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Theodore Robert Hupp

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Jon M. Kaguni
Major professor

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THE INTERACTION OF *Escherichia coli* GENE PRODUCTS WITH MUTANT FORMS OF dnaA PROTEIN

by

Theodore Robert Hupp

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ABSTRACT

THE INTERACTION OF *Escherichia coli* GENE PRODUCTS WITH MUTANT FORMS OF dnaA PROTEIN

by

Theodore R. Hupp

The initiation of DNA replication depends upon the activity of the dnaA initiator protein. Although the mechanism of dnaA protein function has been elucidated, the efficiency and regulation of dnaA protein dependent initiation of replication is not understood. Mutant forms of dnaA protein are altered in some aspect of initiation of replication. Characterization of the alterations associated with these mutant initiator proteins would enlarge our understanding of initiation processes.

Two approaches were used to identify proteins in *E. coli* that interact with mutant forms of dnaA protein. The first relied on the genetic characterization of an extragenic suppressor of the temperature sensitive phenotype of cells which harbor the *dnaA46* mutation. This suppressor, named *dasC*, was mapped to the thioredoxin gene suggesting that thioredoxin may interact with dnaA46 protein to allow initiation of replication to occur at elevated temperatures. The second approach involved the purification and biochemical characterization of a mutant form of dnaA protein, called dnaA5 protein. This mutant initiator protein is inactive in the replication of *oriC* plasmids. Two heat shock proteins, dnaK and grpE, are required for activating dnaA5 protein in replication.

Although it is not known if these gene products - thioredoxin, dnaK protein, and grpE protein - interact with the wild type dnaA protein, these studies have provided a direction for the further understanding of previously unknown aspects of initiation of DNA replication.

this song is dedicated to
my mother
my father
Lisa
Laura
papa
gramma
mamo
grampy
and
old bean

with thanks, honor, and love

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An incomplete expression of joyful gratitude for the many, worthy people who were all, in every way, the catalyst of this document, will be sonorated in the lines below.

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List of Abbreviations

DTT	dithiothreitol
BSA	bovine serum albumin
SSB	single-stranded binding protein
SDS	sodium dodecylsulfate
TLC	thin layer chromatography
PVA	polyvinylalcohol
kd	kilodalton
ng	nanograms
ug	micrograms
ul	microliters
hsp	heat shock protein

Chapter I

LITERATURE REVIEW

I. The physiology and genetics of DNA replication

A. Structure of the chromosomal origin of replication.

The chromosome of *E. coli* is a closed circular duplex molecule (1) with a molecular weight of 4,000 kilobase pairs (2). The knowledge that DNA is a double helix composed of complementary strands (3) resulted in the prediction and subsequent confirmation that DNA is replicated semi-conservatively (4). The importance of chromosomal replication in the life of a cell has resulted in emphasis being placed on understanding the mechanisms of DNA replication and its regulation.

Understanding the mechanisms of macromolecular synthesis in relation to both growth rate and age was important in demonstrating the elegant regulatory mechanisms which a cell utilizes to control its life. The amounts of some macromolecules, including ribosomes and DNA, change exponentially as a function of the growth rate (5). Analyzing the relative concentrations of macromolecules as a function of the cell size has led to the formation of mathematical relationships which describe global cell composition in terms of DNA, RNA, and protein content (6).

The amount of DNA within a cell is subjected to a stringent regulatory system which allows for the initiation of chromosomal replication at a specific time during the cell cycle to ensure that each daughter cell obtains a copy of the chromosome. DNA synthesis is cell cycle regulated in that a certain cell mass or volume must be achieved in order for DNA synthesis to be initiated (7). The initiation of DNA synthesis requires *de novo* protein synthesis (8,9) and RNA synthesis (10) corroborating the idea that initiation factors must be synthesized and accumulate to allow for the initiation of replication to be triggered during the cell cycle.

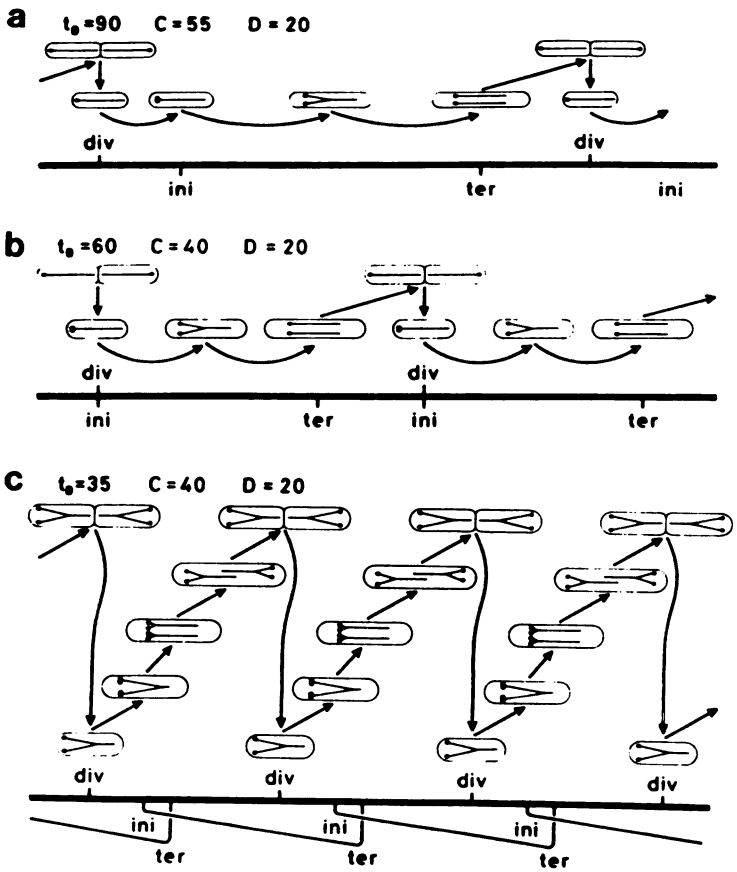
Cooper and Helmstetter determined that, although the concentration of DNA can change as a function of the growth rate, there are biological constants that describe the relationship between the frequency of initiation of replication and cell division (11). The constants in operation were termed C and D, where C represents the time required for the chromosome to be replicated and D describes the time elapsed between termination of DNA replication and cell division. The C and D periods are 40 and 22 minutes, respectively, at a given growth rate.

If the C and D periods are true constants, then how can the total time required for the C and D periods to elapse be longer than the life cycle of a rapidly growing cell? To address this question, they hypothesized that more than one initiation event occurs during the cell cycle in response to a rapid growth rate to account for the fact that bacteria can divide in a time shorter than that elapsing during the C and D periods (Figure 1). The values for C and D were recently determined in slowly growing cells using flow cytometric analysis (13) and were in agreement with the data obtained 15 years earlier (11).

Although the initiation of DNA replication could be measured experimentally, it was not known if the initiation site was unique or if initiation occurred at a random site on the chromosome during successive generations. Jacob (14) proposed a mechanism of initiation of DNA synthesis based on a mechanism known to operate during the regulation of protein synthesis (15). In the latter case, a negative regulator influences the expression of a gene product by binding to an operator element in the DNA to inhibit its synthesis. By analogy and subsequent experimentation, it was proposed that a positive control circuit affects the initiation of DNA replication. In this model, the chromosome is assumed to be a circular structure with two genetic determinants. One being a structural gene giving rise to a diffusible element, the initiator, and the second being a cis-acting operator-like DNA

Figure 1. A scheme describing the C and D periods in the *E. coli* cell cycle (13, 1).

The time required for chromosomal DNA replication (C period) and the time elapsing between the termination of chromosomal DNA replication and cell division (D period) is depicted as a function of differing growth rates; (A) 90 minute doubling time, (B) 60 minute doubling time and (C) 35 minute doubling time. 'ini' is the initiation of DNA replication, 'ter' is the termination of DNA replication, and 'div' is cell division.


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

element that can be acted upon by the initiator possibly to unwind the duplex DNA and lead to DNA synthesis after the formation of a primer.

The existence of this cis-acting locus, the chromosomal origin of replication, *oriC* (16,17), was confirmed, indicating that replication always initiates at a specific site during the cell cycle. More precise experimentation further localized *oriC* to the *ilv* region of the chromosome (18). By the quantification of the relative abundance of marker genes after the initiation of DNA synthesis in a synchronized cell culture, the bi-directional mode of initiation was proven (19-22). The cloning of *oriC* onto plasmids allowed for restriction site analysis and provided for a more precise physical map of *oriC* (23).

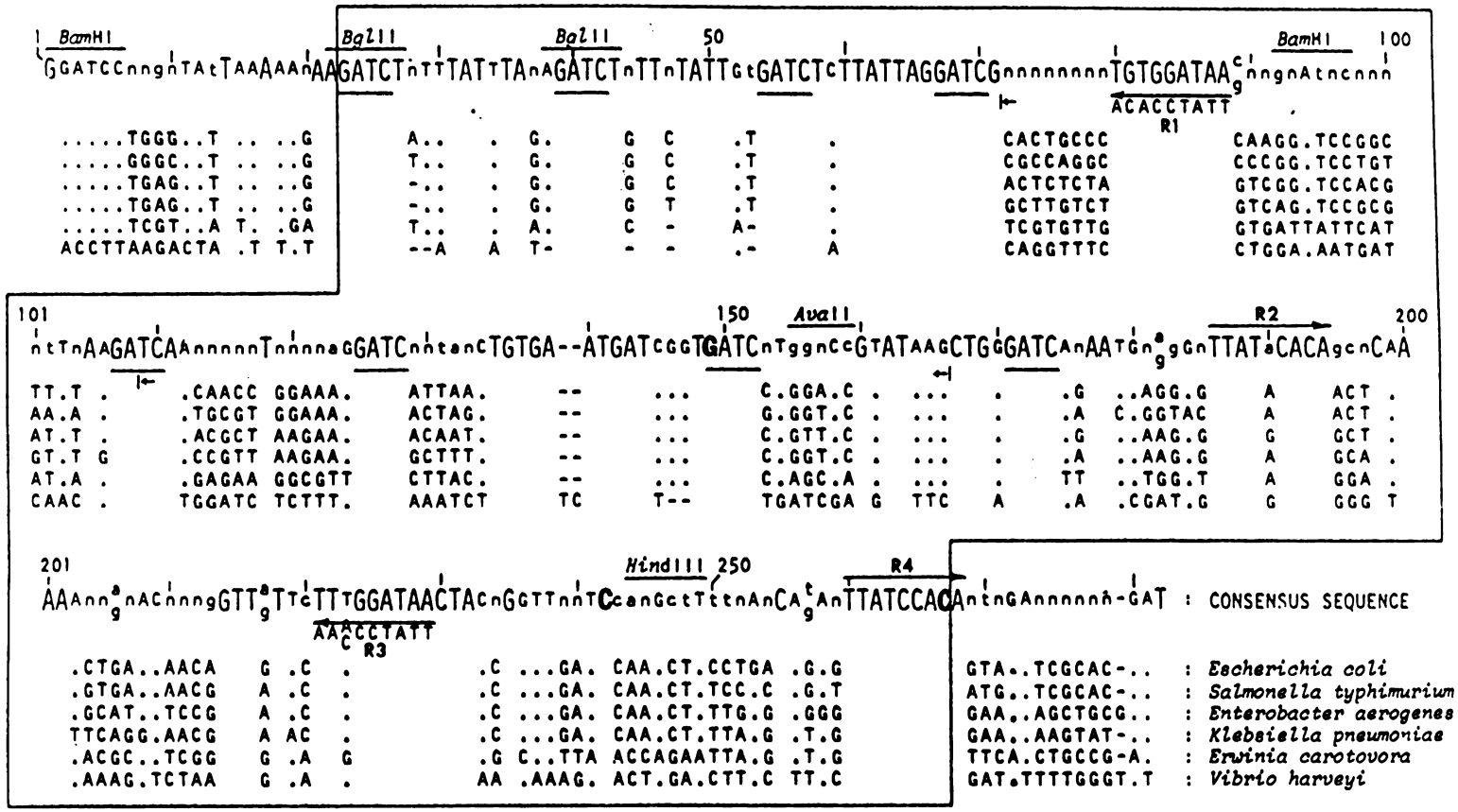
Recombinant DNA techniques were used to clone *oriC* based on its ability to confer autonomous replicating properties to recombinant DNAs (24) and based on its linkage to the *asn* locus (25). The minimal functional segment required for conferring replication activity to vector DNAs is 245 base pairs (26).

Subsequent DNA sequencing of the minimal origin fragment (27, 28) identified the presence of extensive structural motifs, including A-T rich regions, GATC methylation sites, and inverted and repeated sequences (29). Some mutations within *oriC* that perturb the primary sequence, including base substitutions, insertions and deletions, alter the origin activity (30). In addition, the evolutionary conservation of the sequence motifs among enterobactericeae (Figure 2) (31) and the ability of *oriC* from a marine bacterium to function in *E. coli* (32) provided evidence that the structure of the origin is important in directing enzymes and proteins to initiate replication.

Each structural motif is involved in origin activity through its interaction with various cellular enzymes. Methylation at the sequence GATC, which is present 11 times within the minimal origin, by the *dam* methylase gene product (33) is thought to be important in maintaining origin integrity and function. The *dam* gene product is not essential for the growth of *E. coli*, as *dam* mutants are viable (34). This implies that methylation of the GATC motifs is not an essential step in *oriC* directed DNA replication, but this idea

Figure 2. The evolutionary sequence conservation of *oriC* DNA (32).

The sequences from the origin of DNA replication from six bacteria are aligned to maximize visual sequence identity. The minimal origin required to confer activity is enclosed within the box and bold capitol letters indicate where base substitutions inactivate origin function. Conserved motifs are identified as follows: GATC repeats are underlined; contiguous left to right arrows are beneath the A-T rich 13-mer sequences; R1-R2-R3-R4 mark the location of the *dnaA* consensus sequence; a large capital letter indicates conservation of the base in all six origins; a small capital letter indicates the base is present in five of the six origins examined; a lower case letter indicates the base is present in three or four origins; and 'n' is inserted where there seems to be no sequence homology, or there are insertions or deletions within the region.


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

contradicts the results showing that *oriC* minichromosomes are unable to replicate in *dam* cells (35,36). The initiation of DNA replication in *dam* cells may occur at sites on the chromosome other than *oriC*, but this has not been clarified. Although *dam* cells are viable, they are inefficient in the timing of initiation of DNA synthesis during the cell cycle (37). This demonstrates that the timing of initiation, whether from *oriC* or from other sites on the chromosome, is dependent upon the state of methylation of the DNA.

Evidence supporting the importance of the methylation state of *oriC* , in the regulation of origin activity, was obtained by studying the ability of hemi-methylated *oriC* DNA to function. Hemi-methylated *oriC* plasmids are incapable of being replicated in *dam* cells (38), suggesting that full methylation of the origin is required for initiation to occur. DNA containing *oriC* binds to the outer membrane of *E. coli* only when in the hemi-methylated state (39), indicating that the state of methylation may affect the ability of the chromosomes to segregate during cell division. Taken together, it seems that hemi-methylation may prevent unnecessary initiations and at the same time allow for adhesion of the DNA to the membrane ensuring that chromosomal segregation will occur.

The initiator protein, dnaA, binds to four 9 base-pair repeats present within the origin (40, 41) and, in an ATP dependent reaction, it unwinds the duplex DNA at the A-T rich region of *oriC* to facilitate the nucleation of other enzymes involved in replication (42).

Promoters and terminators of transcription have been proposed to exist within *oriC* , given the essential role of RNA polymerase in initiation (43). Promoters within *oriC* have been detected (44), transcriptional termination sites have been mapped (45, 47) and RNA-DNA transitions have been observed within the origin region (46). The chromosomal origin has proven to be a complex structure suitable for interacting with the many enzymes that direct initiation of DNA replication and, possibly, its regulation.

B. Gene products that function in the initiation of DNA replication.

Several investigators have isolated conditionally lethal mutants which are defective in DNA synthesis in an attempt to characterize gene products involved in replication. Such mutants are termed *dna* and are principally defined as gene products involved directly in DNA synthesis. The first class of conditionally lethal mutants isolated (48, 49), later termed *dnaA*, were screened based on their inability to synthesize DNA at elevated temperatures. The refined characterization of one mutant , T46, demonstrated that it exhibited a specific defect in the initiation of DNA synthesis (50). Since then, many mutations in the *dnaA* locus have been isolated and characterized (51). Thirteen different independently isolated mutations were genetically mapped and categorized according to classes which include; 1) cold sensitivity in the haploid or merodiploid state and 2) the ability to be suppressed by *rpoB* mutations (52). There is a correlation between the location of the mutation within the *dnaA* gene and its phenotype indicating that individual domains of the *dnaA* gene product may be involved in different cellular functions.

The *dnaA* gene has been cloned into pBR322 (53) and specialized lambda transducing phages (54). A structural analysis of the region indicated that *dnaN* , *gyrB*, and *recF* genes lie contiguous with *dnaA* (55, 57). The nucleotide sequence of the *dnaA* gene was determined (58, 59) and the predicted molecular weight of the gene product was reported to be 52,800 daltons with a theoretically basic pI of 9.8. dnaA protein autoregulates its own expression *in vivo* (60,61) and *in vitro* (62) by binding to the promoter region and repressing transcription by RNA polymerase. The autoregulatory mechanism of *dnaA* expression confirmed the hypothesis of Hansen et al. (63) that the initiator regulates its own synthesis.

Mutations affecting the initiation of replication have been mapped to genes other than *dnaA* and include *dnaC* (64), *dnaB* (65), *dnaK* (66), and *dnaG* (67). Although the *dnaB* and *dnaC* gene products are involved in initiation, they are also involved in the propagation

of the growing replication fork (68, 69). DNA gyrase is also involved in initiation as inhibitors of DNA gyrase activity affect the initiation of DNA synthesis (56).

The requirement for RNA polymerase during initiation was inferred from studies demonstrating that initiation is sensitive to rifampicin, a known inhibitor of RNA polymerase activity (70, 71, 72). Subsequently, a conditionally lethal mutation residing in the *rpoC* locus was isolated (73), indicating an essential role for RNA polymerase in initiation. An additional mutant was isolated (74) which increases the initiation frequency at elevated temperatures. Based on genetic studies, the enzymes required for initiation were shown to function sequentially (75). The prediction that RNA polymerase acts before dnaA protein which in turn functions prior to dnaC protein was confirmed from biochemical studies (42, 181).

Exhaustive efforts were focused on isolating novel *dna* mutants conditionally lethal for DNA synthesis and this resulted in the acquisition of *dnaA* and *dnaC* mutants. It seemed that new screening methods were needed to find previously unidentified gene products involved in replication. Thus, a novel approach was taken which involved screening for extragenic suppressors of *dnaA* mutations (76). Six suppressor loci were identified in one screening (77). One mapped within the *rpoB* locus, while the other suppressors did not reside in any known gene and they were named *dasA, dasB, dasC, dasF,* and *dasG.*

The best characterized extragenic suppressors of *dnaA* map in a RNA polymerase subunit gene (78) and the allele specific suppression (79-81) was interpreted to mean that *dnaA* and RNA polymerase interact in a transcriptional event at *oriC.*

Cells with mutations in RNase H, which are allelic with *dasF* (82), by-pass the requirement for dnaA protein and *oriC* (83). In RNase H mutant cells, initiation of DNA synthesis occurs at sites other than *oriC* (84), indicating the essential role that RNase H plays in maintaining *oriC-dnaA* dependent initiation of DNA replication (85).

A few of the *dnaA* suppressors have been mapped to specific loci, but the mechanism of suppression is not known. The extragenic suppressor *dasC* (77) is allelic with *trxA* (86)

and a mutation in *dnaA* suppresses a *dnaZ* mutation (87), suggesting that thioredoxin and DNA polymerase III holoenzyme interact with the *dnaA* dependent replication machinery.

The heat shock gene product, *groE* , suppresses the temperature sensitive defect of *dnaA46* cells when present at high copy number in the chromosome (88) or on a high copy number plasmid (89). Under these conditions the overproduction of *groE* may stabilize the mutant protein so that it can function at elevated temperatures. No biochemical information exists describing the interaction between *dnaA* and *groE*, *trxA*, or *dnaZ*, however, it is likely that the *dnaA* dependent replication machinery is composed of many components that coordinately function to initiate DNA replication.

C. Regulation of the initiation of DNA replication.

DNA replication initiates at a specific site on the chromosome, the genetic locus being named *oriC*, and it is dependent upon the activity of the initiator protein, dnaA (90). Although the initiation event occurs at a specific time during the cell cycle, the mode of its regulation is not known. Models that describe the regulation of initiation have been proposed and include a positively and a negatively modulating circuit.

A positive control circuit invokes the requirement for an initiator which activates initiation in response to cell growth (91), possibly as a result of the accumulation of the initiator during the cell cycle. Such an initiator was proposed to autoregulate its own expression to ensure that a balance is maintained between cell growth and initiation (63). The *dnaA* gene product fulfills all of these criteria (60).

An extension of the model invoking the requirement for an initiator incorporates the activity of an inhibitor that competes with the initiator (92). In the early stages of the cell cycle the concentration of the inhibitor is high and prevents the initiator from functioning. As the cell cycle progresses, the cell mass increases, effectively diluting the concentration of the constitutively expressed inhibitor and liberating the initiator to allow initiation to

occur. This idea was supported by the finding of an unstable inhibitory factor that competes with dnaA protein in initiation *in vivo* (93, 94).

The initiation of DNA synthesis is tightly coupled to an increase in the cell mass during the cell cycle (95), possibly due to the increasing abundance of the initiator during growth. The accumulation of the initiation potential has been observed in two differing but related experiments. Protein synthesis is required immediately before the initiation of DNA replication since initiation in a synchronized culture is blocked by inhibitors of protein synthesis (16). In situations where DNA synthesis is inhibited but protein synthesis can continue, there is an unusually high rate of initiation of DNA synthesis upon removal of the block to DNA synthesis (96). The accumulation of this initiation potential has been correlated with an increase in the concentration of dnaA protein in response to an increase in the growth rate of a cell culture (97) and the idea is confirmed by the fact that overproduction of dnaA protein from expression vectors increases the frequency of initiation (98-100). Interpretations of these results include the idea that dnaA protein is the limiting factor in initiation and that its levels determine directly the frequency of initiation during cell growth. However, another rate limiting factor may exist as overinitiation of DNA replication through the overproduction of dnaA protein is abortive (102, 98) and the levels of dnaA protein appear to remain constant during the cell cycle (101). These data indicate that dnaA protein may not be the sole factor involved in the regulation of initiation of DNA replication.

Mutations in *dnaA* have been well categorized and a sub-class of *dnaA* mutants is deficient in the timing of initiation (103). Using a flow cytometer, the concentration of origins in an individual cell can be determined (104) and this technique was used to show that the *dnaA* gene product functions synchronously to initiate DNA synthesis at a certain ratio of cell mass to DNA. The *dnaA5* and *dnaA46* gene products are asynchronous in their ability to initiate DNA replication. Initiations occur randomly throughout the cell cycle indicating that the signal allowing for *dnaA* dependent initiation is not operating. The

explanation for this has not been determined, but it is possible that there may be a defect in the activity of the mutant dnaA protein itself or it may be unable to respond properly to a signal that normally couples cell growth to dnaA protein dependent initiation of replication. Understanding the mechanism underlying this phenomenon may provide information regarding the regulation of initiation.

The involvement of membranes in regulating DNA replication was proposed based on the evidence that DNA must physically associate with some factor in the membrane during fission to ensure proper segregation of the chromosomes (14). Cell membrane fractions are enriched in DNA containing the chromosomal origin, *oriC*, (105) indicating that the site of chromosomal attachment to the membrane may occur at the site of initiation of chromosomal replication. A 10 kd protein derived from membrane fractions has a high affinity for *oriC* DNA (106) indicating that the association of *oriC* with the membrane may occur via this 10 kd protein.

Cardiolipin activates an inactive form of dnaA protein bound to ADP (107), indicating that there may be a possible role for lipid in regulating the initiation of replication through the modulation of dnaA protein activity. This idea is supported by the fact that the gene encoding the enzyme involved in cardiolipin synthesis cannot be deleted from the chromosome unless the cell is supplied with a copy of the gene from a plasmid (108).

The factors regulating replication may be numerous, and it was proposed that dnaA protein is not the rate limiting factor regulating initiation of replication from *oriC* (109) but there may be a sequential requirement for many enzymes, none of which is individually rate limiting, all of which are required for the events leading to initiation.

II. The biochemistry of DNA replication.

A. The mechanism of DNA polymerase function.

Three distinct DNA polymerases exist in *E. coli* and have been named, in the order of their discovery, DNA polymerase I (110), DNA polymerase II (111, 112), and DNA polymerase III holoenzyme (113).

DNA polymerase I was the first polymerase discovered and purified (114). The enzymatic activities embodied by this prototypical DNA polymerase include a 5'-3' reversible polymerase activity (115,116), a 3'-5' exonuclease activity, and a 5'-3' exonuclease activity.

The 5'-3' polymerase activity can be observed provided that three fundamental constraints are met (117); 1) a hydroxyl primer terminus (almost always the 3' hydroxyl of the ribose moiety), 2) a template DNA anti-parallel to the primer terminus strand and 3) a specific dNTP complementary to the base residing in the template strand.

The 3'-5' exonuclease activity was thought to be a contaminating polypeptide for years (118) until it was proven to be an innate corrective proofreading property that results in the excision of a nucleotide improperly base paired to the template strand (119). This ability of DNA polymerase to remove its own error would prevent the rapid accumulation of mutations which would otherwise lead to cell sickness and death.

The 5'-3' exonuclease activity degrades duplex DNA into mono and polynucleotides from the free 5' end of a duplex hybrid (120). This activity is thought to be responsible for the removal of RNA from the Okazaki fragments formed during lagging strand DNA synthesis (see below) through its nick-translation activity (121). This enzymatic function is believed to be required for DNA repair (122) since cells harboring certain mutations in the gene encoding DNA polymerase I are sensitive to UV irradiation. The production of UV induced thymine dimers and the subsequent scission of a DNA strand by uvrABC

endonuclease (123) could provide the free 5'-end required for DNA polymerase I to remove the damaged nucleotide stretch through its nick-translation activity.

DNA polymerase II was the second DNA polymerase identified from a strain of *E. coli* which was defective in DNA polymerase I. This was also the first indication that DNA polymerase I is not essential for cell viability and that some other polymerase (DNA polymerase III holoenzyme, see below) is the major replicative enzyme (124). The gene encoding DNA polymerase II (*polB*) has been mapped (125) and cloned (128) proving that the gene has a locus distinct from DNA polymerase I (*polA*) (126), and the alpha subunit of DNA polymerase III holoenzyme (*dnaE* , see below). Only recently were there published studies describing the characteristics of purified DNA polymerase II. A novel DNA polymerase, presumably DNA polymerase II, is induced in cells that are subjected to UV irradiation (127). This novel DNA polymerase exhibits distinct enzymatic and immunological properties compared to DNA polymerase III holoenzyme and DNA polymerase I. The SOS dependent expression of this DNA polymerase distinguishes it from the other polymerases and this may indicate that it has an important role in DNA repair.

DNA polymerase III holoenzyme is the major replicative enzyme of the cell (129) and it is the enzyme required for efficient *in vitro* DNA replication of phages, plasmids, and minichromosomes (130-132). The enzyme is composed of at least 10 subunits based on the number of polypeptides that co-purify with DNA polymerase activity (133). The function of every subunit is not known, however, the biochemical contribution of some of them have been determined. The alpha subunit, the gene product of *dnaE* (134), is the polypeptide responsible for DNA synthesis (135). The epsilon subunit, encoded by *dnaQ* or *mutD* (136), contains the 3'-5' exonuclease activity (137) and confers a high processivity to the alpha subunit (138). This two subunit enzyme assembly is the minimal complement of polypeptides required to achieve reasonable DNA polymerization activity *in vitro*. The beta subunit, encoded by *dnaN* (139), confers an even higher processivity to

the alpha-epsilon assembly\ (140). The tau subunit causes the core DNA polymerase to dimerize (141) and may facilitate the assembly of the holoenzyme at the replication fork (142). The tau and delta' subunits accelerate assembly of the holoenzyme onto its template and the reconstitution of these five polypeptides, alpha, epsilon, beta, tau, and delta', produces an enzyme with catalytic properties indistinguishable from the 10 subunit DNA polymerase III holoenzyme (142). Perhaps the other subunits function in the cell in some aspect of DNA repair or a higher order protein complex that is required for faithful DNA synthesis *in vivo*.

The physiological role of DNA polymerase III holoenzyme has been elucidated genetically, as genes encoding for various subunits of the holoenzyme alter the physiology of the cell (143). Mutations in the genes *dnaQ*, *dnaN*, and *dnaE* are conditionally lethal or prevent proper DNA repair after DNA damaging agents have been exposed to the cells (144).

The exposure of cells to UV light results in the accumulation of mutations, often leading to cell death, due to errors in DNA replication. The three DNA polymerases that exist in *E. coli* all contribute to DNA repair to some extent, as mutations in any of the three genes results in some lethal or damaging phenotype.

B. A biochemical model describing the enzymatic requirements at a replication fork.

The enzymes and proteins in *E. coli* required for chromosomal DNA replication were identified and studied by examining the components required for viral DNA replication. Some viral DNAs, being small, do not encode many proteins and rely on host encoded enzymes for the replication of their genome. Three well characterized phages, M13, ΦX174, and G4, were used as model systems for the identification of *E.coli* enzymes involved in DNA replication and the priming of DNA synthesis (Figure 3, (145)).

The viral DNA of M13 exists in the phage coat as a single-stranded molecule (146) and in the first stage of its replication cycle, it is converted to the duplex form by *E. coli* enzymes. The requirement for RNA polymerase in the initial stages of viral DNA replication *in vivo* was inferred from the fact that the reaction is sensitive to rifampicin (147), a known inhibitor of RNA polymerase. The rifampicin sensitive RNA polymerizing event could be duplicated *in vitro*, demonstrating for the first time (148), a biochemical role for RNA polymerase in the priming of DNA replication. An RNA primer is synthesized near a hairpin loop in the DNA at the origin of replication and, in the presence of SSB, DNA polymerase III holoenzyme synthesizes an entire DNA strand complementary to the original parental viral DNA (149).

Another viral DNA, G4, exists in a single stranded form in the phage coat (146), and the mechanism responsible for its replication is differentiated from M13 by the fact that the RNA priming of its replication is not dependent upon *E. coli* RNA polymerase, but by another RNA priming enzyme called primase (150), encoded by *dnaG* . This rifampicin resistant RNA polymerase forms an RNA primer at the G4 origin of replication (151), and in the presence of SSB, DNA polymerase III holoenzyme replicates the parental DNA strand.

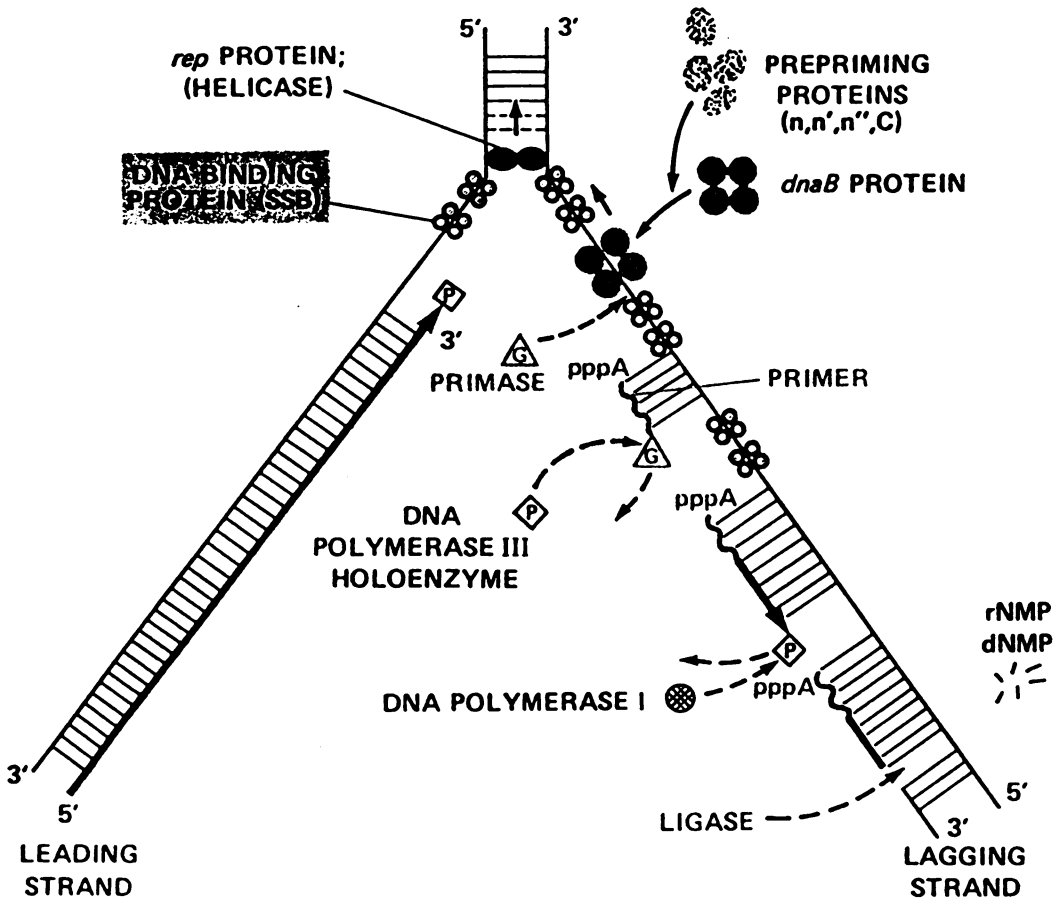
The two bacteriophages described above utilize two different enzymes to prime DNA replication at the origin of replication. The mechanism of RNA priming utilized by the single-stranded bacteriophage ΦX174 (146) is the most complex of the three phages in that at least eight *E. coli* enzymes are required to complete the process (152). These proteins have been purified and characterized *in vitro* and include dnaB, dnaC, dnaT (153), primase, SSB, n, n', n". The priming event is initiated in the presence of SSB after the binding of a DNA dependent ATPase, n' (154), to a sequence motif near the origin of DNA replication. This single binding event allows for the nucleation of n, n", dnaC, dnaB, and dnaT. The addition of primase to this initiation complex, termed the primosome, results in the formation of RNA primers at various intervals along the single-stranded DNA as the

primosome translocates along the template (155). These primers can be utilized by DNA polymerase III holoenzyme to synthesize DNA complementary to the template strand.

The characteristics of the *E. coli* enzymes involved in viral DNA replication are thought to reflect their function in the host chromosomal replication. A model describing the propagation of a replication fork has been formed from such studies and is shown in Figure 3. The replication can be divided into two stages, leading and lagging strand DNA synthesis. On the leading strand, DNA is synthesized continuously in the 5'-3' direction by DNA polymerase III holoenzyme. On the lagging strand, DNA is synthesized discontinuously (156) as the primosome translocates along the single-stranded DNA exposed at the replication fork synthesizing RNA primers and DNA polymerase III holoenzyme extends the primers replicating the template. The removal of RNA from RNA-DNA hybrids and DNA gap-filling by DNA polymerase I, followed by nascent chain closure by DNA ligase terminates the process.

The enzymes proposed to function at the growing replication fork are topoisomerases and helicases which can relieve torsional strain and unwind the duplex DNA. The involvement of the rep helicase was surmised from its known biochemical role in unwinding DNA with flanking 3' single stranded DNA regions (157) in the ΦX174 rolling circle mode of replication (158). The hydrogen bonds between bases at a replication fork must be broken to allow for the propagation of DNA chains. Therefore, the inclusion of a helicase in this model is required to augment authentic replication fork movement. The *rep* gene product is not essential for *E. coli* viability (159), indicating that it is not the only helicase functioning at the replication fork. However, mutations in *rep* decrease the rate of replication fork movement and increase the number of replication forks per cell (160), indicating it is involved in some aspect of DNA replication *in vivo*.

Figure 3. A model describing the enzymatic requirements at a replication fork (145).


5'
3'
rep PROTEIN;
(HELICASE)
PREPRIMING
PROTEINS
(n,n',n'',C)
DNA BINDING
PROTEIN (SSB)
dnaB PROTEIN
P
3'
G
PRIMASE
pppA
PRIMER
G
P
DNA
POLYMERASE III
HOLOENZYME
pppA
rNMP
dNMP
P
DNA POLYMERASE I
pppA
3'
5'
LEADING
STRAND
LIGASE
5'
3'
LAGGING
STRAND

Other helicases discovered since then (161) may replace the function of rep helicase in this model describing the propagation of a replication fork. uvrD or Helicase II (162) can unwind DNA with flanking 3' single-stranded regions in a manner similar to rep helicase. Mutations in *rep* or *uvrD* do not result in cell death, but double mutations in *rep* and *uvrD* are lethal to the cell (163) indicating that at least one of them is required for replication fork propagation and cell viability.

The unwinding of the duplex DNA into single strands at a replication fork relays a positive torsional strain into the double helix in the vicinity of the growing replication fork. DNA gyrase can introduce negative supercoils into covalently closed DNA (164) thus relieving the torsional strain which would have hindered fork movement and the rate of DNA replication. The immediate cessation of DNA synthesis *in vivo* after inhibiting the activity of DNA gyrase by antibiotics (165) or after a temperature upshift in cells containing a temperature sensitive *gyr* allele (166) supports the idea that DNA gyrase is required for progressive fork movement in DNA replication (167).

C. The initiation of DNA synthesis from *oriC* plasmids catalyzed by purified enzymes.

Understanding the mechanisms of viral DNA replication led to the identification of the many cellular enzymes involved in chromosomal replication. These studies gave information concerning the propagation of a replication fork, but information was not obtained on the mechanism of initiation of replication from *oriC* or of its regulation.

The cloning of the chromosomal origin, *oriC*, into plasmid vectors (24, 25) provided the substrate needed to identify enzymes required for the initiation of replication *in vitro*. A technological breakthrough (90) led to the isolation of a crude enzyme fraction that could authentically initiate DNA synthesis from *oriC* . The requirements of initiation in this *in vitro* system mimic those *in vivo*. These include 1) rifampicin sensitivity in initiation

demonstrating the requirement of RNA polymerase, 2) sensitivity to inhibitors of DNA gyrase and 3) a bidirectional mode of DNA synthesis (168).

The essential and unique requirement for the *dnaA* gene product also exists in the *in vitro oriC* replication system. A crude enzyme fraction from a *dnaA* mutant strain could not support the replication of *oriC* plasmids unless supplied with extracts or fractions containing elevated levels of dnaA protein. This complementation assay was used to purify dnaA protein to near homogeneity (169).

Further refinement of the replication system employing a crude enzyme fraction led to the formation of a reconstituted enzyme system composed of purified enzymes and proteins that could initiate DNA synthesis from *oriC* (132). The minimal complement of enzymes required for the initiation and propagation of DNA chains was divided into three sets; initiation factors (which include the proteins dnaA, HU, dnaB, dnaC, DNA gyrase, and RNA polymerase), elongation factors (which include the proteins dnaB, dnaC, primase, SSB, DNA gyrase, and DNA polymerase III holoenzyme) and specificity factors (topoisomerase I and RNase H) which maintain *oriC* dependent initiation of replication.

The reductionist approach was used to dissect this complex system into more simple components in an effort to understand the role of dnaA protein in initiation. dnaA protein binds cooperatively to *oriC* DNA and other DNAs containing the consensus sequence TTATCCACA (40). The ability of dnaA protein to bind to *oriC* DNA implied that it was one of the first participants in the cascade leading to DNA synthesis.

dnaA protein binds nucleotides with a very high affinity (170). This form of dnaA protein binds to *oriC* DNA with an affinity equivalent to the non-nucleotide form of dnaA protein, indicating that its high affinity binding to ATP does not affect its affinity for DNA and may influence other properties of the protein. Subsequent studies (171) demonstrated that, in the presence of ATP, dnaA protein locally unwinds an A-T rich region at one end of the chromosomal origin to allow for the formation of single-stranded regions that are utilized as substrates by the dnaB-dnaC complex (Figure 4) (42). dnaB and dnaC proteins

form a complex in the presence of ATP (172) that results in the dnaC protein dependent delivery of dnaB helicase to single-stranded DNA (173). The binding of dnaB protein results in the release of dnaC protein and this activates dnaB helicase (174). Further unwinding by the helicase leads to the formation of a small bubble capable of being utilized as a substrate by primase which can subsequently lead to DNA synthesis (175). The regulation of this event may occur at the site of dnaA protein action, in that the ADP form of dnaA protein cannot initiate DNA replication because it is unable to unwind *oriC* DNA (107). Acidic phospholipids, like cardiolipin, reverse the ADP inhibition of dnaA protein, suggesting that a role exists for phospholipids in modulating dnaA protein activity in the cell (176).

This *oriC* unwinding model system is incomplete due to the fact that it excludes other proteins, like RNA polymerase, topoisomerase I, and RNase H, all known to be present in the cell and involved in *oriC*-dnaA protein dependent replication *in vitro* (177, 178). RNase H degrades RNA in DNA-RNA hybrids and it is believed that RNase H removes RNA synthesized by RNA polymerase at sites other than *oriC* (85) and thus maintains *oriC* specific initiation of DNA synthesis. This idea is confirmed by the observation that RNase H mutant cells lose *oriC* dependent initiation of replication and initiation occurs at other sites on the chromosome (84,179).

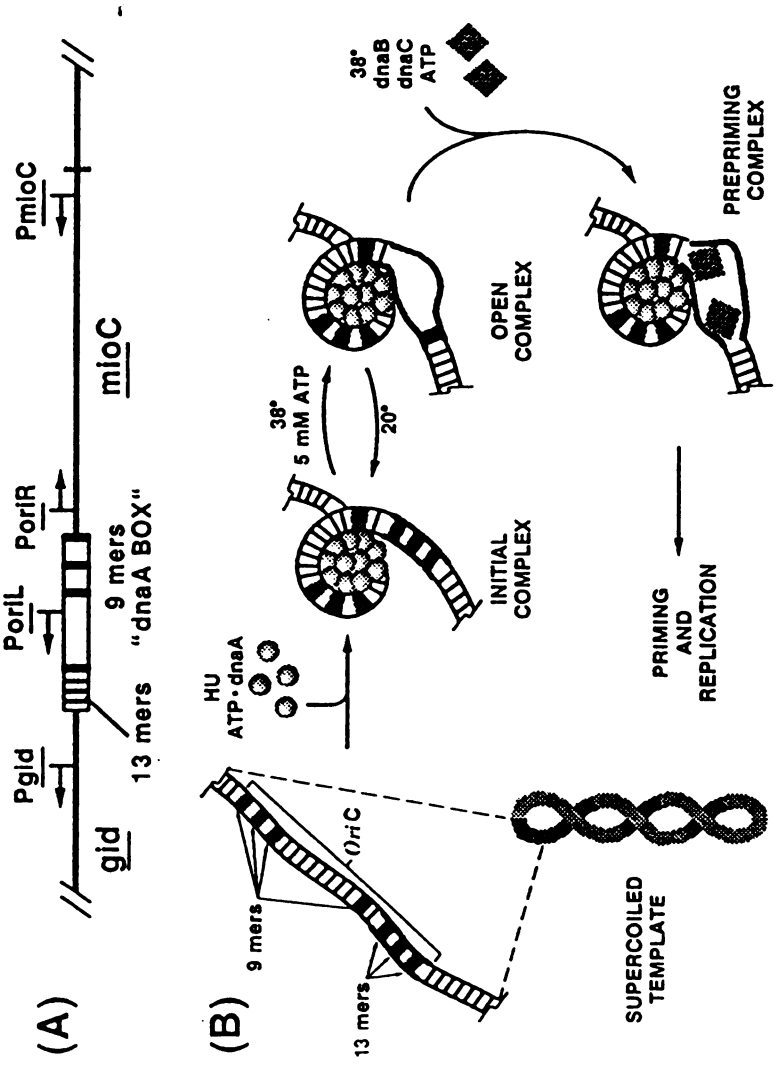
The requirement for RNA polymerase was thought to be in forming a RNA molecule in concert with dnaA protein to prime DNA synthesis initiating at *oriC* (79). This was supported by the results describing the existence of promoters within *oriC* , RNA transcripts terminating within *oriC* , and the existence of RNA-DNA transition sites homologous to origin DNA (44-47). A recent study demonstrated that a RNA polymerase dependent RNA transcript does not prime DNA synthesis at *oriC*, but transcriptional activation by RNA polymerase aids dnaA protein in melting the helix in the vicinity of the A-T rich region near *oriC* (180). However, a more complex role for RNA polymerase cannot be eliminated.

Figure 4. (A) A linear sequence of the origin region (180).

The bold line represents DNA contiguous to the origin region (boxed). Promoters within and proximal to the origin region are labeled according to the direction of transcription and locus. Consensus sequences required for origin function are represented as filled rectangles within the origin box.

(B) A model describing the stages in dnaA protein dependent initiation (180).

The diagram depicts four stages in the dnaA protein dependent initiation process; 1) binding of dnaA protein in the presence of ATP and HU protein to *oriC*, 2) formation of an open complex at elevated temperatures, 3) entry of the dnaC-dnaB helicase and 4) RNA priming and DNA synthesis.


(A)
gid
PgId
13 mers
PorIL
PorIR
9 mers
"dnaA BOX"
mioC
PmioC
(B)
9 mers
13 mers
OriC
HU
ATP·dnaA
INITIAL COMPLEX
38°
5 mM ATP
20°
OPEN COMPLEX
38°
dnaB
dnaC
ATP
PREPRIMING COMPLEX
PRIMING AND REPLICATION
SUPERCOILED TEMPLATE

III. The heat shock response

A. The heat shock proteins of *E. coli.*

The heat shock response is thought to function as a protective mechanism preventing cellular damage in response to enviromental stress (181). However, growing evidence indicates that heat shock proteins play a role in normal cell physiology (182, 183). In *E. coli*, the expression of heat shock proteins is under the control of the *htpR* (*rpoH*) gene product (184), a sigma factor that associates with RNA polymerase. When growing cells are transferred from 30°C to 42°C the expression of the *htpR* gene product is induced and it directs the synthesis of at least seventeen heat shock proteins (185, 186). The rate of synthesis of the heat shock proteins can increase from 5 to 20 times and, 5 to 10 minutes after the heat shock is induced, a new steady state level of expression is achieved.

Seven of the seventeen heat shock proteins from *E. coli* correspond to known gene products and are named *lon, rpoD, groEL, groES, dnaK, dnaJ*, and *grpE*. The *lon* gene product is an ATP dependent protease (187, 188) that is not required for cell viability, but it is required in maintaining a healthy SOS response by proteolytically cleaving a cell division inhibitor (189). The *rpoD* gene encodes sigma 70, the RNA polymerase subunit that directs transcription from most *E. coli* promoters (190). It is thought that an increase in the levels of sigma 70 after heat shock ensures that the cell will be able to rapidly return to normal steady state gene expression and growth.

The other five heat shock gene products described above, were first identified as mutants unable to support lambda phage propagation. *groEL* and groES form an operon and are required for lambda phage body assembly (191, 192) and phage Mu development (193). When *groE* is present at an elevated copy number the thermolability of a *dnaA*

temperature sensitive mutant is suppressed (88,89). The role of *groE* in *E. coli* metabolism is not well understood, but some mutants are defective in RNA and DNA synthesis at elevated temperatures (194). Recently, *groE* was shown to be essential for *E. coli* viability (195).

Mutations in *dnaK* (196, 197), *dnaJ* (198), and *grpE* (199) restrict lambda DNA synthesis and affect the global synthesis of *E .coli* RNA, DNA and protein. These three gene products function in a diverse series of reactions in combination or singly. *dnaK* is required for mini-F DNA replication (203), while *dnaK*, *dnaJ* and *grpE* are required for phage P1 DNA replication (200) and for the initiation of lambda DNA synthesis (201, 202). A novel *dnaK* mutant is specifically defective in initiation of DNA replication indicating a possible role for *dnaK* in that process (66). *dnaK* (204) and *grpE* (205) are required for normal cell growth and they both were shown to interact *in vivo* and *in vitro* (206). The diverse associations amongst different heat shock proteins reflects the cells ability to respond to a wide variety of stimuli.

B. The biochemical role of the 70 kd heat shock proteins.

Bacteria, yeast, insects, plants and mammals all respond to heat shock by synthesizing a set of proteins highly conserved within a cell and among other organisms (207). Antibodies to a 70 kd heat shock protein from chicken cross react with proteins in yeast and humans (208, 209). The 50% homology in the amino acid sequence between a 70 kd heat shock protein from yeast and Drosophila (210) confirmed the high homology that exists between heat shock proteins from different organisms. Another common feature of 70 kd heat shock proteins, which may be required for biochemical activity (see below), is the high affinity for ATP (211).

The biochemical role of the *E. coli* 70 kd heat shock protein, dnaK protein, is not known; however, its biochemical function in lambda phage DNA replication is known

(Figure 5) and its role in that process may provide clues concerning its function in normal *E. coli* growth. Lambda phage DNA replication *in vivo* (212) and *in vitro* (213-215) requires phage encoded and host encoded gene products to authentically initiate bi-directional DNA synthesis (216) from the phage origin of replication, *oriL*. Lambda encodes two of its own initiation proteins, lambda O and P proteins (217-218), that form an initiation complex at *oriL* with the dnaB helicase (219). This initiation complex containing dnaB helicase is similar to the initiation complex at *oriC* containing dnaB protein (173), except that in lambda replication, the activity of dnaB helicase is inhibited when bound by lambda P protein. dnaK protein functions in the presence of dnaJ protein to dissociate lambda P protein from the initiation complex which results in the activation of the dnaB helicase activity (220). This leads to further duplex strand unwinding and subsequent DNA synthesis.

grpE protein is absolutely required for lambda DNA replication *in vivo*, but it is dispensible *in vitro* (221). Although dnaK protein alone can dissociate lambda P protein from the *oriL* initiation complex, grpE protein reduces by ten fold the level of dnaK protein required to dissociate lambda P protein. The mechanism of grpE protein action is unknown, but it was postulated that grpE protein could bind to the dissociated form of lambda P protein. This would reduce the level of dnaK protein required for the lambda P dissociation reaction by preventing non-specific binding of free lambda P protein to dnaK protein. grpE and dnaK proteins can form a complex that is stable in the presence of high levels of salt, but it is unlikely that the two proteins from a complex in initiation, as the reaction contains high levels of ATP and the grpE-dnaK protein complex is dissociated by ATP (202). The role of dnaK in dissociating protein-protein complexes mimics the biochemical role of heat shock proteins in eucaryotic cells.

In *Saccharomyces cerevisiae* a family of 70 kd heat shock proteins is involved in post-translational import of proteins into the membranes of organelles (Figure 6A). A series of heat shock protein mutants accumulates unprocessed precursors of proteins destined for the

mitochondria or the endoplasmic reticulum (222). The heat shock proteins are thought to affect the conformation of proteins, thus the transport of the precursors into the mitochondria requires that the transported protein maintain a conformation suitable for translocation (223). Proposals for the mechanisms of heat shock protein function include 1) the disassembly of aggregates formed from untranslocated precursor proteins, 2) the alteration of the tertiary structure of proteins prior to insertion into membranes and 3) binding to unfolded domains within proteins to prevent the acquisition of conformations incompatible with translocation.

A *S. cerevisiae* cytosolic protein, shown to be a member of the 70 kd heat shock protein family, stimulates the translocation of prepro-alpha factor across microsomal membranes (224). The rate of *in vitro* protein import, independent of the heat shock protein, could be increased upon urea denaturation of the precursor protein. This denaturation stimulated translocation of prepro-alpha factor mimics the role of the heat shock protein, suggesting that the mechanism of action is through a denaturation of the precursor protein prior to insertion into the membrane.

Another well documented biochemical function of heat shock proteins was observed in mammalian systems (Figure 6B). A 70 kd polypeptide from bovine brain, subsequently shown to be a heat shock protein (225), dissociates clathrin from coated vesicles in an ATP dependent manner (226, 227). This heat shock protein, called uncoating ATPase, hydrolyzes ATP in the process of dissociating clathrin from coated vesicles and it is bound in molar amounts to the dissociated form of clathrin. The uncoating ATPase recognizes a specific conformation in clathrin, as only the form of clathrin bound to coated vesicles is able to elicit its ATPase activity (228, 229).

During heat stress, proteins are thought to become partially denatured resulting in the exposure of hydrophobic domains that interact to form aggregates. Heat shock proteins may bind to such exposed domains to limit aggregation and to promote refolding of the protein to restore its native structure (Figure 6B). During normal cellular metabolism,

protein complexes that are naturally occurring, yet exhibit faces similar to heat denatured proteins, may be acted upon by the heat shock proteins to facilitate the acquisition of a specific conformation (230).

Figure 5. A model describing the stages and enzymatic requirements of initiation of DNA replication from the lambda phage origin of replication (221).

Stage 1, binding of lambda O protein to the origin; Stage 2, binding of the lambda P protein-dnaB helicase to the initiation complex; Stage 3, binding of dnaJ protein to the initiation complex; Stage 4, binding of dnaK protein to the initiation complex; Stage 5, dissociation of lambda P protein from the initiation complex to activate dnaB helicase activity; Stage 6, RNA priming and DNA synthesis.

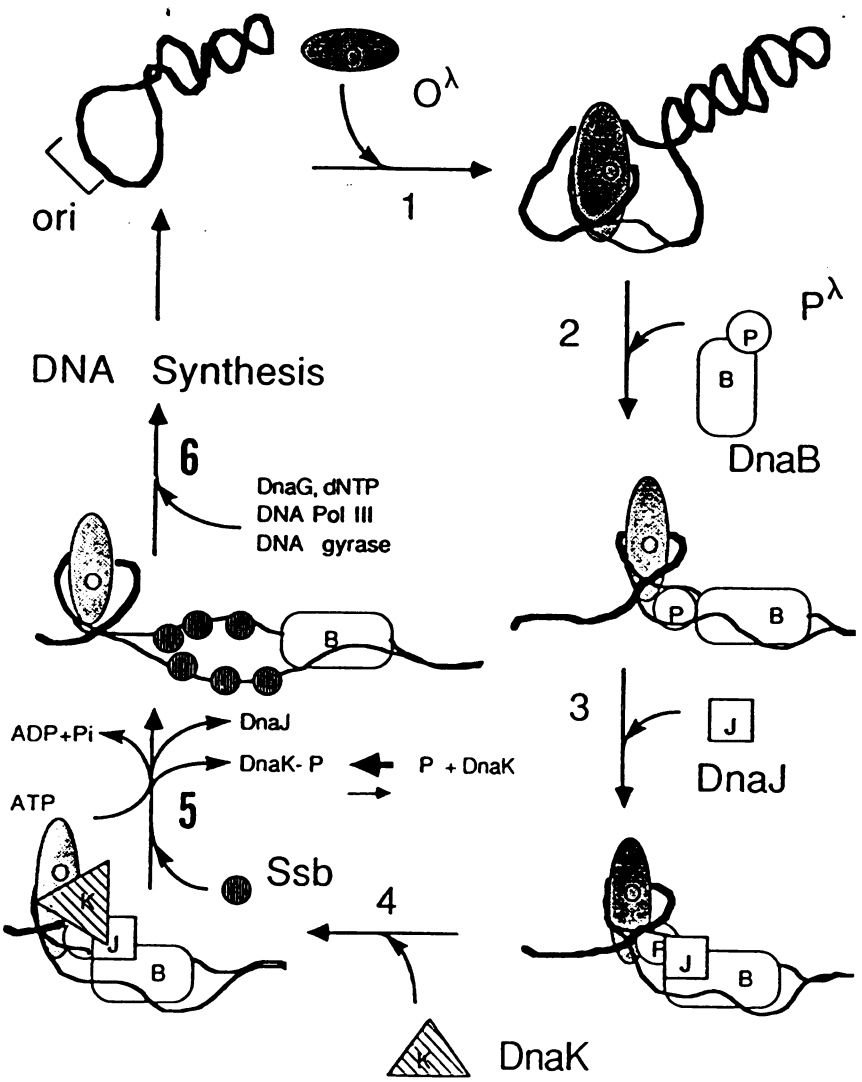
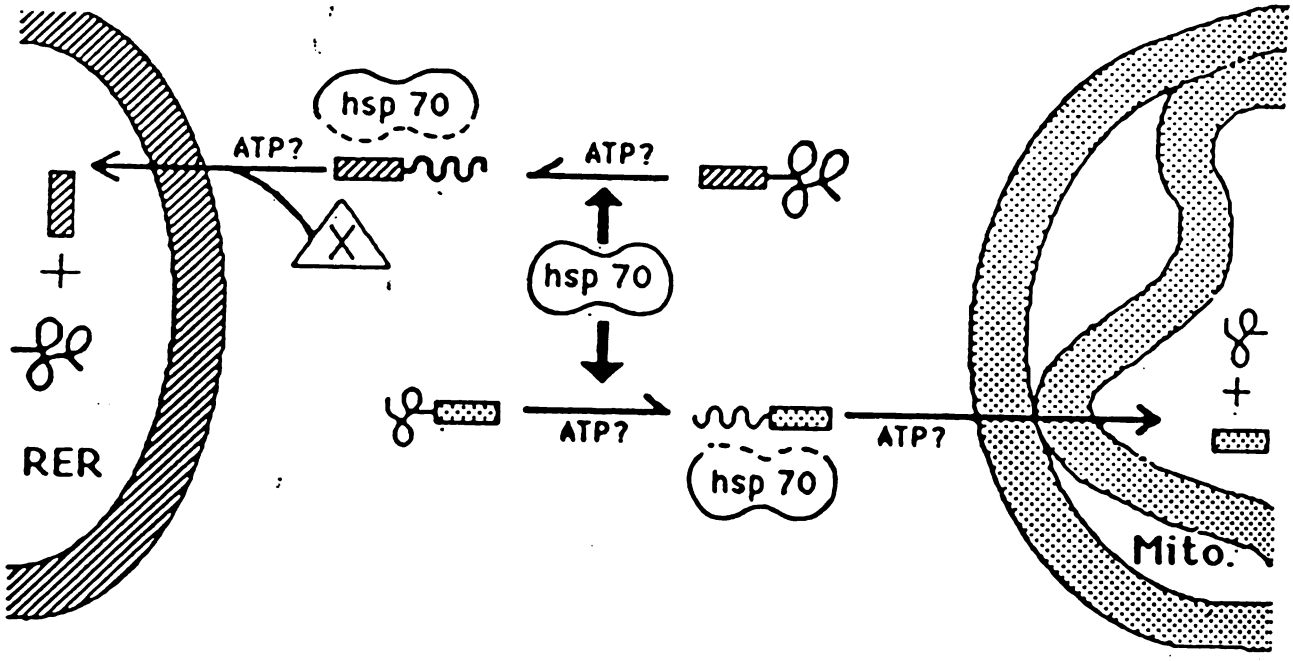
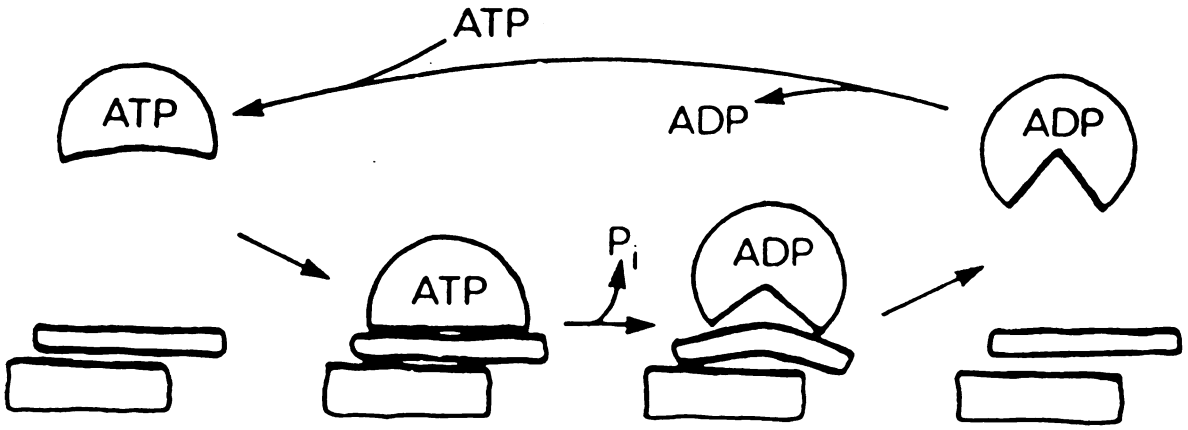

ori
O
O^λ
1
2
P^λ
P
B
DnaB
DNA Synthesis
6
DnaG, dNTP
DNA Pol III
DNA gyrase
3
J
DnaJ
ADP+Pi
ATP
DnaJ
DnaK- P
P + DnaK
5
Ssb
4
K
DnaK

Figure 6. A model describing heat shock protein function in eucaryotic cells.

(A) Hsp 70, in the presence of ATP, binds to unprocessed proteins destined for the endoplasmic reticulum or the mitochondria. Hsp 70 alters the conformation of the protein to catalyze its insertion into membranes (223).

(B) Hsp 70, in the presence of ATP, binds to protein-protein complexes to catalyze the dissociation of specific proteins from the complex (230).


hsp 70
ATP?
ATP?
X
hsp 70
RER
ATP?
ATP?
hsp 70
Mito.


ATP
ATP
ADP
ADP
ATP
Pi
ADP

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Chapter II

SUPPRESSION OF THE *Escherichia coli dnaA46* MUTATION BY A MUTATION IN *trxA*, THE GENE FOR THIOREDOXIN

ABSTRACT

The *dasC* mutation was mapped by P1 transduction near the *rep*, *rho* and *trxA* region of the *E. coli* chromosome. The *dasC* mutation could not be separated from *trxA* by P1 transduction indicating that the *dasC* mutation maps within *trxA*. Multicopy plasmids containing an intact *trxA* gene were able to reverse the suppressive effect of the *dasC* mutation on the *dnaA46* phenotype. Introduction of a frameshift mutation into the cloned *trxA* coding region abolished the ability of the recombinant plasmids to reverse the suppressive effect. These results indicate that *dasC* and *trxA* are allelic.

INTRODUCTION

Initiation of DNA replication from the *Escherichia coli* chromosomal origin, *oriC*, depends on numerous gene products identified by genetic and biochemical analyses. Physiological and genetic studies have indicated that the gene products of *dnaA*, *dnaB*, *dnaC*, *dnaG*, *dnaK*, and *gyrB* are required during the initiation process (1-4). An RNA polymerase mediated event is also required as inferred from the inhibition of initiation of DNA synthesis by rifampicin, a specific inhibitor of RNA polymerase (5, 6).

Biochemical studies of dnaA protein indicate that it acts early in the replication of recombinant plasmids containing the *oriC* sequence and that it binds to DNA sequences within *oriC* (7-9). Other studies have revealed different mechanisms of priming of DNA replication by primase, the product of the *dnaG* locus, by RNA polymerase, or by both enzymes (10, 11). Formation of a pre-priming complex has been observed in replication primed by primase (12). The requirement for proteins, dnaA, dnaB, dnaC, and DNA gyrase suggests their interaction in complex formation and substantiates observations from in vivo studies. A determination of additional properties of dnaA protein and proteins it may interact with will further the understanding of initiation of DNA replication.

A genetic approach to analysis of complex processes has been to select and to characterize extragenic suppressors of mutations in genes whose products are known to be involved in that process (13). This approach has also been used in the isolation of mutants which have not been obtained by conventional genetic approaches (14, 15). Such extragenic suppressors may either interact functionally with the gene product of the primary mutation, alter the levels of the gene product of the primary mutation, or by-pass the requirement of the primary gene product.

This approach has been applied to the analysis of the initiation of DNA replication. Temperature resistant derivatives of thermosensitive alleles of the *dnaA* gene have been isolated (14, 16, 17). One of the first characterized extragenic suppressors of *dnaA* mutants

was mapped in the *rpoB* gene which encodes the B subunit of RNA polymerase (14, 17). Allele specific suppression has been observed in that only some *rpoB* alleles are capable of suppressing particular *dnaA* alleles. Based on this evidence, it has been proposed that RNA polymerase and the *dnaA* gene product interact in a transcriptional event required for the initiation of DNA replication.

Six additional *dnaA* suppressors have been isolated and mapped (14). One of these extragenic suppressors, *dasF*, is allelic with the gene encoding RNaseH (18). This suppression appears to by-pass the normal requirement for *dnaA* mediated initiation of DNA replication at *oriC*, by allowing *dnaA* independent initiations to occur at other sites on the *E. coli* chromosome (19). A second suppressor, *dasC*, has been mapped by the use of specialized transducing phages to the *ilv* region of the chromosome (14). This region is well characterized genetically and includes the *rep* gene which encodes a helicase required fro the replication of bacteriophages, the *rho* gene which encodes a transcriptional termination factor, and *trxA* ,the gene encoding thioredoxin (20-22) . This study extends the characterization of *dasC* by implicating the involvement of thioredoxin in suppression.

EXPERIMENTAL PROCEDURES

Bacterial strains, phages, and plasmids. The bacterial strains used or constructed for this study are listed in Table 1. Phages P1*vir*, M13*Gori*1 (23), and T7, and plasmid pBR322 were laboratory stocks. pJG31 (24) was a gift from D. Calhoun (Mount Sinai School of Medicine).

Strain constructions. The *dnaA46* allele was transduced from K0778 into AB1157 by linkage to *tnaA::Tn10* (25). A tetracycline-resistant, temperature-sensitive transductant named ABA46 was obtained and used in the indicated experiments. The *trxA::kan* allele was transduced from A179 into ABA46. A kanamycin-resistant, temperature-sensitive transductant named TRA46 was obtained. The *trxA::kan* allele was transduced from TRA46 into TC382. A temperature-sensitive isolate (TRH202) was obtained. A temperature-resistant isolate was obtained which resulted from the reversion of *dnaA46* to *dnaA*$^+$. This temperature resistant isolate, TRH204, is isogenic with TRH2 through 7 and TRH9 through 18 (Table 2). F1::*Tn10* from K603 was transferred into TC187 and TC382 with selection by plating the mating mixture onto LB plates containing tetracycline (30 ug/ml) and streptomycin (25 g/ml). LB media and minimal A media supplemented with 0.5% glucose and the required L-amino acids were prepared as described. Where indicated ampicillin (30 ug/ml) and tetracycline (12 ug/ml) were added to the above media.

Plasmid constructions. Plasmid pJGΔKpn was constructed by digestion of pJG31 DNA (Figure 1) with KpnI followed by recircularization of the larger fragment with T4 DNA ligase (New England Biolabs) to delete coding sequences for *rho* and *trxA*. Plasmid pRH023 was constructed by digestion of pJG31 with PvuII and HindIII, followed by isolation of the 3.2 kilobase (kb) fragment containing the *rho* and *trxA* genes. Fragment purification from agarose gels was performed by electrophoresis onto Whatman DE81 paper, elution from the paper by incubation in 1.7 M NaCl, 10 mM Tris-HCl (pH 8), and 1 mM EDTA at 37^{o}C for 2 hr and concentration by ethanol precipitation. This fragment was

ligated to the 2.3 kb PvuII-HindIII fragment of pBR322 containing the replication origin and B-lactamase gene. Self-ligation of the vector was minimized by treatment with calf intestinal phosphatase (Boehringer Mannheim). Plasmid pRH0117 was constructed by linearization of pRH023 at the single *BclI* site in the *rho* gene with BclI restriction enzyme. The cohesive termini were end-filled with the large fragment of DNA polymerase I (New England Biolabs) and religated to produce a frameshift mutation in the *rho* coding region. To form pRH0206, a 12 base pair synthetic BamH1-EcoR1 linker (Worthington) was inserted into pRH023 DNA at the *BclI* site. pTXA2 was constructed by restriction of pRH0206 with BamH1 and recircularization of the larger fragment containing the pBR322 origin of replication, the B-lactamase gene, and the *trxA* gene. pTXA114 and pTXA115 were constructed by cleavage of pTXA2 with ClaI. The cohesive termini were end-filled with the large fragment of DNA polymerase I and religated to introduce a frameshift mutation in the *trxA* coding region. In the above constructions, transformants were initially screened for plasmids with the appropriate structure by restriction analysis of DNA prepared by the method of Davis et al. (26). Recombinant DNAs with the appropriate structure were purified by the cleared-lysate procedure (27) in $CsCl_2$-ethidium bromide gradients and the structure was reconfirmed by restriction analysis. Gradient purified DNAs were used for the above constructions. Restriction enzyme digestions, treatment with calf intestinal phosphatase, end-filling with the large fragment of DNA polymerase I, and transformation of $CaCl_2$-treated competent cells were performed as described (28).

Table 1.

Bacterial Strains

Strain	Genotype	Construction, source, or reference
TC187	*dnaA46 thi argH metB his trp pyrE lac xyl tsx rpsL tna ilvY uhp +*	(14)
TC382	*dasC382a*	(14)
TC383	*dasC383a*	(14)
BW6159	*ilv::Tn10 thil spoT1 relA1 Hfr*	CGSCd
K0778	*flac bglR proA trpB9700 trp::Tn9 his thi supF81 rpsL tsx dnaA46 tnaA::Tn10*	CGSC
AB1157	*thr-1 ara-14 leuB6 fl(gpt-proA)62 lacY1 tsx-33 supE44galK2 - hisG4 rpsL31 xyl-5 mtl-1 argE3 thi-1*	CGSC
ABA46	*dnaA46 tnaA::Tn10b*	This study
A179	*HfrC supD trxA::kan*	(31)
TRA46	*dnaA46 tnaA::Tn10 trxA:: kan*	This study
TRH202	*dnaA46 trxA::kan*	As in Table 2 from an analogous experiment.
TRH204	*trxA::kan* (isogenic with TRH2)	As in Table 2 from an analogous experiment.
TRH2-TRH7, TRH9-TRH18	*trxA::kan*	Table 2
TRH1, TRH8	*dnaA46 dasC trxA::kan*	Table 2
K603	*Fl::Tn10 thr-1 leuB6 trpE63 thi-1 ara-14 lacY1 galK2 galT22 xyl-5 mtl-1 supE44*	CGSC
RK4349	*pro-3 entA403 his-218 ilvC7 metE163::Tn10 flac6 xyl-5 or xyl-7 rpsL109 supE4 hsdR rpsL metB1*	CGSC
C600	*thi-1 thr-1 leuB6 lacY1 tonA21 supE44 -hsdR*	Lab collection
JM103	*F'traD36 fl(lacpro) thi strA supE endA sbc hsdR proAB lacIQZflM15*	Lab collection
A307	*HfrC sup+ fltrxA307 phoA(Am)*	(42)

a isogenic at other loci with TC187
b isogenic at other loci with AB1157
c isogenic at other loci with TC382
d *E. coli* Genetic Stock Center

RESULTS

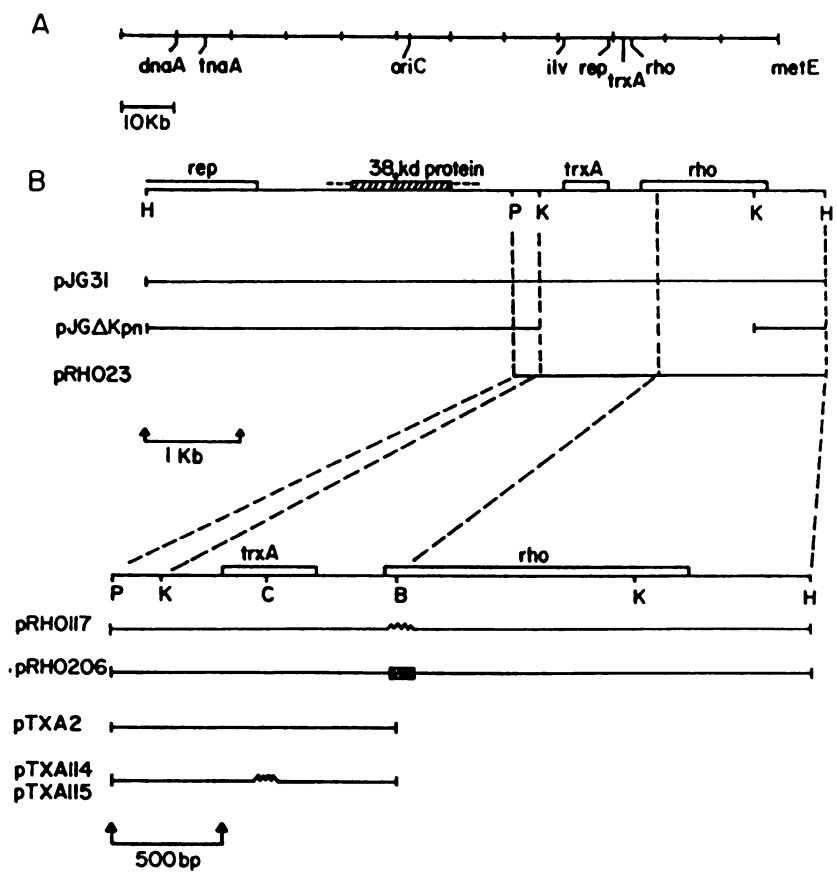
Mapping of dasC by P1 transduction.

The *dasC* mutations in TC382 and TC383 which suppress the temperature sensitive phenotype of the *dnaA46* mutant were reported to be 20-50% linked to the *ilv* locus near 84 minutes on the *E. coli* genetic map ((14); Figure 1). To confirm the map position of the *dasC* mutation, P1 transductions were performed. P1 lysates from BW6159 (relevant genotype, *ilv*::*Tn10*) were used to transduce the *dasC, dnaA46* strains TC382 and TC383 to tetracycline resistance at 30°C. Drug-resistant transductants containing *ilv::Tn10* should also obtain the wild type *dasC* gene with a frequency proportional to the distance between the two loci. Of the 96 tetracycline-resistant transductants of TC382 examined, 68 became temperature sensitive at 40°C presumably due to introduction of the wild type *dasC* gene (71% cotransduction frequency). Transduction of *metE::Tn10* from RK4349 (*metE::Tn10*) into TC382 (*dasC, dnaA46*) resulted in 14 temperature-sensitive colony isolates out of 96 drug-resistant transductants (15% cotransduction frequency). The cotransduction frequencies between *dasC* and *ilv::Tn10, dasC* and *dnaA46* (data not shown), and *dasC* and *metE*::*Tn10* correlates well with the physical map of Figure 1A indicating that the *dasC* mutation maps near the *ilv* operon. It was not possible to demonstrate linkage of *dasC* to *ilv*::*Tn10* with the strain TC383. Work with TC383 was not continued.

Transduction of a trxA null mutant into TC382.

Experiments performed concurrently (described below) indicated that the *dasC* mutation was located in the *trxA* gene which encodes thioredoxin. The insertion mutation in A179 (*trxA*::*kan*) was used to test whether *trxA* and *dasC* were allelic. Because of the proximity of the *dnaA* gene to *trxA*, the *trxA*::*kan* allele was transduced into ABA46 (*dnaA46*) followed by selection for kanamycin-resistant transductants. A temperature-

Figure 1. Physical map of the *trxA* region (31, 32, 35). (A). Relevant genes near *trxA*. (B). Genes contained in the 7 kb Hind III fragment include the C-terminal coding region of *rep*, the *trxA* and *rho* gene (open boxes), and the region encoding a 38 kilodalton (kd) protein of unknown function (crosshatched box). DNA fragments contained in the different recombinant plasmids (solid line) as well as regions which have been deleted are indicated. Mutations introduced by end-filling of cohesive termini by DNA polymerase I (large fragment) and ligation of blunt-ends (⋀⋁), or by insertion of a *BamH1-EcoR1* linkers (▨) are indicated. Restriction enzyme sites : H, *HindIII*; P, *PvuII*; K, *KpnI*; C, *ClaI*; B, *BclI*.


A
dnaA
tnaA
oriC
ilv
rep
trxA
rho
metE
10Kb
B
rep
38kd protein
trxA
rho
H
P
K
K
H
pJG31
pJGΔKpn
pRH023
1 Kb
trxA
rho
P
K
C
B
K
H
pRH0117
pRH0206
pTXA2
pTXA114
pTXA115
500bp

sensitive, kanamycin-resistant transductant, TRA46 (*dnaA46*, *trxA*::*kan*), was obtained and used to transduce the insertion mutation *trxA*::*kan* into the *dasC*, *dnaA46* strain TC382. Of 576 kanamycin-resistant transductants, 558 were temperature sensitive for growth due to the replacement of *dasC* by the *trxA*::*kan* mutation. This suggests that a *trxA* null mutant cannot suppress the temperature-sensitive phenotype of *dnaA46* and that *dasC* and *trxA* might be closely linked. However, further analysis of the remaining 18 temperature-resistant transductants designated TRH1 through TRH18 indicated that they arose either by reversion of *dnaA46* to *dnaA*$^+$, or by an unusual event of gene duplication. The inability to separate *dasC* from *trxA* indicates that they are allelic.

Transduction of dnaA46 into TRH1 through TRH18.

Each of the temperature-resistant transductants, TRH1 through TRH18, was used as a recipient in transduction of *tnaA*::*Tn10* and the linked *dnaA46* mutation from ABA46. Tetracycline-resistant isolates from this experiment were screened for temperature sensitivity. Sixteen of the eighteen transductants named TRH1 through TRH18 appeared to have acquired the *dnaA46* mutation in this experiment near the expected cotransduction frequency (90-95%) with *tnaA*::*Tn10* (Table 2A). These results indicate that the temperature-resistant transductants TRH2 to TRH7, and TRH9 to TRH18, arose due to reversion of the *dnaA46* mutation to wild type. As a control, transduction of *tnaA*::*Tn10* and the linked *dnaA46* mutation into the parental strain TC382 (*dasC*, *dnaA46*) did not result in a high proportion of temperature-sensitive transductants (Table 2B). This reversion rate (16 temperature-resistant isolates of 576 kanamycin-resistant transductants obtained by transduction of *trxA*::*kan* into TC382) is abnormally high relative to the reversion of *dnaA46* to temperature resistance in other strains. This may be due to a deleterious effect of the *trxA*::*kan* allele on growth when introduced into TC382. Such strains grow poorly with a generation time of 65 min in comparison to 35 min for TC382.

Table 2

Transduction of *dnaA46* into TRH1 through TRH18

recipient	tetracycline-resistant colonies	temperature-resistant colonies
A. TRH1	24	22
TRH2	24	0
TRH3	24	3
TRH4	24	0
TRH5	24	1
TRH6	24	1
TRH7	24	0
TRH8	24	21
TRH9	24	0
TRH10	24	3
TRH11	24	0
TRH12	24	2
TRH13	24	0
TRH14	24	0
TRH15	24	0
TRH16	24	0
TRH17	24	0
TRH18	24	1
B. TC382	48	47

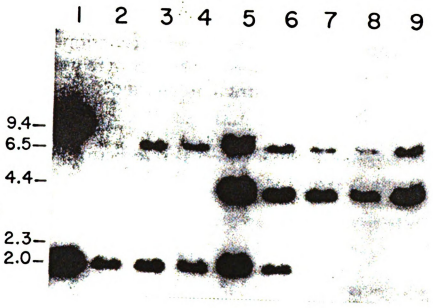
The *dnaA46* allele was cotransduced with *tnaA::Tn10* from ABA46 into the indicated recipient strains.Tetracycline-resistant transductants were obtained and screened for temperature resistance by growth on LB plates at 40°C compared to 30°C.

In contrast, transduction of *tnaA::Tn10* and the linked *dnaA46* mutation from ABA46 into TRH1 or TRH8 resulted in most tetracycline-resistant isolates remaining temperature resistant (Table 2). This may suggest that *dasC* is located close to but not within *trxA* with a linkage of 99.6% (558 temperature-sensitive isolates of 560 kanamycin-resistant transductants). Using the formula derived by Wu (49), this cotransduction frequency may suggest a physical distance of 60 bp separating the two markers . By further examination, TRH1 and TRH8 appeared to have arisen by an unusual tandem duplication to retain both the intact *trxA* gene, presumably *dasC*, and *trxA::kan* based on three lines of evidence.

First, if *trxA::kan* and *dasC* are linked with a cotransduction frequency of 99.6%, P1 transduction of *ilv::Tn10* from BW6159 into TRH1 or TRH8 should result in a cotransduction frequency of about 70% between *ilv::Tn10* and *dasC*$^+$. Of 153 tetracycline-resistant transductants of TRH1 obtained by this cross, only 7 were temperature-sensitive for growth with a linkage of 4.5%. Of 63 tetracycline-resistant isolates of TRH8 examined, 11 were temperature sensitive for growth indicating a linkage of 17.5%. These cotransduction frequencies suggest that *dasC* and *ilv::Tn10* are not as closely linked as expected.

Second, Southern analysis (47) of chromosomal DNA from TRH1 through TRH8 digested with KpnI restriction enzyme indicated the presence of both the *trxA::kan* allele and the *trxA* gene, presumably containing the *dasC* mutation (Figure 2). In this analysis, pTXA2, a pBR322 derivative containing the *trxA* gene in a 1.3 kb chromosomal DNA fragment (Figure 1B), was nick-translated with DNA polymerase I and [α-^{32}P] dCTP and used as a probe. Plasmid pJG31 contains a 7 kb HindIII fragment of chromosomal DNA in pBR322 (24). KpnI digestion of this plasmid produced fragments of 2 kb containing *trxA*, and 11 kb containing the vector and remaining chromosomal DNA (lane 1). Both fragments are homologous to the probe. Two fragments of about 2, and 8.5 kb were detected in KpnI-digested chromosomal DNA from AB1157, TC187 (*dnaA46*), and TC382 (*dasC, dnaA46*) (lanes 2-4). The 2 kb fragment comigrates with the smaller KpnI

Figure 2. Plasmid (30 ng) and chromosomal DNA (1 ug unless indicated) purified (48) from the strains listed were restricted with Kpn I, electrophoresed on a 0.7% agarose gel and transferred to nitrocellulose by the method of Southern (47). Southern analysis was performed using ^{32}P-labeled pTXA2. Lane 1, pJG31; 2, AB1157; 3, TC187; 4, TC382; 5, TRH1 (3 ug); 6, TRH8; 7, TRA46; 8, TRH202; 9, TRH204. Hind III-digested DNA was visualized by ethidium bromide staining prior to transfer and used as a molecular weight marker (sizes indicated in kb)


1 2 3 4 5 6 7 8 9
9.4–
6.5–
4.4–
2.3–
2.0–

fragment from pJG31 and contains the *trxA* gene. The larger fragment of about 8.5 kb corresponds in size with the 8.5 kb KpnI fragment located to the left of the chromosomal KpnI fragment containing *trxA* (Figure 1, (29)) and homologous to the left portion of the inserted fragment in pTXA2. This fragment appears less intense presumably due to the short region of homology. Southern analysis was performed with chromosomal DNA from TRA46 (*dnaA46*, *trxA*::*kan*), TRH202 (*dnaA46, trxA::kan*), and TRH204 (*trxA::kan*) (lanes 7-9). TRH202 and TRH204 are isogenic at other loci with TC382 (Table 2). In addition to the 8.5 kb KpnI fragment from the left of *trxA*, a 3.8 kb fragment was observed corresponding in size to that expected (4 kb) by replacement of *trxA* in the chromosome with the *trxA::kan* allele (31). The 2 kb fragment containing *trxA* was not observed. In contrast, Southern analysis of KpnI-digested chromosomal DNA from TRH1 and TRH8 indicated the presence of the 2 kb fragment presumably containing the *dasC* mutation in *trxA*, the 3.8 kb fragment containing *trxA::kan*, and the 8.5 kb fragment located to the left of *trxA* (lanes 5, 6). Gene duplications such as this involving *trxA*, and *trxA::kan* have been observed following general transduction and are unstable (30).

A similar analysis of HindIII digested chromosomal DNA from these strains confirmed that both *trxA::kan* and *trxA* are present in TRH1 and TRH8 (data not shown). A 7 kb chromosomal HindIII fragment from pJG31, AB1157, TC187 (*dnaA46*), or TC382 (*dasC, dnaA46*) was observed using 32P-labeled pTXA2 as a probe. The kanamycin-resistance gene in *trxA::kan* contains a single HindIII site (31). Chromosomal fragments of about 3.5 kb and 5.2 kb were observed from TRA46 (*dnaA46, trxA::kan*), TRH202 (*dnaA46, trxA::kan*), and TRH204 (*trxA::kan*). These fragment sizes correspond approximately to the fragment sizes expected (5.5 and 3.5 kb) in which *trxA::kan* has replaced *trxA*. The presence of the 7, 3.5, and 5.2 kb fragments in chromosomal DNA from TRH1 and TRH8 not only indicates the presence of *trxA* and *trxA::kan* but also that the event of gene duplication does not involve insertion into the 7 kb HindIII fragment. In addition, other genes in this region also have been duplicated.

The third line of evidence that *trxA* and *trxA::kan* are present in TRH1 and TRH8 is that both kanamycin-resistant isolates support T7 phage growth which requires an intact *trxA* gene (data not shown, see below). From the above experiments, we were unable to separate *dasC* from *trxA::kan* by P1 transduction unless a gene duplication event occurred. These results indicate that *trxA* and *dasC* are allelic.

Mapping of dasC with recombinant plasmids.

The *dasC* mutation was originally mapped by use of *ilv* transducing phages to the right of the *ilv* operon of the *E. coli* chromosome (14). Introduction of particular *ilv* transducing phages into the *dasC, dnaA46* strain TC382 reversed the suppressive effect of the *dasC* mutation and conferred temperature sensitivity. This region of the chromosome has been well characterized and includes *rep*, *rho*, *trxA*, and a gene encoding a 38 kilodalton protein of unknown function (Figure 1B, (21, 22, 32, 33)). Experiments were performed to correlate the *dasC* gene to one of the known genes in the region by an independent method. This approach relied on introduction of a multicopy number plasmid containing wild type chromosomal DNA from this region into TC382, the *dnaA46* strain suppressed by *dasC*. By a gene dosage effect, the wild type gene product encoded by the plasmid may interfere with the suppressor function of the *dasC* gene product to make the transformed strain become temperature sensitive.

The recombinant plasmid, pJG31 (24), containing 7 kb of wild type chromosomal DNA from the *dasC* region inserted into pBR322 (Figure 1B), was used to determine whether the presence of this plasmid in TC382 would confer temperature sensitivity to this *dasC, dnaA46* strain. Cultures of TC382 either lacking a plasmid, transformed with pJG31, or with the vector pBR322 were tested for the ability to grow at 30°C and 39°C. The *dasC, dnaA46* strain containing pJG31 was more temperature sensitive than either TC382 lacking a plasmid, or TC382 containing pBR322 (Table 3). This result indicated

Table 3

Interference of *dasC* function by multicopy number plasmids containing wild type chromosomal DNA.

Expt. A.	Transforming Plasmid	Efficiency of Plating
	--	0.75
	pBR322	0.63
	pJG31	3.5×10^{-2}
	pJGΔKpn	0.81
	pRH023	2×10^{-2}
	pRH0117	1×10^{-4}
	pTXA2	7×10^{-3}

Expt. B.	Transforming Plasmid	Efficiency of Plating
	pTXA2	4.1×10^{-2}
	pJGΔKpn	0.71
	pTXA114	1.0
	pTXA115	0.45

The *dasC, dnaA46* strain TC382 was transformed by the indicated plasmids. Ampicillin-resistant transformants grown at 30°C were then plated after dilution onto LB plates containing 30 ug/ml ampicillin and incubated at 30°C and 39°C (Expt. A) or at 30°C and 40°C (Expt. B) for 1 day. TC382 lacking a plasmid was plated on LB plates. The data are expressed as the efficiency of plating at 39°C or 40°C relative to 30°C. In comparable experiments, the efficiency of plating of TC187, the isogenic *dnaA46* strain not suppressed by the *dasC* mutation, was $<10^{-5}$ at 40°C relative to 30°C.

that this 7 kb chromosomal fragment contained in pBR322 was responsible for the increase in temperature sensitivity.

Derivatives of pJG31 were then constructed to determine the region within this 7 kb chromosomal fragment essential for reversing the suppressive effect of *dasC* on the *dnaA46* mutation. The recombinant plasmid, pJGΔKpn, was constructed which lacks sequences encoding thioredoxin and rho protein (Figure 1B). TC382 (*dasC, dnaA46*) containing this plasmid was able to grow at 30°C and 39°C as well as either the untransformed strain or this strain harboring pBR322 (Table 3). This result indicates that *rho* and/or *trxA* are required for reversing the suppressive effect of the *dasC* mutation. It is also possible that an unknown gene product encoded by this region was responsible for the observed phenotype.

The *rho* and *trxA* genes from this region were subcloned from pJG31 to form the plasmid pRH023 (Figure 1B). As was observed with transformants of TC382 containing pJG31, this *dasC, dnaA46* strain harboring pRH023 was temperature sensitive for growth. This result indicates that the region lacking in pJGΔKpn and contained in pRH023 was required for reversing the suppressive effect of *dasC*.

Recombinants mutationally altered in the *rho* gene or lacking most of the *rho* coding sequence were prepared. These include pRH0117 containing a frame-shift mutation, and pTXA2 containing the N-terminal coding region of the *rho* gene (Figure 1B). The indicated plasmids altered in the *rho* gene were able to reverse the suppressing effect of the *dasC* mutation (Table 3). This indicates that the wild type *rho* gene in intact form apparently is not required for the increase in temperature sensitivity of the plasmid-containing *dasC, dnaA46* strain.

Based on DNA sequence information (34, 35) and restriction analysis, the recombinant pTXA2 contains the entire thioredoxin gene in active form (see below), the *rho* promoter, and coding sequences for 13 amino acids of the N-terminal region of the *rho* polypeptide. Upon transformation into TC382 (*dasC, dnaA46*), pTXA2 was able to counteract

the suppressive effect of the *dasC* mutation (Table 3).

The coding region for thioredoxin in pTXA2 was altered by cleavage with ClaI restriction endonuclease, and conversion of the cohesive termini to blunt ends by the large fragment of DNA polymerase I. The blunt ends were religated together which is expected to produce a frame-shift mutation to inactivate thioredoxin (see below). This construction also interrupts an open reading frame predicted to direct the synthesis from the coding strand of *trxA* of a polypeptide of 132 amino acids. As this coding sequence which would be complementary to the *trxA* message lacks even an infrequently used initiating codon (36), it is unlikely that this open reading frame, if transcribed, is translated. The resultant plasmids pTXA114 and pTXA115, as well as the vector pBR322 were unable to reverse the suppressive effect of the *dasC* mutation when introduced into TC382 (*dasC, dnaA46*) (Table 3).

Thioredoxin is the only intact gene known to be present in the inserted fragment of pTXA2. These results indicate that the *dasC* gene is allelic with *trxA*, the gene for thioredoxin. When contained in MC1061, a strain wild type for the *dasC* and *dnaA* genes, plasmids pJG31, pJGΔKpn, and pRH023 did not affect the growth of this strain at either 30°C or 39°C (data not shown). This indicates that conferral of temperature sensitivity by plasmids containing *trxA* is specific for the strain TC382.

The dasC mutation supports T7 and M13 growth .

Thioredoxin has been shown to be essential for M13 and T7 phage propagation (37, 38). The ability of these phages to form plaques on the *dasC, dnaA46* strain was determined in an attempt to detect an alteration in thioredoxin activity. T7 phage plated with approximately the same efficiency on TC382 (*dasC, dnaA46*), the isogenic parent strain TC187 (*dnaA46*), and on C600 (Table 4).

Table 4

Growth of phages T7 and M13*Gori*1 on *dasC*, and *trxA* mutant strains.

Strain (plasmid)	Relevant Genotype of Host	T7 Phage	M13*Gori*1 Phage
TC187	*trxA*$^+$	+	+*
TC382	*dasC*	+	+*
C600	*trxA*$^+$	+	ND
JM103	*trxA*$^+$	ND	+
A307	*trxA307*	-	-
A307(pJG31, *trxA*$^+$)	Δ*trxA307*	+**	+
A307(pTXA2, *trxA*$^+$)	Δ*trxA307*	+	+
A307(pTXA114, *trxA*$^+$)	Δ*trxA307*	-	-
A307(pTXA115, *trxA*$^+$)	Δ*trxA307*	-	-
A307(pBR322)	Δ*trxA307*	-	-

T7 or the filamentous phage M13*Gori*1 were plated on the strains listed and on A307 containing the indicated plasmids. LB plates were incubated overnight at 33°C for the indicated strains except for A307 which was incubated at 37°C. ND, not determined; +, sensitive to plaque formation; -, insensitive to plaque formation ($<10^{-5}$ for T7, $<10^{-8}$ for M13*Gori*1).

*, *F1::Tn10* derivatives of TC187 and TC382 were used.

**, T7 plaque formation on this strain was 50-fold lower relative to C600.

The ability of M13 to form plaques was tested after introduction of an F factor from K603 into TC382 and the parent strain TC187 (Experimental Procedures). The filamentous phage M13Goril was able to form plaques with approximately equal efficiencies on F factor-containing derivatives of TC382 (*dasC*, *dnaA46*), TC187 (*dnaA46*), and on JM103. Both T7 and M13Goril were not able to form plaques on the thioredoxin deletion mutant A307 unless this strain harbored the recombinant plasmid pJG31, or pTXA2 (Table 4). The *trxA* deletion mutant A307 containing pTXA114 or pTXA115 altered by a 2 bp insertion in the *trxA* coding region remained insensitive to plaque formation with either M13 or T7 phage. These results indicate that only recombinant plasmids containing an intact *trxA* gene could complement the *trxA* deletion mutation to support T7 and M13 phage growth.

The result that both T7 and M13Goril can form plaques with approximately equal efficiencies on a wild type strain and on the *dasC*, *dnaA46* strain TC382 indicates that the *dasC* mutation does not dramatically decrease the ability of thioredoxin to function in T7 or M13 growth.

DISCUSSION

We have determined that a mutation, *dasC*, capable of suppressing the temperature-sensitive phenotype of the *dnaA46* mutation maps in the gene encoding thioredoxin, a protein initially isolated as a cofactor for the reduction of ribonucleoside diphosphates by ribonucleotide reductase (39). This conclusion is based on the inability to separate *dasC* from a *trxA::kan* mutation by P1 transduction and on the ability of multicopy number plasmids containing the *trxA* gene to reverse the suppressive effect of *dasC* on the *dnaA46* mutation. In contrast, alteration of the *trxA* coding region in the plasmid by a frameshift mutation abolished the ability of the recombinant plasmid to interfere with *dasC* function in suppression.

Thioredoxin is required for T7 phage growth as an essential subunit of T7 DNA polymerase (37, 40). The phage-encoded gene 5 protein and thioredoxin in a 1 to 1 stoichiometry comprise the active form of this enzyme. Whereas T7 cannot be propagated in mutants of *E. coli* lacking thioredoxin, *trxA* mutants which produce thioredoxins lacking redox activity will support T7 phage growth (33). Biochemical studies with mutant thioredoxin-gene 5 protein complexes indicate that while the reduced form of thioredoxin is necessary to reconstitute T7 DNA polymerase, DNA synthesis by this enzyme does not require further reductant (41).

Thioredoxin is also required as a host factor for filamentous phage assembly (38). Studies indicate that mutant forms of thioredoxin which are inactive as redox proteins will support filamentous phage growth (42). Thioredoxin is not apparently required as a reductant for phage assembly.

The studies reported here indicate that the *dasC* mutation in *trxA* does not alter the ability of this protein to function in growth of either T7 or M13. Russel and Model (42) described an in vivo assay of thioredoxin redox function. Mutants of thioredoxin inactive as redox proteins cannot grow on media containing methionine sulfoxide when the normal

methionine generating pathway is blocked. TC382 containing mutations in *metB* and *dasC* plated with equal efficiency on minimal media supplemented with either methionine sulfoxide or methionine and other required amino acids indicating that the *dasC* mutation does not appear to affect the redox activity of thioredoxin (unpublished data). Preliminary studies with partially purified preparations of thioredoxin from this *dasC* strain also indicated that it is active in reduction of 5,5'-dithiobis-(2-nitrobenzoic acid).

Eklund et al. (43) have described domains of thioredoxin which may be involved in interactions between thioredoxin and other proteins in oxido-reduction. Mutations in *trxA* which affect filamentous phage assembly have been mapped at or near these domains (42). Some of these mutations encode proteins which are active in redox reactions indicating a functional interaction between these mutant forms of thioredoxin and thioredoxin reductase. It is not known whether the *dasC* mutation maps in one of these domains.

Of its many activities, thioredoxin has been described as a protein disulfide reductase to regulate activity of enzymes involved in CO_2 fixation in plants (20). No evidence exists to indicate that the activity of dnaA protein is influenced by the redox state of its thiol groups. The existence of viable *E. coli* mutants lacking thioredoxin argues against a role of thioredoxin in modulating the activity of dnaA protein unless this effect is not absolutely required or unless another activity in *E. coli* can replace the function of thioredoxin. With regard to the latter possibility, it is thought glutaredoxin and glutaredoxin reductase function in the synthesis of deoxyribonucleotides in the absence of thioredoxin (44).

Thioredoxin has also been observed to function as an efficient protein disulfide isomerase to catalyse the refolding of denatured RNase (45). While no evidence exists to indicate that the native conformation of monomeric dnaA protein is stabilized by disulfide bonds or that its cooperative binding to dnaA protein binding sites requires disulfide bond formation between monomers of dnaA protein, it is possible that the form of thioredoxin encoded by the *dasC* mutation effectively maintains dnaA46 protein in an active form at the normally nonpermissive temperature.

We have not been able to transduce the *dasC* mutation with phenotypic expression from TC382 into isogenic and nonisogenic *E. coli* strains harboring the *dnaA46* allele. This inability suggests that an unlinked mutation unstable in the absence of selection is also required for *dasC* to function as a suppressor.

Attempts to identify the position of the *dasC* mutation by DNA sequence analysis have not been successful. Based on a cloning strategy described by Russel and Model (31), chromosomal DNA from TC382 (*dasC, dnaA46*) was inserted into an M13 vector followed by transformation of the thioredoxin deletion mutant A307 and selection for plaque formation. This cloning strategy requires that the *trxA* gene in the M13 recombinant complements the host *trxA* deficiency to allow phage growth. Sequence analysis of several recombinants obtained by this method indicated that the promotor and *trxA* coding region were identical to the wild type sequence. Under conditions which did not select for thioredoxin function in M13 phage growth, M13 recombinants identified by plaque hybridization (46) and analyzed by DNA sequencing or by restriction enzyme mapping lacked the C-terminal coding region of the *trxA* gene as well as a portion of the vector at the site of insertion. These results indicate that the deletion event occurred during propagation of the recombinant phage. Under identical conditions, the wild-type *trxA* gene inserted into M13 was not deleted. These results suggest that the *dasC* mutation reverts to wild type under conditions of selection. In the absence of selection, *dasC* may be deleterious to cell growth when present in multicopy. Cells capable of maintaining M13 recombinants may arise by deletion of a portion of the *trxA* coding region.

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Chapter III

THE *Escherichia coli* dnaA5 PROTEIN IS INACTIVE IN THE REPLICATION OF *oriC* PLASMIDS IN RECONSTITUTED ENZYME SYSTEMS

ABSTRACT

The purification of dnaA5 protein allowed for its characterization in comparison to dnaA protein. The biochemical characteristics of dnaA5 protein include: 1) a high affinity for DNA containing *oriC* and the *dnaA* promoter, 2) an ability to form proper protein-DNA contacts at the dnaA boxes within the *rpoH* and *dnaA* promoters, 3) the formation of an altered complex with *oriC*, 4) inactivity in *oriC* unwinding assays, 5) inactivity in *oriC* plasmid replication systems containing purified enzymes, 6) inhibition of dnaA protein replication activity suggesting that mixed complexes which form are inactive and 7) thermolabile replication activity in *oriC* plasmid replication systems containing a crude enzyme fraction.

INTRODUCTION

The initiation of DNA replication in *E. coli* is a highly regulated event occurring at regularly timed intervals within the cell cycle (1). The activity of the initiator protein, dnaA, is essential for replication initiating from the chromosomal origin, *oriC*, both *in vivo* (2) and *in vitro* (3). Other enzymes involved in the initiation stage have been identified genetically and biochemically, and include dnaB protein, dnaC protein, DNA gyrase, primase, RNA polymerase, topoisomerase I, and RNase H (4). A *dnaK* mutant defective in the initiation of DNA replication *in vivo* has been identified (5), indicating that there is a requirement for the *dnaK* gene product during initiation.

The biochemical characterization of the *dnaA* dependent initiation process has led to the reconstitution of purified enzymes that function to replicate *oriC* plasmids *in vitro* (3, 6, 7). Early events in the replication process result in the conversion of supercoiled *oriC* plasmid DNA into topologically altered forms by the actions of dnaA protein, and other initiation proteins (8), demonstrating the specific function provided by dnaA protein during a stage prior to DNA synthesis. Additional studies have demonstrated that dnaA protein can function as a site-specific helicase to locally unwind DNA at *oriC* (9), allowing for the formation of single-stranded regions capable of being bound by pre-primosomal enzymes.

The individual biochemical activities of dnaA protein which are required for its replication activity include 1) sequence specific binding to DNA containing the consensus sequence TTAT (C/A)CA (A/C)A, (10), 2) ATP binding with a K_d of 0.03 uM (11) which converts it to a form active for replication, 3) ADP binding which inhibits its replication activity, and 4) cardiolipin activation of the ADP form of dnaA protein (12, 13). The initial event in initiation involves dnaA protein binding to *oriC* through the interaction with four dnaA boxes to form a nucleoprotein complex which precedes its function in the ATP dependent duplex strand unwinding stage (14, 15). The sequence specific DNA binding activity of dnaA protein is also involved in transcriptional repression of certain genes. dnaA

protein binds to the dnaA box within its own promoter autoregulating its transcription (16-18) and to two dnaA boxes within the *rpoH* promoter to inhibit transcription initiating from *rpoH*3P and *rpoH*4P (19).

The identification of conditionally lethal *dnaA* mutants that are defective in the initiation of replication has led to the genetic mapping of the mutations and the positions were shown to be clustered within three regions of the *dnaA* coding region (20). The *dnaA* mutants were placed into three classes and the specific phenotypes associated with each class were categorized in order to associate a domain of the *dnaA* gene product with a specific function. The *dnaA* mutants that were categorized are temperature sensitive for cell growth presumably due to the inactivation of the *dnaA* gene product at elevated temperatures. Some of these conditionally lethal mutants, which include *dnaA46* and *dnaA5*, are cold sensitive in the merodiploid state and asynchronous in the frequency of initiation of DNA replication at permissive temperatures (21).

In a rapidly growing cell there is more than one initiation event during the cell cycle which results in an increase in the number of *oriC* origins. In response to the signal demanding that replication begin, initiations at all of the *oriC* origins occur simultaneously indicating that initiation is synchronous. In some *dnaA* mutants, initiation does not occur at each *oriC* origin as it does in the wild type *dnaA* cell indicating that the mutant has lost the ability to ensure that all *oriC* sites are simultaneously utilized. This un-coordinated initiation is termed asynchronous. The altered timing of initiation, under conditions in which the mutant protein is active, may reflect an intrinsic defect in its initiation activity or a regulatory deficiency.

The biochemical characterization of these mutant forms of dnaA protein may lead to a greater understanding of the role of the *dnaA* gene product in initiation of DNA replication. The purification and characterization of the biochemical deficiencies of dnaA5 protein are reported with the goal of understanding the mechanisms related to the physiological defects associated with *dnaA5* cells.

EXPERIMENTAL PROCEDURES

Reagents and chemicals. Reagents were obtained from the following sources: ribonucleotides, tRNA, polyvinyl alcohol (PVA) (type II), phosphocreatine, heparin, ampicillin, L-arabinose and calf thymus DNA, Sigma; deoxynucleotides and sepharose 4-B, Pharmacia-PL Biochemicals; HEPES, Tris Base, and dithiothreitol (DTT), Calbiochem-Behring; (α-^{32}P) ATP (800 Ci/mmol or 3000Ci/mmol and (γ-^{32}P) ATP (6000 Ci/mmol), DuPont-New England Nuclear Corp.; (^{3}H) TTP, ICN Radiochemicals; hydroxylapatite, Biorad; acrylamide, Boehringer Manheim; TB media contains per liter, 12 g yeast extract (Difco), 24 g tryptone (Difco), 100 mM sodium phosphate (pH 7.0), and 4% glycerol; DNA cellulose was prepared according to the published methods (22) ; and Blue Dextran agarose was a gift from Dr. J. Wilson of this University.

Enzymes and proteins. Enzymes and proteins were obtained from the following sources: bovine serum albumin, Sigma; T4 polynucleotide kinase, restriction endonucleases AvaI, HinfI, ClaI, and TaqI, New England Biolabs; calf intestinal phosphatase (CIP), Klenow fragment, restriction endonucleases AccI and EcoRI, Boehringer Manheim; restriction endonuclease BamHI, Bethesda Research Laboratories.

Highly purified replication proteins were obtained as described (23), dnaA protein (fraction IV, 2 x 10^5 units/mg); dnaA5 protein (fraction V, 6 x 10^4 units/mg) (Chapter III); dnaB protein (fraction V, 6 x 10^5 units/mg) ; dnaC protein (fraction VI, 3 x 10^6 units/mg); primase (fraction V, 2 x 10^6 units/mg); single stranded DNA binding protein (SSB) (fraction IV 4 x 10^4 units/mg); DNA polymerase III holoenzyme (fraction V, 2 x 10^5 units/mg); DNA gyrase subunit A (fraction III, 2 x 10^5 units/mg); DNA gyrase B subunit (fraction V, 1 x 10^5 units/mg) and RNA polymerase (fraction V, 250 munits/mg). RNase H (fraction IV, 8 x 10^5 units/mg); topoisomerase I (fraction IV, 5 x 10^4 units/mg) and HU protein (fraction IV, 5 x 10^4 units/mg) were gifts from Dr. A. Kornberg, Stanford

University. repA protein (fraction V, 8 x 10^5 units/mg) (29) was a gift from Dr. A. Abeles.

Bacterial strains and plasmid DNAs. Escherichia coli WM433 was obtained from Dr. W. Messer (24); K37 *HfrC supD* (lambda) is a laboratory stock; TD2, is a derivative of MC1061 containing *tnaA::TN10* and the *dnaA5 allele*; M13*oriC*26 (25) contains *oriC* and adjacent *E. coli* chromosomal DNA in M13*Gori*1; M13*oriC*2LB5 (3) contains *oriC* on a 345 bp EcoRI fragment in M13*Gori*1; pTSO182 (27) contains *oriC* and adjacent chromosomal DNA on a HaeIII fragment in pBR322 ; M13P1*ori*49 contains the P1 phage replication origin cloned into M13mp18 (31); pDS105 (23) contains the *dnaA5* gene cloned under the control of the *araB* promoter in the vector pING1 (26); pBF1509 contains the *dnaA* gene cloned into the *BamH1* site of pAD329 (10); and pFN42 contains the promoter of the *rpoH* gene and a portion of the N-terminal coding region of *rpoH* cloned into the *PstI* site of pBR322 (19). Plasmid DNAs were prepared by the cleared lysis method followed by sedimentation in $CsCl_2$-ethidium bromide gradients (32).

DNA replication assays. DNA replication reactions that employ a crude enzyme fraction deficient in dnaA protein were performed as previously described (28). DNA replication assays using *oriC* plasmid DNA and a crude enzyme fraction capable of sustaining DNA synthesis upon the addition of dnaA protein were performed in a 25 ul reaction volume containing: 25 mM HEPES-KOH, pH 7.8 ; ATP, 2 mM; CTP, UTP, and GTP, each at 0.5 mM; dATP, dCTP, dGTP, and (^{3}H) TTP (25 to 40 cpm/pmol), each at 100 uM; magnesium acetate, 11 mM; PVA, 8 % (w/v); phosphocreatine, 40 mM; creatine kinase, 100 ug/ml; supercoiled M13*oriC*26, 200 ng; and Fraction II from WM433 (28), 160 ug to 200 ug of protein. The reaction mixtures were assembled at 0°C and subsequently incubated at 30°C for 25 minutes unless otherwise indicated. The incorporation of tritiated nucleotide into trichloroacetic acid precipitable DNA was quantitated by liquid scintillation counting (23). One unit of replication activity represents one pmol of nucleotide incorporated into DNA per minute at the indicated temperature.

The conditions used to demonstrate DNA replication initiating from P1 phage origin DNA templates involved the use of a replication system that employs a crude enzyme fraction, dnaA protein and phage encoded repA protein as previously described (30). The conditions used were identical to those used for the *oriC* plasmid replication assay employing WM433 Fraction II as the crude enzyme source, except 100 ng of M13P1*ori*49 plasmid DNA and purified repA protein were used.

DNA replication reactions using purified enzymes were performed as previously described (23). The 25 ul reactions contained: HEPES-KOH, pH 7.8, 25 mM; Tris-HCl, pH 7.5, 20 mM; sucrose 4% (w/v); ATP, 2mM; CTP, GTP, and UTP, each at 0.5 mM; dATP, dCTP, dGTP, and (^{3}H) TTP (25 to 40 cpm/pmol) each at 100 uM; magnesium acetate, 11 mM; phosphocreatine, 2 mM; DTT, 5 mM; creatine kinase, 100 ug/ml; BSA, 0.08 mg/ml; SSB, 220 ng; HU, 25 ng; topoisomerase I, 2.5 units, and RNase H, 3 ng, were used only in the RNA polymerase dependent reactions; gyrase A subunit, 470 ng; gyrase B subunit, 600 ng; primase, 10 ng; dnaB protein, 100 ng; dnaC protein, 25 ng; RNA polymerase, 500 ng, where noted; DNA polymerase III holoenzyme, 270 ng; M13*oriC*2LB5 supercoiled DNA, 200 ng; and the indicated amounts of dnaA protein or dnaA5 protein. The reactions were assembled at 0°C and the incubations were performed at 30°C for 30 minutes unless otherwise indicated. The total nucleotide incorporation was measured as previously described (26)

DNA binding assays. DNA fragment retention assays on nitrocellulose filters followed by gel analysis to visualize the DNA have been previously described (10). The binding of dnaA protein or dnaA5 protein to DNA fragments was performed by the addition of the indicated amounts of protein at 0°C in a 25 ul reaction volume containing; 100 ng of pTSO182 DNA restricted with TaqI endonuclease and end-labeled with (α–^{32}P) dCTP and DNA polymerase I (large fragment); 40 mM HEPES-KOH, pH 7.8; 5 mM $MgCl_2$; 2 mM DTT; and 50 mM KCl. Following the incubation at 30°C or 38°C for 10 minutes, the samples were filtered through Millipore HAWP nitrocellulose filters (0.22um pore size)

and washed with 250 ul of the reaction buffer. The DNA bound to the filters was eluted by incubating the filter in elution buffer (0.5% (w/v) SDS, 500 mM magnesium acetate, 50 mM Tris-HCl (pH 8.0), and 10 mM EDTA) at 65°C for 20 minutes. The DNA was precipitated from the fractions contained in the flow through and bound samples by the addition of tRNA to 100 ug/ml, NaCl to 100 mM, ethanol to 70% (v/v), and incubation at -70°C for 10 minutes. The samples were centrifuged at 12, 000 x g for 15 minutes and the pellets were resuspended in 25 ul of gel loading buffer (10% glycerol, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0)). Portions of each reaction were separated by electrophoresis in a 6% polyacrylamide gel in TBE. The gels were dried on a Hoefer Scientific gel dryer (model SE 540) and exposed to Kodak XAR-5 film at -70°C using a Crona Quanta III intensifying screen.

The binding affinity of dnaA5 protein for DNA containing the *dnaA* promoter and for *oriC*, in comparison to dnaA protein, involved quantitating the amount of DNA bound to nitrocellulose filters following the methods which are described above. A 463 bp AvaI DNA fragment from M13*oriC*26, which contains *oriC* , and a 390 bp BamHI/EcoRI DNA fragment from pBF1509 containing the *dnaA* promoter fragment were separately treated with CIP and end-labeled with (γ-^{32}P) ATP and T4 polynucleotide kinase. In the binding reaction, 15 fmol of either end-labeled DNA fragments was incubated at 30°C or 40°C for 10 minutes in a 25 ul volume containing; 150 fmol of unlabeled pBR322 DNA restricted with HinfI; 40 mM HEPES-KOH (pH 8.0); 5 mM $MgCl_2$; 2 mM DTT; 25 mM $(NH_4)_2SO_4$ and the indicated amounts of dnaA or dnaA5 proteins. Following the incubations, the samples were filtered through Millipore HAWP nitrocellulose membranes and washed with 250 ul of pre-warmed reaction buffer. The radioactive DNA bound to the filter was quantitated by liquid scintillation counting.

ATP binding assays. The conditions used to demonstrate ATP binding by dnaA protein have been previously described (11, 31). A 25 ul reaction volume contained: 2 pmol of dnaA protein or dnaA5 protein; 0.5 uCi of (α-^{32}P) ATP; the indicated concentrations of

ATP; 0.5 mM magnesium acetate; 5 mM DTT; 15 % (v/v) glycerol; 0.01% (v/v) triton X-100; and 50 mM Tris-HCl, pH 8.0. The incubations were performed at 0°C for 15 minutes, filtered through Millipore HAWP nitrocellulose membranes and the filters were washed with 500 ul of the reaction buffer. The radioactive ATP bound to the nitrocellulose filter was quantitated by liquid scintillation counting.

DNase I protection assays. The conditions used to perform DNase I protection experiments have been previously described (10). A standard 10 ul reaction mixture contained; DNase I binding buffer (10 % glycerol (v/v), 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.2 mM EDTA (pH 7.0), 2 mM DTT, and 100 ug/ml BSA), 50 mM $(NH_4)_2SO_4$ unless otherwise indicated, 25 fmol of (α-^{32}P) end-labeled DNA (*oriC* was a 463 bp AvaI restriction fragment from M13*oriC*26 end-labeled and restricted with HinfI; the *dnaA* promoter was a 390 bp BamHI/EcoRI fragment from pBF1509 end-labeled and restricted with HinfI; and the *rpoH* promoter was labeled as described (19)) and the indicated amounts of dnaA protein or dnaA5 protein. The incubations were performed at 30°C for 10 minutes, after which 0.25 ul of a DNase I solution (prepared by diluting a 2 ug/ml solution of DNase I 200 fold in DNase I binding buffer containing 200 mM NaCl) was added. The incubations were continued at 30°C for 30 to 60 seconds and the reactions were quenched by the addition of 12 ul of DNase I stop buffer (0.1 % (w/v) SDS, 300 mM sodium acetate, 20 mM EDTA, and 100 ug/ml tRNA). The reaction products were precipitated by the addition of two volumes of ethanol and incubation at -70°C for 15 minutes. Following centrifugation at 12, 000 x g, the pellets were resuspended in DNase I gel loading buffer (80 % formamide (v/v), 10 mM NaOH, 0.01% (w/v) Bromophenol Blue, and 1 mM EDTA). The samples were boiled for two minutes before electrophoresis in a 7 M urea/ 6 % (w/v) polyacrylamide sequencing gel. The gels were dried under a vacuum and exposed to Kodak XAR-5 X-ray film at room temperature.

Immunological methods. The ELISA was performed as previously described (33) in order to localize dnaA5 protein during chromatography. Rabbit serum containing antibodies to

dnaA protein was obtained after the injection of the protein (Mono S fraction V) into the popliteal lobes of rabbits as previously described (33). Portions of the fractions that eluted during chromatography were incubated for one hour at room temperature in microtiter wells (Nunc) containing 100 ul of 50 mM sodium borate (pH 9.0). The wells were washed four times with PBS containing 0.05 % (v/v) tween 20, 100 ul of a 1:2000 dilution of dnaA anti-serum (in PBS containing 0.05 % (v/v) tween 20 and 0.2 % (w/v) BSA) was added to the microtiter wells and the incubations were continued for one hour at room temperature. The wells were washed four times with PBS containing 0.05 % (v/v) tween 20, 100 ul of a 1:1000 dilution of goat-anti rabbit HRP (Biorad) (in PBS containing 0.05 % (v/v) tween 20 and 0.2 % (w/v) BSA) was added to the microtiter wells and the incubations were continued for one hour at room temperature. The wells were washed four times with PBS containing 0.05% (v/v) tween 20 and HRP activity was detected as previously described (33).

Protein determination. Protein determinations were performed by the method of Bradford (23).

RESULTS

Overproduction of dnaA5 protein.

dnaA5 protein was purified from TD2 cells (*dnaA5*, *tnaA::TN10*) harboring the plasmid pDS105 which contains the *dnaA5* gene cloned into the protein overproducing vector pING1 (26). This vector contains a cloning site downstream of the *araB* control region and the induction of cloned genes can occur after the addition of arabinose to the cell culture.

The initial experiments involved maximizing the expression of dnaA5 protein in mid-log phase cell cultures containing pDS105 by the addition of arabinose to 0.75 % (w/v). A time course of the induction of dnaA5 protein was performed to determine the time required for maximal expression. The extent of dnaA5 protein overproduction was quantitated by immunological detection of antigenic material from induced whole cell lysates (data not shown) and by determining the levels of dnaA5 protein dependent replication activity present in crude lysates obtained from the induced cells. dnaA5 protein activity in replication systems that utilize a crude enzyme fraction was detected in lysates obtained from cells which were grown in TB media, induced with arabinose and grown for an additional 3 to 4 hours (data not shown). Activity was not detected in lysates obtained from cells grown and induced in LB media under identical conditions, demonstrating the importance of the media and the state of the cell in obtaining elevated quantities of dnaA5 protein (data not shown).

Purification of dnaA5 protein.

The chromatographic steps utilized during the purification of dnaA5 protein were similar to those used during the purification of dnaA protein (23). Presumably, the missense mutation in dnaA5 protein would not have altered its chromatographic characteristics in comparison to dnaA protein. The purification of dnaA protein involves: 1) lysis of cells

which contain the *dnaA* gene cloned into pING to obtain a soluble protein fraction; 2) chromatography on heparin agarose; 3) chromatography on gel filtration resins; and 4) anion exchange chromatography on Mono S. Of these resins, only heparin agarose could be used in the purification of dnaA5 protein because its elution characteristics were drastically different in comparison to dnaA protein. In general, dnaA5 protein eluted broadly from the resins examined and sufficient purification was not achieved. Alternative methods were used for the purification of dnaA5 protein are summarized in Table 1.

TD2 cells harboring the plasmid pDS105 were grown in 12 L of TB media, containing 30 ug/ml of ampicillin, at 30°C to an O.D.595 of 0.5 and arabinose was added to a final concentration of 0.75 % (w/v). The cells were grown for an additional 3.5 to 4 hours with vigorous aeration and harvested by centrifugation at 5,000 x g for 15 minutes. The cells were resuspended in TS buffer (50 mM Tris-HCl (pH 8.0) and 25% sucrose (w/v)) to an O.D. 595 of 200 to 250 and frozen in liquid nitrogen. The purification listed in Table 1 was initiated using two 12 L preparations of cells.

The frozen cells were thawed at 4°C and the following components were added to facilitate lysis: KCl to 500 mM, spermidine-HCl to 20 mM, DTT to 5 mM, and lysozyme to 0.3 mg/ml. After the addition of these reagents, the cells were incubated at 0°C for 30 minutes followed by quick-freezing in liquid nitrogen. The lysate was thawed at 2°C, centrifuged at 15, 000 x g for 12 minutes, and the soluble protein fraction was recovered (Fraction I). Fraction I was diluted five fold with Buffer A (40 mM HEPES-KOH (pH 7.6), 20 % (v/v) glycerol, and 5 mM DTT) to a conductivity equivalent to Buffer A containing 130 mM KCl. The protein was batch adsorbed to the heparin agarose by adding 200 ml of pre-equilibrated resin to the protein solution and the protein was allowed to bind to the resin by stirring for one hour at 4°C. The resultant slurry was packed into a solid column support and washed with Buffer A containing 130 mM KCl. The bound protein was eluted with a ten column volume linear gradient extending from 130 mM to 1 M KCl in Buffer A. The physical presence of dnaA5 protein was detected using an ELISA (see

Table 1

A summary of dnaA5 protein purification

fraction	volume (ml)	protein (mg)	activity (units)	specific activity (units/mg)	activity yield (%)
I. Lysate	51.0	1926	240, 000	127	(100)
II. heparin agarose*	72.0	76.0	62,000	842	26
III. Blue Dextran agarose	26.4	8.2	66,000	8,250	27
IV. DNA Cellulose	23.2	0.32	N.D.	N.D.	N.D.
V. hydroxylapatite	1.21	0.24	14, 080	64,000	5.8

N.D. not determined

* One half of fraction II was used for subsequent chromatographic steps. The values listed for fractions III-V are corrected for by a factor of two.

Experimental Procedures). The fractions containing dnaA5 protein were pooled (Fraction II) and assayed for replication activity. The units recovered and the specific activity of the fractions are summarized in Table 1.

The heparin agarose fractions of dnaA5 protein were dialyzed against Buffer A until the conductivity was equivalent to Buffer A containing 150 mM KCl. The protein was applied to a Blue Dextran agarose column (5 ml), equilibrated in Buffer A containing 150 mM KCl and the bound protein was eluted with a linear gradient extending from 150 mM KCl to 1 M KCl in Buffer A. A substantial purification of dnaA5 protein was achieved because most of the proteins present in Fraction II flowed through the resin. dnaA5 protein was detected in the gradient eluent fractions using an ELISA and it was found to elute broadly in fractions with a conductivity equivalent to Buffer A containing 450 mM KCl. The fractions containing elevated levels of dnaA5 protein were pooled (Fraction III) and assayed to determine the recovery of replication activity (Table 1).

The fractions of dnaA5 protein that eluted from the Blue Dextran agarose column were 25% to 40% pure. A DNA cellulose column was used to further purify dnaA5 protein to near homogeneity. Fraction III was dialyzed against Buffer A until the conductivity was equivalent to Buffer A containing 100 mM KCl and it was applied to a single-stranded DNA cellulose column, equilibrated in the same buffer, at 5 mg of protein per ml of resin. The protein that bound to the resin was eluted with a 20 column volume linear gradient extending from 100 mM to 1 M KCl in Buffer A. An ELISA was used to localize dnaA5 protein and it was found to elute broadly during the later part of the gradient (data not shown). It is possible that this purification was efficient because dnaA5 protein had a tendency to aggregate, which effectively retarded its elution from the resin compared to the bulk of the contaminants. dnaA5 protein obtained from this column was too dilute to assay and it was concentrated using hydroxylapatite. The fractions containing dnaA5 protein (Fraction IV) were loaded directly onto a 0.2 ml hydroxylapatite column, equilibrated with Buffer A containing 50 mM KCl, and the bound protein was eluted using Buffer A

containing 500 mM $(NH_4)_2SO_4$. Hydroxylapatite fractions (Fraction V) were sufficiently concentrated to assay directly in replication reactions and the specific activity of the purified protein was determined to be 64,000 units/mg (Table 1). The purity of the individual fractions obtained during the chromatography is shown in Figure 1.

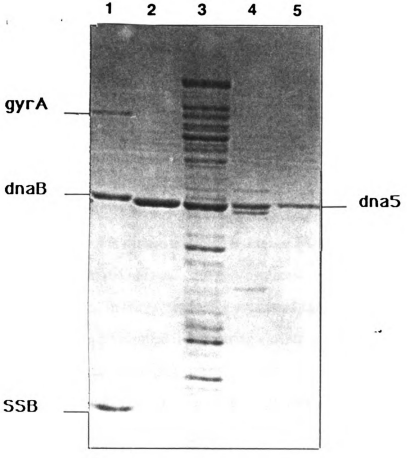
dnaA5 protein is temperature sensitive in the replication of oriC plasmids in a system containing a crude enzyme fraction.

An *in vitro oriC* plasmid replication system which specifically initiates DNA synthesis from *oriC* has been previously reported (29). The activity of dnaA protein in this replication system can be measured using a crude enzyme fraction deficient in dnaA protein. A titration of dnaA protein in this assay is shown in Figure 2A. The addition of 50 ng of 200 ng of dnaA protein resulted in initiation activity as measured by DNA synthesis. dnaA protein dependent replication activity was observed over temperatures ranging from 25°C to 38°C (Figure 2A), with an increasing temperature giving rise to increasing extents of DNA synthesis.

Purified dnaA5 protein (Fraction V) was active in this replication system at temperatures of 25°C and 30°C (Figure 2B). The specific activity of dnaA5 protein at 30°C was reduced 2 to 4 fold compared to the specific activity of dnaA protein and the dependence upon recombinant plasmids containing *oriC* was observed (data not shown).

Genetic studies demonstrated that cells harboring the *dnaA5* allele exhibit, among other characteristics, a temperature sensitive defect in the initiation of DNA replication (20). Cells are able to initiate DNA synthesis at 30°C, but initiation is blocked at 40°C, presumably due to the thermal inactivation of the dnaA5 polypeptide. The ability of purified dnaA5 protein to initiate DNA synthesis at higher temperatures was tested to determine if it exhibited a thermolabile initiation defect *in vitro*. Although 100 ng or 200 ng of dnaA5 protein could sustain DNA synthesis at 25°C or 30°C, DNA synthesis was not

Figure 1. SDS polyacrylamide gel electrophoresis of dnaA5 protein fractions. Various fractions of dnaA5 protein obtained after chromatography were separated by electrophoresis in a 10% polyacrylamide gel. The fractions which are represented include; lane 1, molecular weight markers, gyrase A subunit (105 kd), dnaB protein (54 kd), and SSB (18 kd); lane 2, dnaA protein (fraction IV); lane 3, dnaA5 protein (fraction II); lane 4, dnaA5 protein (fraction III); and lane 5, dnaA5 protein (fraction V).


1
2
3
4
5
gyrA
dnaB
dnaG
SSB

observed after adding dnaA5 protein into the replication assays that were incubated at 35°C or 38°C (Figure 2B). This indicates that the thermolabile defect associated with *dnaA5* cells is due directly to the thermolability of dnaA5 protein in initiation.

dnaA5 protein is active in the replication of P1 phage origin containing plasmids.

An *in vitro* DNA replication system has been developed for P1 phage (30). The reaction requires the presence of the P1 origin on a recombinant plasmid, phage encoded repA protein, host encoded dnaA protein, and a crude enzyme fraction capable of sustaining DNA synthesis. Other enzymes required for the replication of P1 phage DNA, which are present in the crude enzyme fraction, include DNA gyrase, RNA polymerase, DNA polymerase III holoenzyme, dnaB, dnaC, dnaJ, dnaK, grpE, and dnaG proteins (34).

A titration of repA protein in this system is shown in Figure 3A. The addition of 50 ng to 200 ng of purified repA protein did not result in DNA synthesis in the absence of exogenously added dnaA protein. In the presence of a presumed excess of dnaA protein (90 ng), the addition of repA protein resulted in increasing extents of DNA synthesis until approximately 20 % of the input DNA template was replicated.

The dependence of P1*ori* plasmid replication upon dnaA protein is shown in Figure 3B. The reaction contained saturating levels of repA protein (200 ng) as determined from the assay described in Figure 3A. The addition of increasing levels of dnaA protein resulted in increasing extents of DNA synthesis demonstrating the dnaA protein dependence in the reaction.

Assays were performed in parallel in which dnaA protein was titrated into an *oriC* plasmid replication system to examine the relative amounts required for initiation, in comparison to the levels of dnaA protein required for observing DNA synthesis in the P1*ori* replication system (Figure 3B). The similar amounts of dnaA protein required for

observing high levels of DNA synthesis in both reactions indicates that dnaA protein may provide the same function in both systems.

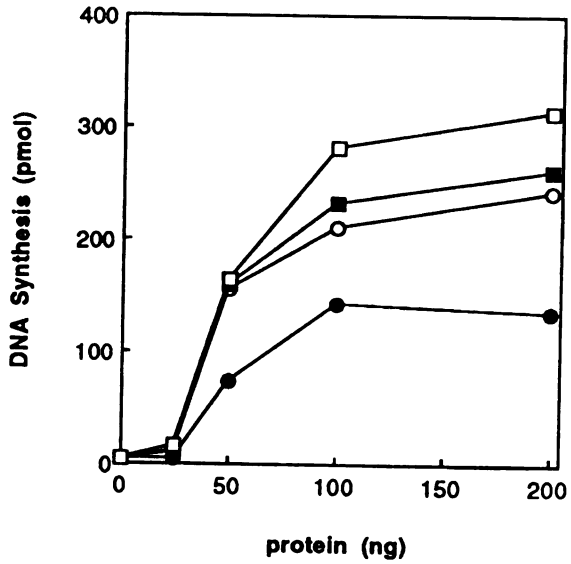
It is known that the ATP form of dnaA protein is active in *oriC* plasmid replication (11) while the ADP form of dnaA protein is inactive. In the P1*ori* plasmid replication system it is not known if differing biochemical activities of dnaA protein are required in comparison to the *oriC* plasmid replication system. It is possible that dnaA5 protein may represent a form of dnaA protein which is deficient in the biochemical properties required for the replication of *oriC* plasmids, but not P1*ori* plasmids.

The replication system dependent upon the P1 origin was examined to see if dnaA5 protein was active in the initiation of DNA synthesis (Figure 4A). The titration of dnaA protein in the presence of saturating levels of repA protein resulted in the production of up to 140 pmol of DNA synthesis. The titration of dnaA5 protein in this system resulted in up to 85 pmol of DNA synthesis indicating that it is capable of initiating DNA synthesis from the P1*ori* templates. As with its activity in *oriC* plasmid replication, dnaA5 protein activity in P1*ori* plasmid replication is 2 to 4 fold lower in comparison to dnaA protein.

In an attempt observe a biochemical distinction between dnaA5 protein and dnaA protein function in the P1*ori* plasmid replication system, the temperature dependence of DNA synthesis was examined. If dnaA5 protein activity is not thermolabile in P1*ori* plasmid replication, like it is in *oriC* plasmid replication, then this would indicate that a biochemical function utilized during the *oriC* dependent initiation reaction is not used during the initiation of P1*ori* plasmid replication. dnaA protein can function in the initiation of DNA replication of *oriC* plasmids over a wide temperature range, while dnaA5 protein is temperature sensitive (Figure 2B). dnaA protein remained active in the P1*ori* dependent replication system at elevated temperatures (Figure 4B), while dnaA5 protein was inactive. The thermolability exhibited by dnaA5 protein indicates that a labile activity operates during the initiation stage of both *oriC* and P1*ori* plasmid replication.

Figure 2. The temperature dependent replication activity of dnaA5 and dnaA proteins. DNA replication reactions employing a crude enzyme fraction were assembled according to the experimental procedures. dnaA protein (A) or dnaA5 protein (B) was titrated into the reactions and incubations were performed at 25°C (●), 30°C (○), 35°C (■), or 38°C (❑) for 25 minutes. The extents of DNA synthesis were quantitated according to the experimental procedures.

A.


400
300
200
100
0
DNA Synthesis (pmol)
0
50
100
150
200
protein (ng)

B.

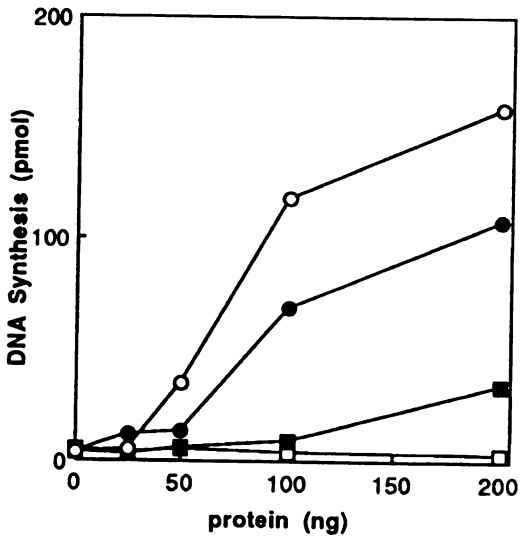
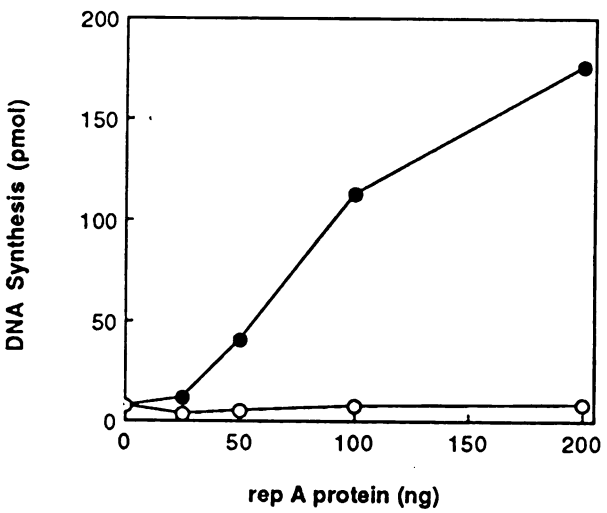

200
100
0
DNA Synthesis (pmol)
0
50
100
150
200
protein (ng)

Figure 3. The requirements for the replication of P1*ori* plasmids. Replication reactions that detect DNA synthesis dependent upon repA protein, dnaA protein, and P1*ori* plasmids were assembled according to the experimental procedures. (A) repA protein titration in the presence (●) and absence (○) of 90 ng of dnaA protein. (B) dnaA protein titration in the *oriC* plasmid replication system (●) and the P1*ori* plasmid replication system containing 200 ng of repA protein (○). The incubations were performed at 30°C for 25 minutes and the extents of DNA synthesis were quantitated according to the experimental procedures.

A.


DNA Synthesis (pmol)
200
150
100
50
0
0
50
100
150
200
rep A protein (ng)

B.

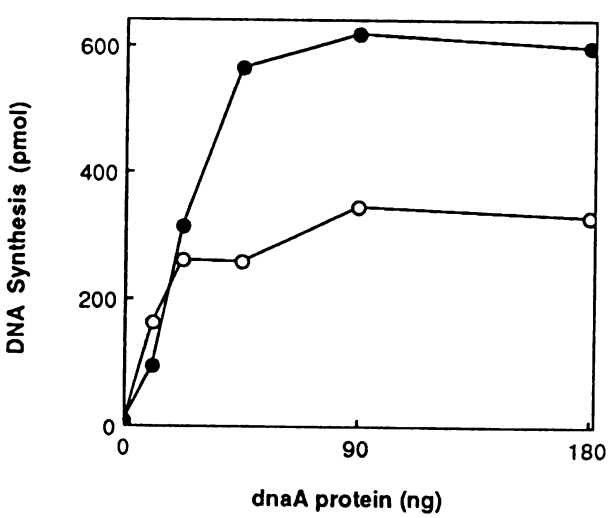
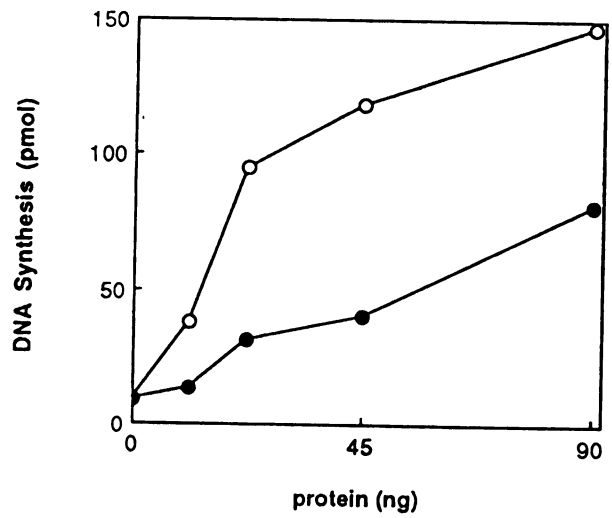
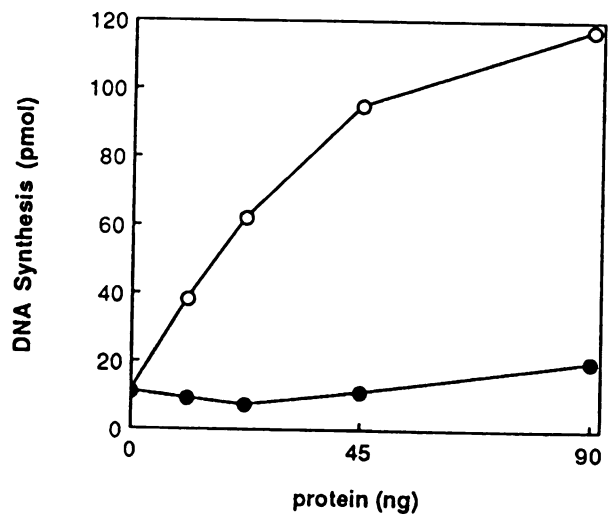

DNA Synthesis (pmol)
600
400
200
0
0
90
180
dnaA protein (ng)

Figure 4. The temperature dependent replication activity of dnaA5 and dnaA proteins in a P1*ori* plasmid replication system. P1*ori* plasmid replication assays containing 200 ng of repA protein were assembled according to the experimental procedures. (A) dnaA5 protein (●) and dnaA protein (○) were added and the reactions were incubated at 30°C for 25 minutes. (B) dnaA5 protein (●) and dnaA protein (○) were added and the reactions were incubated at 38°C for 25 minutes. The extents of DNA synthesis were quantitated according to the experimental procedures.

A.


150
100
50
0
0
45
90
DNA Synthesis (pmol)
protein (ng)

B.


120
100
80
60
40
20
0
0
45
90
DNA Synthesis (pmol)
protein (ng)

dnaA5 protein retains affinity for DNA containing oriC at permissive temperatures.

Nitrocellulose filter binding assays demonstrated that dnaA protein is a sequence specific DNA binding protein that recognizes DNA containing a dnaA box (10). The binding affinity of dnaA5 protein was examined to determine the influence of the mutation on this activity.

Nitrocellulose filter binding assays in conjunction with polyacrylamide gel electrophoresis allows for the direct visualization of DNA fragments which are bound by protein. Figure 5A represents a titration of dnaA protein at 30°C into DNA binding reactions containing pTSO182 DNA restricted with TaqI and end-labeled. The DNA retained by dnaA protein on the nitrocellulose filter was visualized after eluting the DNA from the filter, separating the fragments by electrophoresis in a 6% polyacrylamide gel, and detecting the radioactive fragments by autoradiography. At low amounts of dnaA protein (50 ng), only the DNA fragment containing *oriC* was retained on the filter (lane 3) while most other DNA fragments flowed through (lane 2). At higher amounts of dnaA protein added, most of the DNA containing *oriC* was retained along with DNA fragments containing the *mioC* promoter and the pBR322 origin of DNA replication. The latter two fragments each contain one dnaA box (18).

At a temperature of 30°C, which is the permissive temperature for cell growth, dnaA5 protein is active in the replication of *oriC* plasmids (Figure 2B). dnaA5 protein at this temperature bound to DNA fragments containing *oriC* (Figure 5A). The addition of elevated amounts of dnaA5 protein also resulted in the retention of DNA fragments containing the *mioC* promoter and the pBR322 origin of DNA replication.

There was an increase in the non-specific DNA binding affinity of dnaA5 protein in comparison to dnaA protein. A DNA fragment which migrated near the DNA fragment containing the pBR322 origin of replication was retained at high levels of dnaA5 protein added to the reaction. dnaA5 protein appeared to bind specifically to DNA containing *oriC*

indicating that the mutation residing in *dnaA5* does not drastically affect DNA binding property of dnaA5 protein.

The apparent binding affinity of dnaA5 protein for *oriC* DNA was quantitated using the nitrocellulose filter binding assay containing 15 fmol of ^{32}P end-labeled *oriC* DNA fragment and 150 fmol of unlabeled competitor DNA (Figure 6A). The addition of increasing amounts of dnaA protein resulted in the retention of the *oriC* DNA fragment with its near complete binding observed at the highest level added. Similarly, the addition of dnaA5 protein to the reactions at 30°C resulted in the retention of the *oriC* DNA fragment with an affinity comparable to dnaA protein.

dnaA5 protein retains affinity for DNA containing oriC at the nonpermissive temperature.

At a temperature of 42°C, due to the thermolability of dnaA5 protein, cells harboring the *dnaA5* allele do not grow (20). At 38°C, dnaA5 protein exhibited a thermolability in the initiation of DNA synthesis *in vitro* (Figure 2B). Although the binding affinity of dnaA5 protein to DNA containing *oriC* is similar to dnaA protein at 30°C, the mutation residing in *dnaA5* may result in a protein with thermolabile DNA binding affinity, which could then explain its absolute thermolability in replication.

Nitrocellulose filter binding assays were used to quantitate the amounts of *oriC* DNA fragment bound by dnaA5 protein at 38°C. dnaA protein binding at 38°C is shown in Figure 5B. The results are similar to that observed at 30°C in that after the addition of lower amounts of dnaA protein, only the DNA fragment containing *oriC* was bound demonstrating its preference for this sequence. dnaA5 protein binding to *oriC* was somewhat thermolabile compared to dnaA protein binding, however there was a preference for the DNA fragment containing *oriC* .

The binding affinity of dnaA and dnaA5 proteins at elevated temperatures was quantitated using a nitrocellulose filter binding assay containing 15 fmol of ^{32}P labeled *oriC* DNA fragment and 150 fmol of unlabeled competitor DNA. The addition of dnaA5

protein into the reactions at 38°C resulted in a two-fold decrease in the amount of DNA bound in comparison to dnaA protein (Figure 6B). The most notable difference in binding was observed at levels of 12 ng and 25 ng of dnaA5 protein added. However, substantial binding of the *oriC* DNA fragment by dnaA5 protein at this temperature indicates that the absolute thermolability in replication activity (Figure 2B) cannot be attributed solely to a thermolability in *oriC* DNA binding.

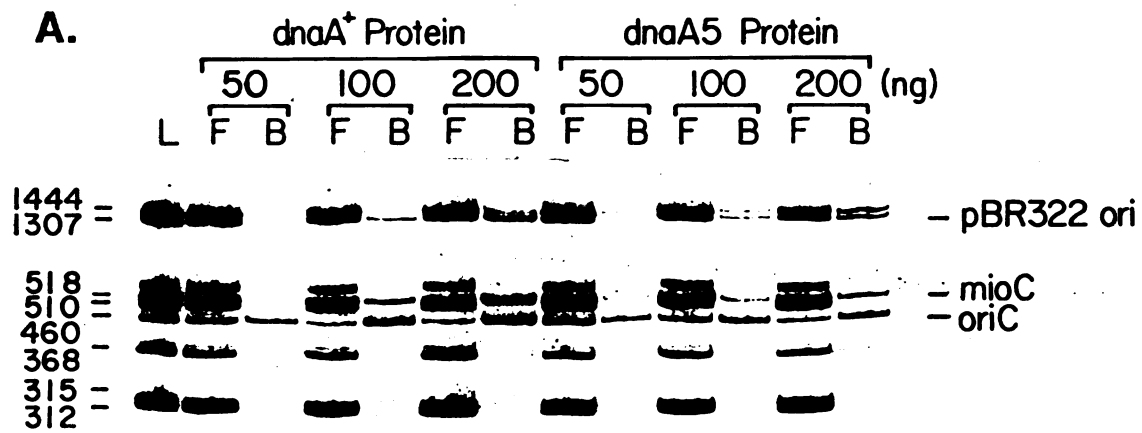
dnaA5 protein retains its affinity for DNA containing the dnaA promoter.

dnaA protein not only binds to four dnaA boxes within *oriC* to catalyze the initiation of DNA replication (10), but also to a single dnaA box within the *dnaA* promoter to autoregulate its own transcription (16-18). The binding affinity of dnaA5 protein to its own promoter containing one dnaA box was examined in comparison to dnaA protein (Figure 7).

With dnaA protein, the retention of DNA containing the *dnaA* promoter was observed at amounts added from 11 ng to 270 ng, which is similar to the levels observed previously (18). Higher amounts of dnaA protein are required for binding to the *dnaA* promoter in comparison to DNA containing *oriC* presumably due to the presence of four dnaA boxes in *oriC*. dnaA5 protein binding to DNA containing the *dnaA* promoter under identical conditions is shown in Figure 7. As with dnaA protein, the titration of 11 ng to 270 ng of dnaA5 protein resulted in an increasing retention of DNA containing the *dnaA* promoter.

These nitrocellulose filter binding experiments demonstrate that the binding affinity of dnaA5 protein to DNA containing *oriC* and the *dnaA* promoter is similar to dnaA protein. In comparison to dnaA protein, dnaA5 protein also exhibits an identical binding affinity to the two dnaA boxes in the *rpoH* promoter (19), (data not shown). The binding to DNA fragments containing one, two or four dnaA boxes demonstrates that the mutation in *dnaA5* does not alter the affinity of dnaA5 protein for its consensus DNA binding sequence.

Figure 5. The binding of dnaA5 and dnaA proteins to *oriC*. Nitrocellulose filter binding reactions containing linearized and ^{32}P end-labeled pTSO182 DNA containing *oriC* were assembled according to the experimental procedures. The amounts of dnaA protein and dnaA5 protein were added as indicated and the reactions were incubated at (A) 30°C or (B) 38°C for 10 minutes. The samples were applied to nitrocellulose filters and the flow through (F) and bound (B) samples were processed according to the experimental procedures. The letter, L, represents labeled fragments as molecular weight markers.


A.
dnaA+ Protein
dnaA5 Protein
50 100 200 50 100 200 (ng)
L F B F B F B F B F B F B
1444
1307
pBR322 ori
518
510
460
368
315
312
mioC
oriC

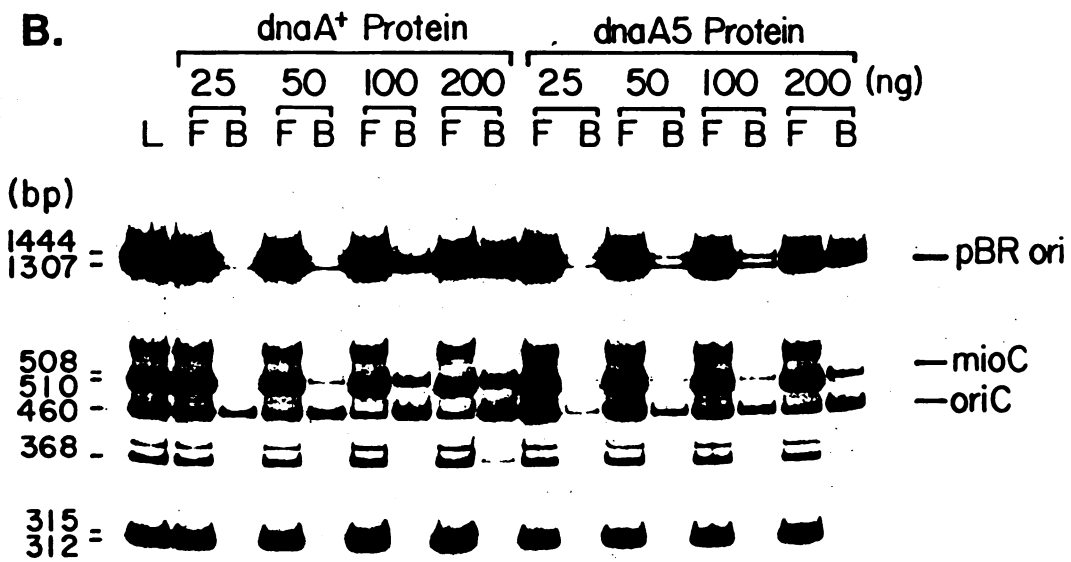
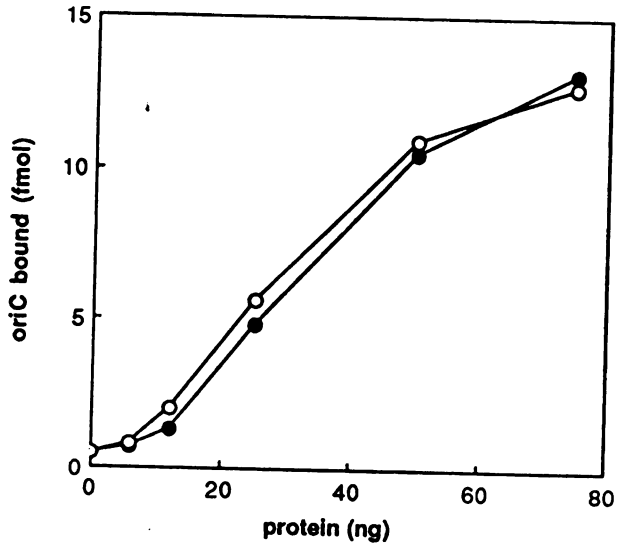

B.
dnaA+ Protein
dnaA5 Protein
25 50 100 200 25 50 100 200 (ng)
L F B F B F B F B F B F B F B F B
(bp)
1444
1307
pBR ori
508
510
460
368
315
312
mioC
oriC

Figure 6. dnaA5 protein and dnaA protein affinity to *oriC* . Nitrocellulose filter binding assays containing a radioactively labeled *oriC* restriction fragment were prepared according to the experimental procedures. (A) dnaA5 protein (●) or dnaA protein (○) was added and the incubations were performed at 30°C for 10 minutes. (B) dnaA5 protein (●) or dnaA protein (○) was added and the incubations were performed at 38°C for 10 minutes. The reaction products were filtered through nitrocellulose and radioactively bound *oriC* DNA was quantitated according to the experimental procedures.

A.


15
10
5
0
oriC bound (fmol)
0
20
40
60
80
protein (ng)

B.

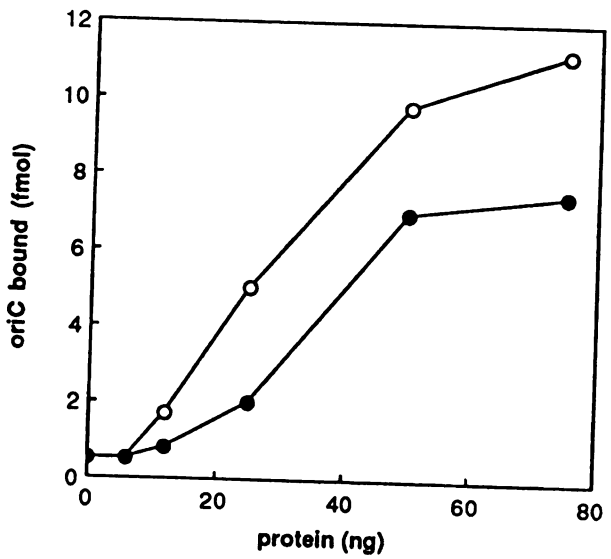
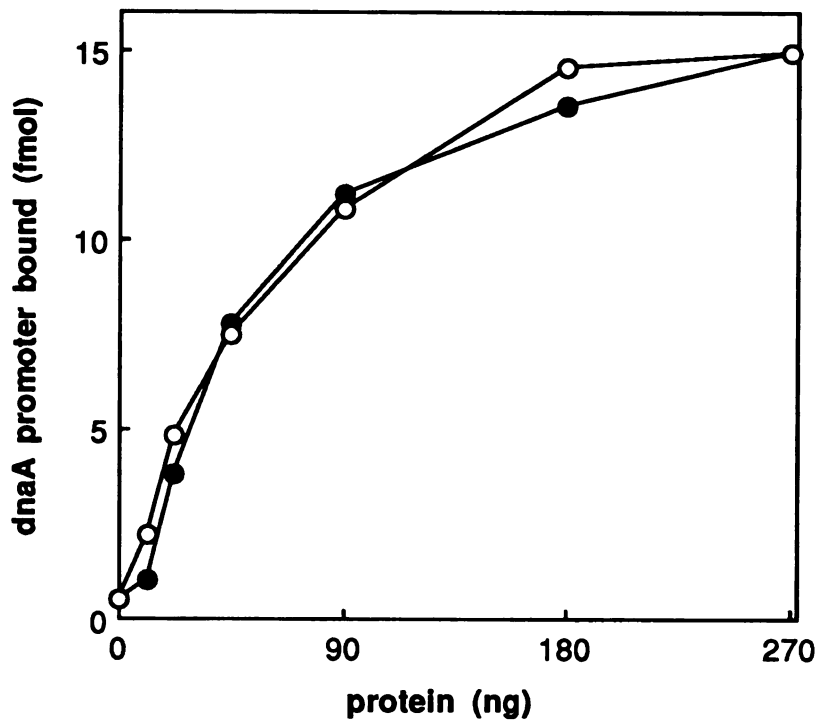

12
10
8
6
4
2
0
oriC bound (fmol)
0
20
40
60
80
protein (ng)

Figure 7. dnaA5 protein and dnaA protein binding to the *dnaA* promoter fragment. dnaA5 protein (●) or dnaA protein (○) was incubated with radioactively labeled DNA containing the *dnaA* promoter at 30°C for 10 minutes. DNA complexed to protein was filtered through nitrocellulose and the quantitation of DNA bound by protein was performed according to the experimental procedures.


dnaA promoter bound (fmol)
15
10
5
0
0
90
180
270
protein (ng)

dnaA5 protein retains specificity in binding to its consensus DNA sequence.

DNase I protection experiments were performed to determine if the specificity of dnaA5 protein in binding to the dnaA box is altered. Although the mutation in dnaA5 protein does not change its affinity for DNA containing the dnaA box (Figure 7), the protein-DNA contacts may be altered.

The DNase I protection pattern of the *dnaA* promoter region when bound by dnaA and dnaA5 proteins is shown in Figure 8. With increasing amounts of dnaA protein added protection from DNase I cleavage was observed within and adjacent to the dnaA box (hatched box) and hypersensitive sites were induced within the same region. In comparison, the region protected from DNase I cleavage by dnaA5 protein and the hypersensitive sites induced within the dnaA box region were nearly identical. This indicates that the mutation in *dnaA5* does not alter the ability of dnaA5 protein to form a proper protein-DNA complex at the dnaA box within the *dnaA* promoter.

Similar results were obtained in DNase I protection experiments with the *rpoH* promoter region (Figure 9). With a fixed level of dnaA and dnaA5 proteins, known to be present at saturating levels (unpublished data) in DNase I protection reactions containing two different amounts of ammonium sulfate, the areas within the *rpoH* promoter protected from cleavage by DNase I were nearly identical. These experiments demonstrate that the interaction of dnaA5 protein with DNA containing one or two dnaA boxes is not dramatically affected indicating that the mutation does not seem to affect the ability of the protein to form proper protein-DNA contacts in the respective regions.

The interaction of dnaA5 protein with DNA containing oriC is altered.

The affinity of dnaA5 protein for DNA containing *oriC* , the *dnaA* promoter and the *rpoH* promoter was similar to dnaA protein (Figure 6 and Figure 7) and the sequence specific binding to the dnaA boxes within the *dnaA* and *rpoH* promoters was similar to dnaA protein (Figures 8 and 9). DNase I protection experiments were performed using ^{32}P end-

labeled DNA containing *oriC* to probe the structure of *oriC* when bound by dnaA5 protein in comparison to dnaA protein (Figure 10).

At low levels of dnaA protein protection from DNase I cleavage was observed at some regions, specifically near dnaA boxes 3 and 4. Higher levels of dnaA protein resulted in a more elaborate pattern of DNase I cleavage to yield the periodic pattern of hypersensitive sites and protected regions characteristic of a dnaA protein-*oriC* complex (10, 37). The areas bound by dnaA protein included sequences immediately to the left of dnaA box 4 and sequences throughout *oriC*, most notably near dnaA box 2 and dnaA box 1 where a strong hypersensitive region exists. The binding did not appear to extend into the 13-mer region, yet the poor resolution of the DNase I ladder prevents a precise determination of the end-point of dnaA protein binding. This area of strong dnaA protein dependent hypersensitive site induction and protection from DNase I cleavage is near the point at which the protein is known to function as a helicase locally unwinding the DNA (9).

dnaA5 protein formed an unusually different nucleoprotein complex at *oriC* in comparison to dnaA protein in the region encompassing dnaA box 1 (Figure 10). At low amounts of dnaA5 protein protection from DNase I cleavage and induction of hypersensitive sites were nearly identical to that observed with dnaA protein. Higher amounts of dnaA5 protein resulted in the elimination of some hypersensitive sites and a more extensive and general protection from DNase I cleavage was observed. The most notable difference in the binding between dnaA and dnaA5 proteins was in the region encompassing dnaA box 1. dnaA5 protein did not yield a nucleoprotein structure similar to dnaA protein. This difference may account for its lack of *oriC* dependent replication activity in systems containing purified enzymes (described below).

dnaA5 protein is defective in ATP binding.

ATP binding by dnaA protein is essential for its replication activity *in vitro* (11) and it is required for the destabilization of the double helix in the A-T rich region of *oriC* (8, 9).

ATP binding by dnaA protein was measured (Figure 11). The Kd was calculated to be 0.03 uM and the stoichiometry of binding was 0.17 ATP per monomer, both of which are near the values previously reported (11, 23). In contrast, ATP binding by dnaA5 protein was not measurable under these conditions. The nitrocellulose filter binding assay can only detect stable protein-nucleotide complexes and it is possible that dnaA5 protein has some affinity for ATP that could not be detected using this assay.

dnaA5 protein is inactive in the replication of oriC plasmids in systems containing purified enzymes.

An *oriC* plasmid replication system utilizing purified enzymes was developed (3). The dependence of dnaA protein in two different reconstituted enzyme systems reflects the essential role of dnaA protein as the initiator. One replication system is dependent upon primase for the RNA priming of DNA synthesis (6). The activity of dnaA5 protein was compared to dnaA protein in this replication system (Figure 12). The addition of 50 ng to 100 ng of dnaA protein resulted in the replication of greater than 40% of the input DNA template. In contrast, the addition of up to 200 ng of dnaA5 protein did not result in any detectable DNA synthesis.

The second replication system contains primase and RNA polymerase as well as topoisomerase I, RNase H, and other replication enzymes. RNase H and topoisomerase I function to maintain the template specificity of the reaction. The addition of 45 ng to 90 ng of dnaA protein catalyzed maximal extents of DNA synthesis, while the addition of up to 200 ng of dnaA5 protein did not result in DNA synthesis (Figure 13).

Due to the essential role of ATP in dnaA protein function (11) and the requirement for a proper association of dnaA protein with *oriC* (10), the demonstration that dnaA5 protein is defective in both of these activities may explain its inactivity in *oriC* plasmid replication. This result is in contrast with data demonstrating that dnaA5 protein is active in the replication system employing a crude enzyme fraction (Figure 2B). The reason for this

Figure 8. The DNase I protection pattern of the *dnaA* promoter when bound by dnaA and dnaA5 proteins. DNase I protection reactions containing the *dnaA* promoter and the indicated levels of protein (lane 1, no protein; lane 2, dnaA protein (50 ng); lane 3 dnaA protein (100 ng); lane 4, dnaA protein (200 ng); lane 5, dnaA protein (300 ng); lane 6, dnaA5 protein (50 ng); lane 7, dnaA5 protein (100 ng); lane 8, dnaA5 protein (200 ng); and lane 9, dnaA5 protein (300 ng)) were assembled and processed according to the experimental procedures. The regions within the *dnaA* promoter that were protected from DNase I cleavage are represented by the hatched boxes and DNase I hypersensitive sites are indicated by the arrows.

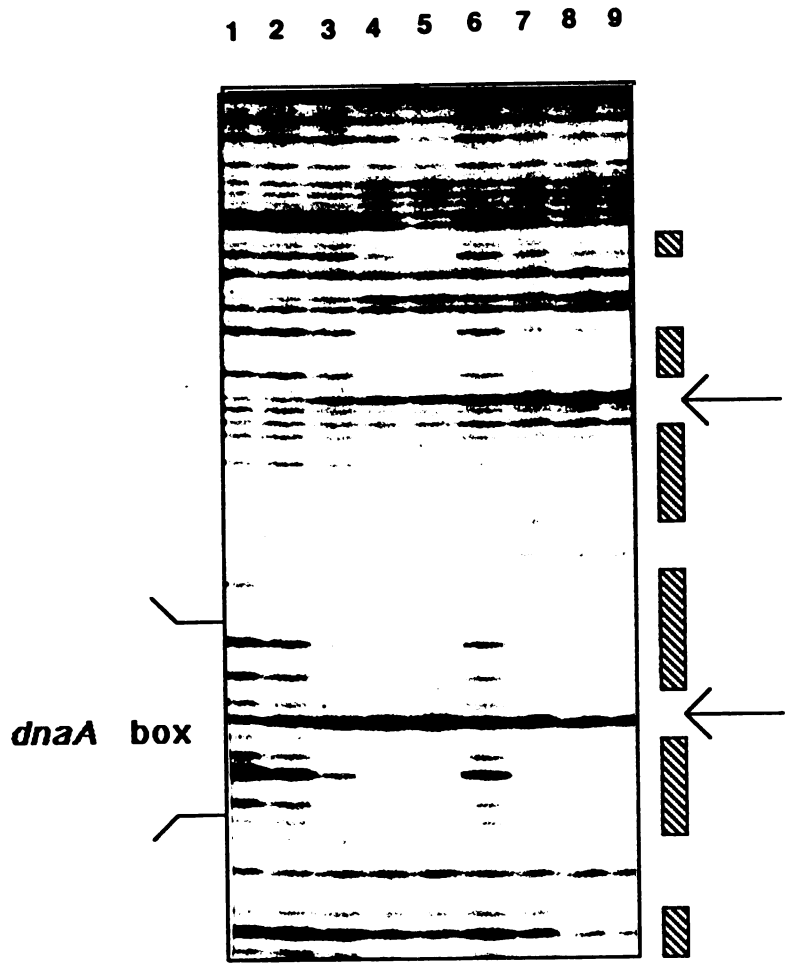

1 2 3 4 5 6 7 8 9
dnaA box

Figure 9. The DNase I protection pattern of the *rpoH* promoter when bound by dnaA and dnaA5 proteins. DNase I protection reactions containing the *rpoH* promoter and the indicated amounts of salt (lanes 1-3, 25 mM ammonium sulfate; lanes 4-6, 50 mM ammonium sulfate) and protein (lanes 2 and 5, dnaA protein (50 ng); lanes 3 and 6, dnaA5 protein (50 ng)) were assembled and processed according to the experimental procedures. The regions within the *rpoH* promoter that were protected from DNase I cleavage are represented by the hatched boxes.

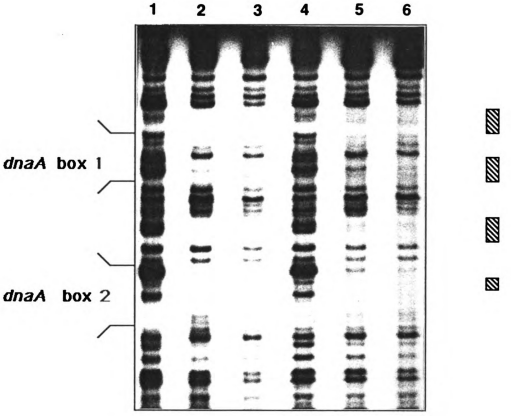

1
2
3
4
5
6
dnaA box 1
dnaA box 2

Figure 10. DNase I protection pattern of *oriC* when bound by dnaA and dnaA5 proteins. DNase I protection reactions containing an *oriC* fragment and the indicated amounts of protein (lane 1, no protein; lane 2, dnaA protein (25 ng); lane 3, dnaA protein (50 ng); lane 4, dnaA protein (100 ng); lane 5, dnaA protein (200 ng); lane 6, dnaA5 protein (25 ng); lane 7, dnaA5 protein (50 ng); lane 8, dnaA5 protein (100 ng); and lane 9, dnaA5 protein (200 ng)) were assembled and processed according to the experimental procedures except that the salt concentration was elevated three fold. The regions within *oriC* that were protected from DNase I cleavage by dnaA and dnaA5 protein are represented by filled boxes and hatched boxes, respectively. Differences observed in the induction of hypersensitive sites by dnaA or dnaA5 proteins are indicated by an asterisk.

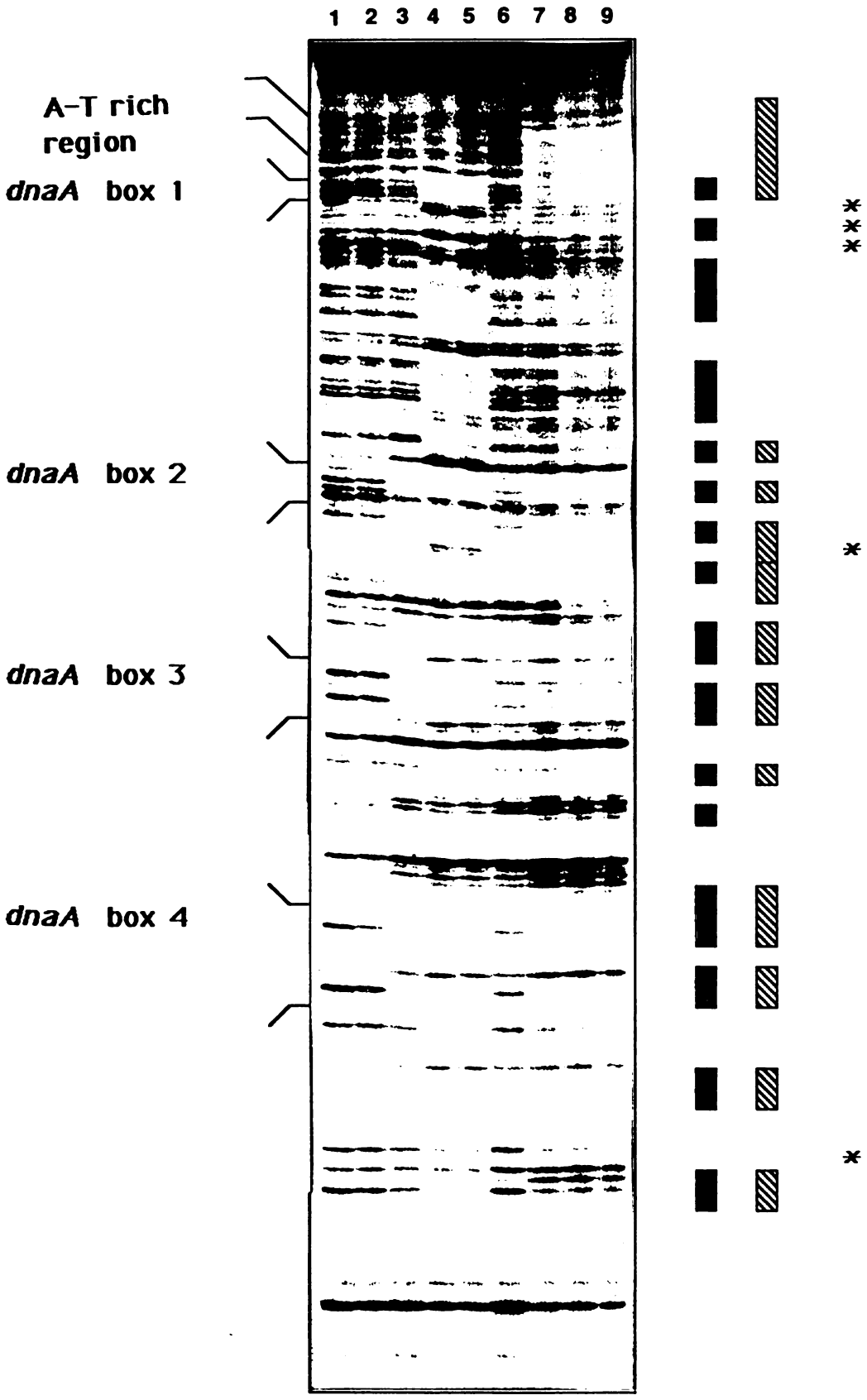

1 2 3 4 5 6 7 8 9
A-T rich region
dnaA box 1
dnaA box 2
dnaA box 3
dnaA box 4

Figure 11. dnaA5 protein and dnaA protein binding affinity to ATP. dnaA5 protein (●) or dnaA protein (○) was incubated with various concentrations of (α-^{32}P) labeled ATP for 15 minutes at 0^{o}C according to the experimental procedures. The reaction products were filtered through nitrocellulose and radioactively labeled ATP bound to protein was quantitated according to the experimental procedures.

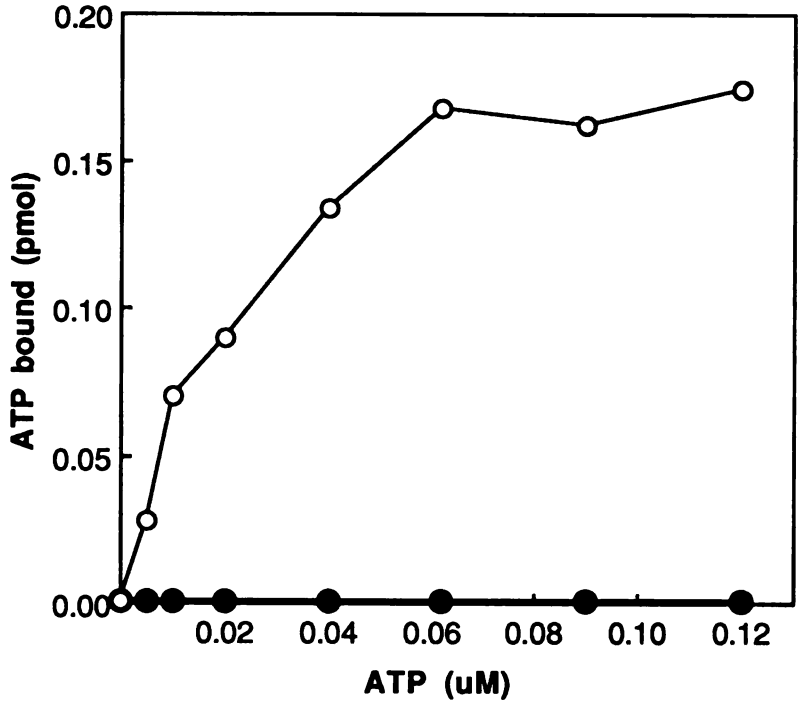

0.20
0.15
0.10
0.05
0.00
ATP bound (pmol)
0.02
0.04
0.06
0.08
0.10
0.12
ATP (uM)

Figure 12. dnaA5 protein is inactive in the reconstituted enzyme system containing primase as the sole priming enzyme. DNA replication reactions containing purified enzymes (which include primase as the sole priming enzyme and the BSA-sucrose solvent system) were assembled according to the experimental procedures. dnaA protein (○) or dnaA5 protein (■) was added and the incubations were performed at 30°C for 30 minutes. The extents of DNA synthesis were quantitated according to the experimental procedures.

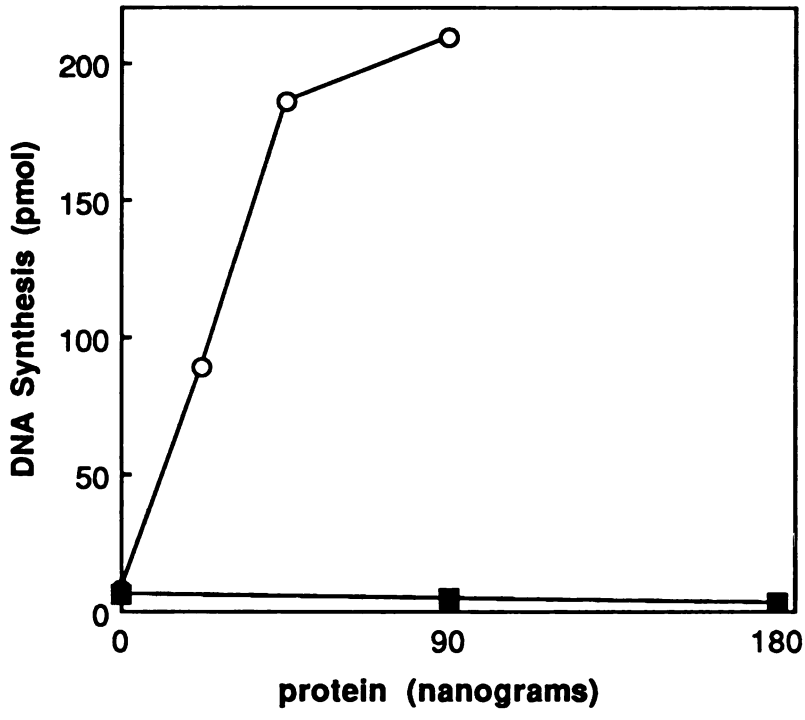
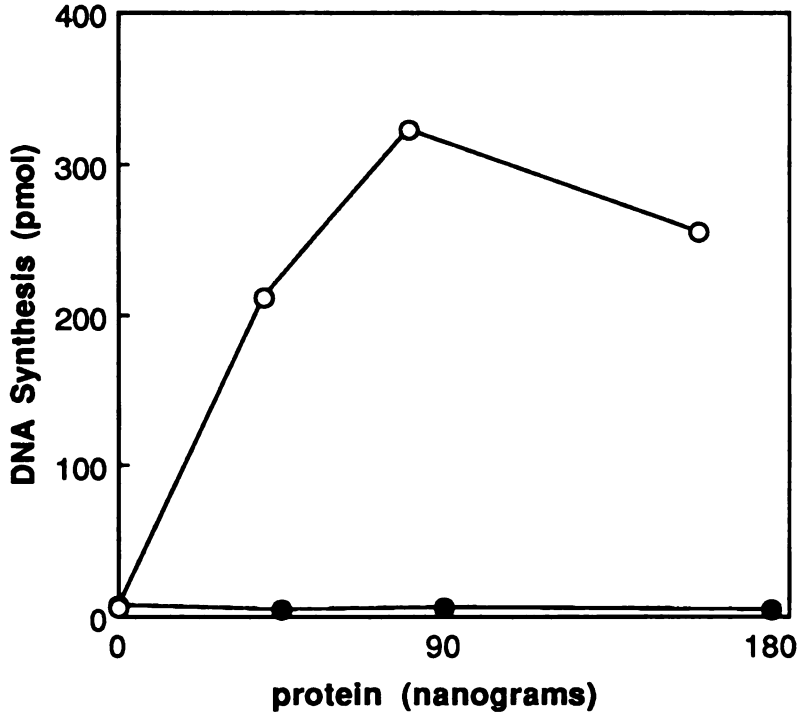

DNA Synthesis (pmol)
200
150
100
50
0
0
90
180
protein (nanograms)

Figure 13. dnaA5 protein is inactive in the RNA polymerase dependent reconstituted enzyme system. The replication system containing RNA polymerase and primase as priming enzymes were assembled according to the experimental procedures. Various levels of dnaA protein (○) and dnaA5 protein (●) were added and the reactions were incubated at 30°C for 30 minutes. The extents of DNA synthesis were quantitated according to the experimental procedures.


400
300
200
100
0
DNA Synthesis (pmol)
0
90
180
protein (nanograms)

discrepancy is not due to the different solvents used in the reaction. The replication system utilizing the crude enzyme fraction contains and requires PVA at levels of 6% to 8% (28). The reconstituted enzyme system containing RNA polymerase and the specificity factors, topoisomerase I and RNase H, was originally developed using PVA at levels of 7% in the reactions (3). The addition of PVA to the reconstituted enzyme systems did not restore activity to dnaA5 protein under conditions in which dnaA protein was active (data not shown, Chapter IV (35)). These results suggest that a factor in the crude enzyme fraction is responsible for activating dnaA5 protein in replication. This factor may be absent in the reconstituted enzyme system, which presumably accounts for the inactivity of dnaA5 protein.

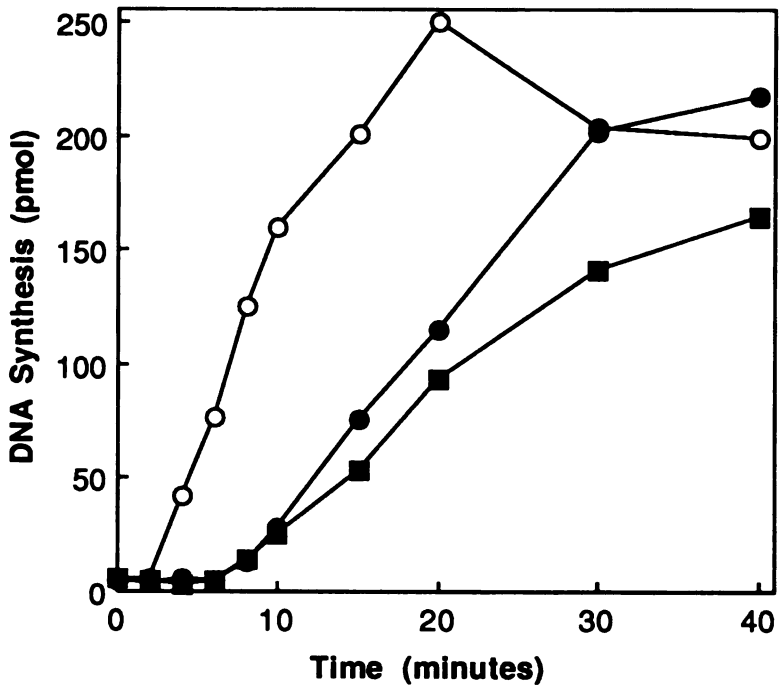
An extended lag precedes dnaA5 protein activity in a replication system employing a crude enzyme fraction.

A time course of DNA synthesis in the replication system utilizing a crude enzyme fraction, dependent upon the addition of dnaA protein or dnaA5 protein, is shown in Figure 14. A characteristic 5 minute lag preceded DNA synthesis at the amount of dnaA protein added. In contrast, a 7 to 10 minute lag preceded dnaA5 protein dependent initiation of DNA synthesis in this system. In view of the fact that dnaA5 protein is inactive in a reconstituted enzyme system possibly due to deficiencies in *oriC* binding (Figure 10) or ATP binding (Figure 11), but it is active in a replication system employing a crude enzyme fraction, the extended lag may represent the time required for activation of dnaA5 protein by some factor present in the crude fraction.

dnaA5 protein inhibits dnaA protein activity in a replication system containing purified enzymes.

A characteristic of *dnaA5/dnaA* merodiploids is that their growth is cold sensitive (20). Colonies can form at temperatures ranging from 37°C to 42°C, but very small or no

Figure 14. Time course of dnaA5 protein and dnaA protein activity in the replication system containing a crude enzyme fraction. DNA replication reactions containing a crude enzyme fraction were assembled according to the experimental procedures. dnaA protein, 50 ng (○), dnaA5 protein, 100 ng (●) or dnaA5 protein, 200 ng (■) were added and at the indicated times the reactions were stopped by the addition of 10% TCA/ 0.1 M Sodium Pyrophosphate. The extents of DNA synthesis were quantitated according to the experimental procedures.


250
200
150
100
50
0
DNA Synthesis (pmol)
0
10
20
30
40
Time (minutes)

colonies form at 30°C. To gain possible insight into this physiological phenomenon, mixing experiments were performed to study the effects of mixed complexes between dnaA and dnaA5 proteins *oriC* plasmid replication.

dnaA5 protein was added into a reconstituted enzyme system containing fixed levels of dnaA protein (50 ng) (Figure 15). The addition of equimolar amounts of dnaA5 protein inhibited the reaction by 50%, with the addition of higher amounts proportionally inhibiting DNA synthesis. As a control, the addition of up to 200 ng of dnaA protein did not significantly reduce DNA synthesis to the extents observed after the addition of dnaA5 protein. The inhibition is not dependent upon the order of addition or temperature (data not shown). Assuming that this inhibition occurs prior to DNA synthesis, this suggests that the mutation in the dnaA5 protein does not alter the ability of the protein to associate with dnaA protein in a cooperative manner. Mixed complexes which form are subsequently inactive because the conformation of the initiator protein complex which forms is not suitable for initiation.

dnaA5 protein inhibits dnaA protein dependent unwinding at oriC.

To further subdivide the dnaA5 protein dependent inhibition of dnaA protein activity to a stage preceding DNA synthesis, a second replication system was utilized which detects dnaA protein activity in a stage preceding DNA synthesis. The addition of the proteins dnaA, dnaB, dnaC, HU, SSB and DNA gyrase to supercoiled *oriC* plasmid DNA results in the unwinding and conversion of the FI DNA to a highly single-stranded and more negatively supercoiled structure (FI* DNA) (8). The stable intermediate produced after the reaction is the substrate capable of supporting RNA priming and DNA synthesis.

A dnaA protein titration in this system is shown in Figure 16A. Eleven ng of dnaA protein did not result in the conversion of FI DNA to FI* DNA. However, the addition of 45 ng to 180 ng of dnaA protein catalyzed the conversion of up to 95 % of FI DNA to FI*

DNA. dnaA5 protein lacks unwinding activity in this system, as the addition of up to 180 ng of dnaA5 protein did not result in the production of FI* DNA.

The ability of dnaA5 protein to form mixed complexes with dnaA protein as detected by the inhibition of dnaA protein unwinding activity is shown in Figure 16B. Forty-five ng of dnaA protein resulted in a conversion of FI DNA to FI* DNA. The addition of increasing amounts of dnaA5 protein to the reactions containing fixed levels of dnaA protein (45 ng) resulted in the inhibition of FI* DNA formation. The addition of equimolar amounts of dnaA5 protein inhibited the reaction by 40%, and higher levels inhibited the reaction in a proportional manner.

dnaA5 protein interferes with the formation of a structure characteristic of an active dnaA protein-oriC complex.

To associate the dnaA5 protein dependent inhibition of dnaA protein activity to a structural perturbation of a normal dnaA protein-*oriC* complex, a DNase I protection experiment was performed on *oriC* DNA bound by dnaA protein (45 ng) in the absence and presence of increasing amounts of dnaA5 protein (Figure 17). The addition of 3 ng to 11 ng of dnaA5 protein did not interfere with the characteristic DNase I protection pattern dependent upon dnaA protein. However, the addition of 22 ng to 90 ng of dnaA5 protein did interfere with the dnaA protein dependent DNase I protection pattern within *oriC*. The specific loss of the unique dnaA protein dependent induction of hypersensitive sites near the dnaA box 1 region may reflect the ability of mixed complexes of dnaA and dnaA5 protein to form and subsequently become abortive in the initiation of DNA replication. The cold sensitivity of the merodiploids may be due to the abortive dnaA5-dnaA protein complexes that form in the cell.

Figure 15. dnaA5 protein inhibition of dnaA protein replication activity. Reconstituted enzyme systems containing primase as the sole priming enzyme were assembled according to the experimental procedures. To each reaction, 50 ng of dnaA protein was added followed by the titration of either dnaA protein (○) or dnaA5 protein (●). The reactions were incubated at 30°C for 30 minutes and the extents of DNA synthesis were quantitated according to the experimental procedures.

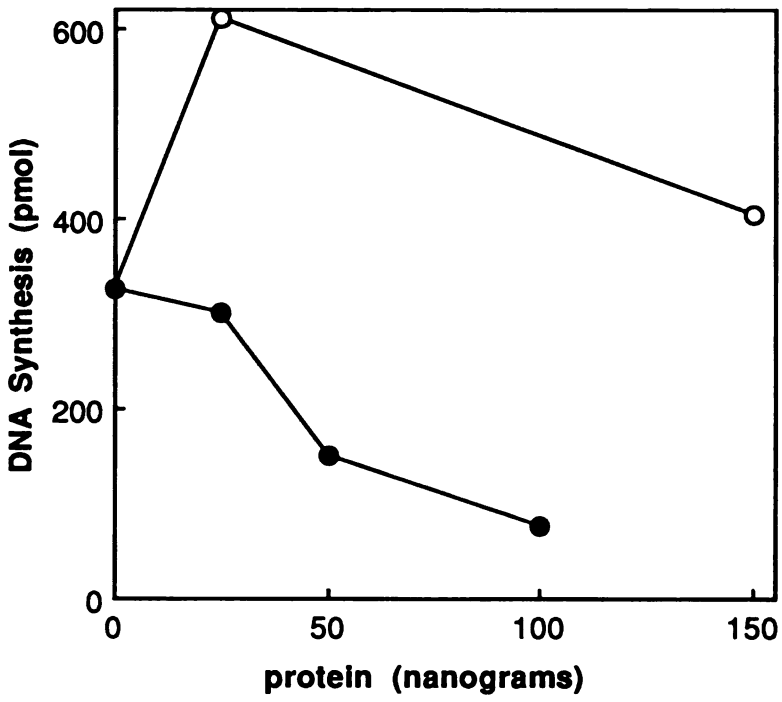
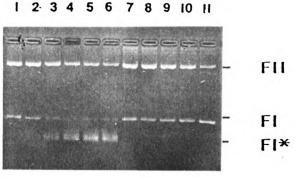

600
400
200
0
DNA Synthesis (pmol)
0
50
100
150
protein (nanograms)

Figure 16. dnaA5 protein and dnaA protein influence on FI* formation. *oriC* unwinding reactions were assembled as previously described (8). A 25 ul reaction volume contained: 25 mM HEPES-KOH (pH 7.6); 12% glycerol; 0.2 mM ATP; 1 mM calcium chloride; 0.2 mM EDTA; 0.1 mg/ml BSA; 100 ng of M13*oriC*2LB5 supercoiled plasmid; HU protein (25 ng); SSB (640 ng); dnaB protein (90 ng); and dnaC protein (25 ng). dnaA protein and dnaA5 protein were added as indicated and incubations were performed at 33°C for 30 minutes. The reactions were placed on ice and the following components were added: gyrase A (500 ng); gyrase B (450 ng); ATP to 10 mM; and magnesium acetate to 20 mM. The incubations were continued at 24°C for 8 minutes, 6 ul of SDS stop buffer (2% SDS, 50 mM EDTA) were added to each reaction, and the samples were heated at 65°C for 2 minutes. The samples were separated by electrophoresis at 20 volts for 16 hours in a 1% agarose gel containing 0.5 ug/ml of ethidium bromide in TBE. (A). A titration of dnaA and dnaA5 proteins. Lane1, no dnaA protein; lanes 2-6, 11 ng, 22 ng, 45 ng, 90 ng, 180 ng of dnaA protein, respectively; lanes 7-11, 11 ng, 22 ng, 45 ng, 90 ng, and 180 ng of dnaA5 protein, respectively. (B). dnaA5 protein inhibition of dnaA protein unwinding activity. Lane 1, no dnaA or dnaA5 protein; lane 2, dnaA protein (45 ng); lanes 3-7 contain dnaA protein (45 ng) and increasing amounts of dnaA5 protein (11 ng, 22 ng, 45 ng, 90 ng, and 180 ng, respectively).

A.



B.

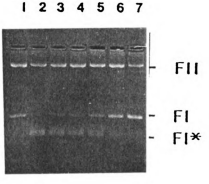
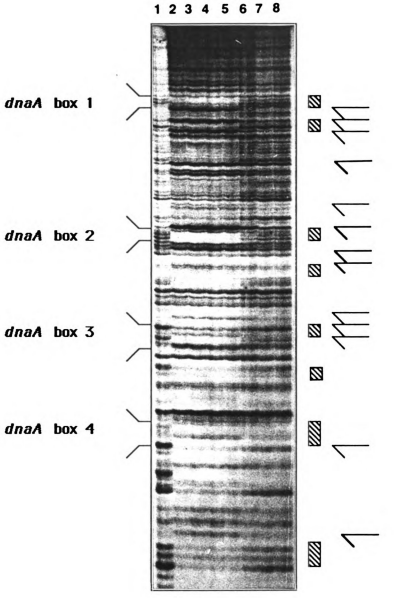


Figure 17. dnaA5 protein inhibition of dnaA protein binding to *oriC*. DNase I protection reactions were assembled and performed at 30°C according to the experimental procedures. Lane 1 no protein; lane 2, dnaA protein (45 ng); lanes 3-8, dnaA protein (45 ng) and increasing levels of dnaA5 protein (6 ng, 12 ng, 22 ng, 45 ng, 90 ng, and 180 ng, respectively). The dnaA protein induced DNase I pattern within *oriC* was altered by dnaA5 protein as represented by hatched boxes and arrows.


1 2 3 4 5 6 7 8
dnaA box 1
dnaA box 2
dnaA box 3
dnaA box 4

DISCUSSION

Highly purified preparations of dnaA5 protein have allowed for its biochemical characterization in comparison to dnaA protein. The biochemical properties of dnaA5 protein include: 1) high-affinity binding to DNAs containing the dnaA protein consensus sequence in the *dnaA* promoter, the *rpoH* promoter, and *oriC*, 2) normal protein-DNA contacts within the recognition sequences in the *dnaA* and *rpoH* promoters, 3) altered protein-DNA interactions at *oriC* , 4) no detectable ATP binding, 5) inactivity in *oriC* unwinding (FI *), 6) inactivity in replication systems which utilize purified enzymes, 7) ability to form mixed, inactive complexes with dnaA protein and 8) thermolabile activity in replication systems which employ a crude enzyme fraction dependent upon *oriC* or P1*ori* plasmids.

The mutational alteration within dnaA5 protein, presumably giving rise to a conformational change, was expected to correlate with one or more biochemical activities contained in wild type dnaA protein. The most straightforward possibility is one in which the amino acid substitution affects one activity of a protein permitting a correlation to be formed between mutational change and function. However, the defect in dnaA5 protein leads to multiple changes in biochemical activities, and immediate cause and affect relationships are more difficult to interpret. ATP binding, interaction with *oriC*, DNA strand unwinding activity, and initiation activity are affected by the mutation. Complex as the alterations are, some conclusions can be formulated.

The properties of dnaA protein known to be essential for its replication activity include ATP binding (11) and sequence specific DNA binding (10). Kinetic data led to the interpretation that ATP influences the activity of dnaA protein during two separate stages in initiation. One stage involves the requirement for low levels of ATP to allow for the formation of an open complex, and the second stage requires a high level of ATP to allow for the formation of a pre-priming complex with dnaB and dnaC proteins (8, 9, 12).

Given the essential role of ATP in promoting dnaA protein dependent replication, the inability of dnaA5 protein to bind ATP was expected to influence negatively its replication activity. This is the case as dnaA5 protein is inactive in all replication systems containing purified enzymes. The biochemical explanation for the inactivity is not definitive, but once binding to *oriC* occurs, the simple defect in ATP binding may prevent duplex strand opening at *oriC* and subsequent initiation of DNA synthesis. It is possible that dnaA5 protein exhibits a low affinity for ATP that was not detected using the assay described. Since dnaA5 protein is not active in the reconstituted enzyme systems containing a high concentration of ATP, it is unlikely that it exhibits a low affinity for ATP that could be utilized for initiation.

The inability of dnaA5 protein to bind ATP does not alter its affinity for DNAs containing the dnaA boxes in a variety of DNA fragments. In addition, protein-DNA contacts in various DNAs are equivalent to that obtained with dnaA protein. The functional independence of the biochemical activities of dnaA protein have been reported (40). The ability of a partially proteolyzed form of dnaA protein to retain ATP binding properties despite elimination of its *oriC* binding and replication activity suggests that these individual biochemical activities of dnaA protein are distinct. Communication may exist within or between molecules of dnaA protein as inferred from the influence of ATP on strand unwinding by dnaA protein (4). However, the ability of dnaA5 protein to retain a high affinity in binding to DNAs containing dnaA boxes implies that communication does not exist between the ATP binding and DNA binding functional domains to facilitate recognition of its consensus sequence.

The essential role of structural motifs residing in DNA bound by dnaA protein is well documented (38). Deletions of various regions of *oriC* (39) or of the A-T rich region at the left end of *oriC* (9) inactivate the cis-acting contribution of *oriC* to dnaA protein dependent replication. The requirement for the dnaA boxes is presumed to be in allowing for the formation of a proper nucleoprotein structure and the A-T rich region is required as it is

thermodynamically less stable and can be melted to allow for the formation of a pre-priming complex (38). dnaA5 protein binds into the A-T rich region, distinguishing it from dnaA protein. This may prevent strand unwinding in this essential region of the origin and lead to dnaA5 protein inactivity in reconstituted enzyme systems. It is unlikely that the defect in ATP binding directly influences the formation of a proper *oriC*-dnaA5 protein complex as detected by DNase I protection, since ATP, ADP or the nucleotide free forms of dnaA protein retain similar affinities for *oriC* DNA and exhibit indistinguishable DNase I protection patterns (11).

The physiological defects associated with *dnaA5* cells include: 1) asynchrony in initiation of DNA replication at the permissive temperature, 2) thermolability in initiation of DNA replication at the nonpermissive temperature, and 3) cold sensitivity in the merodiploid state (20). The association of the genetic defect exhibited by *dnaA5* cells to biochemical characteristics of dnaA5 protein may further our understanding of initiation of DNA replication.

The cold sensitive phenotype associated with *dnaA5/dnaA* merodiploids was proposed to occur through the association of dnaA5 and dnaA proteins to form mixed complexes which are inactive in initiation (20). The biochemical explanation for this process may relate to the ability of dnaA5 and dnaA proteins to form mixed complexes that are inactive in replication. The amounts of dnaA5 protein required for the loss of dnaA protein activity in *oriC* unwinding assays (FI*) or in replication assays suggests that a 1:1 association can occur. The mechanism of inhibition appears to be the disruption of *oriC* binding near dnaA box 1. In addition, the mutation in dnaA5 protein does not appear to alter its ability to associate cooperatively with dnaA protein.

The defects of dnaA5 protein in ATP binding, in *oriC* binding, and in its inactivity in reconstituted enzyme systems contrasts with its activity in replication systems which utilize a crude enzyme fraction. This replication activity is preceded by an uncharacteristically long lag phase in comparison to dnaA protein. Presumably, this extended lag reflects the

time required for the activation of dnaA5 protein in initiating DNA replication. The hypothesis that a factor in the crude enzyme system, which is lacking in the reconstituted enzyme system, acts to restore activity to dnaA5 protein was confirmed by subsequent experimentation (Chapter IV(35)). Two *E. coli* heat shock proteins, grpE and dnaK, are required for this activation of dnaA5 protein in *oriC* plasmid replication (Chapters IV and V(35, 36)). These activators may restore the biochemical defects associated with dnaA5 protein to allow initiation to occur or they may allow dnaA5 protein to function despite its' handicaps.

Thermolabile cell growth (20) is a characteristic of a subclass of *dnaA* mutants, including *dnaA5*. The replication activity of dnaA5 protein is thermolabile in systems utilizing a crude enzyme fraction, confirming the hypothesis that the thermolabile defect in *dnaA5* cells was due to its replication activity. It is possible that the temperature sensitivity relates to an inherent instability within dnaA5 protein or to some unknown secondary defect. This thermolability is not due to the loss of dnaA5 protein binding affinity to *oriC* at elevated temperatures, as the the binding affinity is reduced only two-fold at temperatures where its replication activity is completely blocked. From studies to be discussed (Chapter IV and V (35, 36)), it is apparent that dnaA5 protein is inherently unable to initiate DNA replication from *oriC* plasmids. The interaction between dnaA5 protein and the activating proteins restores its DNA replication activity. This interaction appears to be the point at which temperature sensitivity is observed (Chapter V(36)), indicating that the thermolability resides in the activation stage of dnaA5 protein function.

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Chapter IV

THE *Escherichia coli* dnaA5 PROTEIN IS ACTIVATED IN *oriC* PLASMID REPLICATION BY grpE AND dnaK HEAT SHOCK PROTEINS.

ABSTRACT

An assay was devised that could detect the activation of dnaA5 protein in the replication of *oriC* plasmids using a reconstituted enzyme system. The activation of dnaA5 protein was dependent upon factors from a crude enzyme fraction obtained by fractionation of a cell lysate. The purification of the activating factors resulted in the demonstration that two heat shock proteins, grpE and dnaK, are required for the activation of dnaA5 protein. The participation of dnaK protein in the activation reaction was examined based on studies demonstrating that it stimulates the replication activity of dnaA46 protein in replication assays containing a crude enzyme fraction (21). Although dnaK protein alone could not activate dnaA5 protein, it was one participant in the activation reaction which required at least one other protein. The inclusion of dnaK protein in reconstituted enzyme systems allowed for the partial purification of the second activating protein. grpE protein was deduced to be the second activating protein based on three criteria: 1) a protein with a molecular weight similar to grpE protein was co-incident with activity, 2) the activity was co-incident with a protein that reacted to grpE antibody, and 3) purified preparations of grpE protein could replace the partially purified fractions of the second activating protein in activation reactions.

INTRODUCTION

The biochemical properties of dnaA protein which are required for the initiation of DNA replication include ATP binding (1) and sequence specific DNA binding (2), both of which are needed for unwinding of the duplex DNA in the vicinity of *oriC* (3). The ATP dependent unwinding of *oriC* DNA in the vicinity of the A-T rich region (4) is essential for the nucleation of other replication proteins required for events subsequent to dnaA protein function. dnaA protein binding to ATP induces a conformational change in the protein (5), presumably yielding the energy for strand unwinding activity.

Biochemical evidence describing factors that may regulate dnaA protein include the observation that ADP inhibits the replication activity of dnaA protein (6). This inhibition does not relate to an altered affinity to the chromosomal origin, but to the inability of dnaA protein to unwind *oriC* DNA. The reactivation of the ADP form of dnaA protein can occur through the action of acidic phospholipids, specifically cardiolipin (7), which displaces the bound ADP to allow dnaA protein to bind ATP. Membrane involvement in *oriC* dependent DNA replication has been implied from various studies (8, 9) and the phospholipid involvement in the regulation of dnaA protein activity *in vitro* may be related to a possible mode of dnaA protein regulation in the cell.

Physiological studies of DNA replication in *E. coli* have revealed a coupling between cell growth and chromosomal replication (10), indicating that initiation of replication is highly regulated. It has been proposed that the regulation of initiation of DNA replication occurs through the regulation of the activity of the *dnaA* gene product (11). Quantitation of the levels of the *dnaA* gene product in the cell indicated that it remains constant throughout the cell cycle (12). Other studies have shown that the levels of the *dnaA* gene product influence the accumulation of initiation potential (13, 14) and is therefore a rate-limiting factor in initiation of DNA replication. The existence of another rate-limiting factor has also been proposed (15).

Mutations in *dnaA*, including *dnaA5* and *dnaA46*, are conditionally lethal. Cells exhibit phenotypes of temperature sensitivity in initiation of DNA replication at non-permissive temperatures (16) and asynchrony in the frequency of initiation of replication at permissive temperatures (17). The altered timing of the initiation of DNA synthesis in *dnaA* mutant cells may result from an inefficient regulation of the mutant dnaA protein activity or from a deficient interaction with the replication machinery during the initiation process.

The biochemical understanding of the physiological defects associated with *dnaA* mutants has been initiated through the purification and characterization of the *dnaA46* and *dnaA5* gene products (18, 19). Both mutant proteins were shown to be deficient in one or more of the biochemical activities known to be required for dnaA protein function. dnaA46 protein is defective in its affinity for both ATP and *oriC* DNA (20). dnaA5 protein is defective in its affinity for ATP, and though it retains a high affinity for *oriC* DNA, it forms altered contacts in the A-T rich region (19). The biochemical deficiencies associated with the mutant forms of dnaA protein are reflected in the inability of dnaA46 and dnaA5 proteins to initiate DNA replication in a system composed of purified enzymes. This observation contradicts results demonstrating that dnaA46 and dnaA5 proteins are active in initiation of DNA synthesis in a replication system which utilizes a crude enzyme fraction. This indicates that there may be an activating factor in crude enzyme fraction, which is absent in the replication system composed of purified enzymes. In the replication system employing the crude enzyme fraction, dnaA5 and dnaA46 proteins exhibited a pronounced lag in comparison to dnaA protein. This may represent the time required for the activation to occur.

dnaA46 protein activity in replication systems containing a crude enzyme fraction is stimulated by the prior incubation of dnaA46 protein with dnaK protein and another unidentified factor in a crude extract (21), indicating that there may be a possible role for dnaK protein in the initiation of *oriC* plasmid replication. The discovery of a *dnaK* mutant

specifically deficient in initiation of DNA replication *in vivo* provides genetic evidence of the involvement of the *dnaK* gene product in initiation (22).

This study extends the characterization of dnaA5 protein by demonstrating that it is activated by two proteins. These two activating proteins were purified based on an assay containing a reconstituted enzyme system which replicates *oriC* plasmids (23). In the absence of these two proteins, dnaA5 protein was inactive. Two heat shock proteins, grpE and dnaK, were shown to be essential for activating dnaA5 protein in replication. This activation of dnaA5 protein may relate to the physiological defects associated with dnaA5 cells. This is addressed in the accompanying Chapter (24).

EXPERIMENTAL PROCEDURES

Reagents and enzymes. Commercials enzymes, solvents, chemicals and radioactive isotopes were obtained as described in Chapter III, (19). Trypsin, trypsin inhibitor, and ATP-agarose, Type III, were obtained from Sigma; Affigel-10 was obtained from Biorad; Mono Q, Mono S, and Superose 12 FPLC were obtained from Pharmacia P-L Biochemicals.

Replication assays. DNA replication assays containing purified enzymes were as described in Chapter III (19), except that EDBS Buffer was replaced by an 8% (w/v) solution of PVA. WM433 Fraction II was prepared as described in Chapter III (19). A highly purified fraction of grpE protein used as a molecular weight marker was obtained from Dr. M. Zylicz, University of Utah.

Bacterial strains and plasmids. Bacterial strains were as described in Chapter III (19); *E.coli* RLM893 containing the *dnaK* gene on a high copy number plasmid (25) was a gift from Dr. Roger McMacken, Johns Hopkins University; *E.coli* DA31 (relevant genotype *dnaK103*) containing the *grpE* gene in a high copy number plasmid (26) was a gift from Debbie Ang, University of Utah.

Buffers. Buffer A was described in Chapter III (19). Buffer B contains 10 mM Imidazole-HCl, pH 7.0, 10 mM $MgCl_2$, 10% (w/v) sucrose, and 5 mM DTT.; Buffer C contains 25 mM HEPES-KOH, pH 7.6, 5 mM DTT, and 20% (v/v) glycerol; Buffer D contains 500 mM KCl, 80 mM $CaCl_2$, 100 mM HEPES-KOH, pH 7.0, and 5 mM DTT; Buffer E contains 25 mM HEPES-KOH, pH 7.6, and 5 mM DTT.

Purification of dnaK protein. Purification of dnaK protein was performed by a modification of the published protocol (25). 12 liters of *E. coli* RLM893 was grown in LB media containing 10 ug/ml of chloramphenicol at 30°C until the O.D.$_{595}$ was 0.5. At this point, an equal volume of LB media pre-heated to 55°C was added to the culture and the cells were incubated an additional three hours at 42°C. Cells were harvested, frozen, and

lysed to obtain a soluble Fraction I as previously described in Chapter III, (19). To the soluble supernatant (Fraction I), solid ammonium sulfate was added over a 15 minute period to a final concentration of 0.4 grams/ml, and stirred for an additional hour at 4°C. The suspension was centrifuged at 15,000 x g for 20 minutes at 4°C and the ammonium sulfate pellet was dialyzed against Buffer E until the conductivity was equivalent to Buffer E containing 400 mM KCl to obtain Fraction II. Six hundred mg of Fraction II protein was dialyzed for 12 hours at 4°C against Buffer B. The protein was applied at 4 column volumes per hour to a 5 ml ATP-agarose column equilibrated in Buffer B. The column was washed sequentially with 10 ml of Buffer B, 30 ml of Buffer B containing 1 M KCl, and with 25 ml of Buffer B. Bound protein, highly enriched for dnaK protein protein (greater than 95% pure), was eluted with Buffer B containing 20 mM $MgCl_2$ and 10 mM ATP. Fractions containing dnaK protein were pooled, precipitated by the addition of an equal volume of saturated ammonium sulfate, and sedimented at 15,000 x g for 15 minutes at 4°C. The pellet was resuspended in Buffer A, and dialyzed against Buffer A containing 50 mM KCl for 16 hours at 4°C. ATP-free dnaK protein was obtained by chromatography of the ATP eluent fractions on Mono Q. Fractions containing dnaK protein eluted from ATP agarose were directly injected onto Mono Q and ATP was removed by washing with Buffer A containing 150 mM KCl. Bound protein was eluted with Buffer A containing 600 mM KCl, and dialyzed for 16 hours at 4°C against Buffer A containing 50 mM KCl.

Preparation of a dnaK protein affinity column. dnaK protein was coupled to Affigel-10 according to the manufacturers' suggestions. Twenty mg of dnaK protein in 2 ml (ATP-agarose fractions) were dialyzed against Buffer D for 16 hours at 4°C. The protein was mixed with 1.5 ml of Affigel-10 resin for 24 hours at 4°C. The resin was washed with 20 ml of Buffer A containing 50 mM KCl, then with 10 ml of Buffer A containing 2 M KCl, and equilibrated in Buffer A until no protein eluted from the column. A coupling efficiency of 60% was achieved. The final wash with a buffer containing no salt was an important step in the column preparation, since some of the dnaK protein that was retained on the

Affigel resin was not bound covalently and the wash without salt may have aided in the disruption of hydrophobic aggregates between coupled and uncoupled dnaK protein. The column was stored at 4°C in Buffer C for one day prior to use.

Immunological methods. Polyclonal antibodies to dnaK protein were obtained according to standard procedures (27). Protein fractions (Fraction III) were used for production of dnaK antibodies (21). Polyclonal antibody to grpE protein was a gift of Debbie Ang, University of Utah. The ELISA was performed according to the experimental procedures described in Chapter III (19). Serum containing antibodies to grpE or dnaK proteins was diluted 1:1000 in PBS containing 0.05% tween 20 and 0.2% BSA prior to use.

Protein determinations. Protein determinations were performed as described (18).

RESULTS

The activation of dnaA5 protein in a DNA replication system containing purified enzymes.

dnaA5 protein activity can be detected in replication systems dependent on a crude enzyme fraction, but not in systems utilizing purified replication enzymes (19). This suggests that dnaA5 protein is inherently inactive in initiating DNA synthesis and that some factor(s) in the crude enzyme fraction, required for activation of dnaA5 protein, is absent in the replication system composed of purified enzymes.

Cells which harbor the *dnaA5* mutation are thermolabile in initiation of DNA replication (16) and are defective in the proper timing of initiation of DNA replication at the permissive temperatures (17). The physiological properties may relate to the rate-limiting activation of dnaA5 protein. The identification of the factor(s) required for dnaA5 protein activity may indicate how initiation of DNA replication is regulated. An assay was developed which was dependent on factor(s) from a crude enzyme fraction which conferred replication activity to dnaA5 protein in the reconstituted enzyme system. This assay would allow for the purification of the factor(s) and characterization of the activation reaction.

dnaA5 protein was shown to be inactive in replication systems composed of purified enzymes, which include BSA and sucrose as protein stabilizing agents (19). Initial experiments designed to detect activation of dnaA5 protein involved the replacement of BSA and sucrose with PVA, an essential component in replication assays utilizing the crude enzyme fraction. This difference in the reaction conditions cannot account for activation of dnaA5 protein (data not shown, Table 1) as it is inactive in a replication system composed of purified enzymes in the presence of PVA. The most complex replication system containing purified enzymes contains PVA at 8% and 12 highly purified enzyme fractions, including RNA polymerase, topoisomerase I, and RNase H (23). This complement of replication enzymes and reaction conditions comes the closest at mimicking the conditions of the replication system employing a crude enzyme fraction where dnaA5

protein activity can be detected. Replication assays which contain purified enzymes including RNA polymerase, RNase H, and topoisomerase I, are thought to authentically reflect the nature of initiation of replication in the cell (28), and are more physiologically relevant. For these reasons, this assay system was used to test for activation of dnaA5 protein.

The addition of Fraction II from WM433, a resuspended ammonium sulfate precipitate of a cell lysate, to this replication system resulted in the activation of dnaA5 protein replication activity (Table 1). The addition of either dnaA5 protein or Fraction II from WM433 alone to the replication assay did not result in DNA synthesis. The titration of Fraction II into reactions containing fixed amounts of dnaA5 protein resulted in proportionately increasing levels of DNA synthesis. Presumably, this assay reflects the activation of dnaA5 protein in the initiation of DNA synthesis. The activation of dnaA5 protein required PVA and RNA polymerase dependent replication reactions (data not shown). Other assay conditions could not be used to detect activation of dnaA5 protein.

The activating factor is heat sensitive.

Fraction II containing the activating factor contains many nucleases and other uncharacterized inhibitors of *in vitro* DNA replication reactions. Heat treatment of Fraction II was attempted in order to remove inhibitors from the assay and to examine the heat sensitivity of the activating factor. Fraction II was incubated at various temperatures and the resultant fractions were assayed in the replication system containing purified enzymes and dnaA5 protein (Table 2). The incubation of Fraction II at 55°C resulted in a 3-fold increase in the specific activity of the factor. Incubations at higher temperatures resulted in complete inactivation of the factor. The temperature sensitivity of the factor suggests that it is a protein.

Assays were performed to address whether the DNA synthesis observed with the 55°C heat treated Fraction II (called Fraction III) was dependent upon dnaA5 protein (Table 3).

Addition of various amounts of either Fraction III or dnaA5 protein alone resulted in background levels of DNA synthesis. The combination of the two fractions resulted in increasing levels of DNA synthesis indicating that dnaA5 protein was being activated. The relative heat stability of the factor resulted in the removal of a large proportion of the contaminating protein and this step was incorporated into the purification regime (see below).

The activating factor is a protein.

Trypsin treatment of Fraction III was performed to determine if the activating factor is a protein (Table 4). Fractions were assayed for activation in the replication system composed of purified enzymes in the presence of dnaA5 protein. The addition of Fraction III, which was treated with buffers only, resulted in 201 pmol of DNA synthesis. This is ten times the level of DNA synthesis observed without the addition of any crude fraction. The treatment of Fraction III by first adding trypsin inhibitor then trypsin resulted in 119 pmol of DNA synthesis, or a two-fold reduction in activity compared to the untreated control. The treatment of Fraction III by the addition of trypsin followed by trypsin inhibitor completely inactivated the factor, as background levels of DNA synthesis were observed. The trypsin sensitivity of the factor indicates that it is a protein.

dnaK protein is one of the activating proteins.

Purification of the activating protein from Fraction III as the starting material was unsuccessful. The activating protein did not bind to resins including; heparin agarose, DNA cellulose, CM sepharose, Mono S, or Blue Dextran agarose (data not shown). The only resin capable of binding the activating protein at neutral pH was Mono Q. The elution profile of the protein from Mono Q was broad and the activity recovered after chromatography was very poor (data not shown). In an attempt to obtain a foothold on the

purification of the activating protein, the effect of purified dnaK protein on the activation of dnaA5 protein was examined.

dnaK protein (in addition to an unidentified factor in a crude cell lysate) stimulates the replication activity of dnaA46 protein 3 to 4 fold in a replication system containing a crude enzyme fraction (21). The biochemical mechanism for this stimulation has not been clarified. It seemed conceivable that the mechanism of dnaK protein action in the stimulation of dnaA46 protein activity was similar to the mechanism of activation of dnaA5 protein in the replication system composed of purified enzymes.

The influence of dnaK protein in the activation of dnaA5 protein was investigated by the addition of various amounts of Fraction III into the activation reactions in the absence and presence of a presumed excess of dnaK protein (Figure 1). The addition of Fraction III resulted in extents of DNA synthesis previously observed. However, in the presence of 2 ug of dnaK protein, the level of Fraction III required for activation of dnaA5 protein decreased 5-fold, indicating that there may be more than one activating protein.

A titration of dnaK protein was performed in the presence and absence of Fraction III to confirm that activation of dnaA5 protein requires both enzyme fractions (Figure 2). The addition of up to 5 ug of dnaK protein alone did not result in DNA synthesis indicating that dnaK protein alone cannot activate the replication function of dnaA5 protein. However, in the presence of low levels of Fraction III, the amounts of dnaK protein required for complete activation of dnaA5 protein were 1 ug to 2 ug. These results indicate that dnaK protein is one participant in the activation reaction and at least one other factor is required for activation of dnaA5 protein.

The second activating factor is a protein.

Fraction III was treated with either trypsin, trypsin inhibitor, or buffer only, as described above in order to examine the trypsin sensitivity of the second activating factor in the activation of dnaA5 protein (Figure 3). Fraction III treated with buffer only resulted in

up to 189 pmol of DNA synthesis. The treatment of Fraction III with trypsin inhibitor followed by trypsin resulted in similar levels of DNA synthesis. However, Fraction III treated first with trypsin then with trypsin inhibitor resulted in a complete loss of activity. This indicates that the second factor required for activation is a protein.

The second activating protein is heat sensitive

The heat stability of the second activating protein was examined with the goal of finding a unique property of the protein that could be used to facilitate its purification. The second activating protein is somewhat heat resistant at 55°C since Fraction III (Fraction II heated at 55°C) is the source material used. Fraction II was incubated at various temperatures and 6 ug of protein from each heat treated fraction was added to the reconstituted enzyme system containing dnaA5 protein and 2 ug of dnaK protein. At temperatures of 55°C or 60°C, substantial soluble activity was recovered, but this activity was inactivated at higher temperatures (Table 5).

The partial purification of the second activating protein.

The inclusion of dnaK protein in the reconstituted enzyme system containing dnaA5 protein allowed for the partial purification of the second activating protein (Table 6). Upon gel filtration chromatography of this activity on Superose 12, four polypeptides in the peak fractions were co-incident with activity based on resolution in a SDS polyacrylamide gel stained with Coomasie Blue (data not shown). The difficulty in purifying the second activating protein to near homogeneity resulted in attempts at finding alternative methods for determining its identity. An attempt was made to correlate the protein of interest to a known *E. coli* protein by comparing the sizes of these four polypeptides to that of a known *E. coli* protein. One of these four polypeptides was a protein with a molecular weight of 24 kd. dnaK protein is known to form a complex with another heat shock protein, the 24 kd grpE protein (29).

Antibody to grpE protein was obtained and an ELISA was performed to see if there was a protein in the Superose 12 fractions that would cross react to the grpE antibody. An ELISA profile of grpE protein and dnaK protein (also present in these Superose 12 fractions) is shown in Figure 5B. The peak of dnaK protein trailed that of activity, while the peak of grpE protein was coincident with activity. This result suggested that the second activating protein was a known *E. coli* protein, grpE protein.

The purification of grpE protein

To confirm whether the second activating protein was grpE protein, it was purified from an overproducing strain by published methods (29). The ability of grpE protein to replace the Superose 12 gel permeation fraction in the activation of dnaA5 protein would result in the identification of the second activating protein.

The purification of grpE protein is a one step procedure which involves the ability of grpE protein to form a salt-resistant, ATP-sensitive complex with dnaK protein (29). A dnaK protein affinity column was prepared and used according to the Experimental Procedures. *E. coli* DA31, which overproduces the grpE protein, was grown in 5 L of LB media at 37°C to an O.D.595 of 1.0 and the cells were harvested, frozen, and lysed. A Fraction II was prepared according to published methods (32).

Fraction II (45 mg), containing elevated levels of grpE protein, was diluted to a conductivity equivalent to Buffer C containing 50 mM KCl and loaded at four column volumes per hour onto a dnaK protein Affigel-10 column equilibrated in the same buffer. The column was washed successively with 6 ml Buffer C containing 50 mM KCl, 8 ml of Buffer C containing 2 M KCL, and Buffer C containing 50 mM KCl until protein in the eluent was not detected. The bound protein was eluted with Buffer C containing 50 mM KCl, 20 mM $MgCl_2$, and 10 mM ATP at a flow rate of 0.5 ml per hour. The protein in the ATP eluent fraction, grpE protein Fraction III, was analyzed by SDS polyacrylamide gel electrophoresis to demonstrate that a protein of 24 kd, corresponding to grpE protein, was

present (Figure 6, lane 7). A band at the 24 kd position migrated at the position of grpE protein obtained from another laboratory (Figure 6, lane 3), indicating that this purification resulted in highly purified fractions of grpE protein. In addition, this fraction of grpE protein contains a minor contaminant of 68 kd, which corresponds in molecular weight to dnaK protein. Apparently, some dnaK protein non-covalently bound to the dnaK protein affinity resin was eluted by the buffer containing ATP. The contaminating dnaK protein did not originate from the Fraction II obtained from the grpE overproducing cells. The strain used, harboring the *dnaK103* allele, does not synthesize a stable full length *dnaK* gene product (29). Densitometric scanning of the lanes containing purified grpE protein indicated a purity of 85%. This highly purified fraction of grpE protein was tested for its ability to activate dnaA5 protein in the reconstituted enzyme systems containing dnaK protein.

grpE protein is the second activating protein.

The addition of purified grpE protein and a Superose 12 peak fraction containing the second activating protein to activation assays is shown in Figure 7. The specific activities of the Superose 12 peak fraction and grpE protein in the activation assay were 2,095 and 42,600 units per mg of protein, respectively. Based on the specific activity of the highly purified grpE protein preparation, the amount of grpE protein present in the Superose 12 peak fraction was estimated to be 5%, which correlates well with the physical abundance of the 24 kd protein in this fraction. These results indicate that, in addition to dnaK protein, grpE protein is required for the activation of dnaA5 protein.

The amounts of dnaK protein and grpE protein required for activation of dnaA5 protein.

The optimal amounts of dnaK and grpE heat shock proteins required for the activation of dnaA5 protein were examined. Purified dnaK protein was optimal at 5 ug of protein added to a reconstituted enzyme system containing fixed levels dnaA5 protein (100 ng) and grpE

protein (100 ng) (Figure 8A). Purified grpE protein was optimal at 120 ng of protein added to a reconstituted enzyme system containing fixed levels of dnaA5 protein (100 ng) and differing levels of dnaK protein (Figure 8B). Activation was dependent on the addition of both dnaK and grpE proteins. Based on the molar quantities of each protein required for activation, the ratio of dnaA5 protein: grpE protein: dnaK protein in the activation reaction is 1: 2: 40.

Figure 1. The influence of dnaK protein on Fraction III dependent activation of dnaA5 protein. Replication reactions containing purified enzymes and dnaA5 protein (90 ng) were assembled according to the experimental procedures. Fraction III was titrated into the reactions in the presence (●) and absence (○) of 2 ug of dnaK protein. The incubations were performed at 30°C for 60 minutes and the extents of DNA synthesis were quantitated according to the experimental procedures.

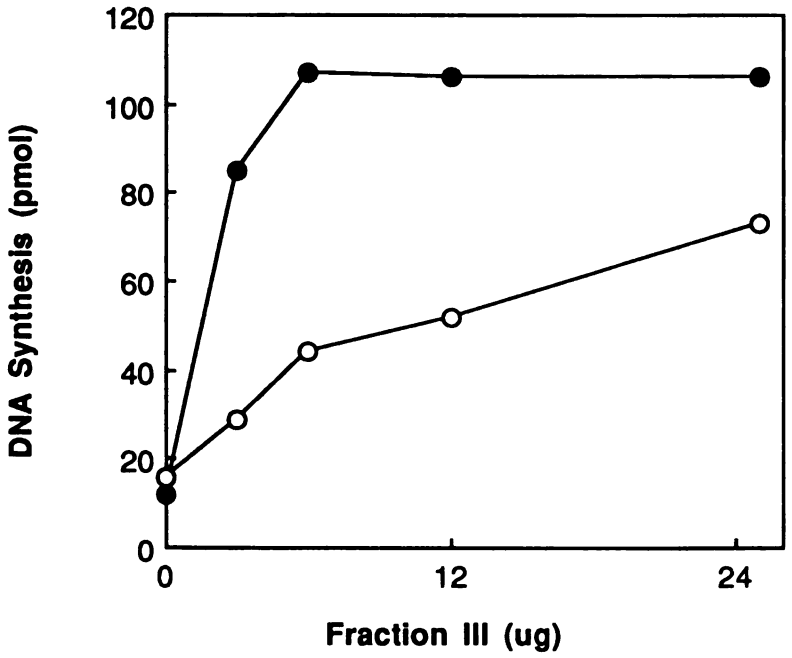

120
100
80
60
40
20
0
0
12
24
DNA Synthesis (pmol)
Fraction III (ug)

Figure 2. Titration of dnaK protein in activation reactions. Replication reactions containing purified enzymes and dnaA5 protein (90 ng) were assembled according to the experimental procedures. dnaK protein was titrated into the reactions in the presence (●) and absence (○) of 12 ug of Fraction III. The incubations were performed at 30°C for 60 minutes and the extents of DNA synthesis were quantitated according to the experimental procedures.

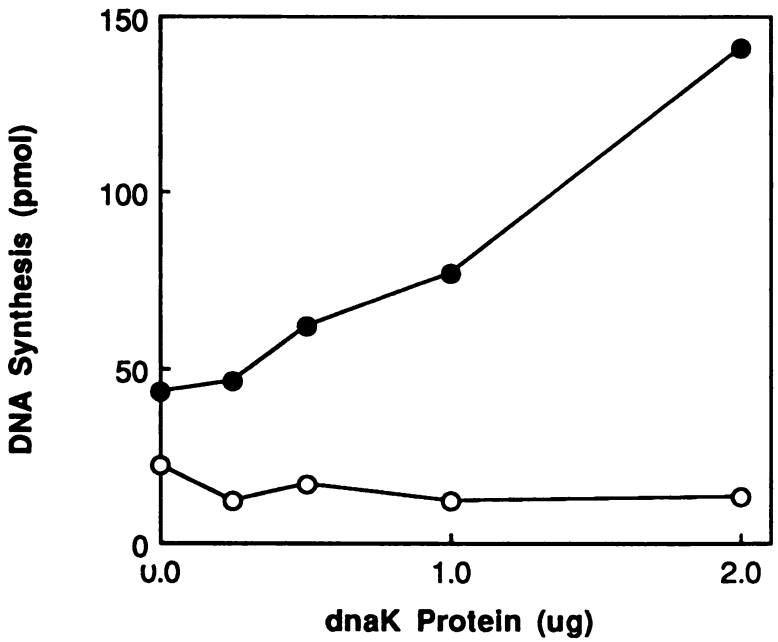

150
100
50
0
DNA Synthesis (pmol)
0.0
1.0
2.0
dnaK Protein (ug)

Figure 3. The second activating factor is trypsin sensitive. Fraction III was treated with trypsin (○), trypsin inhibitor (■), or buffer only (●) and incubated at 30°C for 10 minutes. The fractions were then treated with trypsin inhibitor (○), trypsin (■), or buffer only (●), respectively, and incubated at 30°C for 10 minutes. Replication reactions containing purified enzymes and 90 ng of dnaA5 protein were assembled and the indicated amounts of the treated fractions were added. The incubations were performed at 30°C for 60 minutes and the extents of DNA synthesis were quantitated according to the experimental procedures.

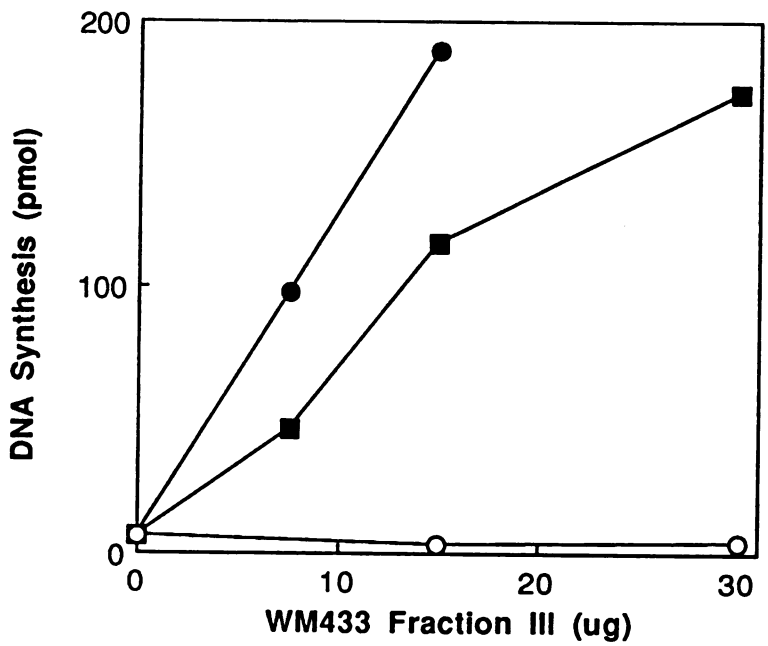

200
100
0
DNA Synthesis (pmol)
0
10
20
30
WM433 Fraction III (ug)

Figure 4. Mono Q chromatography of the second activating protein. Fraction III was injected onto a Mono Q column (8 cm x 1 cm) as stated in Table 6. The activation of dnaA5 protein was measured using a replication reaction containing purified enzymes, 90 ng of dnaA5 protein, and 2 ug of dnaK protein. The extents of DNA synthesis (●) and protein determinations (○) were determined according to the experimental procedures.

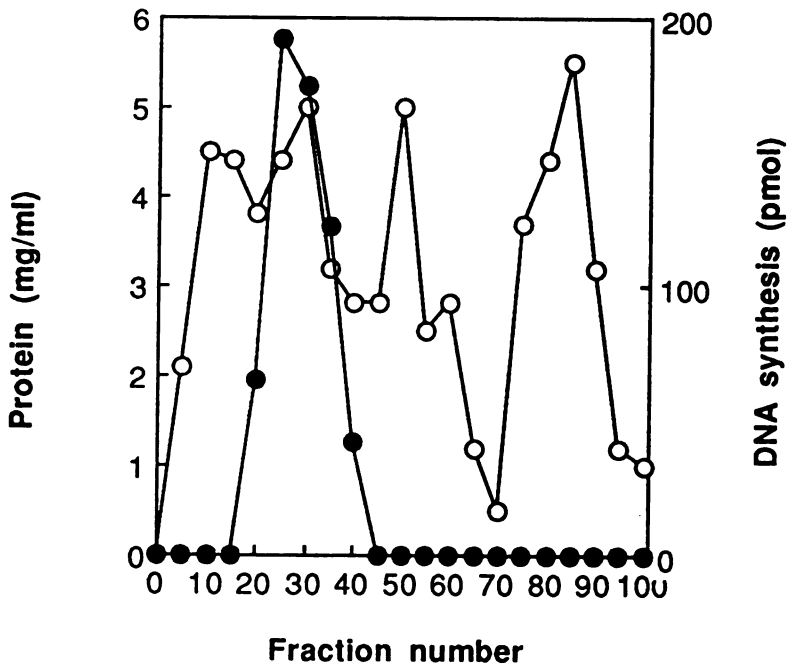
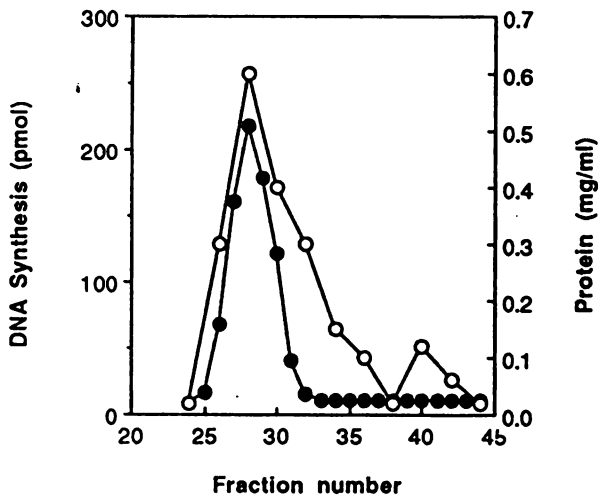

Protein (mg/ml)
DNA synthesis (pmol)
Fraction number
0
1
2
3
4
5
6
100
200
0 10 20 30 40 50 60 70 80 90 100

Figure 5. (A). Superose 12 chromatography of Fraction IV. Three hundred ug of Mono Q fraction number 25 was injected onto a Superose 12 column (25 cm x 1 cm) equilibrated in Buffer A containing 250 mM KCl as described in Table 6. Fractions (500 ul) were collected and 1 ul was assayed in replication reactions containing purified enzymes, 90 ng of dnaA5 protein and 2 ug of dnaK protein. The reactions were assembled according to the experimental procedures and incubated at 30°C for 60 minutes. The extents of DNA synthesis (●) and protein determinations (○) were performed according to the experimental procedures. (B). ELISA of Superose 12 fractions. Two ul of each fraction were assayed in an ELISA according to the experimental procedures. grpE protein (●) and dnaK protein (○) levels were quantitated according to the experimental procedures.

A.


DNA Synthesis (pmol)
Protein (mg/ml)
Fraction number
300
200
100
0
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
20
25
30
35
40
45

B.

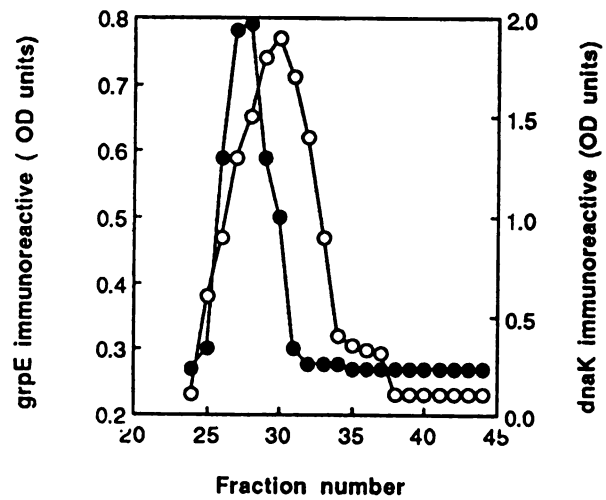

grpE immunoreactive (OD units)
dnaK immunoreactive (OD units)
Fraction number
0.8
0.7
0.6
0.5
0.4
0.3
0.2
2.0
1.5
1.0
0.5
0.0
20
25
30
35
40
45

Figure 6. SDS polyacrylamide gel of grpE protein containing fractions. Protein samples were separated by electrophoresis in a 12% polyacrylamide gel and stained with Coomasie Blue. The fractions represented are: lane 1, molecular weight markers, gyrase subunit A (105 kd), dnaB protein (54 kd) and SSB, (18 kd); lane 2, dnaK protein (Fraction III); lane 3, purified grpE protein from Dr. M. Zylicz; lane 4, Mono Q fraction 25 containing the second activating protein; lane 5, Superose 12 fraction number 28 containing the second activating protein; lane 6, Fraction II from a grpE overproducing cell strain; and lane 7, grpE protein eluted from a dnaK protein affinity column.

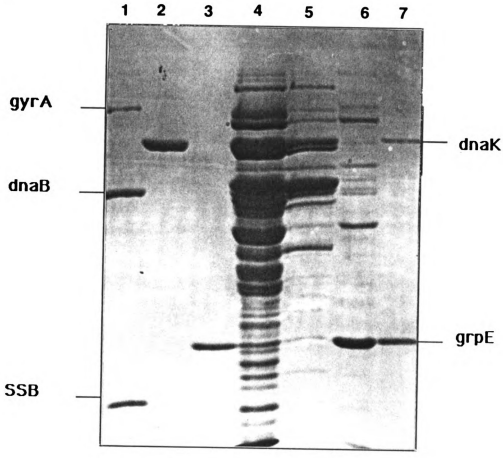

1
2
3
4
5
6
7
gyrA
dnaB
SSB
dnaK
grpE

Figure 7. grpE protein replaces a Superose 12 fraction in the activation of dnaA5 protein. Replication reactions containing purified enzymes, 90 ng of dnaA5 protein, and 2 ug of dnaK protein were assembled according to the experimental procedures. grpE protein (●) or Superose 12 fraction number 28 (○) was added and the incubations were continued at 30°C for 60 minutes. The extents of DNA synthesis were quantitated according to the experimental procedures.

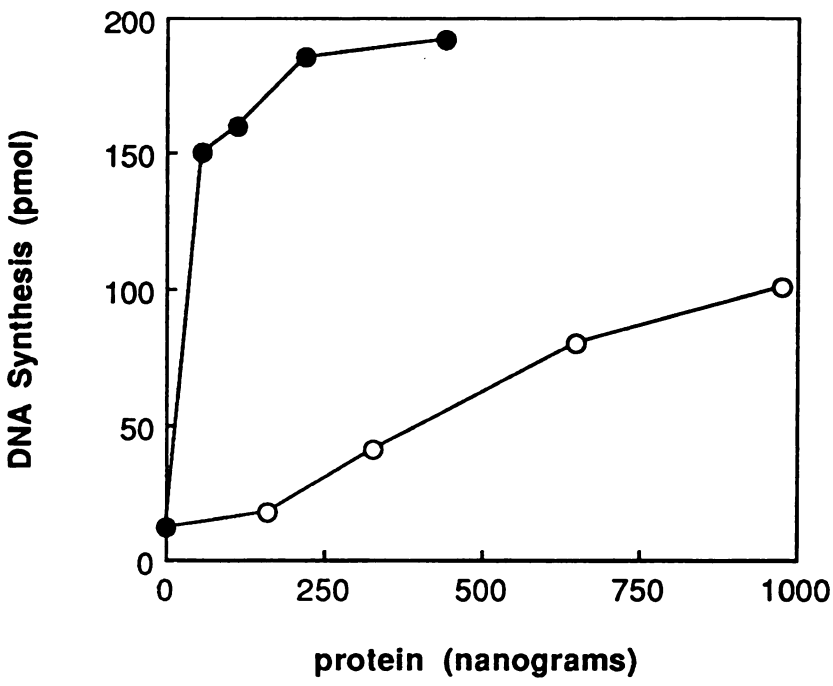
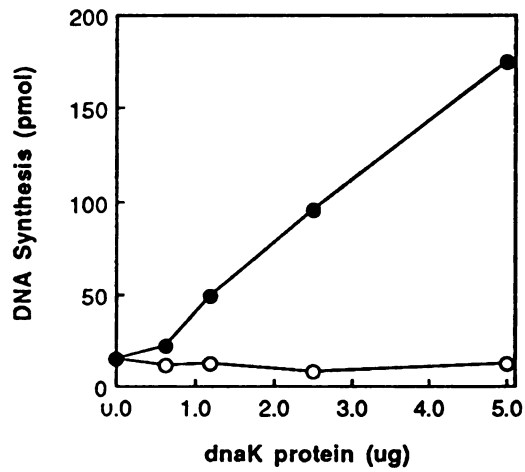

DNA Synthesis (pmol)
200
150
100
50
0
0
250
500
750
1000
protein (nanograms)

Figure 8. (A). dnaK protein titration in activation reactions. dnaK protein was added into replication reactions containing purified enzymes and 90 ng of dnaA5 protein without grpE protein (○), or with 100 ng of grpE protein (●). (B) grpE protein titration in activation reactions. grpE protein was titrated into replication reactions containing purified enzymes and 90 ng of dnaA5 protein without dnaK protein (○), with 1.2 ug of dnaK protein (■), or with 2.5 ug of dnaK protein (●). The reactions were assembled according to the experimental procedures and incubated at 30°C for 60 minutes. The extents of DNA synthesis were quantitated according to the experimental procedures

A.


200
150
100
50
0
DNA Synthesis (pmol)
0.0
1.0
2.0
3.0
4.0
5.0
dnaK protein (ug)

B.

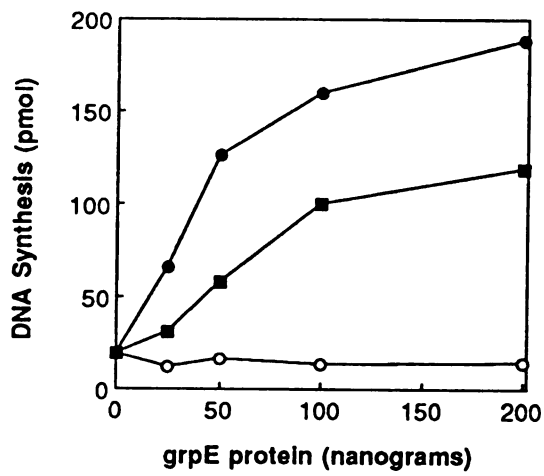

200
150
100
50
0
DNA Synthesis (pmol)
0
50
100
150
200
grpE protein (nanograms)

Table 1

The activation of dnaA5 protein by WM433 Fraction II

Components added	Fraction II (ug)	DNA synthesis (pmol)
dnaA5	-	19
Fraction II + dnaA5	25	18
Fraction II + dnaA5	50	70
Fraction II + dnaA5	100	184
Fraction II	50	8
Fraction II	100	7

DNA replication reactions containing purified enzymes and 90 ng of dnaA5 protein, where indicated, were assembled according to the experimental procedures. The indicated components were added and incubations were performed at 30°C for 45 minutes. The extents of DNA synthesis were quantitated according to the experimental procedures.

Table 2

The activating factor is heat sensitive

Fraction added	protein (ug)	incubation temperature	DNA synthesis (pmol)
-	-	-	6
II (diluted)	12	0°C	36
II (diluted)	25	0°C	102
IIIa	6	55°C	79
IIIa	12	55°C	151
IIIb	6	71°C	9
IIIb	12	71°C	10
IIIc	6	91°C	6
IIIc	12	91°C	5

WM433 Fraction II was diluted two fold with Buffer A and portions were incubated at the indicated temperatures for 7 minutes. Insoluble material was removed by centrifugation at 12, 000 x g for 5 minutes and the indicated amounts of protein were added to replication reactions containing purified enzymes and 90 ng of dnaA5 protein. The incubations were performed at 30°C for 45 minutes. The extents of DNA synthesis were quantitated according to the experimental procedures.

Table 3

dnaA5 protein is required to observe Fraction III dependent replication activity

dnaA5 protein (90 ng)	Fraction III (ug)	DNA Synthesis (pmol)
+	-	19
+	6	77
+	12	139
-	6	4
-	12	5

Replication reactions containing purified enzymes were assembled according to the experimental procedures. The indicated components were added and incubations were performed at 30^{o}C for 45 minutes. The extents of DNA synthesis were quantitated according to the experimental procedures.

Table 4

The activating factor is trypsin sensitive

dnaA5 protein	Fraction III (50 ug)	treatment	DNA Synthesis (pmol)
+	-	-	20
+	+	buffer	201
+	+	try/ti	4
+	+	ti/try	119

Fraction III (15 ul) was treated with Buffer A only, 2.5 ug of trypsin (try) or 2.5 ug of trypsin inhibitor (ti) and incubated at 30°C for 10 minutes. The reactions were then treated, respectively, with Buffer A only, 2.5 ug of trypsin inhibitor, or 2.5 ug of trypsin and incubated at 30°C for 10 minutes. The indicated amounts of the treated fractions were added to replication reactions containing purified enzymes and 90 ng of dnaA5 protein. The reactions were incubated at 30°C for 60 minutes and the extents of DNA synthesis were quantitated according to the experimental procedures.

Table 5

The second activating protein is heat sensitive

Fraction added	protein (ug)	incubation temperature	DNA Synthesis (pmol)
-	-	-	48
IIIa	12	55°C	197
IIIb	12	60°C	169
IIIc	12	65°C	95
IIId	12	70°C	46

WM433 Fraction II was diluted two fold with Buffer A and portions were incubated at the indicated temperatures for 7 minutes. The insoluble material was removed by centrifugation at 12, 000 x g for 5 minutes. The indicated components were added to replication reactions containing purified enzymes, 90 ng of dnaA5 protein, and 2 ug of dnaK protein. The reactions were incubated at 30°C for 60 minutes and the extents of DNA synthesis were quantitated according to the experimental procedures.

Table 6

The partial purification of the second activating protein

Fraction	volume (ml)	protein (mg)	units x 10^3	specific activity (units/mg)	unit yield (%)	fold purification
I	62.0	1220	N.D.	N.D.	N.D.	N.D.
II	7.50	795	48	60	N.D	N.D
III	29.0	272	369	1354	(100)	22
IV	16.5	64.4	286	4440	77	74
V*	66.0	38.1	267	6915	67	115

* 1/33 of fraction IV was used to produce Fraction V and the values obtained were corrected for by a factor of 33.

WM433 Fraction II was diluted two-fold with Buffer A, DNase I was added to a final concentration of 0.01 ug/ml, and an incubation was performed at 37°C for 20 minutes. The inclusion of DNase I resulted in the degradation of contaminating DNA that interfered with the replication assay after Mono Q chromatography (data not shown). The fraction was incubated at 55°C for 7 minutes, followed by centrifugation to remove insoluble material. An additional heat step was incorporated to remove additional contaminating protein as well as to inactivate residual DNase I. The resultant soluble fraction, Fraction III, was diluted to a conductivity equivalent to 50 mM KCl in Buffer A and applied to a Mono Q column (8 cm x 1 cm) equilibrated in the same buffer. Bound protein was eluted with a twenty ml linear gradient extending from 50 mM KCl to 600 mM KCl in Buffer A. Every fifth fraction was assayed in the reconstituted enzyme system containing dnaA5 protein and 2 ug of dnaK protein. The protein and activity profiles are summa-

rized in Figure 4. The activity eluted between 250 mM and 350 mM KCl. A 90% yield of activity was obtained (Fraction IV) with a 3-4 fold purification. The second activating protein did not bind to DNA cellulose, heparin agarose, Mono S, or Blue Dextran agarose (data not shown). Gel filtration was performed in order to determine the molecular weight of the protein as well as to aid in its purification. Fraction number 25 from Mono Q was injected onto a Superose 12 gel filtration column equilibrated in Buffer A containing 250 mM KCl. The activity profile is shown in Figure 5A. The fractions containing activity are not highly enriched for one particular polypeptide (Figure 6) and the purification was only two-fold compared to the starting material. The recovery of units was 85% (Fraction V) and the activity detected was also dependent upon exogenously added dnaK protein (data not shown).

DISCUSSION

dnaA5 protein is inactive in replication systems containing purified enzymes (19). By contrast, it is active in a replication system employing a crude enzyme fraction suggesting that an activating factor in the crude enzyme fraction is absent in the reconstituted enzyme system. The extended lag preceding DNA replication dependent on dnaA5 protein in the crude enzyme system may reflect the time required for this activation to occur (19). An assay was developed which detects the ability of a factor from a crude enzyme fraction to activate dnaA5 protein in the replication system containing purified enzymes.

dnaA protein involvement in the *in vitro* replication of *oriC* plasmids was first demonstrated using crude enzyme fractions obtained from *dnaA* mutants (32). A feature of this system was the requirement for an ATP regenerating system and PVA. Such crude enzyme fractions, deficient in dnaA protein activity, could support bidirectional initiation of DNA replication from *oriC* upon the addition of dnaA protein (33); this was used as an assay to purify dnaA protein (34).

Reconstitution of the replication system containing a crude enzyme fraction into purified components led to the formation of a system composed of 12 highly purified enzyme fractions, including RNA polymerase, topoisomerase I, and RNase H (23). This reconstituted enzyme system supports *oriC* plasmid replication dependent upon dnaA protein. Simplification of this replication system dependent upon RNA polymerase has led to the discovery of an RNA polymerase independent replication system, where primase is the sole priming enzyme (30, 31). Further refinement resulted in the discovery that dnaA protein functions to unwind the double helix at A-T rich regions in the vicinity of *oriC* (4), facilitating the formation of a pre-priming intermediate including dnaB and dnaC proteins (3).

The replication system containing RNA polymerase, topoisomerase I, and RNase H is thought to be more physiologically relevant (28). The influence of these proteins on dnaA

protein activity is not completely understood. A transcriptional event by RNA polymerase is thought to alter the topology of DNA through transcriptional activation (35). This topological alteration may facilitate dnaA protein dependent unwinding of *oriC*. Topoisomerase I and RNase H maintain *oriC* dependence by preventing nonspecific transcription that leads to *oriC* independent initiation of DNA synthesis (23).

The activation of dnaA5 protein was not observed in RNA polymerase dependent replication assays in the absence of PVA, in replication systems where primase is the sole priming enzyme, or in *oriC* unwinding assays (FI* formation) (unpublished data). The assay used to detect activation of dnaA5 protein was the RNA polymerase dependent replication system containing PVA. The requirement for RNA polymerase in the replication reaction may indicate that the activated form of dnaA5 protein requires RNA polymerase to facilitate unwinding of *oriC*.

grpE and dnaK heat shock proteins are both required for activation of dnaA5 protein replication activity. Initially, purification of the activator was unsuccessful. The demonstration that one of the activators was dnaK protein allowed for the partial purification of the second factor, subsequently shown to be grpE protein.

The observation that the second protein required for the activation of dnaA5 protein was grpE protein was deduced from the following evidence: 1) a 24 kd protein, isolated by gel filtration and resolved in a SDS polyacrylamide gel stained with Coomasie Blue, was coincident with activity, 2) the active fractions obtained by gel filtration were coincident with a protein that cross reacted to grpE antibody and 3) purified fractions of grpE protein replaced a Superose 12 fraction which activated dnaA5 protein with a similar efficiency.

The heat shock proteins dnaK, dnaJ, and grpE (36) function at an early step in lambda phage DNA replication (37-39). The dnaK protein involvement in initiation of lambda DNA replication occurs at a stage after the formation of a lambda O-lambda P-dnaB-dnaJ initiation complex at the origin (40-44). Its action results in the displacement of lambda P protein from dnaB protein (46-49), permitting dnaB protein to function as a helicase. grpE

protein is dispensable in the *in vitro* lambda replication system composed of purified enzymes as its role is to reduce the levels of dnaK protein required by 10-fold (45).

grpE and dnaK proteins form a complex (54) that is dissociated by ATP (29). The levels of ATP present in the lambda replication reaction are sufficiently high to maintain them in the dissociated state. grpE protein may function to reduce the level of dnaK protein required for initiation of lambda DNA replication by sequestering free lambda P protein to prevent its reassociation with dnaB helicase (50). In the absence of grpE protein, the elevated levels of dnaK protein may be required to, similarly, prevent reassociation of lambda P protein with the dnaB helicase.

The activation of dnaA5 protein absolutely requires both grpE and dnaK protein; the addition of either protein alone, even up to 10 ug of dnaK protein (unpublished data), results in no dnaA5 protein activation. This seems to differ from the involvement of dnaK and grpE proteins in lambda DNA replication, as dnaK protein can function in the absence of grpE protein. The activation of dnaA5 protein may proceed through a readily reversible process during which dnaA5 protein can assume the inactive form. dnaK protein alone may be unable to prevent the reaction reversal, and by analogy to the lambda DNA replication system, grpE protein may prevent the activation reaction from reversing and thus maintain dnaA5 protein in its active state.

grpE and *dnaK* mutants are temperature sensitive for *E. coli* growth (51, 52) demonstrating their essential role in cellular metabolism. A *dnaK* mutant defective in initiation of DNA replication (22) indicates its participation in this process *in vivo*. Until recently, no direct biochemical evidence existed which demonstrated a role for dnaK protein in *oriC* plasmid replication *in vitro*. A form of wild type dnaA protein that is inactive in *oriC* DNA replication systems composed of purified enzymes, elutes as an aggregate upon gel filtration (34) and contains elevated levels of phospholipid (53). This form of dnaA protein becomes active upon incubation with dnaK protein or phospholipase A2, indicating that dnaK protein may regulate the pool of dnaA protein in the cell by

converting it from the aggregate to the monomeric state by the dissociation of phospholipid (53). dnaA5 protein was not activated in replication by phospholipase A2 (data not shown), indicating the mechanism of activation may be different from the activation of the aggregated form of dnaA protein .

The physiological defects associated with *dnaA5* cells may relate to the activation of dnaA5 protein by heat shock proteins. The mechanism involving activation and its relation to physiological defects will be discussed in Chapter V (24).

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Chapter V

THE *Escherichia coli* dnaA5 PROTEIN IS ACTIVATED BY grpE AND dnaK HEAT SHOCK PROTEINS PRIOR TO ITS INTERACTION WITH *oriC* DNA

ABSTRACT

The replication activity of dnaA5 protein in a reconstituted enzyme system is restored through the combined actions of grpE and dnaK heat shock proteins (Chapter IV). The activation was divided into two stages which separated the activation event from DNA synthesis. The minimal components necessary during Stage I for the efficient activation of dnaA5 protein included: ATP at concentrations greater than 0.25 mM, PVA at levels of 8% or glycerol at concentrations of at least 10%, 50 ng to 100 ng of grpE protein, and dnaK protein at concentrations of at least 0.1 mg/ml. The subsequent addition of the activated form of dnaA5 protein to the reconstituted enzyme system (Stage II) resulted in high levels of replication with a lag preceding DNA synthesis characteristic to that of wild type dnaA protein. Although grpE and dnaJ proteins stimulate the ATPase activity of dnaK protein ten-fold, the presence of grpE and dnaA5 proteins had no effect on its ATPase activity. Using a monoclonal antibody that differentiates dnaA5 from dnaA protein a conformational change in dnaA5 protein was detected in the ATP dependent activation reaction. The interaction between dnaA5 protein and the heat shock proteins was thermolabile suggesting that the thermolability of *dnaA5* cells may be related to the interaction of the *dnaA5* gene product with the heat shock proteins.

INTRODUCTION

The initiation of DNA replication, *in vivo* (1) and *in vitro* (2), requires the function of the dnaA initiator protein. dnaA protein functions at an early stage in the initiation of replication of *oriC* plasmids, during which duplex DNA in the vicinity of *oriC* is converted to a single-stranded form (3). This intermediate is capable of supporting RNA priming and DNA synthesis (4). The individual biochemical properties of dnaA protein required for its replication activity include ATP binding (5) and sequence specific DNA binding to *oriC* (6).

The *dnaA* dependent initiation of replication occurs at a specific time within the cell cycle (7) indicating that chromosomal DNA replication is tightly coupled to cell growth. It is not known if or how the replication activity of dnaA protein is modulated *in vivo*, but accumulating evidence suggests that it may be under some form of regulation. dnaA protein bound to ADP is inactive in replication (8). The regulation of dnaA protein activity *in vivo* has been proposed to occur through the phospholipid reactivation of the ADP form of dnaA protein (9). The concentration of dnaA protein affects the initiation of replication *in vivo* in that overproduction of dnaA protein from expression vectors containing the *dnaA* gene increases the rate of initiation of DNA synthesis (10), suggesting that dnaA protein is the rate-limiting factor in initiation. However, other rate-limiting factors may exist as these initiation events are abortive (11). The concentration of dnaA protein per cell increases linearly with the growth rate (12), possibly on the demand for increasing the rate of initiation events in more rapidly growing cells. Clearly, there is a correlation between the levels of dnaA protein in the cell and the frequency of initiations, but the mechanism responsible for the coupling of dnaA protein activity in chromosomal DNA replication to cell growth is not known.

Cells harboring mutations in *dnaA*, including *dnaA5* and *dnaA46*, are temperature sensitive (13) and are asynchronous in the initiation of replication (14). An understanding

of the biochemical deficiencies associated with mutant forms of dnaA protein may allow for a greater understanding of initiation of DNA replication and its regulation. For this reason, mutant forms of dnaA protein, including dnaA5 (15) and dnaA46 (17, 18), have been purified and characterized in comparison to wild type dnaA protein. These mutant forms of dnaA protein are unable to bind ATP and interact inefficiently with *oriC* DNA resulting in their inability to initiate DNA synthesis in replication systems composed of purified enzymes (15, 17, 18). However, dnaA5 and dnaA46 proteins were observed to be active in replication systems employing a crude enzyme fraction and it was thought that components in the crude fractions were able to restore replication activity to the defective proteins. This hypothesis was confirmed by designing an assay which measured the activation of dnaA5 protein in replication systems composed of purified enzymes (16). Further purification of these activators has demonstrated that two heat shock proteins, grpE and dnaK, are required for the activation of dnaA5 protein.

DNA replication initiating from the lambda origin, *oriL*, requires phage encoded lambda O and P proteins (19, 20) and host encoded heat shock proteins including grpE, dnaJ, and dnaK (21). Biochemical studies on the replication of plasmids containing *oriL* has resulted in the reconstitution of the enzymes and proteins required for the process. The lambda O initiator protein binds to its consensus sequence within *oriL* and unwinds the duplex DNA in the vicinity of the origin (22). Lambda P protein interacts with lambda O protein and forms a complex with dnaB protein at the lambda origin (23). The requirement for dnaJ and dnaK heat shock proteins occurs after events leading to the formation of a lambda O-lambda P-dnaB nucleoprotein complex at *oriL*. In the presence of dnaJ protein, dnaK protein dissociates the lambda P protein from the initiation complex (24) in an ATP dependent manner to activate the helicase activity of dnaB protein (25). grpE protein is dispensable in this replication system containing purified enzymes (21) as its function is to reduce the level of dnaK protein required for initiation of DNA replication (26).

The identification of a *dnaK* mutant which is conditionally defective in initiation of DNA replication *in vivo* (27) indicates that dnaK protein is involved in the initiation of replication from *oriC*. The demonstration that dnaK and grpE proteins are required for activation of dnaA5 protein in *oriC* plasmid replication (16) provides a new system for the study of the function of heat shock proteins and a possible direction in understanding the regulation of DNA replication. This study extends the characterization of the heat shock protein mediated activation of dnaA5 protein by providing evidence that dnaK and grpE proteins function prior to dnaA5 protein association with *oriC* DNA. This interaction is temperature sensitive indicating that the thermolability in cell growth is related to the interaction between the *dnaA5* gene product and the *dnaK* and *grpE* gene products and not with an inherent instability of dnaA5 protein at elevated temperatures. The timing defect associated with *dnaA5* and *dnaA46* cells may be related to the inherent inactivity of the mutant initiator protein in initiating DNA replication. A rate limiting reaction involving the grpE and dnaK heat shock proteins may be required for the accumulation of initiation potential *in vivo* giving rise to an asynchronous initiation phenotype.

EXPERIMENTAL PROCEDURES

Reagents and enzymes. Commercial enzymes, solvents, chemicals, and radioactive isotopes were obtained as described (15). Polyethyleneimine TLC plates were obtained from Macherey-Nagel. dnaJ protein was a gift of Dr. M. Zylicz, University of Utah.

DNA replication assays. RNA polymerase dependent *oriC* plasmid replication assays which include PVA at 8% (w/v)were performed as previously described (16). The protein fractions used include; dnaA protein (fraction V, 2 x 10^5 units/mg) (17); dnaA5 protein (fraction III, (15)); dnaK protein (fraction IV, (16)); and grpE protein (fraction III, (16)).

Staging activation. The activation of dnaA5 protein in the RNA polymerase dependent replication system containing PVA was divided into two stages. Stage I contained the following components in a 5 ul volume: PVA at 8%, 40 mM HEPES-KOH (pH 7.6), 40 mM creatine phosphate, 0.4 mg/ml creatine kinase, 2 mM ATP, 10 mM magnesium acetate and, unless otherwise indicated, 100 ng of dnaA5 protein (fraction III or IV, (15)), 2 ug of dnaK protein (fraction IV, (16)), and 100 ng of grpE protein (fraction III, (16)). The reactions were incubated at 30°C for one hour, unless otherwise indicated. During Stage I incubations, Stage II components were assembled at 0°C in a 20 ul volume and contained the following reagents: PVA (8%) (w/v), 40 mM HEPES-KOH (pH 7.6), 40 mM creatine phosphate, 2 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.5 mM UTP, 1 mM dNTP (^{3}H-dNTP at 20 to 40 cpm/pmol nucleotide), 0.4 mg/ml creatine kinase, 10 mM magnesium acetate, 200 ng of M13*oriC*2LB5 plasmid, and purified replication enzymes and proteins as described in Chapter III (15). After Stage I, the Stage I reactions were placed on ice and 20 ul of a mixture containing Stage II components were added. The incubations were continued at 30°C for 25 minutes unless otherwise indicated. The extents of DNA synthesis were determined according to published procedures (15).

ATPase assays. The methods used for assembling the reactions were previously described (47) and are indicated in Figure 7. The products of the reaction were chromatographed on

polyethyleneimine TLC plates by spotting a one ul aliquot onto the origin, drying under a heat lamp, and developing the chromatogram in 1 M Formate / 500 mM LiCl. After the TLC plates were dried, the positions of the radioactive species were visualized by exposing the plates to X-ray film. The radioactive products were excised and the radioactivity was quantitated by liquid scintillation counting.

Immunological methods. Monoclonal antibodies to dnaA protein were produced (data not shown) and purified on protein A Superose resin according to standard procedures (46). Quantitating the levels of monoclonal antibody bound to its antigen in an ELISA was performed by the incubation of a 1:1000 dilution of goat anti-mouse HRP (Biorad) in PBS buffer containing 0.05% tween 20 and 0.2% BSA for one hour at room temperature. Following four washes with PBS containing 0.05% tween 20, the levels of goat anti-mouse HRP present were quantitated as previously described (46).

RESULTS

The time course of DNA synthesis in activation reactions.

The activation of dnaA5 protein in replication depends upon grpE and dnaK proteins (Chapter IV (16)). This coupled assay measures the activation of dnaA5 protein as detected by its ability to initiate DNA synthesis dependent upon other replication enzymes. An extended time course of activation (Figure 1A) revealed that a lag of about 30 minutes preceded DNA synthesis, then proceeded linearly for up to 100 minutes. Presumably, the 30 minute lag preceding DNA synthesis reflects the time required for activation of dnaA5 protein by the combined action of grpE and dnaK proteins.

This extended lag is not characteristic of DNA synthesis initiated by dnaA protein (2). The initiation of DNA synthesis dependent upon dnaA protein (Figure 1B) occurred much earlier in this replication system, which is expected, since it is thought that dnaA protein is in an active form when isolated from the cell and does not need grpE and dnaK proteins for activity. Once replication was initiated by both the dnaA protein dependent and the dnaA5 protein dependent systems, DNA synthesis proceeded linearly for up to 60 minutes.

Staging the activation of dnaA5 protein.

The extended lag required for dnaA5 protein to initiate DNA synthesis in a replication system composed of purified enzymes (Figure 1A) may reflect the time required for activation to occur. Presumably, the assay measures two events that are independent; the activation of dnaA5 protein by the heat shock proteins and subsequent initiation of replication catalyzed by the activated form of dnaA5 protein. The reaction was initially divided into two stages which involved the incubation of dnaA5 protein with a presumed minimal complement of proteins and reaction substrates. In the first stage (Stage I), dnaA5 protein was incubated with grpE and dnaK proteins at 30°C for 1 hour in a 5 ul reaction mixture as described in the Experimental Procedures. The second stage (Stage II) involved

the addition of a 20 ul reaction mixture containing *oriC* DNA, ^{3}H-dNTP, and replication enzymes to the Stage I mixture followed by incubations at 30°C for 30 minutes. The results are summarized in Table 1A. Control reactions containing only dnaA5 protein, or a sub-set of the three proteins (dnaA5, grpE and dnaK), showed that DNA synthesis did not occur. The addition of dnaA5, grpE, and dnaK proteins to Stage I resulted in high levels of DNA synthesis. The control reactions which involved the omission of dnaA5, grpE and dnaK proteins in Stage I but their addition to Stage II did not result in DNA synthesis. This is expected since the activation of dnaA5 protein in the coupled system requires that at least 30 minutes elapse before any detectable DNA synthesis occurs (Figure 1 A). These results indicate that grpE and dnaK proteins activate dnaA5 protein at a stage preceding binding to *oriC* DNA and can occur in the absence of general replication enzymes.

It is possible that one of the two heat shock proteins function during Stage I and the other functions during an early part of Stage II. To demonstrate that grpE and dnaK proteins are both required during Stage I, experiments were performed which involved the incubation of dnaA5 protein with one of the two heat shock proteins in Stage I and subsequent addition of the omitted component to Stage II (Table 1B). The incubation of dnaA5 protein with grpE or dnaK proteins in a Stage I reaction mixture and subsequent addition of either dnaK or grpE proteins to Stage II, respectively, did not result in DNA synthesis. The control reaction in which all three components were incubated together during Stage I resulted in high levels of DNA synthesis. These results indicate that one of the two heat shock proteins cannot function individually during Stage I, but both grpE and dnaK proteins are required to observe activation of dnaA5 protein.

The time course of the staged activation of dnaA5 protein.

To determine the time course of DNA synthesis (Stage II), dnaA5, grpE and dnaK proteins were incubated in a Stage I reaction mixture at 30°C for 60 minutes, the time required for efficient activation of dnaA5 protein. Stage II components were added to the

Stage I reactions and the incubations were performed at 30°C from 0 to 60 minutes (Figure 2A). A lag of 5 minutes preceded DNA synthesis, which is linear for up to 20 minutes. This short lag phase preceding DNA synthesis is similar to that observed with dnaA protein in the same replication system (Figure 1B) (2). The 30 to 40 minute reduction in the lag phase preceding dnaA5 protein dependent initiation of DNA synthesis in the staged system compared to the coupled activation reaction (Figure 1B) demonstrates that efficient and complete activation of dnaA5 protein can occur before its interaction with the general set of replication enzymes and *oriC* DNA. The extents of DNA synthesis observed also indicate that the specific activity of the activated form of dnaA5 protein is similar to dnaA protein.

The time required for the activation of dnaA5 protein to occur during Stage I is described in Figure 2B. dnaA5 protein was incubated with dnaK and grpE proteins at 30°C in a Stage I reaction mixture for the indicated times, after which Stage II components were added and incubations were continued at 30°C for 25 minutes. The activation of dnaA5 protein is linear from 10 to 30 minutes with a low level of increased activity observed with extended times of incubation.

dnaK protein exhibits a concentration dependence in staged activation reactions.

The optimal amounts of dnaK and grpE proteins required for dnaA5 protein activation in the coupled activation reaction (Chapter IV, (16)) were 5 ug and 120 ng, respectively. The reduction in volume from 25 ul in the coupled activation reaction to 5 ul in Stage I reactions may result in a change in the levels of the heat shock proteins required for activation to proceed. Preliminary experiments demonstrated that five fold less dnaK protein was required for the activation of dnaA5 protein in the staged activation reaction (data not shown). Stage I incubations are performed in a 5 ul volume, which is a five fold lower volume than that used during the coupled activation reaction. Therefore, the observation that five fold less dnaK protein was required during the staged activation of dnaA5 protein may be due to the five fold volume reduction.

The replication observed after the titration of dnaK protein into differing volumes of a Stage I reaction and subsequent addition to Stage II is shown in Figure 3A. Maximal activation of dnaA5 protein during Stage I required only 0.5 ug of dnaK protein in 2.5 ul reaction volumes. Doubling the volumes of the Stage I reaction up to 5 ul and 10 ul resulted in increasing amounts of dnaK protein required for the activation of dnaA5 protein. This differs from the amount of dnaK protein required during the coupled activation reaction up to as much as 10 fold. Five ug of dnaK protein are required for maximal activation of dnaA5 protein in the coupled activation reaction (Chapter IV, (16)), while as little as 0.5 ug are required for maximal activation in the staged activation reactions. Under these conditions, there is a decrease in the ratio of dnaK protein to dnaA5 protein from 40:1 to 4:1. These data indicate that the relative amount of dnaK protein required for the activation of dnaA5 protein is not critical, but that the concentration may be important.

grpE protein is required in stoichiometric levels during the activation of dnaA5 protein.

The amount of grpE protein required for the staged activation reaction is shown in Figure 3B. grpE protein dependence was observed from 30 ng to 120 ng of protein added. This level of grpE protein required for the activation of dnaA5 protein is the same as that required in the coupled activation reaction (Chapter IV, (16)). This indicates that the relative molar quantity of grpE protein is essential for activation and is independent of the reaction volume. The amounts of grpE protein required for maximal activation of dnaA5 protein suggest that two molecules of grpE protein are required for the activation of one molecule of dnaA5 protein.

The temperature dependence of the activation reaction.

dnaA5 protein is thermolabile in initiation of DNA synthesis in a replication system containing a crude enzyme fraction (15) and *in vivo* (13). The replication activity of dnaA5 protein depends upon dnaK and grpE proteins in systems containing purified enzymes. It

was of interest to determine whether the thermolabile defect of dnaA5 protein was during Stage I in the interaction with grpE and dnaK proteins or whether it became manifest during Stage II when dnaA5 protein functioned in replication.

The complete Stage I activation reaction (containing dnaA5, grpE, and dnaK proteins), was incubated for one hour at temperatures ranging from 25°C to 38°C followed by Stage II incubations at 30°C for 25 minutes. Increasing the temperature of the Stage I incubations resulted in decreasing replication activity observed during Stage II (Figure 4A). This suggests that the interaction between dnaA5 protein and the heat shock proteins is temperature sensitive.

If the above conclusion is valid, then the activated form of dnaA5 protein should become thermally insensitive. The complete Stage I activation reaction (containing dnaA5, grpE, and dnaK proteins), was incubated at 30°C for one hour, during which complete activation of dnaA5 protein should occur. Aliquots from the Stage I reactions were titrated into Stage II reactions and incubations were performed at 30°C and 38°C for 25 minutes (Figure 4B). The activated form of dnaA5 protein resulted in normal extents of DNA synthesis at 30°C and reasonably high levels of replication activity were observed at 38°C. Although the specific activity of dnaA5 protein was reduced three fold at 38°C compared to 30°C, there was substantial replication activity at elevated temperatures, confirming that the thermolabile deficiency is in the interaction between dnaA5 protein and the heat shock proteins.

The influence of solvents on the activation reaction.

The activation reaction, whether staged or coupled, has included 8% PVA. The initial inclusion of PVA resulted from the knowledge that activation of dnaA5 protein could not be detected in its absence (data not shown). The replacement of PVA by solvents known to stabilize proteins, like glycerol, was initiated in order to understand the affects of solvents on the activation of dnaA5 protein. Although less efficient than PVA, glycerol at 20%

could replace PVA in Stage I (Table 2). The activation of dnaA5 protein in the presence of 20% glycerol was dependent upon grpE and dnaK proteins.

The addition of glycerol in Stage I reactions resulted in background levels of DNA synthesis at concentrations less than or equal to 5% (Figure 5). DNA synthesis increased linearly at levels of glycerol ranging from 10 % to 30%, demonstrating the requirement of stabilizing solvents during Stage I.

Attempts at replacing PVA in Stage II have not been successful (Table 3). dnaA5 protein, activated during Stage I reactions which contain PVA or glycerol was active in Stage II reactions containing PVA (Table 3, experiments 1 and 2). However, dnaA5 protein that was activated in Stage I reactions containing PVA or glycerol was not able to initiate DNA synthesis in Stage II reactions in the absence of PVA (Table 3, experiments 3 and 4). There is some labile event, yet to be understood, which requires the inclusion of PVA during the initiation of DNA replication by the activated form of dnaA5 protein.

ATP is required for the activation of dnaA5 protein.

To determine whether ATP was required during activation, Stage I reactions were assembled according to the Experimental Procedures in the presence of various concentrations of ATP (Figure 6). In the absence of ATP, no activation was observed indicating that the reaction requires ATP. The maximal concentrations of ATP required for efficient activation were from 0.5 mM to 1 mM.

The ATPase activity of dnaK protein is not stimulated by dnaA5 protein.

The heat shock proteins, dnaJ and grpE, are both required to stimulate the ATPase activity of dnaK protein up to 10 fold (46), while grpE or dnaJ proteins alone do not influence the ATPase activity of dnaK protein. The ATPase activity of uncoating ATPase, a 70 kd heat shock protein analogous to dnaK protein, is stimulated when clathrin is bound to coated vesicles. This indicates that a specific conformation is recognized by uncoating ATPase

before catalyzing protein disassembly (34, 35). By analogy, it is possible that dnaA5 protein serves as a substrate for dnaK protein to stimulate its ATPase activity.

The effects of grpE and dnaJ proteins on the stimulation of the ATPase activity of dnaK protein, under conditions used during Stage I incubations, is shown in Figure 7A. The incubation of either grpE or dnaJ proteins with dnaK protein resulted in the level of ATPase activity similar to that catalyzed by dnaK protein alone. ATPase activity was not detectable with grpE and dnaJ proteins in the absence of dnaK protein. In contrast, the presence of both grpE and dnaJ proteins stimulated the rate of ATP hydrolysis by dnaK protein.

The effects of dnaA5 protein on the ATPase activity of dnaK protein is shown in Figure 7B. dnaA5 protein alone did not exhibit ATPase activity and its addition to dnaK protein did not stimulate the ATPase activity of dnaK protein. grpE protein is also essential for activation, yet dnaA5 and grpE proteins did not stimulate the ATPase activity of dnaK protein. This suggests that the mechanism of dnaK, dnaJ, and grpE protein dependent disassembly of the *oriL* initiation complex may be different from the dnaK and grpE protein dependent activation of dnaA5 protein.

dnaA5 protein and dnaA protein react differentially with monoclonal antibodies.

Monoclonal antibodies that recognize dnaA protein were produced by standard procedures (unpublished results). An ELISA was performed to determine if dnaA5 protein exhibited a differential reactivity with a particular monoclonal antibody, when compared to dnaA protein. This would indicate a conformational alteration is associated with the mutant protein.

The affinity of monoclonal antibody, M1, for dnaA and dnaA5 proteins was determined using an ELISA (Figure 8A). The monoclonal antibody, M1, had a similar affinity to dnaA and dnaA5 proteins, indicating that the epitope recognized by M1 is apparently identical in both proteins. In contrast, the monoclonal antibody, A4, exhibited a differential affinity to dnaA5 protein in comparison to dnaA protein. The monoclonal antibody, A4, reacted very

weakly with dnaA protein under these conditions, while it was five to ten-fold more reactive to dnaA5 protein. Presumably, there is a conformational change in dnaA5 protein that results in a greater affinity of A4 to its epitope.

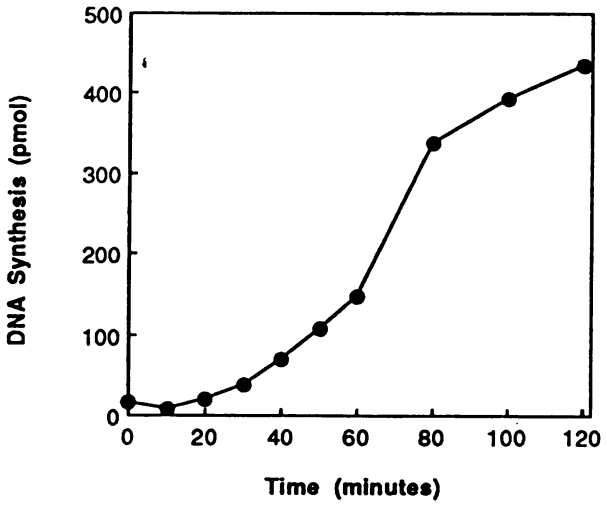
The activation of dnaA5 protein produces a conformational change in the protein.

A prediction derived from these studies on the activation of dnaA5 protein is that the heat shock proteins can induce a conformational change in dnaA5 protein to allow it to assume a conformation more similar to its wild type counterpart. The monoclonal antibody, A4, exhibits a higher affinity for dnaA5 protein than dnaA protein in an ELISA (Figure 8A). If dnaA5 protein undergoes a conformational change after activation, then it may assume a conformation more closely related to dnaA protein and may bind with a lower affinity to monoclonal antibody, A4.

As the activation of dnaA5 protein requires ATP (Figure 6), Stage I reactions (containing dnaA5, grpE and dnaK proteins) were assembled in the presence and absence of ATP and incubated at 30^{o}C for 30 minutes. Presumably, the omission of ATP in Stage I would prevent the heat shock protein dependent activation of dnaA5 protein. An ELISA was performed on the activated and inactivated forms of dnaA5 protein using monoclonal antibody A4 (Figure 8B). In the presence of ATP, during which the presumed conformational change associated with activation of dnaA5 protein occurs, a decrease in the affinity of A4 to its epitope was observed relative to dnaA5 protein under similar conditions but in the absence of ATP. This suggests that a conformational change is induced within dnaA5 protein after activation and that this conformation is more closely related to dnaA protein resulting in a lower affinity to monoclonal antibody, A4.

Figure 1. (A). Time course of activation of dnaA5 protein. DNA replication reactions containing purified enzymes, 100 ng of dnaA5 protein, 2 ug of dnaK protein and 100 ng of grpE protein were assembled according to the experimental procedures. (B). Time course of DNA synthesis dependent upon dnaA protein. DNA replication reactions containing purified enzymes and 90 ng of dnaA protein were assembled according to the experimental procedures. Incubations were performed at 30^{o}C for the indicated times and the extents of DNA synthesis were quantitated according to the experimental procedures.

A.


DNA Synthesis (pmol)
500
400
300
200
100
0
0
20
40
60
80
100
120
Time (minutes)

B.

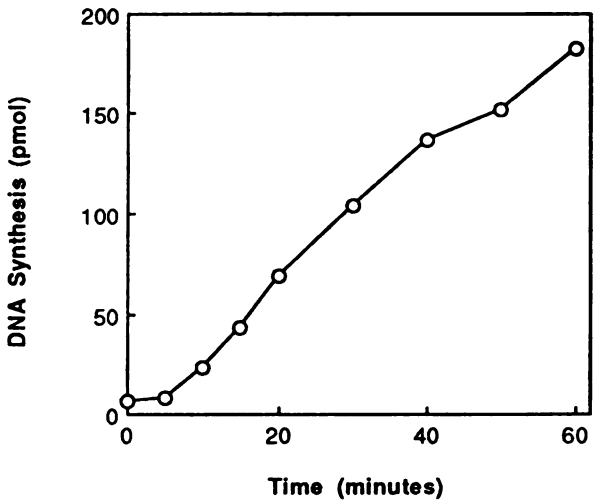
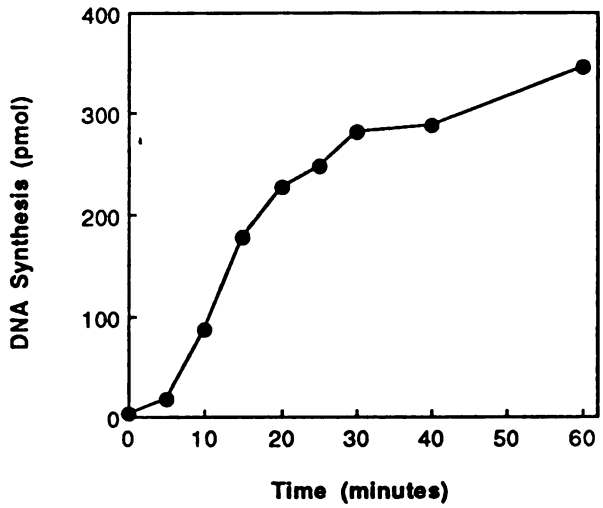

DNA Synthesis (pmol)
200
150
100
50
0
0
20
40
60
Time (minutes)

Figure 2 . Time course of staged activation of dnaA5 protein. (A). Stage I reactions containing 100 ng of dnaA5 protein, 2 ug of dnaK protein, and 100 ng of grpE protein were assembled according to the experimental procedures. The reactions were incubated at 30°C for the indicated times and, after the addition of Stage II components, the reactions were incubated at 30°C for 30 minutes. (B). Stage I reactions containing 100 ng of dnaA5 protein, 100 ng of grpE protein, and 2 ug of dnaK protein were assembled according to the experimental procedures and incubated for one hour at 30°C. Stage II components were added and reactions were incubated at 30°C for the indicated times. The extents of DNA synthesis were quantitated according to the experimental procedures.

A.


400
300
200
100
0
DNA Synthesis (pmol)
0
10
20
30
40
50
60
Time (minutes)

B.

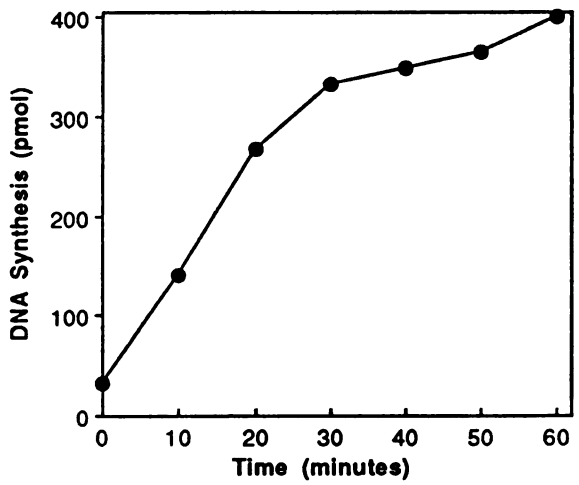
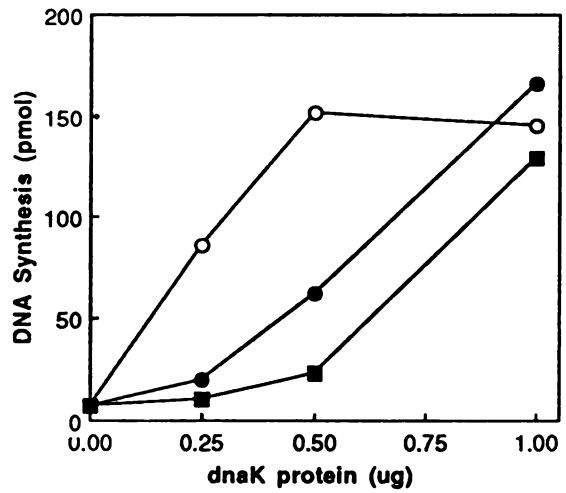

400
300
200
100
0
DNA Synthesis (pmol)
0
10
20
30
40
50
60
Time (minutes)

Figure 3. (A). The amounts of dnaK protein required during Stage I. Stage I reactions containing 100 ng of dnaA5 protein and 100 ng of grpE protein were assembled according to the experimental procedures, except that the final volumes were 2.5 ul (○), 5 ul (●) or 10 ul (■). dnaK protein was titrated into the reactions and incubations were performed at 30°C for one hour. Following the addition of Stage II components such that the final reaction volume was 25 ul, the reactions were incubated at 30°C for 25 minutes. (B) The amounts of grpE protein required during Stage I. Stage I reactions containing 100 ng of dnaA5 protein and 2 ug of dnaK protein were assembled according to the experimental procedures. grpE protein was titrated into the reactions and incubations were continued at 30°C for one hour. Following the addition of Stage II components, the reactions were incubated at 30°C for 25 minutes. The extents of DNA synthesis were quantitated according to the experimental procedures.

A.


200
150
100
50
0
DNA Synthesis (pmol)
0.00
0.25
0.50
0.75
1.00
dnaK protein (ug)

B.

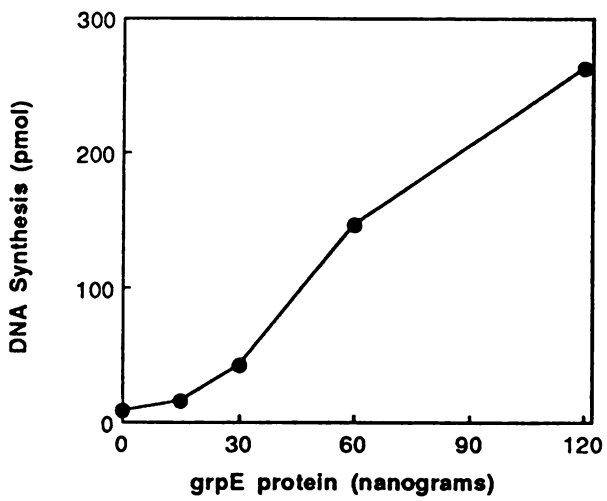
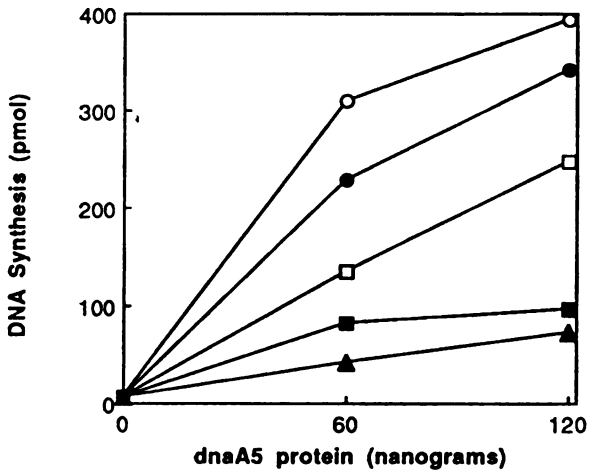

300
200
100
0
DNA Synthesis (pmol)
0
30
60
90
120
grpE protein (nanograms)

Figure 4. (A). Thermolability of Activation. Stage I reactions containing 60 ng or 120 ng of dnaA5 protein, 2 ug of dnaK protein and 100 ng of grpE protein were assembled according to the experimental procedures. The reactions were incubated at 25°C (○), 30°C (●), 33°C (❑), 35°C (■), or 38°C (▼) for one hour. Following the addition of Stage II components, the reactions were incubated at 30°C for 25 minutes, and the extents of DNA synthesis were quantitated according to experimental procedures. (B). Thermoresistance of the activated form of dnaA5 protein. Stage I reactions containing 30 ng, 60 ng, or 120 ng of dnaA5 protein, 2 ug of dnaK protein and 100 ng of grpE protein were assembled according to the experimental procedures. Stage I reactions were incubated at 30°C for one hour, and following the addition of Stage II components, the incubations were performed at 30°C (○) or 38°C (●) for 25 minutes. The extents of DNA synthesis were quantitated according to the experimental procedures.

A.


DNA Synthesis (pmol)
400
300
200
100
0
0
60
120
dnaA5 protein (nanograms)

B.

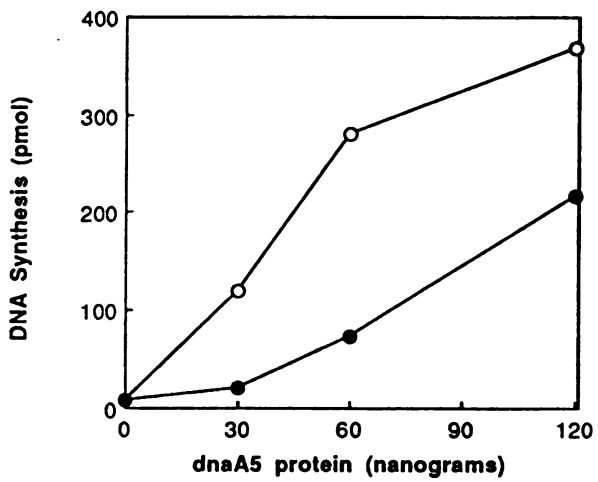

DNA Synthesis (pmol)
400
300
200
100
0
0
30
60
90
120
dnaA5 protein (nanograms)

Figure 5. The concentrations of glycerol in Stage I required for the activation of dnaA5 protein. Stage I reactions containing 100 ng of dnaA5 protein (○); 100 ng of grpE protein and 2 ug of dnaK protein (❑); or 100 ng of dnaA5 protein, 100 ng of grpE protein, and 2 ug of dnaK protein (●) were assembled according to the experimental procedures, except that PVA was replaced by the indicated concentrations of glycerol. The incubations were performed at 30°C for one hour, and following the addition of Stage II components, the incubations were continued at 30°C for 25 minutes. The extents of DNA synthesis were quantitated according to the experimental procedures.

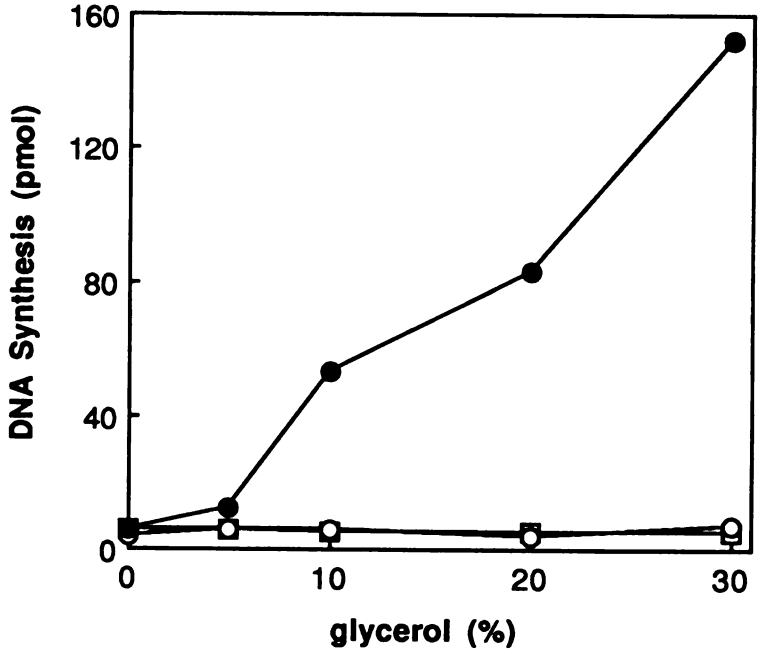

160
120
80
40
0
DNA Synthesis (pmol)
0
10
20
30
glycerol (%)

Figure 6. The concentrations of ATP in Stage I required for the activation of dnaA5 protein. Staged activation reactions containing 100 ng of dnaA5 protein, 100 ng of grpE protein, and 2 ug of dnaK protein were assembled and processed according to the experimental procedures, except that differing levels of ATP were added during Stage I incubations.

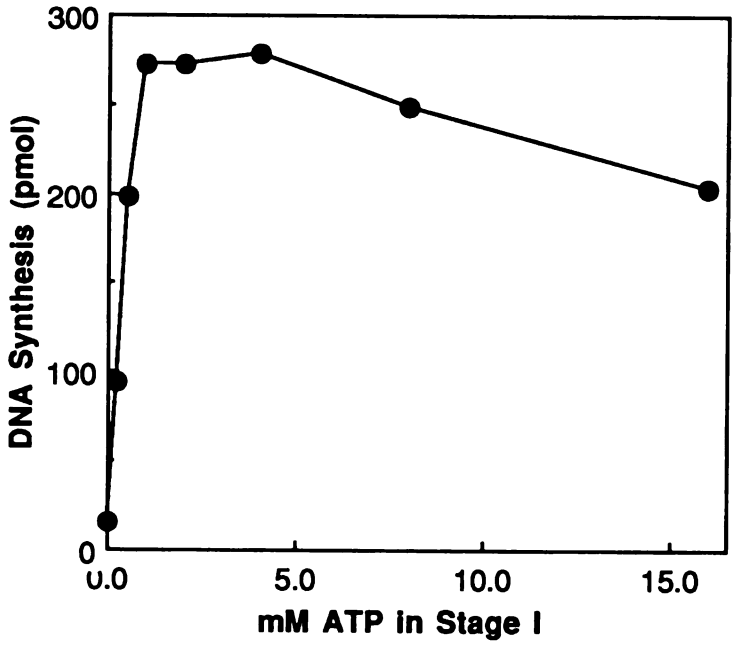
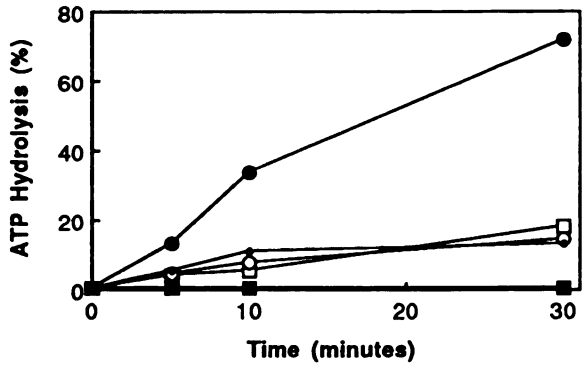

300
200
100
0
DNA Synthesis (pmol)
0.0
5.0
10.0
15.0
mM ATP in Stage I

Figure 7. The influence of dnaA5 protein on the ATPase activity of dnaK protein. Stage I activation reactions containing glycerol at 30 % were assembled according to the experimental procedures, except, ATP was present at only 0.1 mM (with ^{32}P gamma ATP present at 0.1 uCi /ul) and there was not an ATP regenerating system. After the addition of the appropriate protein fractions, the incubations were continued at 30^{o}C for the indicated times. The amounts of hydrolyzed ATP were quantitated according to the experimental procedures. (A.) Stimulation of the ATPase activity of dnaK protein by grpE and dnaJ proteins. grpE and dnaJ (■), dnaK (○), dnaK and grpE (❑), dnaK and dnaJ (◆), and dnaK, grpE, and dnaJ (●). (B). dnaA5 protein does not stimulate the ATPase activity of dnaK protein. dnaA5 protein (◆), dnaK and grpE proteins (❑), dnaK and dnaA5 proteins (▼), dnaK protein (○), dnaK, grpE and dnaJ proteins (●), dnaK, grpE and dnaA5 proteins (■).

A.


ATP Hydrolysis (%)
80
60
40
20
0
0
10
20
30
Time (minutes)

B.

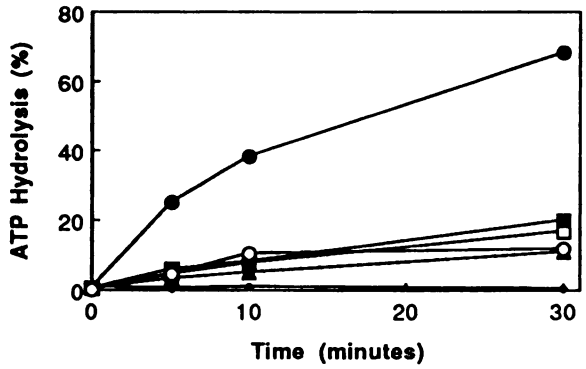

ATP Hydrolysis (%)
80
60
40
20
0
0
10
20
30
Time (minutes)

Figure 8. (A). The affinity of monoclonal antibodies to dnaA and dnaA5 proteins. Various amounts of dnaA or dnaA5 protein were incubated for five hours at room temperature in microtiter plates containing 100 ul of 50 mM sodium borate (pH 9.0). The individual wells were washed four times with PBS containing 0.05% tween 20 and 0.2% BSA. One hundred nanograms of monoclonal antibodies, M1 or A4, were added to the appropriate wells and incubations were continued for one hour. The amounts of monoclonal antibody bound to the antigen were quantitated according to the experimental procedures. dnaA5 protein + M1(■), dnaA protein + M1(❑), dnaA5 protein+ A4 (○), and dnaA protein + A4 (●) are indicated. (B). The affinity of monoclonal antibody A4 to dnaA5 protein after Stage I reactions in the presence and absence of ATP. Stage I reactions containing 30% glycerol, 100 ng of dnaA5 protein, 100 ng of grpE protein, and 2 ug of dnaK protein were assembled without ATP (○) and with 1 mM ATP (●) according to the experimental procedures. The incubations were performed at 30^{o}C for 30 minutes, after which, aliquots of each reaction were titrated into microtiter wells containing 100 ul of 50 mM sodium borate (pH 9.0) and incubated at room temperature for 4 hours. Following four washes with PBS containing 0.05% tween 20 and 0.2% BSA, 100 ng of monoclonal antibody, A4, was added to each reaction and incubations were continued at room temperature for one hour. The levels of monoclonal antibody bound to the antigen were quantitated according to the experimental procedures.

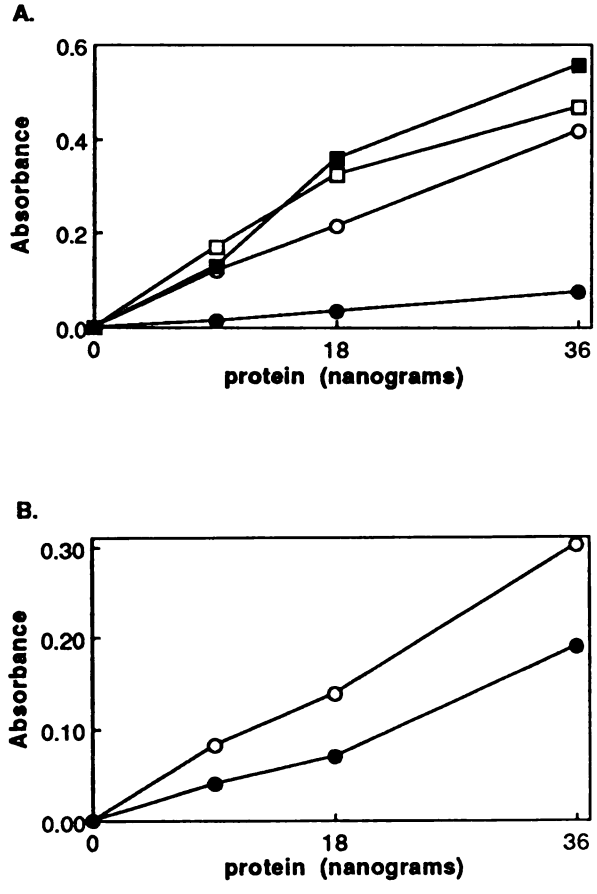

A.
0.6
0.4
0.2
0.0
Absorbance
0
18
36
protein (nanograms)
B.
0.30
0.20
0.10
0.00
Absorbance
0
18
36
protein (nanograms)

Table 1

A. Staged activation of dnaA5 protein

	Stage I addition			Stage II addition			
reaction	dnaA5	grpE	dnaK	dnaA5	grpE	dnaK	DNA Synthesis (pmol)
1	+						16
2		+					8
3			+				10
4		+	+				9
5	+	+					14
6	+		+				24
7	+	+	+				360
8				+	+	+	26

Activation of dnaA5 protein was staged by first incubating the indicated components in a 5 ul reaction volume according to the the experimental procedures (Stage I addition). Following the one hour incubation at 30°C, Stage II reaction components, were added to the Stage I reactions and incubations were continued at 30°C for 30 minutes. The extents of DNA synthesis were quantitated according to the experimental procedures.

B. grpE and dnaK proteins are required during Stage I reactions

	Stage I addition			Stage II addition			
reaction	dnaA5	grpE	dnaK	dnaA5	grpE	dnaK	DNA Synthesis (pmol)
1	+						12
2	+				+	+	18
3	+	+	+				221
4		+	+	+			12
5	+		+		+		17
6	+	+				+	21

Activation of dnaA5 protein was staged as indicated in Table 1A, except one of the two heat shock proteins was omitted during Stage I incubations and the omitted component was added to Stage II. Incubations and quantitations of the extents of DNA synthesis were performed as indicated in Table 1A.

Table 2

Stage I solvent requirements

Stage I solvent system	dnaA5	grpE	dnaK	DNA Synthesis (pmol)
PVA	+			14
PVA		+	+	5
PVA	+	+	+	379
glycerol	+			14
glycerol		+	+	3
glycerol	+	+	+	213

Stage I reactions were assembled according to the experimental procedures except that, where indicated, PVA was replaced by glycerol at a concentration of 20%. Following the addition of the indicated components, the reactions were incubated at 30°C for one hour. Stage II components were assembled according to the experimental procedures, added into Stage I reactions and the incubations were continued at 30°C for 25 minutes. The extents of DNA synthesis were quantitated according to the experimental procedures.

Table 3

Solvent replacements in the staged activation reactions

experiment	components added	Stage I solvent	Stage II solvent	DNA Synthesis (pmol)
1	-	PVA	PVA	8
	dnaA5	PVA	PVA	16
	grpE, dnaK	PVA	PVA	14
	dnaA5, grpE, dnaK	PVA	PVA	360
2	dnaA5	30% glycerol	PVA	6
	grpE, dnaK	30% glycerol	PVA	7
	dnaA5, grpE, dnaK	30% glycerol	PVA	152
3	-	PVA	EDBS	14
	dnaA	PVA	EDBS	195
	dnaA5	PVA	EDBS	8
	dnaA5, grpE, dnaK	PVA	EDBS	5
4	-	30% glycerol	EDBS	10
	dnaA	30% glycerol	EDBS	281
	dnaA5	30% glycerol	EDBS	11
	dnaA5, grpE, dnaK	30% glycerol	EDBS	7

Stage I reactions were assembled with the indicated components according to the experimental procedures, except that PVA is replaced by 30% glycerol where indicated. The incubations were performed at 30°C for one hour. Stage II reactions were assembled according to the experimental procedures with either the PVA or EDBS buffer system. The incubations were continued at 30°C for 25 minutes and the extents of DNA synthesis were quantitated according to the experimental procedures.

DISCUSSION

The activation of dnaA5 protein occurs prior to its interaction with oriC DNA. grpE and dnaK proteins activate dnaA5 protein at a stage preceding its action in initiation, indicating that this modification can occur prior to the stage in which an *oriC* initiation complex is formed. The role for grpE and dnaK proteins seems to be distinct from the role associated with these two proteins in lambda phage DNA replication based on two criteria. First, the activation of dnaA5 protein shows an absolute dependence on grpE protein, whereas in the lambda replication system, grpE protein is dispensable (21), its role being to reduce the level of dnaK protein required for activity. Second, dnaK protein functions after the formation of an *oriL* initiation complex by dissociating lambda P protein from the complex (24), while it participates in the activation of dnaA5 protein before the formation of an *oriC* initiation complex. dnaK protein function in the *oriC* replication system could be similar to its function in the lambda replication system, but this requires a dual role for dnaK protein function. If so, dnaK protein in conjunction with grpE protein first converts dnaA5 protein to a form active for replication. Second, dnaK protein may also function in the dissociation of proteins bound to the *oriC* initiation complex to activate some component involved in subsequent steps in initiation, by analogy with the role of dnaK protein in lambda replication. However, this role for dnaK protein in *oriC* plasmid replication seems unlikely as the lag preceding DNA synthesis dependent on dnaA protein, in which dnaK protein is absent, is similar to the lag preceding DNA synthesis catalyzed by the activated form of dnaA5 protein. The similar kinetics in the lag phase implies that, after dnaA5 protein is "modified", it is fully active and must follow the same pathway as dnaA protein to catalyze the initiation of replication.

The detection of replication activity by the activated form of dnaA5 protein. Activation of dnaA5 protein after its interaction with grpE and dnaK proteins requires that two constraints are met. The first is the requirement for PVA or high concentrations of glycerol for activation of dnaA5 protein during Stage I and the requirement for PVA to detect replication activity in Stage II reactions. These reagents may act by macromolecular crowding (28) or by the stabilization of the protein complexes. This indicates that some labile property of the mutant protein related to its initiation activity is not understood.

The second feature characterizing the activation reaction is the requirement for the RNA polymerase dependent replication system, regardless of whether activation is coupled or staged. The observation that the activated form of dnaA5 protein cannot function in the *oriC* unwinding assay (F1* formation) (unpublished data) in the presence of levels of PVA sufficient for replication activity suggests that RNA polymerase is required to facilitate unwinding of *oriC*.

RNA polymerase is thought to function in *oriC* replication through transcriptional activation (29) which is thought to alter the superhelical density of DNA to facilitate dnaA protein dependent unwinding of *oriC*. The activated form of dnaA5 protein may be more sensitive to the restraints of superhelicity and transcriptional activation may aid it in unwinding *oriC* DNA and lead to initiation of replication. The activity of dnaA protein in unwinding *oriC* DNA under these conditions is unaffected by the presence of dnaK and grpE proteins (data not shown) indicating that the inactivity of dnaA5 protein is not due to an inhibitor in the dnaK or grpE protein preparations.

The mechanism of heat shock protein function. E. coli dnaK protein and its eukaryotic counterpart, the hsp70 family, display a high affinity for ATP and require ATP for function. dnaK protein binds ATP and may hydrolyze ATP in the process of dissociating lambda P protein from an initiation complex (25). Hsp 70 from *S. cerevisieae* catalyzes the translocation of precursor proteins destined for membranes. This process requires ATP (30, 31). In addition, the dissociation of clathrin from coated vesicles by the heat shock

protein, uncoating ATPase (32, 33), requires the hydrolysis of ATP. Only the form of clathrin bound to a vesicle can elicit the ATPase response from this heat shock protein (34, 35) suggesting that a specific conformation in clathrin is required before uncoating ATPase can function. The activation of dnaA5 protein by the *E. coli* heat shock proteins requires ATP indicating the similarity between this system and other systems involving heat shock protein function.

The importance of the dnaJ and grpE protein dependent stimulation in the rate and extents of the ATP hydrolysis by dnaK protein (47) is not known. dnaA5 protein does not influence the ATPase activity of dnaK protein indicating that there may be a mechanistic difference between dnaK protein function in the lambda replication system and the dnaA5 protein dependent *oriC* initiation system. dnaA5 protein does not seem to function in concert with dnaK protein in a mechanism similar to clathrin and uncoating ATPase (34, 35), as dnaA5 protein does not serve as a substrate for increasing the ATPase activity of dnaK protein.

Heat shock proteins have been characterized in many systems and these biochemical studies have revealed insights into their function. Heat shock proteins are thought to alter the conformation of proteins (41) through an ATP dependent, multi-step process involving protein unfolding and refolding. It is possible that dnaK and grpE proteins function by a similar process to unfold and refold a domain of dnaA5 protein required for its initiation activity, thus restoring a conformation suitable for functioning in initiation. Given the known biochemical function of the *E. coli* dnaK protein in lambda DNA replication, it is also possible that activation of dnaA5 protein occurs through a process in which de-aggregation of dnaA5 protein occurs and this form of dnaA5 protein can function to initiate DNA replication.

dnaA5 protein has an altered conformation compared to dnaA protein as determined by its differential affinity to a monoclonal antibody. The differential reactivity may be due to the inherent difference in the epitope due to the amino acid change or dnaA5 protein may

exist in an improperly folded state. If the latter is true, the activated form of dnaA5 protein may have assumed a conformation similar to wild type dnaA protein.

dnaA5 protein is defective in ATP binding (Chapter III (15)) and the heat shock proteins may alter the conformation of the mutant protein to activate ATP binding, which in turn allows for the initiation of DNA replication. Attempts at detecting ATP binding by dnaA5 protein after activation by grpE and dnaK proteins have not been successful (data not shown).

The physiological defects associated with dnaA mutants may involve heat shock protein function. *dnaA5* and *dnaA46* cells exhibit an asynchronous initiation frequency (14) and are thermolabile in the initiation of DNA replication (13). These two distinct physiological defects may be related to the same phenomenon. The activation of dnaA5 protein and dnaA46 protein (data not shown) by both grpE and dnaK proteins demonstrates a role for these two heat shock proteins in modulating the activity of the mutant initiator proteins *in vitro*, suggesting that asynchrony in initiation results from the rate limiting interaction between the heat shock proteins and the mutant forms of dnaA protein. An alternative explanation for asynchrony involves the requirement for RNA polymerase in detecting dnaA5 protein activity after activation by the heat shock proteins. It is possible that the rate limiting step in initiation of replication catalyzed by mutant forms of dnaA protein, though dependent upon heat shock proteins, is the availability and distribution of free RNA polymerase for initiating the transcriptional event at *oriC*.

The activation of dnaA5 and dnaA46 proteins (data not shown) is thermolabile suggesting that the temperature sensitivity of cells harboring *dnaA5* or *dnaA46* mutations is related to the interaction between the *dnaA5* gene product and the heat shock proteins. The inability of the heat shock proteins to activate dnaA5 and dna46 proteins at elevated temperatures suggests that the conformation of the mutant initiator proteins at elevated temperatures may be skewed preventing a proper association with the heat shock proteins.

Heat shock proteins are induced upon the accumulation of foreign or abnormal proteins in the cell (37-39) and they may accelerate the recovery from the possible damage afforded under these conditions through the dissociation and refolding of the abnormal or foreign proteins (40, 41). The *dnaA5* gene product is reversibly denatured at elevated temperatures (42). It is possible that heat shock proteins aid in the restoration of the activity of dnaA5 protein after the transfer to permissive conditions.

Heat shock proteins in *E. coli* are regulated by a complex regulon (43). Recently, it was shown that dnaA protein negatively regulates transcription of the *rpoH* gene (44), involved in the expression of heat shock proteins including grpE and dnaK. It is not known if dnaA protein regulates cell growth by functioning to maintain a balance between the cell mass and synthesis of proteins involved in normal cell physiology. Heat shock proteins may function in the *dnaA* dependent initiation pathway by modifying dnaA protein prior to initiation of DNA replication and dnaA protein may, in turn, regulate the expression of the heat shock proteins through regulation of levels of the *rpoH* gene product. A role for the heat shock proteins in regulating dnaA protein activity in the cell suggests that a self-controlling regulatory circuit may be involved in regulating the initiation of replication.

In regards to this possibility, dnaA protein isolated from a *dnaK* mutant exhibits the same biochemical characteristic as mutant forms of dnaA protein, that being activity in a crude enzymatic replication system, but inactivity in a reconstituted enzyme system (45). This may indicate that heat shock proteins modulate dnaA protein dependent initiation of DNA synthesis.

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Chapter VI

SUMMARY AND PERSPECTIVES

A greater understanding of dnaA protein dependent initiation of replication *in vivo* and *in vitro* was obtained by studying the activity of mutant forms of dnaA protein. The extragenic suppressor of the *dnaA46* mutation, *dasC*, was shown to be allelic with *trxA*.. This indicates that thioredoxin and dnaA46 protein may interact in the cell. The presence of an unstable and unlinked mutation required for *dasC* to suppress the *dnaA46* mutation indicated that another factor was required for suppression to occur and a biochemical understanding of the interaction was not pursued due to the complex genetics of the mutant cell.

Thioredoxin is involved in the reduction of ribonucleotide reductase, it forms an essential subunit of T7 DNA polymerase, and it is involved in M13 phage body assembly. Another research group has shown that the *groE* gene product, a heat shock protein involved in lambda phage body assembly, can suppress the temperature sensitive phenotype of *dnaA46* cells when present at high copy number. These two apparently unrelated gene products, *trxA* and *groE*, are involved in phage morphogenesis and can suppress the temperature sensitive phenotype of *dnaA46* cells under the conditions discussed. If the role of *trxA* and *groE* during lambda phage growth in the processing of higher order protein structures is characteristic of their function in other processes, then their role in suppression of *dnaA46* mutations may be in the assembly of a *dnaA46* complex active for the initiation of replication.

The existence of *dnaA* mutants, well categorized in their physiological defects, provides an excellent foundation for studying mutant forms of dnaA protein. The goal of the project involving the purification of dnaA5 protein was to determine the biochemical alteration(s) in its activity and associate those changes with the physiological defects of *dnaA5* cells. In comparison to dnaA protein, dnaA5 protein was unable to bind ATP and it formed an improper nucleoprotein complex at *oriC*. These deficiencies were reflected in the inability of dnaA protein to catalyze initiation of DNA synthesis in replication assays containing purified enzymes. In contrast, dnaA5 protein was active in replication assays which contain a crude enzyme fraction. This result indicated that there was a factor in the crude fraction able to activate the

replication activity of dnaA5 protein. The purification of the factor resulted in the demonstration that two known heat shock proteins, grpE and dnaK, were required for activation to occur. The heat shock proteins may function to restore the deficient activities of dnaA5 protein. Two avenues of research that will be pursued to understand the activation reaction include 1) determining the changes that occur within the dnaA5 polypeptide to confer replication activity and 2) determining the mechanism of heat shock protein function.

The changes in dnaA5 protein after activation have been difficult to pinpoint. There appears to be a conformational change in the protein after activation as detected by the change in its affinity to a monoclonal antibody. However, a more thorough analysis of these methodologies is required to conclusively support this result. Given the absolute requirement for ATP in the dnaA protein dependent unwinding of *oriC*, the heat shock proteins may restore ATP binding activity to dnaA5 protein. Preliminary experiments resulted in the inability to detect ATP binding by dnaA5 protein after activation. To address whether conformational changes occur in dnaA5 protein after activation, controlled proteolytic digestions may be performed. Alterations in the conformation of dnaA5 protein may expose or hide an epitope that will alter the products of proteolysis.

The mechanism of heat shock protein (hsp) function in the activation of dnaA5 protein is similar to the mechanism of hsp function in other types of cells. Activation of dnaA5 protein requires ATP, which supports models proposed by other investigators that require hsp's utilize ATP during their performances.

Hsp's may function through the denaturation or refolding of proteins, which involves direct contact with the substrate. To determine the ability of dnaK and grpE proteins to function in the refolding of dnaA5 protein, two experiments will be performed. First, the ability of dnaA5 protein to bind to dnaK and/or grpE proteins before and after activation will be examined by the ability of dnaK and/or grpE proteins to co-immunoprecipitate with dnaA5 protein. dnaA5 protein may be bound to dnaK and grpE proteins only in the inactivated state. After activation, dnaK and grpE proteins may dissociate thus liberating dnaA5 protein to catalyze initiation. If

dnaK and grpE proteins co-immunoprecipitate with dnaA5 protein only before activation is completed, then this would indicate that they may only recognize and bind to a specific epitope within dnaA5 protein. The second experiment will involve the ability of dnaK and/or grpE proteins to reactivate a denatured form of dnaA5 or dnaA protein. Dr. M. Zylicz has demonstrated that *E. coli* dnaK protein can activate a heat denatured from of RNA polymerase (personal communication). In a similar manner, it is possible that dnaK and grpE proteins refold a denatured domain within dnaA5 protein.

Cells which harbor the *dnaA5* allele are temperature sensitive and are altered in the timing of initiation of replication. From the studies described in this thesis, it was found that the interaction between dnaA5 protein and the heat shock proteins is temperature sensitive *in vitro*. The thermolabile defect observed *in vivo* may be related to this interaction. The altered timing of initiation may involve a rate-limiting interaction between dnaA5 protein and the heat shock proteins *in vivo*, but this is more difficult to corroborate biochemically.

A model can be formed in which heat shock proteins couple dnaA protein dependent initiation of replication to cell growth. A defect within dnaA protein (such as that exhibited by dnaA5 protein) may prevent it from responding to this heat shock mediated signal. If dnaA protein (or dnaA5 protein) is not rate-limiting *in vivo*, then perhaps the activity of the heat shock proteins is rate-limiting. Experiments that would address this would involve determining the concentration of dnaA (or dnaA5 protein), dnaK and grpE proteins within the cell cycle. If the levels of any of these proteins fluctuate during the cell cycle then this would suggests that they may be the rate-limiting regulatory signal.