely nonspecific.

To demonstrate further that T435M was defective in the ability to bind

Figure 5. T435M is defective in high affinity DNA binding. Fragment retention assays with wild type DnaA protein (●) or T435M (○) and 20 fmol of each DNA fragment were performed as described in Materials and Methods. All reactions, except that indicated with the nonspecific DNA substrate, contained *Hinf* I digested pBR322 (50 ng) as competitor DNA.



specifically to DNA, the effect of competitor DNAs on its binding to a fragment containing *oriC* was examined. In a gel mobility shift assay, it was able to bind to a DNA fragment containing *oriC* (Figure 6). DnaA⁺ protein formed several discrete complexes. These have been shown to result from the ordered binding of DnaA protein to the four DnaA boxes in *oriC* (19). In contrast, T435M formed a smear from the position of free DNA to the wells, suggesting nonspecific binding. Furthermore, at least 8-fold more T435M was required relative to DnaA to shift all of the *oriC* fragment from the “free” position. With this DNA binding assay, the effect of competitor DNAs on the DNA binding activities of DnaA⁺ and T435M was examined.

Binding of fixed amounts of DnaA⁺ or T435M to a radiolabelled *oriC* fragment was measured in the presence of increasing amounts of either the same *oriC* fragment (unlabelled) or a 1,031 bp fragment (unlabelled) from pBluescript (Figure 7). The 1,031 bp DNA fragment lacks a DnaA box motif. Inasmuch as the 1,031 bp fragment was about 2-fold larger in size than the *oriC* fragment (464 bp), it was a poor competitor for binding of wild type DnaA to *oriC*. However, the 1,031 bp fragment was comparable to the unlabelled *oriC* fragment in reducing binding of T435M to labelled *oriC* (a 4-to-4.5-fold excess of unlabelled *oriC* was required relative to a 7-to-7.5-fold excess of the 1,031 bp fragment to compete away 50% of bound radiolabelled *oriC* from T435M). These results indicate that the affinity of T435M for a nonspecific fragment was similar (within 2-fold) to that for *oriC*.

A 4-to-4.5-fold molar excess of unlabelled *oriC* was required to compete away 50% of the radiolabelled *oriC* fragment bound to DnaA protein (or T435M). This was presumably due to the fact that ~95-98% of the radiolabelled fragment was shifted from the “free” position. As complexes II through VI appear to contain more than one DnaA monomer per *oriC* fragment (19), stoichiometric

Figure 6. T435M does not form discrete complexes by gel mobility shift analysis. The DNA binding activity of T435M was measured by use of a gel mobility shift assay as described in Materials and Methods with the indicated amounts of wild type DnaA protein or T435M.

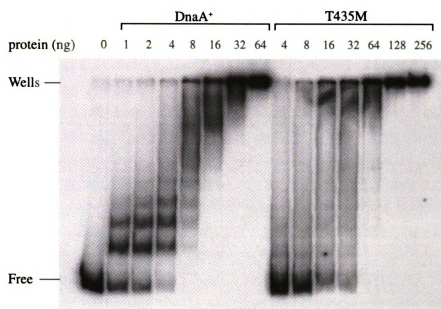
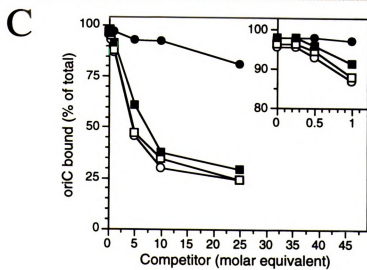
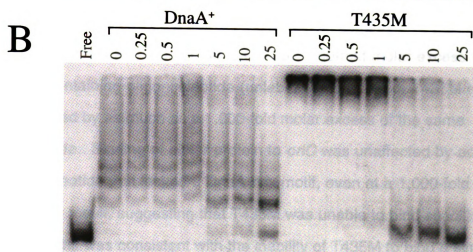
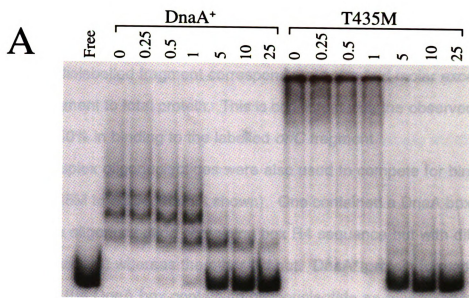


Figure 7. Competitive gel mobility shift analysis of DnaA and T435M. Competitive gel mobility shift assays were performed as described in Materials and Methods. Binding of wild type DnaA protein (1.9 ng) or T435M (18 ng) to 25 fmol of a radiolabelled DNA fragment containing *oriC* (464 bp) was competed by addition of increasing molar equivalents (from 0 to 25X) of either the same unlabelled *oriC* fragment (**A**), or a DNA fragment from pBluescript (1,031 bp) lacking a DnaA box motif (**B**). Quantitation of the percent of radiolabelled *oriC* fragment bound (i.e. counts shifted from the “free” position) relative to the total *oriC* fragment present in that reaction (i.e. total counts in that lane) (**C**) was determined with a Packard Instant Imager. Symbols are: ○, DnaA⁺ competed with cold *oriC* fragment; ●, DnaA⁺ competed with cold nonspecific fragment; □, T435M competed with cold *oriC* fragment; ■, T435M competed with cold nonspecific fragment.



(i.e. 1:1 molar ratio of radiolabelled *oriC* to unlabelled *oriC*) competition was not observed. The requirement for 4-to-4.5-fold more unlabelled *oriC* fragment relative to radiolabelled fragment corresponds to a two-fold molar excess of total *oriC* fragment to total protein. This is consistent with the observed reduction of 50% in binding to the labelled *oriC* fragment.

Two duplex oligonucleotides were also used to compete for binding of DnaA⁺ or T435M to *oriC* (data not shown). One contained a DnaA box motif (similar to the oligonucleotide containing box R4 sequence but with different flanking sequences) whereas the other did not. DnaA⁺ protein formed a single complex with the DnaA box-containing oligonucleotide as measured by gel mobility shift assays (data not shown). Whereas binding of DnaA⁺ protein to radiolabelled *oriC* was reduced by 55-60% by a 50-fold molar excess of the DnaA box-containing oligonucleotide (unlabelled), binding of T435M to *oriC* was unaffected by as much as a 1,000-fold molar excess of the same oligonucleotide. Binding of either protein to *oriC* was unaffected by addition of the oligonucleotide that lacked a DnaA box motif, even at a 1,000-fold molar excess. This result, suggesting that T435M was unable to bind specifically to a DnaA box motif was consistent with the inability of T435M to bind a similar oligonucleotide containing sequence corresponding to box R4 of *oriC* (see Figure 4).

Mutant proteins are poorly active for *in vitro* replication of an *oriC* plasmid. Of the eleven mutant proteins containing C-terminal substitutions, five (R407H, A412P, 412(ΩMA)413, G426D, and T435M-T436M) are inactive for replication from both *oriC* (data not shown) and the pSC101 origin *in vivo* (see Chapter III). Three of the remainder (T435M, A440V, and A428T-A440T) were poorly active for pSC101 replication whereas the other three (V383M, V437M, and A440T) retained replication activity at 30°C.

However, the ability of the latter six mutants to sustain replication of the bacterial chromosome from *oriC* is unknown. As the mutant proteins characterized in this study were defective for DNA binding (Figure 4), we expected that they would also be defective for *in vitro* replication of an *oriC* plasmid.

Of the eleven mutants, eight were assayed biochemically for their ability to initiate replication from *oriC in vitro*. We were unable to purify the others (V383M, A412P, and A428T-A440T) because of proteolytic degradation after induced expression (data not shown).

Whereas DnaA protein sustained replication of an *oriC* plasmid *in vitro*, T435M (see Figure 10 below) and A440V were inactive, even at concentrations as high as 200 ng (Figure 8A). A440T was partially active at 30°C but less so at 40°C, consistent with its temperature sensitive phenotype *in vivo* for pSC101 replication (see Chapter III). T435M was poorly active even when assayed at high concentrations for a prolonged period of time (Figure 8B). Although V437M was fairly active for pSC101 replication *in vivo*, it was inactive for replication from *oriC in vitro*. The inactivity of the mutant proteins in replication of an *oriC* plasmid *in vitro* is presumably due to their defect in DNA binding.

T435M is active for ATP binding. DnaA protein binds ATP and ADP with high affinity (K_D of 0.03 and 0.1 μ M, respectively) (29). Whereas the ATP-bound form is active for *in vitro* replication of an *oriC* plasmid, the ADP-bound form is much less active. To confirm that the defect in DNA binding activity of T435M is specific, the ability of T435M to bind ATP was measured and found to be comparable to DnaA protein (Figure 9). The calculated dissociation constants and estimated stoichiometries (n values) for both T435M (K_D 15 nM and $n=0.42$, respectively) and wild type DnaA (K_D 17 nM and $n=0.14$, respectively) were consistent with those reported previously for the wild type protein (K_D 0.03 μ M and $n=0.48$, respectively) (29).

Figure 8. Mutant forms of DnaA are poorly active for *in vitro* replication from *oriC*. Mutant proteins were assayed for *in vitro oriC* replication activity at 30° (□, ○) and 40°C (■, ●) using the indicated amounts of protein as described in Materials and Methods. **(A)** A440V (inset) was representative of all the mutant proteins except A440T (□) that retained partial replication activity. Results for R407H, G426D, 412(ΩMA)413, T435M (see inset to Figure 10), T435M-T436M, and V437M are not shown. None of the mutant proteins (except A440T) were active for *in vitro* replication at concentrations as high as 200 ng. Wild type DnaA (○) was saturating for *in vitro* replication of *oriC* at levels where A440T was just beginning to show replication activity. **(B)** Time course of wild type DnaA and T435M for *in vitro oriC* replication. 50 ng of wild type DnaA protein (○) or 200 ng T435M (□) were assayed for replication activity. Reactions were incubated at 30° (□, ○) or 40°C (■, ●) and aliquots were removed at the indicated time points and quenched as described in Materials and Methods.

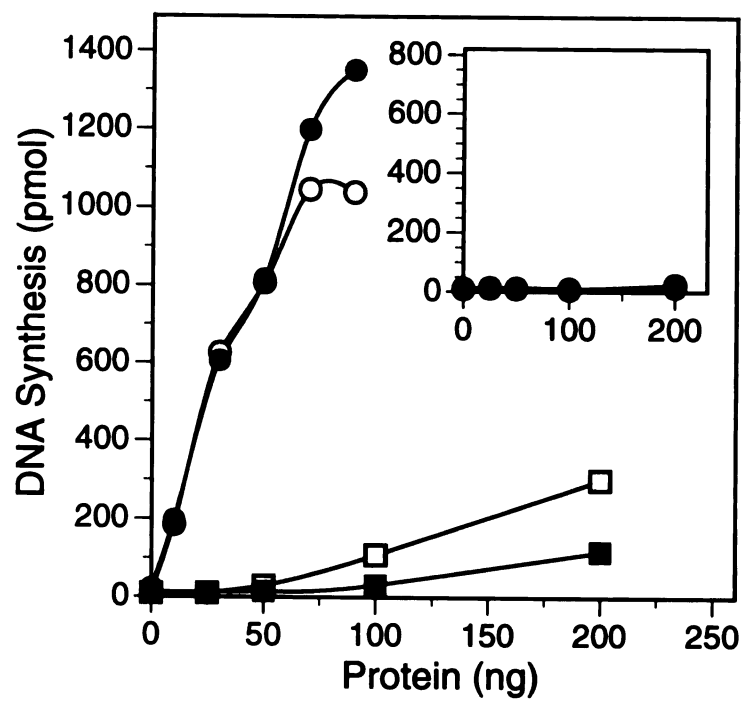
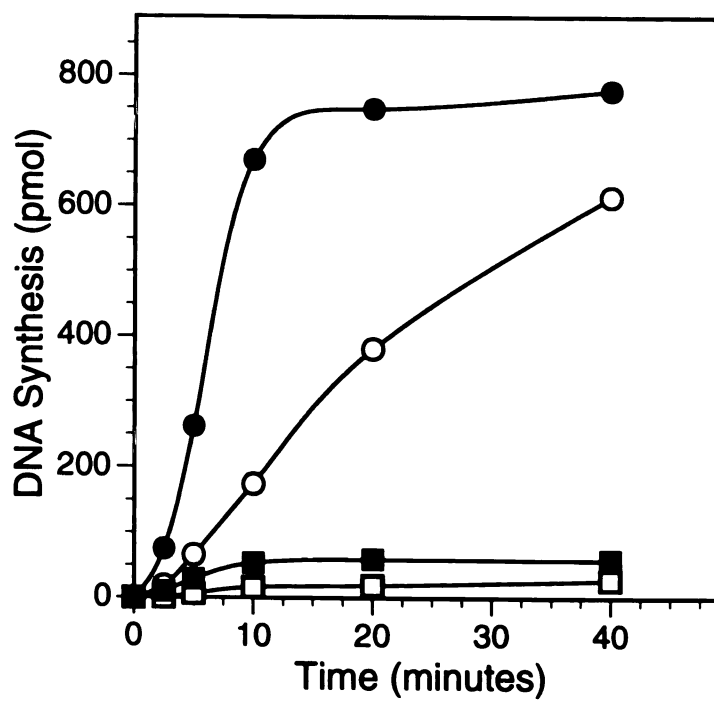
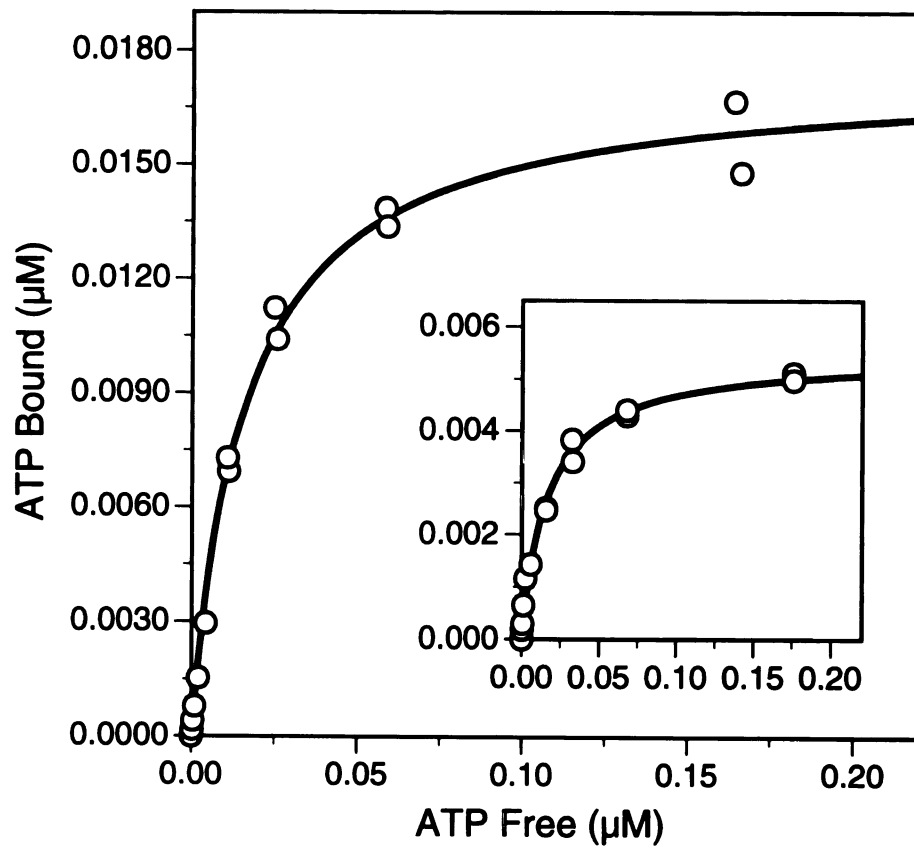
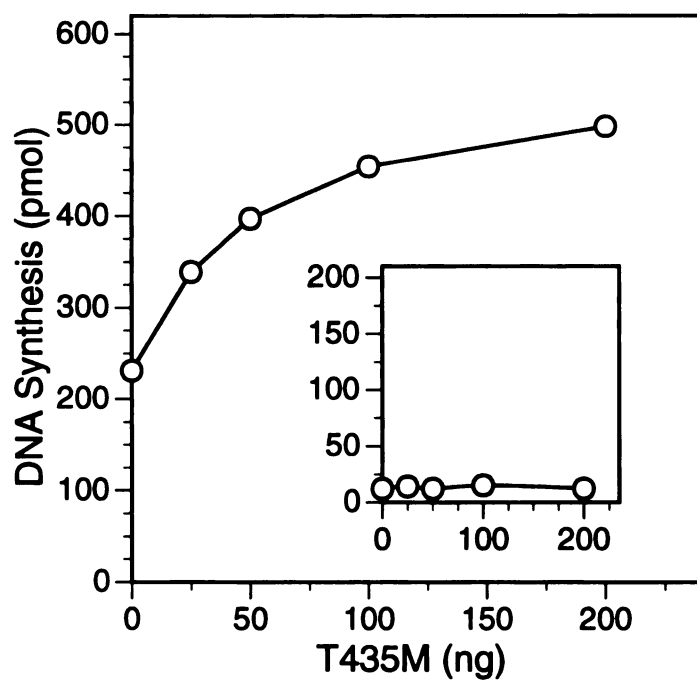
A**B**

Figure 9. T435M binds ATP with high affinity. ATP binding assays were performed with T435M or wild type DnaA protein (inset) as described in Materials and Methods.



T435M augments limiting levels of wild type DnaA for *in vitro* *oriC* replication. By electron microscopy studies, it is estimated that 20-30 monomers of DnaA bind to *oriC* resulting in a large nucleoprotein complex referred to as the initial complex (10). As *oriC* contains only four DnaA boxes, initial binding is speculated to be followed by nonspecific DNA binding and by protein-protein interactions between DnaA monomers. T435M retains activities of ATP binding and binding to DNA nonspecifically, but was inactive in *in vitro* replication. We performed experiments to determine if T435M could augment limiting levels of wild type DnaA protein (Figure 10). Our observations that T435M is inactive alone but augments limiting levels of DnaA protein suggests that monomers of DnaA interact in formation of an active replication complex.

Figure 10. T435M augments limiting levels of DnaA for *in vitro* replication from *oriC*. 10 ng of wild type DnaA protein and the indicated amount of T435M were assayed for *in vitro oriC* replication as described in Materials and Methods. Reactions were incubated at 30°C for 20 minutes. The inset shows the replication activity observed for T435M alone.



Discussion

The DNA binding domain of DnaA protein is within the C-terminal 89 residues of DnaA protein. Two approaches were used to identify amino acid residues of DnaA protein that are involved in sequence-specific DNA binding. The first involved the genetic characterization of novel mutations with an *in vivo* assay of DNA binding (see Chapter IV). Here, expression of *lacZ* was under control of the *dnaA* promoter and DNA binding was measured indirectly by quantitation of β -galactosidase activity. Binding of DnaA protein to a DnaA box within the *dnaA* promoter region results in transcriptional repression (34). With this approach, missense and deletion mutations of *dnaA* were identified that were defective in repression of *dnaA*'-'*lacZ* expression, suggesting that the C-terminus of DnaA protein is involved in DNA binding.

The second approach involved biochemical characterization of these mutant proteins to obtain direct evidence that the C-terminus of DnaA is involved in DNA binding. To complement the internal and C-terminal deletion mutants, N-terminal deletion mutants were constructed. Three different methods to measure DNA binding activity were used. Southwestern analysis offers the advantage that a large number of samples can be analyzed without extensive purification. However, inactivity in this assay may be due to poor renaturation of the protein due to the mutation(s). The two other biochemical methods of nitrocellulose filter binding and gel mobility shift were performed with partially or highly purified proteins. Except for the three mutant proteins that were proteolytically unstable, the results obtained with the latter two methods corroborated the Southwestern analysis, and the results obtained using the genetic assay (see Chapter IV). The proteolysis observed with

V383M, A412P, and A428T-A440T that hindered their purification is likely not related to the phenotypes of these mutants in the genetic assay. Western blot analysis of these mutants expressed from the *dnaA* promoter region indicated that they were present at levels comparable to that observed for wild type DnaA protein (see Chapters III and IV). Taken together, these results not only indicate that sequences within 88 residues of the C-terminus are involved in DNA binding, but also suggest particular amino acid residues that may be involved in contact with DNA.

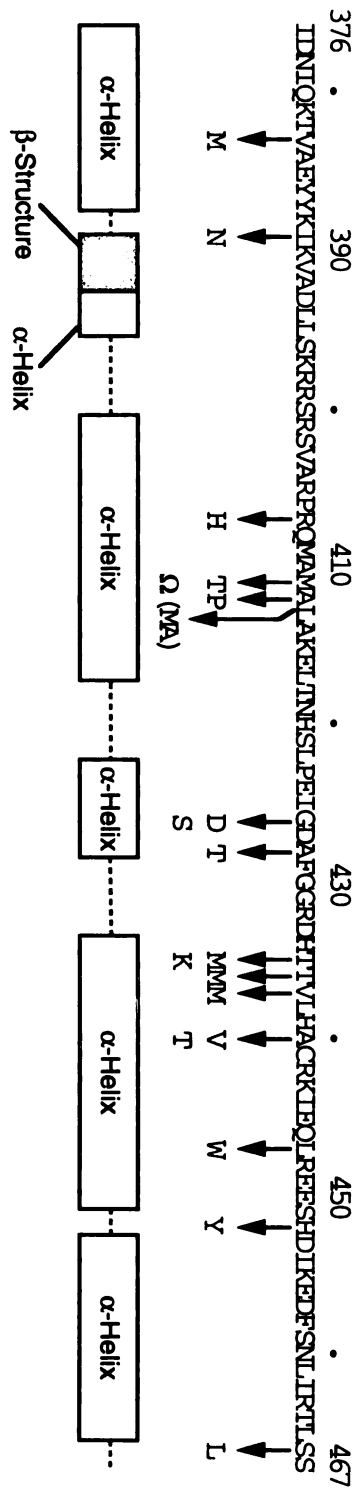
Regarding the last issue, one mutation that encodes the substitution of threonine for methionine at position 435 is particularly interesting. This substitution dramatically reduces the ability of DnaA protein to bind specifically to DNA fragments containing DnaA box motifs. As expected, the mutant protein is inactive in *in vitro* replication by itself but retains its affinity for ATP. These results suggest that threonine 435 plays an important role in recognizing and binding to sequences of the DnaA box. Finally, the finding that this mutant can augment limiting levels of wild type DnaA protein suggests strongly that monomers of DnaA bound to *oriC* interact physically in initiation of DNA replication.

A mutation mapping to position 435 (T435K) was identified by the Walker laboratory based on its ability to suppress the temperature sensitive phenotype of the *dnaX2016*(Ts) allele (14). *dnaX* encodes the γ and τ subunits of DNA polymerase III holoenzyme (16). In light of the biochemical characterization of T435M, the ability of T435K to suppress the temperature sensitive phenotype of the *dnaX2016*(Ts) allele is presumably due to an altered DNA binding activity of the T435K mutant protein. However, biochemical characterization of the DNA binding activity of the T435K mutant would be necessary to substantiate this premise.

Proposed secondary structure for the DnaA binding domain of the *E. coli* DnaA protein. A secondary structure for the DNA binding domain of DnaA protein was obtained using the PHD method (26) (Figure 11). This analysis predicted the presence of six α helical regions and one short stretch of β structure encompassing the C-terminal 92 residues of the protein. The majority of the mutations that affected DNA binding activity mapped to one of three proposed α helical regions encompassing residues 401-451. Although the proposed structure of the DNA binding domain of DnaA protein does not contain a canonical DNA binding domain (for a review see reference 23), it is reminiscent of the helix-turn-helix motif. In a helix-turn-helix type motif, one helix confers specificity and a second helix is involved in nonspecific interactions with the phosphate backbone. Assuming threonine 435 is within a helix that confers specificity, this type of motif is consistent with the DNA binding characteristics of T435M.

Whether the amino acids identified in this report are involved in specific contacts between DnaA protein and the DNA substrate, or that they serve a structural role may be addressed by crystallographic or NMR analysis of the wild type and/or truncated forms of the protein.

Figure 11. Secondary structure prediction of the DNA binding domain of DnaA protein. The proposed secondary structure for the DnaA binding domain of the *E. coli* DnaA protein was generated using the PHD method (26). Positions of mutations and their deduced substitutions for all known *dnaA* mutations (reviewed in reference 21) as well as those described in this thesis (see Chapters II and III) are indicated.



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Chapter VI

Summary and Perspectives

To correlate the structure of DnaA protein to its activity in initiation of chromosomal DNA replication, essential residues were identified in a collection of novel *dnaA* alleles that were obtained using two independent genetic approaches. Both relied on pSC101, a plasmid that is completely dependent on *dnaA* function for its replication (3, 4, 7). Thirty three missense mutants were obtained identifying 36 different residues that when substituted affect the activity of DnaA protein for pSC101 replication. DNA sequence analysis indicated that all but one were novel. In addition to the missense mutations, seven different nonsense mutations, and one allele encoding an in-frame deletion were obtained. Characterization of these alleles using various genetic assays identified four different classes of mutations, suggesting four functionally distinct domains.

Two mutants, *dnaA* Δ 237-378 and *dnaA-am361*, were temperature sensitive for pSC101 replication but were inactive for replication from *oriC*. A nonsense mutation that encodes a stop codon for residue 245 was inactive for pSC101 replication, suggesting that the domains defined by *dnaA* Δ 237-378 and *dnaA-am361* were alternately dispensable for pSC101 replication. The C-terminal 89-to-94 residues of DnaA protein are required for DNA binding (see below and reference 10). Based on these findings, we propose that a region within residues 237 through 360-to-378 is involved in a direct, physical interaction with the pSC101-encoded initiator, RepA. A DnaA-RepA interaction was proposed previously based on the observation that RepA protein can facilitate the binding of DnaA protein to the pSC101 origin (14).

In support of this model, genetic characterization of three *dnaA* alleles that were specifically defective for pSC101 replication indicated that one or more activities of DnaA protein required for replication of pSC101 are not required by *oriC*. We speculated that the residues altered by these alleles

affects a proposed interaction with RepA. This interaction may be through direct contact, or at the functional level. One of the three mutations substitutes glycine 287 with serine that is within the region proposed to interact with RepA protein.

In this thesis, the function of DnaA protein in replication of pSC101 and *oriC* can be distinguished mutationally. A future line of experimentation to investigate these observations at the molecular level involves the development an *in vitro* replication system for pSC101. With this assay, the replication activities of relevant DnaA mutants for *oriC* and pSC101 can be examined to confirm their replicon-specific defect, and to identify specific biochemical defects required for one replicon but not the other. More insight into the mechanism of DnaA protein function in replication of the bacterial chromosome and pSC101 will be obtained.

Mutations mapping to the N-terminal 64 residues were temperature sensitive for replication but did not exhibit a mutant phenotype in other genetic assays. Purification and biochemical characterization of representative mutants from this class will identify their specific defect(s) and elucidate the function(s) of the N-terminus of DnaA protein.

A P-loop motif mapping to residues 172-179 is thought to constitute a portion of the ATP-binding site of DnaA protein (11). Certain *dnaA* alleles described in this thesis mapping within or near this P-loop exhibited a cold sensitive phenotype when contained in a plasmid under control of the *dnaA* promoter region and harbored in an *E. coli* strain with a chromosomally encoded *dnaA*⁺ allele. These mutations were temperature sensitive for pSC101 replication. A similar cold sensitive phenotype has been reported for *dnaA* mutations that contain an alanine-to-valine substitution at position 184 (6). The *dnaA5* and *dnaA46* alleles both contain this substitution and are defective for high affinity ATP binding *in vitro* (8, 9). These observations suggest that the

cold-sensitive heteropolyploid phenotype may be due to an ATP binding defect. Biochemical characterization of these novel mutants will confirm a defect in ATP binding activity. Furthermore, it may be possible to determine the cause of the cold-sensitive heteropolyploid phenotype by correlation of the biochemical defects of those DnaA mutants mapping to the P-loop and do not exhibit the phenotype with those that do.

Normally, rapidly growing bacterial cells initiate replication from multiple origins simultaneously (12). This event is termed synchrony in initiation. *dnaA* mutants exhibit an asynchrony in initiation of replication (13). It is possible to correlate asynchrony with *dnaA* mutations containing the alanine-to-valine substitution. This has led to the proposal that the asynchronous phenotype is the result of an ATP binding defect. Flow cytometry may be employed to study the degree of asynchrony in initiation of P-loop mutants. This analysis, in combination with assays of ATP binding activity, may correlate the asynchrony phenotype to a defect in ATP binding.

Genetic characterization of one class of *dnaA* alleles that contain mutations in the C-terminal 85 residues suggested that this region of DnaA protein is involved in DNA binding. This was concluded based on their inability to repress expression of a *dnaA* promoter'-*lacZ* fusion *in vivo*. The *dnaA* gene is autoregulated (1, 2). Binding of DnaA protein to a DnaA box in the *dnaA* promoter region represses transcription from both *dnaA* promoters (15). Biochemical characterization of truncated forms of DnaA that lacked either N-terminal, C-terminal, or internal sequences confirmed that the C-terminal 89 residues are essential for specific DNA binding. All C-terminal deletions of DnaA investigated were inactive in DNA binding. Roth and Messer recently reported that the C-terminal 94 residues of DnaA protein are sufficient and required for specific DNA binding activity (10).

In conclusion, the identification and characterization of a large collection of *dnaA* alleles has identified distinct functional domains of DnaA protein. The three dimensional structure of DnaA protein would complement these studies in providing a structural understanding for specific biochemical defects. However, DnaA protein has a tendency to multimerize (5) that is an obstacle to its crystallization and to other physical methods to study its structure. Individual domains may prove more amenable to structural studies, as well as biochemical analysis. These studies should provide a basis for a detailed molecular understanding of DnaA protein in DNA replication.

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