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dnaA 46 PROTEIN IN INITIATION OF *in vitro* *oriC* PLASMID REPLICATION

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dnaA46 PROTEIN IN INITIATION OF *in vitro* *oriC* PLASMID REPLICATION

By

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ABSTRACT

dnaA46 PROTEIN IN INITIATION OF *in vitro* *oriC* PLASMID REPLICATION

By

Deog Su Hwang

The alleles of *dnaA46*, *dnaA5*, and *dnaA204* have been cloned from the *E. coli* chromosome into a protein overproducing vector. Under conditions of induced expression, similar amounts of the *dnaA46* and *dnaA5* gene products and less of the *dnaA204* gene product were observed. Facilitated by elevated expression levels, the purification of dnaA46 protein has been obtained. Highly purified preparations of dnaA46 protein have permitted its biochemical characterization in comparison with the activities of wild type dnaA protein. In contrast with the wild type protein, DNA synthesis dependent upon dnaA46 protein showed a pronounced lag before incorporation of deoxyribonucleotides. Whereas the replication activity of dnaA46 protein did not appear to be thermolabile upon a prior incubation at various temperatures, thermolabile replication activity was observed under conditions of coupled DNA synthesis. It has been determined that dnaA46 protein is reduced in its ability to bind to DNA fragments containing *oriC*. This mutant protein was also defective in binding of ATP, and was inactive for replication of *oriC* plasmids in purified enzyme systems. dnaK

protein and another factor(s) have been identified which appear to interact with dnaA46 protein prior to DNA synthesis. These studies indicate that this interaction is thermolabile. Stimulation of DNA synthesis occurs through the reduction of the prolonged lag prior to DNA synthesis.

To

My Parents,

Wife, Son, and Daughter

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

bp	base pair
dnaA box	the 9 bp consensus sequence recognized by dnaA protein
dNTP	deoxyribonucleotide
DTT	dithiothreitol
EDTA	ethylenedinitrilo tetraacetic acid
HEPES	4-(2-hydroxethyl)-1-piperizineethane sulfonic acid
Kb	kilobase pair
KDa	kilodalton
NEM	N-ethylmaleimide
oriC plasmid	plasmid containing the oriC sequence
PVA	polyvinyl alcohol
RF	replicative form
rNTP	ribonucleotide
SDS	sodium dodecyl sulfate
ss	single-stranded
SSB	single strand DNA binding protein
U	unit
u	micro

CHAPTER I

Literature Review

LITERATURE REVIEW

Escherichia coli chromosomal DNA consists of a circular duplex DNA of 4,000 kilo base pairs (1-3). E. coli chromosomal DNA replication begins at a unique site on the E. coli chromosome, the genetic locus oriC, near 83 minutes of 100 minutes of the E. coli genetic map. The replication forks proceed bidirectionally from oriC. The leading strands are synthesized continuously in the 5' to 3' direction and the lagging strands are synthesized discontinuously in the 5' to 3' direction. Replication of the chromosomal DNA terminates at a site, terC, which is located in the region of 30 to 32 minutes on the E. coli genetic map.

Initiation of DNA Replication

E. coli DNA polymerases including DNA polymerase I, DNA polymerase II and DNA polymerase III holoenzyme are unable to synthesize DNA de novo (1-2). The polymerases only extend preexisting primers in a 5' to 3' direction. DNA polymerase III holoenzyme which is composed of at least 7 subunits is the major DNA polymerase which is responsible for synthesizing daughter strands during DNA replication. Because the rate of chain elongation by DNA polymerase III holoenzyme is on-going and relatively constant at 1,000 nucleotides per second, it is believed that the regulation of the DNA replication mainly occurs at an initiation step during which the primer is synthesized. The primer is elongated by

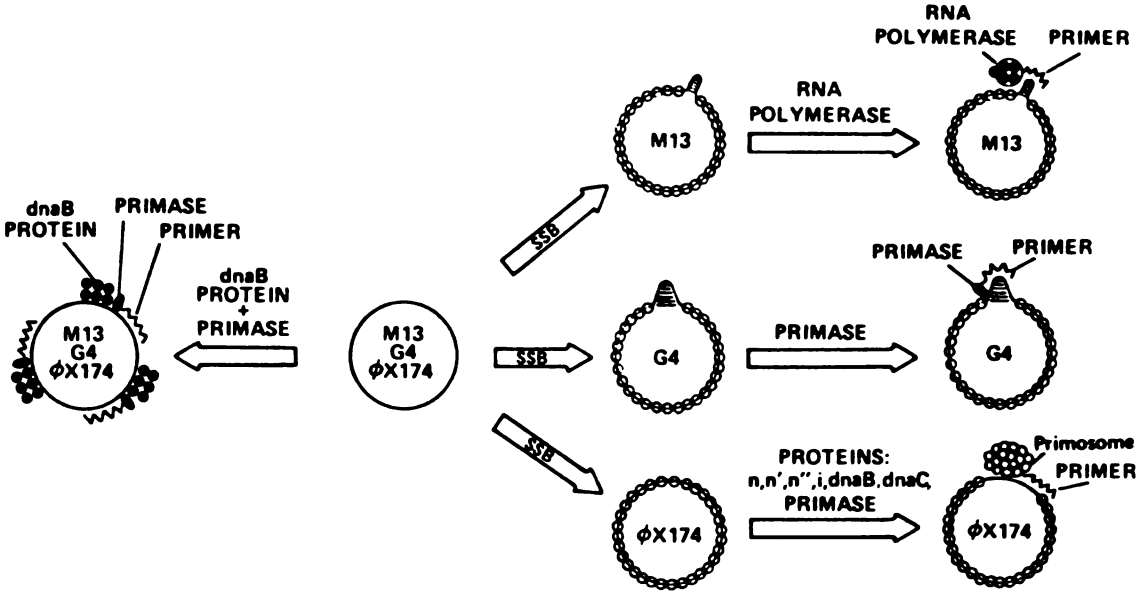
the DNA polymerase III holoenzyme in the elongation stage of DNA replication. The function of an origin DNA sequence and the proteins that interact with it is an important biological problem.

As an example, conversion of single-stranded viral DNA of M13, G4 and øX174 to their duplex RF (replicative form) DNA shows the existence of different mechanisms of initiation of viral DNA replication (Figure 1).

Nonspecific priming

In the absence of SSB protein (single strand DNA binding protein), a general priming reaction occurs which is not specific to the viral DNAs (Figure 1, 1-2,4). dnaB protein, a hexamer of 52 KDa, binds ATP with a K_D of 10 uM and possesses DNA-dependent ATPase activity (5-7). Although free dnaB protein weakly binds to ss DNA, the ATP/dnaB protein complex binds to multiple sites on ss DNA (7). In the presence of ATP or nonhydrolyzable ATP analogs, dnaB protein forms a stable ternary complex of ATP (or the nonhydrolyzable ATP analogs)/dnaB protein/ss DNA (7-8), indicating that ATP hydrolysis is not required for the binding of dnaB protein to ss DNA. ATP hydrolysis is necessary for dissociation of the dnaB protein from the ternary complex. Binding of the ATP/dnaB protein complex to ss DNA appears to result in a conformational change (secondary structure) on the DNA, inferred from the enhancement of ethidium bromide fluorescence. By recognition of the secondary structure on the ssDNA or by interaction with dnaB protein, DNA primase, with a molecular weight of 66 KDa and encoded by dnaG, is placed at the priming site, then synthesizes a primer which can be elongated by DNA polymerase III holoenzyme (8-11). DNA primase can incorporate rNTPs

Figure 1. Nonspecific (general) priming by dnaB protein and primase on uncoated DNA and the specific priming systems for M13, G4 and ϕX174 DNAs coated with SSB (1).


dnaB PROTEIN
PRIMASE
PRIMER
M13 G4 ϕX174
dnaB PROTEIN + PRIMASE
M13 G4 ϕX174
SSB
M13
RNA POLYMERASE
RNA POLYMERASE
PRIMER
M13
SSB
G4
PRIMASE
PRIMASE
PRIMER
G4
SSB
ϕX174
PROTEINS: n,n',n'',i,dnaB,dnaC, PRIMASE
Primosome
PRIMER
ϕX174

(ribonucleotides) and dNTPs (deoxyribonucleotides) into the primer (9-12). Although the primase binds dNTPs more tightly than rNTPs, the rate of incorporation of rNTPs is more efficient. The tight binding of dNTPs to primase may interfere with nucleotide incorporation into the primer. Also, RNA polymerase nonspecifically primes the viral DNAs in the absence of dnaB protein. Transcription by RNA polymerase appears to be coupled to DNA elongation by DNA polymerase III holoenzyme (14).

Specific priming which is origin dependent

Specific priming occurs at the unique origins of the following viral DNAs in the presence of SSB protein, a single strand DNA binding protein (Figure 1).

In vivo conversion of M13 viral DNA to the RF form is sensitive to rifampin which inhibits transcription by RNA polymerase (15), suggesting that RNA polymerase primes DNA replication. The SSB-coated M13 viral DNA is efficiently primed by RNA polymerase holoenzyme in vitro (14-16). RNA polymerase core enzyme which lacks the sigma subunit of the RNA polymerase holoenzyme is inefficient in the priming and is not specific to the M13 viral DNA (16), indicating that the sigma factor recognizes the M13 origin and RNA polymerase transcribes the primer at the origin of the SSB-coated M13 viral DNA.

DNA primase specifically synthesizes the primer at the origin of the SSB-coated G4 viral DNA (9, 17-20). In the absence of dNTPs, primase synthesizes a 29 ribonucleotide long primer at the G4 origin (19-20). At 20 uM rNTPs and 50 uM dNTPs, shorter primers (about 6 nucleotides) are synthesized, but the elongation by the DNA polymerase

III holoenzyme is not affected (20). This suggests that the in vivo primers are short and mixtures of ribo- and deoxynucleotides.

The synthesis of a primer on the SSB-coated øX174 DNA requires a multiprotein complex, the primosome, which is composed of proteins n, n', n'', i, dnaB, dnaC and primase (Figure 1). n' protein, which is 76 KDa, contains sequence specific DNA-dependent ATPase activity (21-22). The effector for this ATPase activity appears to be unique in øX174 viral DNA. n' protein displaces SSB protein on the SSB-coated viral DNA and the displacement is stimulated by ATP (23). n' protein with n and n''proteins binds to the origin of the SSB-coated øX174 DNA and the complex is isolatable on a Bio-Gel A5-m gel permeation column. The functions of n (14 KDa) and n'' (17 KDa) proteins are not clearly understood (24).

In the presence of ATP, dnaB protein forms a stable complex with dnaC protein which is isolable by glycerol gradient sedimentation, gel permeation chromatography or DEAE-cellulose chromatography (25-28). DnaB protein in the complex of dnaB/dnaC protein does not exhibit ATPase activity, which contrasts with the DNA dependent ATPase activity of dnaB protein alone. Although the free dnaC protein is sensitive to N-ethylmaleimide, the dnaC protein in the complex is resistant to N-ethylmaleimide. This indicates that the complex contains the different properties from free dnaB and free dnaC protein. The entry of dnaB/dnaC protein complex is mediated by protein i (29). The protein i has a molecular weight of 20 KDa, the product of dnaT, and has a binding affinity to SSB-coated ss DNAs (29-30). After the entry of dnaB protein into the primosome complex, the proteins dnaC and i appear to dissociate

from the complex (28-29). The function of dnaC and i proteins is to place dnaB protein onto the primosome complex. Subsequently, primase synthesizes the primer which is elongated by DNA polymerase III holoenzyme.

When the priming reaction is not coupled to DNA elongation, multiple primers are formed on the SSB-coated ϕX174 viral DNA due to translocation of the primosome complex (5). The primosome moves in a direction opposite to the DNA elongation, using the energy from hydrolysis of ATP (5,31-34). The movement appears to be performed by the protein n', because the protein n' can displace SSB protein on the template (21-22). Movement occurs with either ATP or dATP. Only n' protein contains dATPase activity among the primosome proteins. Recently, dnaB protein (35-36) and protein n' (37) have been shown to contain an ATP dependent DNA helicase activity which unwinds duplex DNA. This helicase function apparently is not required for the replication of the SSB-coated viral DNAs, because the template is not duplex.

Replication of the SSB-coated ϕX174 viral DNA has been suggested to be a model for the lagging strand synthesis in E. coli chromosomal DNA replication (1,5,31-34,38). During chromosomal DNA replication, the leading strands are synthesized continuously in the direction of the fork movement. In contrast, the lagging strands which are composed of the discontinuous Okazaki fragments need to be synthesized in a direction opposite to fork movement. The movement of the primosome in the direction opposite to DNA elongation solves the polarity problem in lagging strand synthesis.

In addition, replication of E. coli plasmids and other phage DNAs shows different mechanisms and unique requirements for proteins in initiation of DNA replication, depending upon the origin of DNA replication (1,3). Dependence upon oriC and dnaA protein is one of the characteristics of E. coli chromosomal DNA replication.

Physiology of the Initiation of E. coli Chromosomal DNA Replication

Chromosomal DNA replication in E. coli is tightly coordinated to the cell cycle and the regulation of the replication mainly occurs at the initiation step of the DNA replication (1-3,39-41). The time span between initiation and termination of the chromosomal DNA replication as well as the time between termination of the replication and cell division are relatively inflexible in bacteria growing with doubling times shorter than 60 min at 37°C. The major variable seems to be the time required for initiation of new round of replication. In slowly growing cells, the new round of the replication is delayed until after cell division. In contrast, rapidly growing cells initiate new rounds of replication before cell division. This results in the formation of more than one initiation event per cell cycle. By regulating the frequency of the initiation, the cell controls the rate of DNA replication. Flow cytometric analysis (42-43) reveals that initiation of replication on multiple origins in a rapidly growing cell is highly synchronized. These results support the view that the control of chromosomal DNA replication mainly occurs at the initiation step.

To identify the genes required for E. coli chromosomal DNA replication, conditional-lethal mutants have been obtained by mutagenesis and by spontaneous mutation. A number of mutants have been identified which show defects in DNA replication at the nonpermissive temperature, usually above 40°C. The mutants harbor conditionally defective proteins which can function normally at the permissive temperature but are unable to function at the nonpermissive temperature as a result of missence mutations. Through mapping of the mutation locus on the chromosome, genes whose protein products are essential for DNA replication have been identified. These include dnaA, dnaB, dnaC, dnaE, dnaG, dnaK, dnaN, dnaQ, dnaT, dnaZX, gyrB, polA, rpoB, and others (1-2), indicating that many proteins are required for DNA replication.

The dnaA gene has been identified through the isolation of mutants which are unable to initiate chromosomal DNA replication at the nonpermissive temperature, 42°C. Generally, dnaA temperature-sensitive mutants exhibit the same growth rate as $dnaA^+$ strains at the permissive temperature, 30°C (44-49). At the nonpermissive temperature, the mutants are unable to initiate a new round of replication, although the on-going rounds of the replication are completed. This phenotype is distinct from genes whose products are required during the elongation phase of DNA replication. Since this observation, the dnaA gene product has been shown to be essential for the initiation of chromosomal DNA replication in vivo and in vitro (1-3).

The dnaA temperature-sensitive mutants, dnaA5 and dnaA46, accumulate initiation potential for chromosomal DNA replication while at the nonpermissive temperature (47-48). When the growth temperature of

the mutants is shifted from the nonpermissive temperature to the permissive temperature and the synthesis of new mutant proteins is inhibited by treating the cells with chloramphenicol prior to the shift-down, the mutants initiate the chromosomal DNA replication at the permissive temperature. Although the mutant proteins can not function in replication at the nonpermissive temperature, the mutant proteins synthesized at the nonpermissive temperature are active at the permissive temperature, indicating reversible inactivation of the mutant proteins. In contrast, dnaA204 mutants irreversibly lose activity for replication at the nonpermissive temperature (48). The mutation in the dnaA46 allele is a single base substitution, resulting in the replacement of the alanine residue with a valine residue at the amino acid 184 of dnaA protein (50-51). The mutation in the dnaA5 allele is close to the mutation of dnaA46 allele in the coding region (49). The mutation in the dnaA204 allele is 391 nucleotides away from the mutation in dnaA46 allele toward the C-terminus (49).

The dnaA mutation can be phenotypically suppressed by extragenic suppressors. Mutations in rpoB which confer rifampin resistance to RNA polymerase suppress the temperature-sensitive phenotype of dnaA mutants (52-54). The rpoB suppression appears to be allele specific, suggesting that dnaA protein interacts with RNA polymerase in the initiation of the chromosomal DNA replication. Also, a mutation in trxA which encodes thioredoxin also suppresses a mutation in dnaA (55). The requirement for dnaA function in replication can be bypassed by integration of another replicon which does not require dnaA function for its replication. When an origin of DNA replication such as the origin of

plasmid R100.1, R1 and F' is integrated in the chromosomal DNA, replication is independent of dnaA protein by initiating replication at the integrated origin (56-59). The inactivation of the rnh gene, which encodes RNase H, also suppresses dnaA mutations (60-63). RNaseH, which digests RNA in RNA/DNA hybrids, prevents mRNAs hybridized to the chromosmal DNA from being elongated by DNA polymerase. In rnh mutants, mRNAs hybridized to the chromosome are believed to serve as alternative primers for chromosomal DNA replication. The suppression by integration of another origin and the mutation in rnh implies that, once initiation occurs, dnaA function is not required for chromosomal DNA replication.

The timing of initiation of chromosomal DNA replication appears to be precise during the cell cycle in dnaA$^+$ strains. dnaA5 and dnaA46 mutants show a defect in the timing of initiation compared to dnaA$^+$ strains (42-43). The number of chromosomes in a cell was measured by flow cytometry after the treatment of cultures with rifampin which inhibits a new round of the replication but allows for completion of on-going rounds of replication. dnaA$^+$ and dnaA204 cells grown at 29°C contain 2^n (=2, 4 and 8) numbers of the chromosomes with the exception of 2-7% of cells containing 3, 5, 6 or 7 chromosomes, indicating that the initiation of chromosomal DNA replication is synchronized in such cells. In contrast, a large fraction of dnaA5 and dnaA46 mutant cells grown at 29°C contain aberrant numbers of the chromosomes, suggesting that the timing of initiation is disturbed and asynchronous compared to the dnaA$^+$ or dnaA204 strains. The reduction in DNA/mass ratio at 30°C, 10-30%, even with a normal growth was observed in dnaA5 (64) and dnaA46 mutants (65). This implies that dnaA activity in the mutants may be

limiting for the initiation of replication, resulting in asynchrony of initiation in the mutants. The asynchrony of the mutants suggests that dnaA function is involved in timing of the initiation of chromosomal DNA replication.

The DNA replication of plasmids containing the oriC region is dependent upon dnaA protein and other replicative proteins which are required for chromosomal replication (66-71). These plasmids containing the oriC sequence are present at 20 to 40 copies per cell (72-74). Although it is expected that the oriC plasmids may titrate out dnaA protein required for host chromosomal DNA replication, the growth of the host is not disturbed (72-74). The effect of the level of dnaA protein on chromosomal DNA replication was examined by overproduction of dnaA protein in which expression was under the control of the inducible promoters placUV5 (75) or lambda P_L (76) in the plasmid. The induction of dnaA protein by 1mM IPTG for placUV5 or 42°C incubation for lambda P_L does not to lead to a dramatic increase in DNA synthesis. But the copy number of oriC and the expression of atp, a gene close to oriC, increases 2-3 fold (76). These result suggest that the initiation of chromosomal DNA replication at oriC is induced by the increased level of dnaA protein but replication is soon aborted. The observations that there is no disturbance of cell growth by titrating out dnaA protein or that there is no increase on the net DNA synthesis by the overproduction of the dnaA protein suggest that some control mechanism in addition to the dnaA function exists in regulation of the timing of the initiation.

oriC

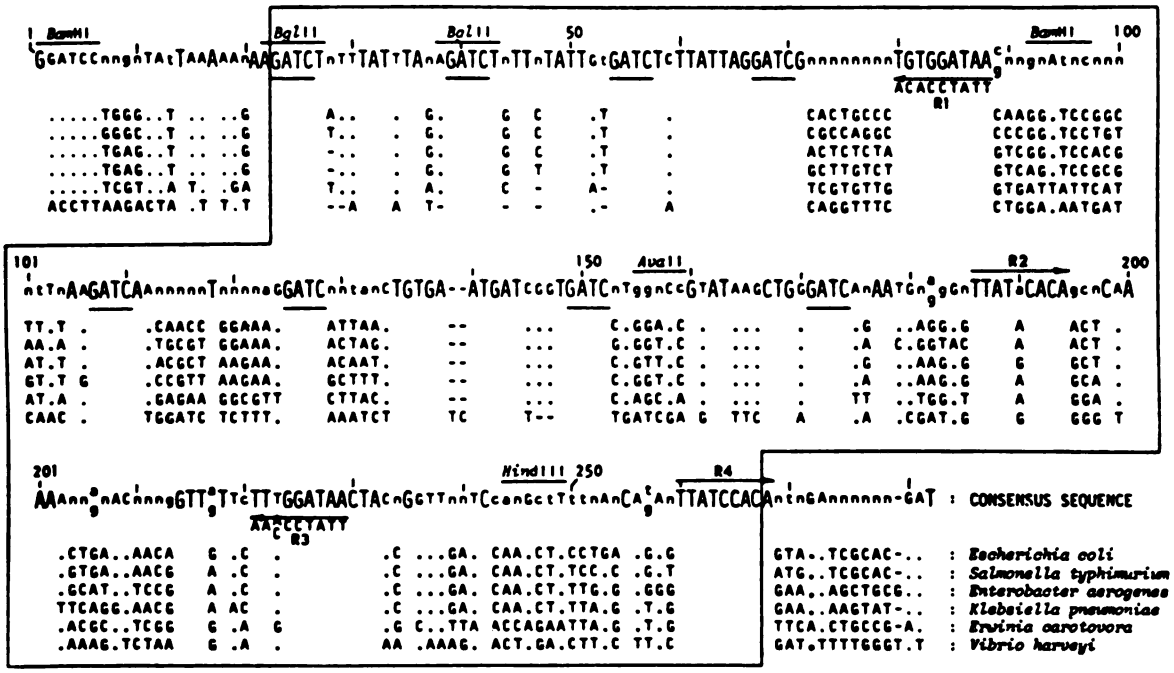
Physiological observations suggested that the replication of E. coli chromosomal DNA is initiated at a unique site located near 83 min (1-3). The origin region of chromosomal replication was cloned by enabling a nonreplicating DNA fragment containing the ampicillin resistance gene, bla, to be autonomously maintained in E. coli (77), indicating that the cloned fragment functions as the origin of DNA replication. It was confirmed that the cloned origin is located at 83 min on the chromosome between uncA and rbsK through genetic mapping. The origin of E. coli chromosomal DNA replication, oriC, is composed of a unique DNA sequence of 245 base pairs (Figure 2), determined by selectively deleting flanking regions of the cloned DNA fragment containing the oriC (78-79). A notable feature of the oriC sequence is the abundance of repetitive sequences (Figure 2).

The 9 base pairs, 5'-TTAT(C/A)CA(C/A)A-3', which is the consensus sequence for the binding of dnaA protein (80) is present 4 times within oriC, arranged as two oppositely oriented pairs. 20 to 40 molecules of dnaA protein cooperatively bind to the oriC region (80). DNase I protection experiments reveal that a repetitive pattern of cleavage enhancement at 8 to 10 bp intervals alternates on both strands of oriC region bound by dnaA protein (80). This suggests that the oriC region is wrapped or folded into a specific nucleoprotein structure by binding of dnaA protein. The phenomenon of dnaA protein aggregation (68) may reflect its ability to bind cooperatively and to form nucleation centers at oriC.

Three tandem repeats of the 13-mer, 5'-GATCTnTTnTTTT-3', exist in the AT rich region at the left edge of oriC. An AT rich region exists

Figure 2. Consensus sequence of the minimal origin of the bacterial chromosome.

The consensus sequence is derived from six bacterial origin sequences (81). A large capital means that the same nucleotide is found in all six origins ; a small capital letter means the nucleotide is present in five of the six sequences; a lower case letter is used when that nucleotide is present in three or four of six bacterial origins but only two different nucleotides are found at that site; and where three or four of the four possible nucleotides, or two different nucleotides plus deletion, are found at a site, the letter n is used. In the individual origin sequences, - means a deletion relative to the consensus sequence is present and a dot indicates that the nucleotide in the bacterial sequence is the same as the nucleotide in the consensus sequence. GATC site are underlined in the consensus sequence, and certain E. coli restriction sites are noted. The minimal origin of E. coli is enclosed within the box. The numbering of the nucleotide position is that used for E. coli, and the 5' end is at the upper left. The four related 9-bp dnaA boxes, R1, R2, R3, and R4, are indicated by the arrows, with the 5' to 3' consensus sequence listed below the arrows for those dnaA boxes in the opposite orientation. A related sequence, which may present a fifth possible dnaA box, R5, is found between E. coli nucleotide positions 135 and 143.


BamHI
BglII
BglII
BamHI
R1
R2
AvaII
R3
HindIII
R4
CONSENSUS SEQUENCE
Escherichia coli
Salmonella typhimurium
Enterobacter aerogenes
Klebsiella pneumoniae
Erwinia carotovora
Vibrio harveyi

in many origins of DNA replication including chromosomal DNAs (81-84), plasmid DNAs (85-86) and phage DNAs (87-88). Binding of replication initiator proteins to the origin may lead to the unwinding of the AT rich region to facilitate the entry of replicative proteins.

The dam methylation site, 5'-GATC-3' in which the adenine residue is methylated by dam methylase, is repeated 11 times within oriC (Figure 2). The first four nucleotides in the three 13-mers is a dam methylase recognition sequence (89-90). These observations suggest a role for dam methylation during the initiation of chromosomal DNA replication. The replicating chromosomal DNA forms transient hemimethylated duplex daughter DNAs in which the template strands are methylated but the newly synthesized strands are not. The undermethylated or hemimethylated oriC appears to be inactive for DNA replication in vivo and in vitro (91-93). This suggests that the dam methylation of the oriC sequence is involved in the regulation of the chromosomal DNA replication.

Mutations in the repetitive sequences of the dnaA protein recognition sequences, 13-mers and dam methylation sites abolish the function of oriC as an origin for DNA replication (94-95). Comparative nucleotide sequence analyses of origins of chromosomal DNA replication from Enterobacteriacea reveal that the nucleotide sequence of these origins is highly conserved (81-84), suggesting the existence of a conserved mechanism and function for proteins which are involved in initiation of chromosomal DNA replication.

In vitro Replication of E. coli Chromosomal DNA

An *in vitro* cell free DNA replication system has been developed in which replication is dependent upon the *oriC* sequence (66-68). This system requires a soluble enzyme fraction of *E. coli* as the enzyme source, ribonucleotides for RNA primer synthesis, deoxynucleotides for DNA synthesis, an ATP regenerating system of creatine kinase and phosphocreatine, and a hydrophilic polymer such as polyethylene glycol or polyvinyl alcohol. The ATP regenerating system maintains the ATP pool in the reaction, and the hydrophilic polymer may increase the local concentration of the components in the reaction by enhancing molecular crowding (96). An electron microscopic study of replicating intermediates demonstrated that replication begins at or near the *oriC* sequence and that the replication forks proceed bidirectionally from *oriC* (67). A soluble enzyme fraction prepared from *dnaA* mutants is unable to replicate *oriC* plasmid DNA without the addition of exogeneous dnaA protein (68), indicating that the dnaA protein is required for *in vitro* replication. Apparently, the mutant dnaA protein in such extracts is inactive. Addition of active dnaA protein complements the defect. Rifampin, which is an inhibitor of RNA polymerase, inhibits the *in vitro* replication as shown *in vivo* (66,97). In addition, replication is dependent upon other key replicative enzymes which have been identified by genetic analysis. Thus, replication of *oriC* plasmid DNA in a crude enzyme system resembles the *in vivo* replication of *E. coli* chromosomal DNA.

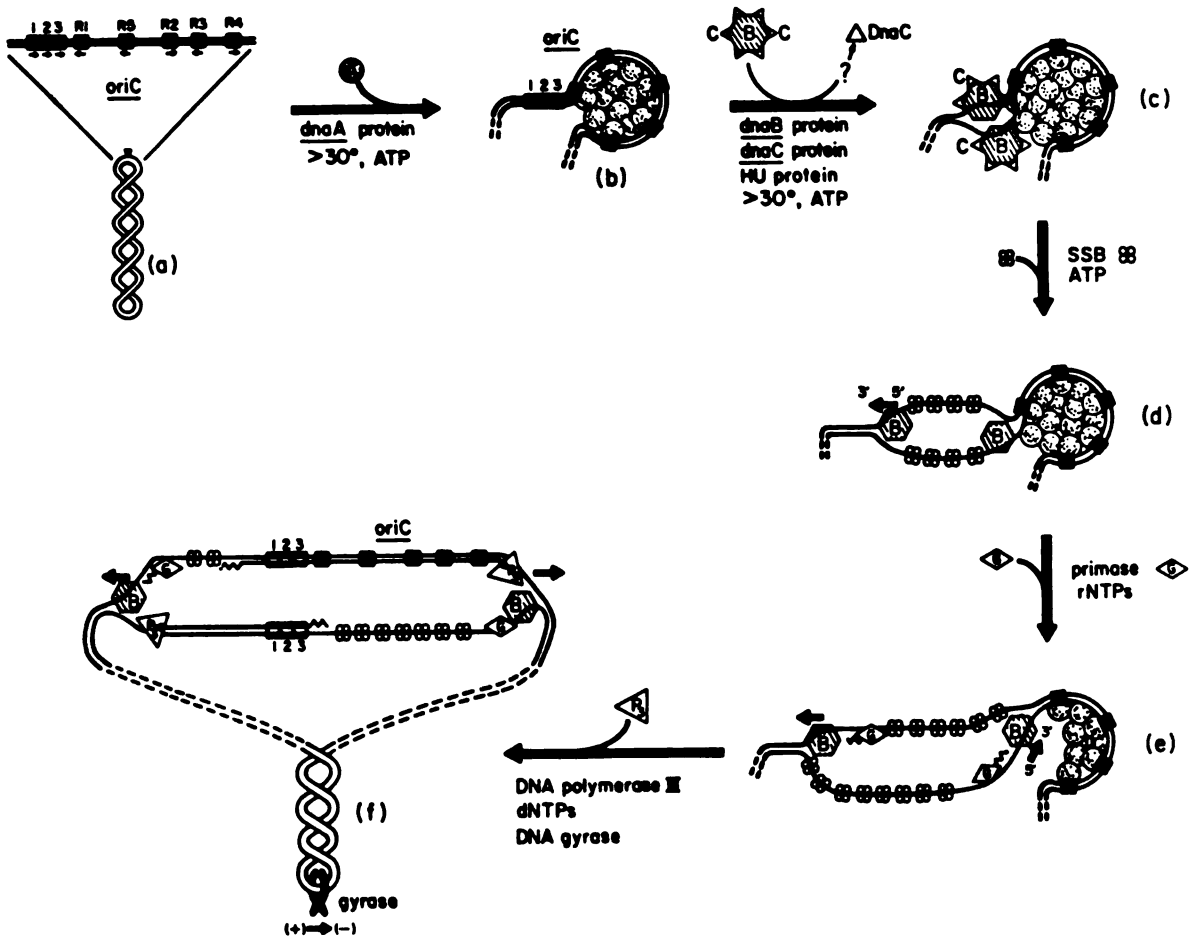
In order to understand the biochemical mechanism of *E. coli* chromosomal DNA replication, the proteins which are required for *oriC* plasmid DNA replication have been fractionated from the soluble enzyme

fraction, and reconstituted *in vitro* (69-71). Replication of *oriC* plasmids in this system has allowed for the formation of a speculative mechanism as described in Figure 3. First, molecules of dnaA protein bind to the dnaA boxes within *oriC*, then 20 to 30 molecules of dnaA protein cooperatively bind to *oriC* region (80). In the presence of ATP, the 13-mers in the AT rich region of *oriC* become sensitive to S1 or P1 nuclease which specifically digests single-stranded DNA (98-100), indicating that binding of dnaA protein to *oriC* in the presence of ATP unwinds the 13-mers. This opened duplex DNA with the bound dnaA protein is termed an open complex. Free dnaA protein or ADP/dnaA protein complex binds to *oriC* as well as ATP/dnaA protein complex, but free dnaA protein or the ADP/dnaA protein complex is unable to form the open complex (99-100), implying that ATP binding of dnaA protein is required to unwind the 13-mers. Replacement of ATP by the nonhydrolyzable ATP analog, ATP-gamma-S, demonstrates that a hydrolysis of ATP is not required for the strand opening. The histone like protein HU appears to induce the unwinding of 13-mers by dnaA protein. dnaB protein complexed with dnaC protein enters the open complex (99-100). ATP hydrolysis is required for the helicase function of dnaB protein to unwind the duplex DNA (35-36). dnaC protein appears to be dissociated from a protein complex at *oriC* because the dnaB protein complexed with dnaC protein is unable to hydrolyze ATP (28).

Although plasmid pBR322 DNA contains one dnaA box near the origin of plasmid DNA replication, its replication is not dependent upon dnaA protein *in vivo* or *in vitro* (101-103). When the recognition sequence of the protein n' near the origin of pBR322 is deleted, DNA replication

Figure 3. Hypothetical scheme for the initiation of bidirectional DNA replication at the E. coli origin in vitro (2).

The closed boxes, R1 through R5, represent recognition sequences for the E. coli dnaA protein. The three open boxes (1, 2, 3) represent 13-mer.


1 2 3 R1 R5 R2 R3 R4
oriC
(a)
dnaA protein
>30°, ATP
oriC
1 2 3
(b)
C B C
ΔDnaC
?
dnaB protein
dnaC protein
HU protein
>30°, ATP
C B C
(c)
SSB
ATP
3' 5'
(d)
primase
rNTPs
oriC
1 2 3
1 2 3
(e)
DNA polymerase III
dNTPs
DNA gyrase
(f)
gyrase
(+) (−)

becomes dependent upon dnaA protein in vivo and in vitro. This suggests that the dnaA protein replaces the function of the proteins n, n', n'' and i allow binding by the dnaB protein complexed with dnaC protein at the origin of DNA replication as shown in replication of the SSB-coated ϕX174 viral DNA replication (Figure 1).

Electron microscopic (98) and P1 nuclease studies (99-100) reveal that, in the presence of ATP, dnaB helicase moves in both directions to unwind the duplex DNA. The ss DNAs produced by dnaB helicase are coated and stabilized by SSB protein. The topological strain generated by the unwinding of the duplex DNA is relieved by DNA gyrase protein which introduces negative superhelicity into a circular duplex DNA. When the prepriming reaction is not coupled to priming by primase and DNA synthesis by DNA polymerase III holoenzyme, extensive unwinding occurs in the presence of gyrB protein (36,98). By subsequent addition of primase and DNA polymerase III holoenzyme, DNA synthesis starts in the ss DNA unwound and coated by SSB protein including oriC, indicating that primer synthesis is not restricted to oriC. On coupling of the prepriming reaction to priming and DNA synthesis, unwinding is rapidly followed by priming and DNA synthesis at or near oriC. Thus, dnaA, dnaB and dnaC proteins play a role in guiding the priming and DNA synthesis to start at oriC in the coupled reaction. The replication forks containing dnaB helicase, primase and DNA polymerase III holoenzyme move in both directions, and new strands are synthesized in both directions (104). DNA gyrase relieves the topological strain on the template during DNA synthesis.

Under conditions of oriC plasmid replication which include a higher levels of protein HU, RNA polymerase is required to stimulate replication (69-71). Transcription by RNA polymerase is thought to activate the template for initiation at oriC. However, allelic suppression of mutations in dnaA by mutations in rpoB which encodes the beta subunit of RNA polymerase has suggested the direct interaction of dnaA protein with RNA polymerase (52-54). To prevent the transcription by RNA polymerase from being coupled to DNA elongation at a nonspecific site which is not oriC, RNase H and topoisomerase I are necessary (70-71,105). RNase H cleaves the RNA transcripts hybridized to DNA template, and seems to inhibit non-oriC RNA transcripts from being elongated by DNA polymerase III holenzyme (60-63). The function of topoisomerase I is not clear.

Although the above model describes DNA replication at oriC using the known properties of the proteins, the variable requirements of some proteins under different reaction conditions raise questions regarding which is the faithful in vivo mechanism. The proteins n, n', n'' and i which have been suggested for lagging strand synthesis are not required for oriC replication in the purified enzyme system. Whether or how the synthesis of lagging strands occurs in the in vitro replication system is not known yet. DnaJ and dnaK proteins are required for bacteriophage lambda DNA replication in vivo and in vitro (106-110) and seem to be necessary for in vivo chromosomal DNA replication (111), but the proteins do not show any influence in the purified enzyme system of oriC plasmid DNA replication.

dnaA Protein

dnaA protein is essential for *E. coli* chromosomal DNA replication *in vivo* and *oriC* plasmid DNA replication *in vitro* (1-3). dnaA protein is a basic protein of 465 amino acid residues with a molecular weight 52.5 KDa predicted by the nucleotide sequence (112-113).

dnaA protein preferentially and cooperatively binds to the DNA fragments containing the dnaA box. These regions include the origins of *E. coli*, pBR322, pSC101, F' and R1, and phage P1, the promoters of *dnaA*, *mioC*, *htpR*, and *dam*, and the inverted repeat of transposon Tn5 (80, 89, 114-115). dnaA protein is indispensible for the DNA replication of the plasmid pSC101 (116-117). The origin of DNA replication in this plasmid contains one dnaA box and one 13-mer (100). It is expected that binding of dnaA protein is required to open the 13-mer region for the plasmid DNA replication as shown in the replication of the *oriC* plasmid. Plasmids R1 and phage P1 appear to require *dnaA* function in DNA replication (118-119), but the plasmid pBR322 does not require *dnaA* function for *in vivo* or *in vitro* replication when the n' recognition sequence is present (101-103).

The *dnaA* gene is located near 82 min on the *E. coli* genetic map (49). It is the first gene in an operon that also contains the *dnaN* gene which encodes the beta subunit of DNA polymerase III holoenzyme (50,113). The regulatory region of the *dnaA* gene contains one dnaA box between the two *dnaA* promoters, *dnaA1P* and *dnaA2P*. Binding of dnaA protein to the dnaA box in between the two *dnaA* promoters represses the transcription of *dnaA* gene in an *in vitro* run-off transcription assay (114). Transcriptional and translational fusions of the *dnaA* promoter

region to the coding region of lacZ or tet gene also showed that the expression of the dnaA gene is autoregulated at the transcriptional level (120-121). dnaA protein also influences the expression of genes, htpR and dam (115,89). The htpR gene product, sigma 32, is a sigma factor of RNA polymerase whose expression increases at elevated temperatures (122-123). This protein appears to be responsible for induction of E. coli heat shock proteins at elevated temperatures (124-125). These include the dnaK, groEL and groES gene products which are essential for propagation of lambda phage. Dam methylase encoded by the dam gene influences chromosomal DNA replication, DNA repair, DNA recombination, and expression of genes in the SOS response and dnaA (89-90).

dnaA protein binds ATP (K_D=0.03 uM) and ADP (K_D=0.1 uM) (99). The ATP or ADP-dnaA protein complex is very stable. Binding of ATP to dnaA protein is required for the unwinding of the 13-mers in the initiation of oriC plasmid replication. Despite binding of ATP to dnaA protein with a K_D of 0.03 uM, the unwinding of the 13-mers requires mM range of ATP (99-100). This may suggest that dnaA protein has an additional site for ATP binding with lower affinity and binding of ATP to this site induces a conformational change in oriC and results in sensitivity of the 13-mers to P1 nuclease. In contrast to the ATP-dnaA protein complex, the ADP-dnaA protein complex is inactive for oriC plasmid replication due to the defect in unwinding of 13-mers in oriC. In the presence of ATP, cardiolipin which is a phospholipid component of the cell membrane displaces ADP in the ADP-dnaA protein complex to allow

binding of ATP (99). The physiological significance of the phospholipid cardiolipin and the ADP-dnaA protein complex is not clearly understood.

The autoregulatory function of dnaA protein on its expression may be to maintain a constant level of dnaA protein throughout the cell cycle. One cell of E. coli harbors 800 to 2,100 molecules of dnaA protein as determined by a Western blot analysis (126). Levels of dnaA protein appear to remain constant throughout its growth phases (127). If the rapidly growing cell contains four replication origins, 80 to 120 molecules of dnaA proteins are necessary for initiation of the chromosomal DNA replication. While dnaA protein is indispensible for initiation of chromosomal DNA replication, its abundance suggests that dnaA protein may not be the only factor responsible for timing initiation of chromosomal DNA replication in vivo. It is possible that the function of dnaA protein in DNA replication is modulated and that other control mechanisms exist for the regulation of chromosomal DNA replication.

This work is focused on examining the in vitro functions of dnaA mutant proteins in comparison with the $dnaA^+$ protein and on investigating how altered function(s) of the mutant protein affects in vitro DNA replication. The results obtained are correlated with the physiological properties of the mutant. These data will provide additional information on role of dnaA function in DNA replication and metabolism.

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CHAPTER II

Purification of dnaA46 Protein

INTRODUCTION

Chromosomal DNA replication in E. coli is tightly coordinated to the cell cycle, and it is regulated at the level of initiation (1-5). Initiation of DNA replication begins at a unique site, oriC, near 83 min on the 100 min of E. coli genetic map (6-8). One of the proteins required for initiation of replication, dnaA protein, has a molecular weight of 52 KDa and it is essential for initiation of DNA replication in vivo and in vitro (3-5). In the presence of ATP, preferential and cooperative binding of dnaA protein molecules to the oriC sequence is followed by opening of the double strands in the 13-mer region of the oriC sequence (9-12). This binding of dnaA protein and subsequent strand opening is thought to be a prerequisite for the initiation of oriC plasmid DNA replication in vitro. Whereas dnaA protein binds ATP with a K_D of 0.03 uM, unwinding of the 13-mers requires mM range of ATP (11-12). The binding of dnaA protein to specific promoters containing a 9 bp consensus sequence results in transcriptional repression of these genes, which include dnaA, mioC, htpR and dam (15-20). This indicates that the dnaA protein plays a role in control of gene expression and in the initiation of DNA replication. dnaA protein also appears to be involved in the timing of initiation of chromosomal DNA replication in vivo (21-22).

The dnaA gene has been identified through the isolation of mutants which are unable to initiate new rounds of chromosomal DNA replication at the nonpermissive temperature, although the mutants complete on-going rounds of replication (3-5,23-24). The inability of dnaA mutants to initiate new rounds of DNA replication indicates that the dnaA gene product is essential for initiation. Among many alleles of dnaA isolated, the dnaA5, dnaA46 and dnaA204 alleles have been well characterized physiologically (4-5,25-27). The dnaA5 and dnaA46 genes encode proteins that reversibly lose the ability to initiate the DNA replication at the nonpermissive temperature (25-28). In contrast, dnaA204 encodes a protein which is irreversibly inactivated (25). The dnaA5 and dnaA46 mutants show asynchrony in initiation events of chromosomal DNA replication at the permissive temperature, compared to the synchronous initiation in $dnaA^+$ and dnaA204 strains (21-22).

This chapter describes purification of dnaA46 protein from a dnaA46 protein overproducing strain. To facilitate the purification of dnaA mutant proteins, the dnaA mutant alleles, dnaA5, dnaA46 and dnaA204, were cloned and subcloned into the protein overproducing vector. Among the three mutant protein overproducing strains, the crude enzyme fraction prepared from the dnaA46 protein overproducing strain contained the highest activity in an in vitro DNA replication assay. In order to ensure an assay throughout the purification, the mutant protein with the highest replication activity was chosen. Of the known activities of $dnaA^+$ protein, the study of the dnaA mutant protein will be useful in correlating its defect in DNA replication.

EXPERIMENTAL PROCEDURES

Materials

Reagents were obtained from the following sources: Ribonucleotides, cycloserine, polyvinyl alcohol (type II), phosphocreatine, heparin, tetracycline, ampicillin, and L-arabinose, Sigma; deoxynucleotides, SalI decanucleotide linkers, and Sepharose 4-B, Pharmacia PL Biochemicals; HEPES, Tris base, and dithiothreitol (DTT), Calbiochem-Behring; [^{35}S]methionine (1100 Ci/mmol), [alpha ^{32}P]ATP (800 Ci/mmol) and [alpha ^{32}P]dTTP (800 Ci/mmol), New England Nuclear Corp.; [^{3}H]dTTP, ICN Radiochemicals; and hydroxylapatite, Biorad.

Enzymes and Proteins

Enzymes and proteins were obtained from the following sources: bovine serum albumin, rabbit muscle creatine kinase, egg white lysozyme, rabbit muscle glycogen phosphorylase b, bovine liver glutamate dehydrogenase, porcine muscle lactate dehydrogenase, bovine erythrocyte carbonic anhydrase, and soybean trypsin inhibitor, Sigma; DNA polymerase I (large fragment), and T4 DNA ligase, New England Biolabs; and restriction enzymes, New England Biolabs, BRL, IBI, and Pharmacia PL Biochemicals.

Bacterial Strains and Plasmid DNAs

Escherichia coli W3110; K37 *HfrC*, *supD*, (lambda); MC1061 *araD139*, Δ(*ara*, *leu*)7697, Δ*lacX74*, *galU*, *galK*, *hsdR*, *rpsL*; an isogenic strain, TD3 *araD139*, Δ(*ara*, *leu*)7697, Δ*lacX74*, *galU*, *galK*, *hsdR*, *rpsL*, *dnaA46*,

tnaA::Tn10, and JM103 Δ(lac pro), thi, strA, supE, endA, sbcB, hsdR, F'traD36, proAB, $lacI^Q$, Z M15 (29) were laboratory strains. E. coli WM448 leu19, pro19, trp25, his47, thyA59, arg28, met55, deoB23, lac11, gal11, strA56, sul1, $hsdS^{K12}$, dnaA46; WM493 (isogenic with WM448 except dnaA5); and WM433 (isogenic with WM448 except dnaA204) were obtained from Dr. Walter Messer (25). MCL22 trkA405, trkD1, thi, Δ(kdp phr)214, rha, Δ(gal uvrB), Δ(srl recA)306, rpsL31 was from Matthew Lorence, University of Texas, Dallas.

M13oriC26 (30), contains oriC, and adjacent E. coli chromosomal DNA in M13Gori1; M13oriC2LB5 contains oriC sequences in M13 ΔE101, an M13 derivative lacking the complementary strand origin (31-32). Plasmids pING1 (33) was from Dr. Dan S. Ray, pBF1509 containing the E. coli $dnaA^+$ gene from pBF1209 (10) inserted into the vector pAD329 (34), and pdnaA/dnaN (35) were from Dr. Arthur Kornberg. Plasmid DNAs were prepared by the cleared lysis method followed by sedimentation in CsCl-ethidium bromide gradients (36).

Buffers

Buffer A contains 25 mM HEPES-KOH (pH 7.8), 0.1 mM EDTA, 2 mM DTT, and 15% glycerol (v/v); buffer B contains 0.12 M potassium phosphate (pH 6.8), 0.1 mM EDTA, 2 mM DTT, and 15% glycerol (v/v); and buffer C contains 50 mM Tris-HCl (pH 8.3), 0.1 mM EDTA, 2 mM DTT, and 15% glycerol (v/v).

Replication Assay

Enzyme fractions (fraction II) from WM433 (relevant genotype, dnaA204) were prepared as described (37) and used in replication reactions of 25 ul containing HEPES-KOH (pH 7.8); ATP, 2mM; CTP, UTP, and GTP, each at 0.5 mM; dATP, dCTP, dGTP and [^{3}H]dTTP (15-45 cpm/pmol), each at 100 uM; magnesium acetate, 11 mM; polyvinyl alcohol, 7% (wt/vol); phosphocreatine, 40 mM; creatine kinase, 100 ug/ml; supercoiled M13oriC26, 200 ng (600 pmol in nucleotide); fraction II from WM433, 200-250 ug. Reaction mixtures were assembled at 0°C and incubated at 30°C. Unless indicated, incubations were for 20 min for dnaA$^+$ protein and 40 min for dnaA46 protein. Total nucleotide incorporation (in pmol) was measured by liquid scintillation counting after trichloroacetic acid precipitation onto glass fiber filters (Whatman GF/C). One unit of replication activity is equal to one pmol of nucleotide incorporated per min at 30°C.

Cloning of dnaA46, dnaA5, and dnaA204 Alleles

Standard recombinant DNA methods for this procedure, summarized in Figure 2 and 3, were as described (38). Chromosomal DNA isolated as described (39) from WM448, WM493, and WM433, was digested with Hind III and XhoI restriction enzymes, electrophoresed in 0.8% agarose gels, and DNA fragments of 2.4-2.7 kilobase pairs (Kbp) were isolated (Figure 1). DNA was purified from agarose by electrophoretic transfer onto DE81 paper (Whatman), elution from the paper by incubation in 1.7 M NaCl, 10 mM Tris-HCl (pH 8), and 1 mM EDTA at 37°C for 2 hr, and concentration by ethanol precipitation. Blunt-ended termini were produced by incubation with DNA polymerase I (large fragment) and deoxyribonucleotides (40).

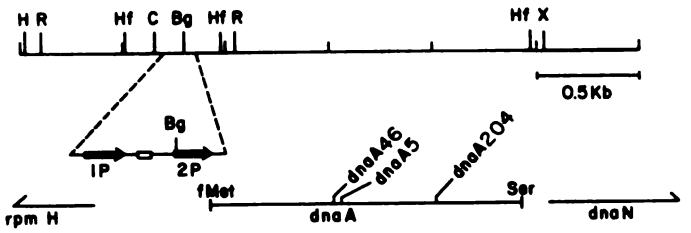
M13G*ori*1 replicative form (RF) DNA was linearized by cleavage at the single PvuII site, ligated to the end-filled chromosomal DNA with T4 DNA ligase, and transfected into *E. coli* JM103. Plaques were transferred to nitrocellulose filters and screened by hybridization with ^{32}P-labeled pBF1509 nick-translated with pancreatic DNase I, DNA polymerase I, and [alpha ^{32}P]dCTP. DNA from plaques homologous to the probe were further characterized by 1) size analysis by agarose gel electrophoresis, 2) orientation analysis by phage hybridization (44) to M13 derivatives containing either strand of the *dnaA*$^{+}$ gene, and 3) restriction analysis of the recombinant RF DNAs. Recombinants of M13G*ori*1 containing the *dnaA5*, *dnaA46*, and *dnaA204* alleles were obtained.

One study indicated that multiploid strains containing the *dnaA46* allele in pBR322 and the chromosomal *dnaA*$^{+}$ gene were cold sensitive for growth (cited in 5). This suggested that elevated levels of dnaA46 protein might be deleterious to cell growth. The temperature lability of the mutant gene products precluded use of vectors in which expression of cloned genes is induced by elevating the temperature of the culture. To circumvent these problems, the different *dnaA* alleles contained in the M13 vector were subcloned into the protein expression vector, pING1 (33). In the appropriate orientation, the *dnaA* allele is placed under control of the inducible *araB* promoter. In the uninduced state, expression from this promoter is tightly repressed by the vector-encoded *araC* gene product. The *dnaA* promoter region contains a BglII site. Restriction by this enzyme cleaves within one of the two *dnaA* promoters and separates the coding region from the promoters and the dnaA protein recognition sequence (Figure 1A, 16). BglII-XhoI restriction fragments

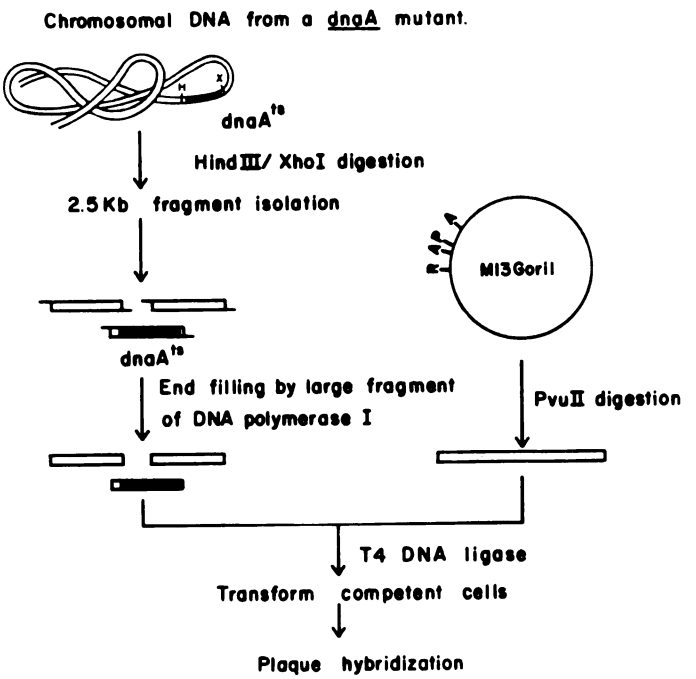
Figure 1. Cloning of the dnaA alleles into an M13 vector.

A. The coding region of the dnaA gene, and the N-terminal coding regions for rpmH, and dnaN are indicated. Arrows denote the direction of transcription. Restriction sites: H, HindIII; R, EcoRl; Hf, Hinfl; C, ClaI; Bg, BglII; X, XhoI. The approximate positions of the dnaA promoters (▬), the dnaA protein recognition sequence (▭), and of mutations corresponding to the different dnaA alleles are indicated (27,41-43). B. Chromosomal restriction fragments containing the dnaA alleles indicated in Figure 1A were cloned into the PvuII site of M13GorilRF as described in Experimental Procedures. Restriction sites: A, AvaII; H, HindIII; P, PvuII; R, EcoRI; and X, XhoI.

A.


H R
Hf C Bg Hf R
Hf X
0.5Kb
Bg
1P
2P
fMet
dnaA46
dnaA5
dnaA204
Ser
rpm H
dnaA
dnaN

B.


Chromosomal DNA from a dnaA mutant.
dnaA^ts
HindIII/ XhoI digestion
2.5Kb fragment isolation
M13Gori
dnaA^ts
End filling by large fragment of DNA polymerase I
PvuII digestion
T4 DNA ligase
Transform competent cells
Plaque hybridization

containing each dnaA allele were isolated by the DE81 paper method described above, end-filled with DNA polymerase I (large fragment), ligated to SalI linkers, digested with SalI restriction enzyme to produce cohesive termini, and inserted into the SalI site of pING1 with T4 DNA ligase (Figure 2).

Recombinants of pING1 were obtained, each containing one of the three dnaA alleles. Restriction analysis indicated that pDS215 (dnaA46), pDS105 (dnaA5), and pDS319 (dnaA204) contained the indicated dnaA alleles in the appropriate orientation for inducible expression of the mutant gene product.

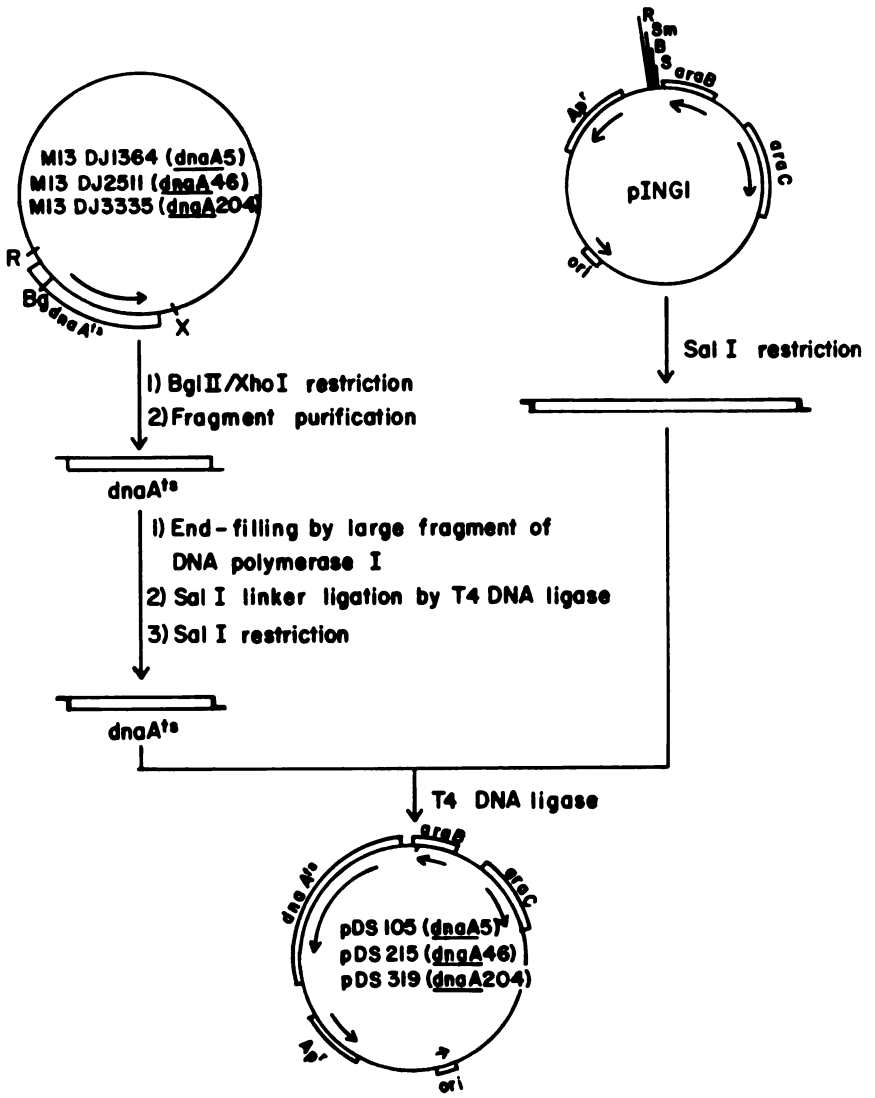
The wild type dnaA gene was also subcloned from plasmid pdnaA/dnaN in the appropriate orientation into the SalI site of pING1 to produce pDS596. Plasmid pdnaA/dnaN contains the $dnaA^+$ gene and adjacent dnaN gene within the inserted 1.0 Kbp and 3.3 Kbp EcoR1 fragments of E. coli DNA (35). The 2.2 Kbp BglII-XhoI fragment containing the $dnaA^+$ gene was isolated, incubated with DNA polymerase I (large fragment) to produce blunt-ended termini, ligated to SalI linkers, cleaved with SalI to produce cohesive termini, and inserted into the SalI site of pING1. Restriction analysis indicated that pDS596 contained the $dnaA^+$ gene in the proper orientation for inducible expression from the araB promoter.

Identification of Plasmid-Encoded Proteins

A modification of the maxicell method of Sancar et al. (45) was used to identify plasmid-encoded proteins. MCL22 containing recombinants of pING1 with the different dnaA alleles were grown to exponential phase in M9 medium (46) supplemented with 1 mM MgSO4, 0.1 mM

Figure 2. Subcloning of the dnaA alleles into the expression vector pING1.

The indicated dnaA alleles in recombinants of M13Gori1 were subcloned into the SalI site of pING1 as described in Experimental Procedures. Recombinants of pING1 contain the different dnaA alleles in the appropriate orientation for inducible expression from the araB promoter. The N-terminal coding region of araB and the araC gene contained in the vector and required for repression and induction from the araB promoter are indicated. Restriction sites: B, BamHI; Bg, BglII; R, EcoRI; S, SalI; Sm, SmaI; and X, XhoI.


M13 DJ1364 (dnaA5)
M13 DJ2511 (dnaA46)
M13 DJ3335 (dnaA204)
R
Bg
dnaA'ts
X
1) BglII/XhoI restriction
2) Fragment purification
dnaAts
1) End-filling by large fragment of DNA polymerase I
2) Sal I linker ligation by T4 DNA ligase
3) Sal I restriction
dnaAts
R
Sm
Sp
araB
Ap'
araC
pING1
ori
Sal I restriction
T4 DNA ligase
araB
dnaA'ts
araC
pDS 105 (dnaA5)
pDS 215 (dnaA46)
pDS 319 (dnaA204)
Ap'
ori

$CaCl_2$, 2 ug/ml biotin, 1 ug/ml thiamine-HCl, 0.4% glucose, 0.4% casaminoacids (Difco) and 50 ug/ml ampicillin. Five ml of each plasmid containing-strain was transfered to a petri dish, and irradiated for 30 sec with a UV lamp (UV Products, UVGL-25) at a dose rate of 0.47 $J/m^2/sec$. The irradiated culture was transferred to a flask containing 5 ml of M9 medium supplemented as above and grown at 32°C for 3 h with aeration. An equivalent amount of cycloserine was then added. After further incubation for 2 h at 32°C, cells were collected by centrifugation at 4,000 rpm for 5 min at 4°C in a Sorvall SS-34 rotor, washed in sulfate-free M9 medium, resuspended in 5 ml of M9 medium equilibrated at 32°C and containing 1% arabinose unless indicated, and incubated at 32°C for 1 h with aeration. [^{35}S]methionine was added to 5 uCi/ml and the culture was incubated at 32°C for 1 h with aeration. The cells were then collected by centrifugation at 4,000 rpm for 5 min at 4°C in a Sorvall SS-34 rotor, resuspended in 1 ml of M9 medium, centrifuged in an Eppendorf microcentrifuge for 15 sec in the cold, and resuspended in 200 ul of a solution containing 1% sodium dodecyl sulfate (SDS), 0.1 M DTT, 10% glycerol, 0.005% bromophenol blue, and 10 mM Tris-HCl (pH 6.8). The sample was incubated at 100°C for 2 min. An equal volume (20 ul) from each sample was applied to an SDS-polyacrylamide gel (4% stacking gel and 12% seperating gel) (47). After electrophoresis, the gel was stained with Coomassie-blue to detect molecular weight markers, and treated as described for fluorography (48).

Protein Determination

Protein was determined by the method of Bradford (49) with bovine serum albumin as a standard.

RESULTS

The dnaA gene, the first gene in an operon also containing the dnaN gene, has been cloned and its DNA sequence determined (42-43,50-52). The positions of control regions, of the coding region, and of base substitutions corresponding to the dnaA46 and dnaA204 alleles and the approximate position of the dnaA5 mutation have been determined (26-27, 43, Figure 1A).

The different dnaA alleles were cloned into a protein-overproducing vector to facilitate purification of the corresponding mutant gene product. To eliminate the possible complication introduced by restriction site polymorphism on the cloning strategy, chromosomal DNA from W3110 (relevant genotype, $dnaA^+$), and the isogenic strains WM493 (dnaA5), WM448 (dnaA46), and WM433 (dnaA204) was digested with HindIII and XhoI restriction enzymes. The digested DNA was size-fractionated by agarose gel electrophoresis, transferred to a nitrocellulose membrane, and annealed to ^{32}P-labeled, nick-translated pBF1509 DNA which contains the dnaA gene. The denatured probe annealed to a single chromosomal DNA fragment of apparently identical size (2.5 Kbp) from each of the four strains (data not shown). This result agrees with the fragment length expected from the DNA sequence of the region

and with the size of the HindIII-XhoI fragment containing the dnaA+ gene from pdnaA/dnaN.

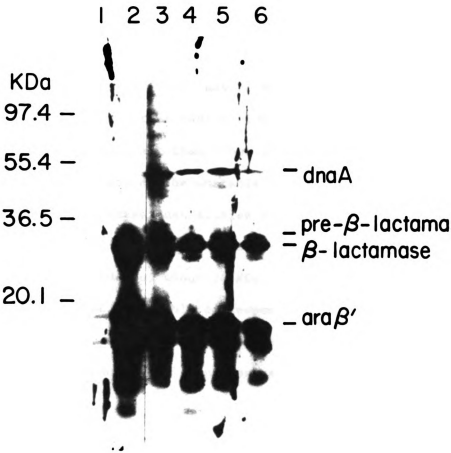
To enrich for the dnaA alleles, chromosomal DNA fragments of 2.2-2.7 Kbp from the above strains were isolated after restriction with HindIII and XhoI enzymes. Each dnaA allele contained in a 2.5 Kbp HindIII-XhoI fragment was cloned into an M13 cloning vector as described in Figure 1. A BglII-XhoI fragment was subcloned into the vector, pING1 as described in Experimental Procedures, and summarized in Figure 2. Properties of this vector include the ability to induce expression of cloned genes from the araB promoter by addition of arabinose. In addition, expression from the araB promoter is repressed by the vector-encoded araC gene product in the uninduced state. Plasmids pDS596 (dnaA$^+$), pDS105 (dnaA5), pDS215 (dnaA46), and pDS319 (dnaA204) were obtained which contain the cloned dnaA alleles in the proper orientation for inducible expression from the araB promoter.

Identification of Plasmid-Encoded Proteins

Maxicell experiments (45) were performed to determine the sizes and relative amounts of plasmid-encoded proteins induced by addition of arabinose (Figure 3). Fluorography of whole cell lysates from induced strains containing each of the above recombinant plasmids resulted in the appearance of a 50 KDa polypeptide which was not observed in lysates from induced strains containing the vector pING1 (Figure 3). This radioactive polypeptide comigrated with purified dnaA protein included as a molecular weight marker and visualized by Coomassie-blue staining of the polyacrylamide gel prior to fluorography (data not shown). The

Figure 3. Identification of plasmid-encoded proteins.

E. coli MCL22 harboring recombinants of pING1 containing the *dnaA* alleles were grown and treated as described in Experimental Procedures. Plasmid-encoded proteins were detected by fluorography. Radioactively-labeled proteins encoded by plasmids from uninduced (lane 1) or induced cultures (lanes 2 to 6) were: lane 1, pING1; 2, pING1; 3, pDS596 (*dnaA*$^{+}$); 4, pDS105 (*dnaA5*); 5, pDS215 (*dnaA46*); 6, pDS319 (*dnaA204*). Molecular weight standards (KDa) were phosphorylase B, 97.4; glutamate dehydrogenase, 55.4; lactate dehydrogenase, 36.5; and trypsin inhibitor, 20.1. The position of dnaA protein, and the deduced identities of proteins encoded by the vector are indicated.


1 2 3 4 5 6
KDa
97.4 –
55.4 –
36.5 –
20.1 –
dnaA
pre-β-lactamase
β-lactamase
araβ′

appearance of this radioactively-labeled 50 KDa polypeptide, presumed to be dnaA protein, was dependent on the addition of arabinose. The slight difference in electrophoretic mobility of the mutant proteins relative to dnaA$^+$ protein has not been observed in other experiments (see below, data not shown). Comparable amounts of dnaA protein were observed with the exception of MCL22 harboring pDS319 (dnaA204). In this circumstance, densitometric analysis of the autoradiogram indicated a 3-fold reduced amount of this polypeptide compared to dnaA$^+$ protein encoded by pDS596. This lesser amount may be due to relatively inefficient radiolabelling as other radioactive polypeptides in this sample also appeared less abundant than those encoded by pDS596 (dnaA$^+$). Alternatively, dnaA204 protein may be unstable. However, maxicell experiments performed with these dnaA alleles in another vector and host strain indicated near equal levels of expression (data not shown). Other radioactive polypeptides of about 28 KDa and 30 KDa were more abundant upon induction and presumably correspond to beta-lactamase and its unprocessed form (33). A polypeptide of about 17 KDa was also present in lysates from induced cells and corresponds to the truncated araB' gene product (33).

Purification of dnaA46 and dnaA$^+$ Protein

Conditions for maximal induction of dnaA+ protein activity from the araB promoter and for lysis were optimized with a strain harboring pDS596 (dnaA$^+$) (data not shown). Activity of dnaA protein from fractions of induced strains containing pDS105 (dnaA5), pDS215 (dnaA46), and pDS319 (dnaA204) was then measured under these optimized conditions.

Table 1. Replication Activity of fractions from strains which overproduce mutant and wild-type forms of dnaA protein.

Plasmid-containing strains were grown at 32°C in 400 ml of LB medium containing 50 ug/ml ampicillin to an OD at 595 nm of 0.5. Arabinose was added to 0.7%, the cultures were grown for 2.5 h, and harvested by centrifugation for 15 min in a Sorvall GSA rotor at 5,000 rpm at 2°C. The cell paste was resuspended in buffer containing 10 mM HEPES-KOH (pH 7.8), 1 mM EDTA, 2 mM DTT, and 10% sucrose to an OD at 595 nm of 150-200, frozen in liquid nitrogen and stored at -80°C. Frozen cells were thawed at 0°C and lysed in 0.25 M KCl, 20 mM EDTA, 20 mM spermidine-HCl, 2 mM DTT, and 0.2 mg/ml egg white lysozyme. The suspension was incubated on ice for 30 min, frozen in liquid nitrogen, and thawed at 0°C to lyse cells. The thawed sample was centrifuged for 20 min at 40,000 rpm in a Beckman Ti50 rotor. Operations at this step and beyond were performed at 0°C. The volumes of the supernatants (fraction I) were determined and solid $(NH_4)_2SO_4$ was added with stirring (0.32 g/ml of supernatant). After an additional 30 min of stirring, the suspension was centrifuged as above. The pellets were resuspended in Buffer A (fraction II), frozen and stored in liquid nitrogen. Replication activity was measured (Experimental Procedures) by incubation at 30°C for 20 min.

Table 1

Strain	Plasmid	Cloned Allele	Units/mg
MC1061	(pDS596)	*dnaA*$^{+}$	5400
MC1061	(pDS105)	*dnaA5*	60
MC1061	(pDS215)	*dnaA46*	270
MC1061	(pDS319)	*dnaA204*	ND[a]
TD3	(pDS215)	*dnaA46*	154

a ND, not detected

Using replication assays specific for dnaA protein, low but detectable activity was measured in fractions from dnaA5 and dnaA46 protein-overproducing strains (Table 1). No activity was detected in fractions from strains which overproduce the dnaA204 polypeptide.

11 liters of E. coli TD3 (dnaA46) harboring pDS215 (dnaA46) was grown at 33°C in a New Brunswick microfermenter and induced by addition of arabinose as described in Table 1. The cell paste, harvested by centrifugation in a refrigerated Sharples continuous flow centrifuge, was resuspended, frozen in liquid nitrogen, and stored at -80°C. Frozen cells, prepared by six runs of fermentation, were thawed at 0°C, and lysed by a freeze-thaw method as described in Table 1 to avoid thermal inactivation possible in the method of heat-produced lysis (53). The thawed sample was centrifuged for 30 min at 40,000 rpm in a Beckman 45Ti rotor. Operations at this step and beyond were performed at 0°C. The supernatant (fraction I) was precipitated with ammonium sulfate as described in Table 1 (Table 2). The precipitate was collected by centrifugation for 30 min at 30,000 rpm in a Beckman Ti 45 rotor and resuspended in Buffer A (fraction II). Fraction II (72 ml) was thawed and dialysed against 2 liters of Buffer A containing 0.1 M KCl for 3 h, diluted to 387 ml by addition of Buffer A to a conductivity equivalent to Buffer A containing 0.1 M KCl, and applied to a heparin-Sepharose 4B column (6.5 cm x 12 cm, 400 ml) equilibrated in Buffer A containing 0.05 M KCl. The column was washed with 3 liters of Buffer A containing 0.1 M KCl, then with 1.5 liters of Buffer A containing 0.22 M KCl. Activity was eluted with a linear gradient (3 liters) of 0.22 M to 1.5 M KCl in

Table 2. Purification of dnaA46 protein.

	Volume (ml)	Protein (mg)	Activity (10-6 U)	Specific Activity (10-3 U/mg)	Yield[a] (%)
I Lysate	390	7800	0.55	0.07	
II $(NH_4)_2SO_4$	72	5580	1.16	0.21	100
III Heparin sepharose 4B	730	240	0.19[b]	0.79[b]	
IV Hydroxyl-apatite	30.5	76.3	0.89	11.7	77
V Selective precipitation[c]	7.6	3.4	0.23	68	20

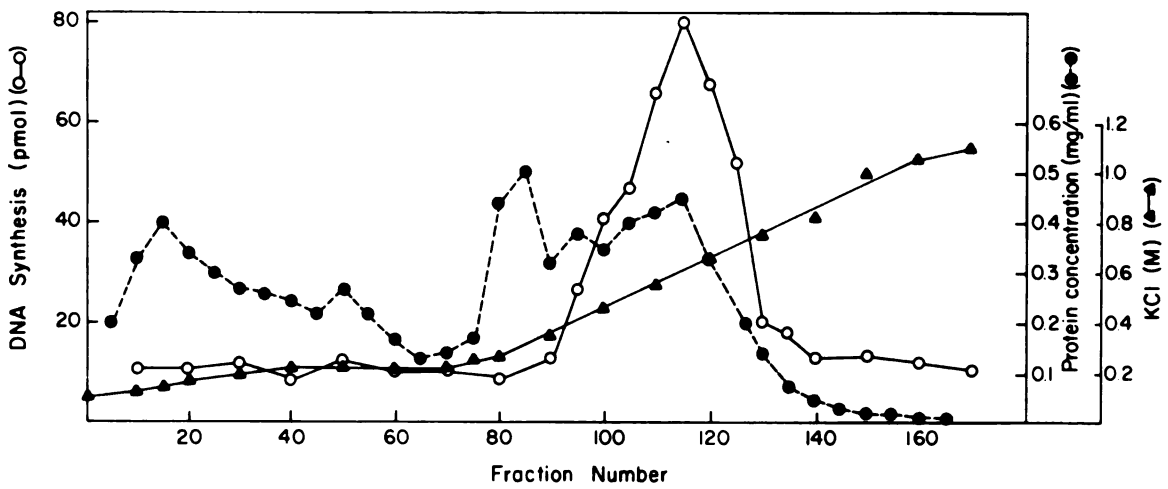
a Yield and purification are based on fraction II. Activity could not be measured reliably in Fraction I.

b Calculated from the activity of every fifth fraction concentrated by ammonium sulfate precipitation.

c 2/61 of Fraction IV was subjected to selective precipitation. The yield of Fraction V has been corrected by a factor of 30.5.

Figure 4. Heparin-sepharose 4B chromatography of dnaA46 protein.

The dialyzed and diluted fraction II was chromatographed on a heparin-sepharose 4B column as described in the text. 50 ul of every fifth column fraction was concentrated with ammonium sulfate and assayed in a DNA replication (see text).


DNA Synthesis (pmol) (o–o)
Protein concentration (mg/ml) (●–●)
KCl (M) (▲–▲)
Fraction Number
80
60
40
20
0.6
0.5
0.4
0.3
0.2
0.1
1.2
1.0
0.8
0.6
0.4
0.2
20
40
60
80
100
120
140
160

Buffer A (Figure 4). It was necessary to concentrate aliquots of column fractions by ammonium sulfate precipitation in order to assay for dnaA46 protein. 50 ul of material which did not bind to the column, and of every fifth column fraction (110 total fractions) was mixed with 1 ul of 20 mg/ml of bovine serum albumin and 100 ul of a neutralized, saturated solution of ammonium sulfate, and kept on ice for 30 min. The precipitates were collected by centrifugation for 15 min with a microcentrifuge (Brinkman), resuspended in 10 ul of Buffer A, and assayed for replication activity. Activity could not be measured accurately at this step, possibly due to inefficient precipitation of dnaA46 protein (Table 2). dnaA46 protein activity eluted in a broad peak between 0.4 and 0.8 M KCl in Buffer A (Figure 4). Active fractions were pooled (fraction III, 730 ml). Fraction III was applied to a hydroxylapatite column (2.8 cm x 6.5 cm, 40 ml) equilibrated in 20 mM potassium phosphate (pH 6.8), 0.2 M KCl, 2 mM DTT, and 15% glycerol (v/v). The column was washed with 120 ml of Buffer A containing 0.1 M KCl, then with 120 ml of Buffer B. Activity was eluted with Buffer B containing 0.4 M $(NH_4)_2SO_4$ (Figure 5). No activity was observed in subsequent elution of the column with Buffer B containing 1 M $(NH_4)_2SO_4$. Fractions with activity were pooled (fraction IV, 30.5 ml) and stored in liquid nitrogen. This concentrated fraction, containing dnaA46 protein as determined by replication activity and by SDS-polyacrylamide gel electrophoresis, was refractory to further purification by conventional and modern chromatographic methods (see Discussion).

Conditions of selective precipitation of dnaA46 protein were found by reducing the ionic strength of fraction IV by dialysis followed by

Figure 5. Hydroxylapatite chromatography of dnaA46 protein.

The 730 ml of fraction III (pool of the heparin-sepharose fraction) was loaded on a hydroxylapatite column (40 ml) and washed as described in the text. Proteins were eluted by step gradients: A, Buffer B; B, Buffer B containing 0.4 M $(NH_4)_2SO_4$; C, Buffer B containing 1 M $(NH_4)_2SO_4$.

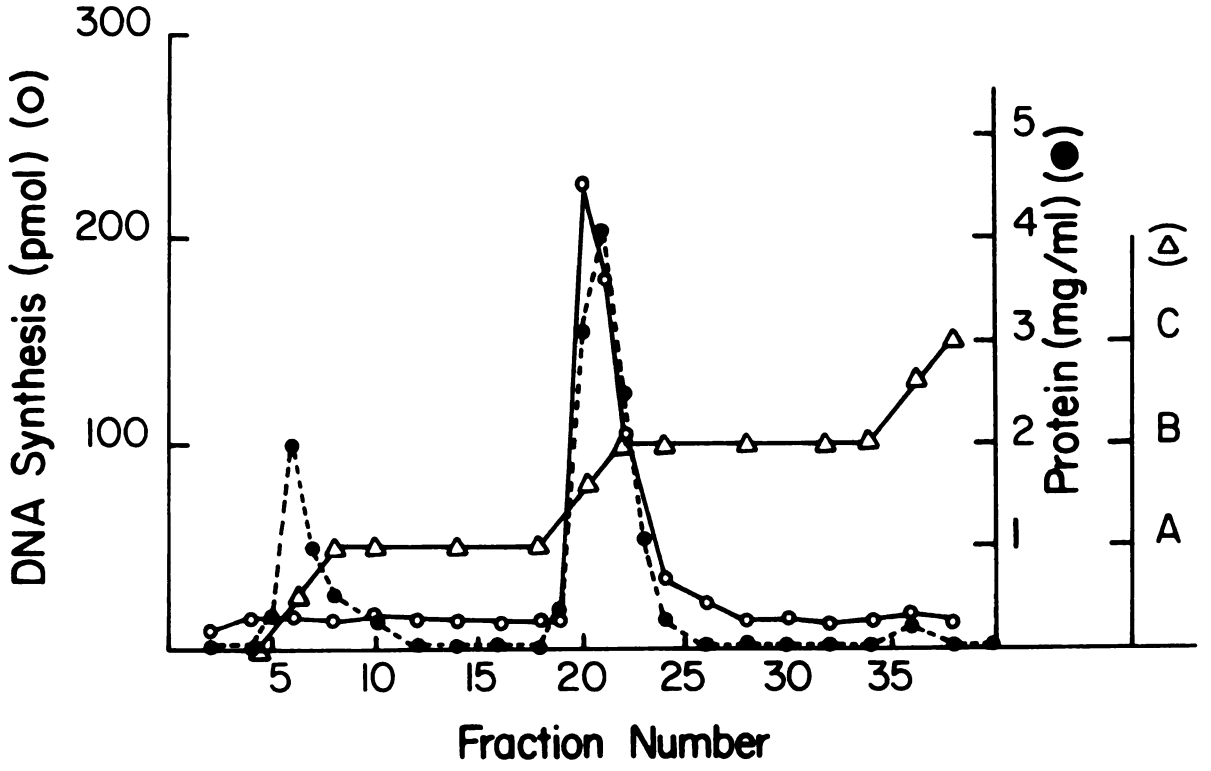
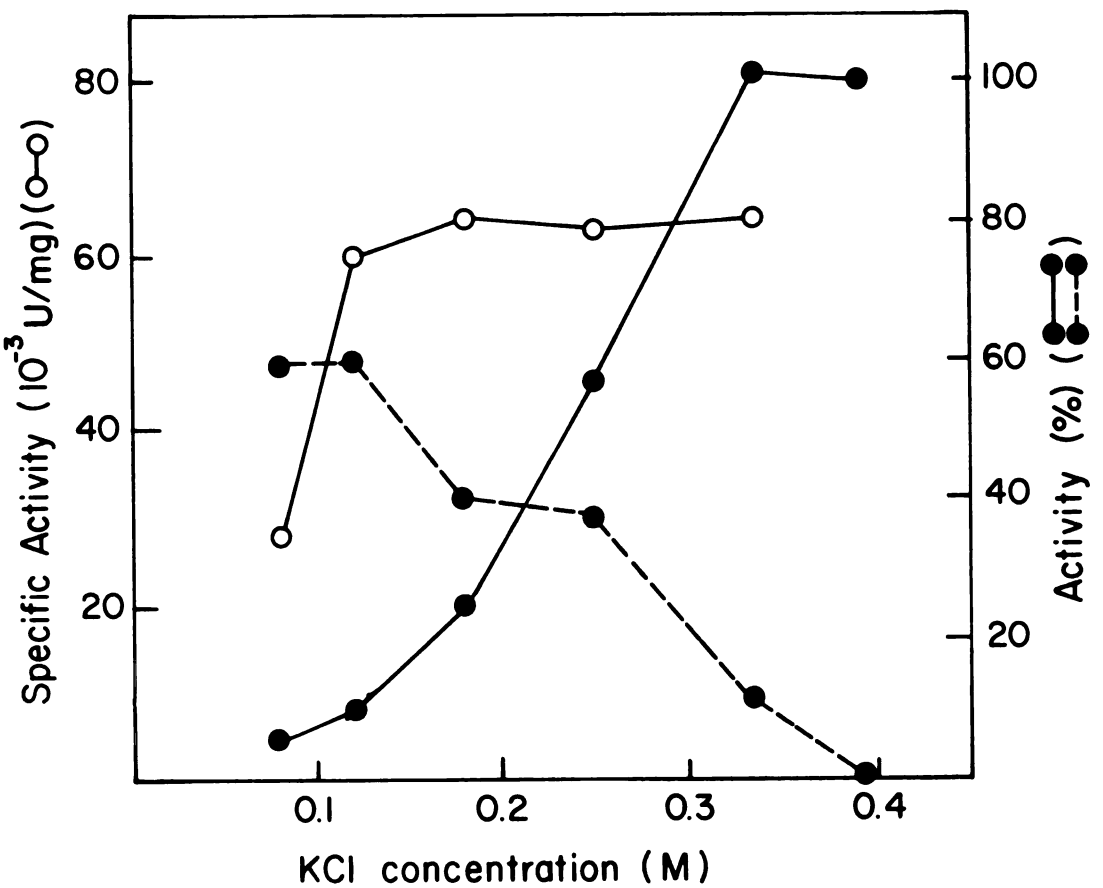

300
200
100
DNA Synthesis (pmol) (o)
5
4
3
2
1
Protein (mg/ml) (●)
(Δ)
C
B
A
5
10
15
20
25
30
35
Fraction Number

Figure 6. Selective precipitation of dnaA46 protein.

Fraction IV (2 ml) was dialysed against 0.5 liter of Buffer C containing 0.1 M KCl. At ionic strengths equivalent to the indicated KCl concentrations, samples were removed and kept on ice. At an ionic strength equivalent to 0.25 M KCl, the dialysis buffer was replaced by 0.5 liter of Buffer C. Samples of the dialysate were centrifuged in a microcentrifuge for 10 min in the cold. Precipitates were resuspended overnight in the cold on an oscillating rocker in 30 ul of Buffer B containing 1 M ammonium sulfate and centrifuged for 5 min as above to remove insoluble material. Replication activity was measured from the first supernatant (●—●) and from the resuspended precipitate (● - -●) and are expressed as the percent of the total activity of the dialysed sample prior to centrifugation. Specific activities of the resuspended precipitate (o—o) were calculated after determination of protein concentration.

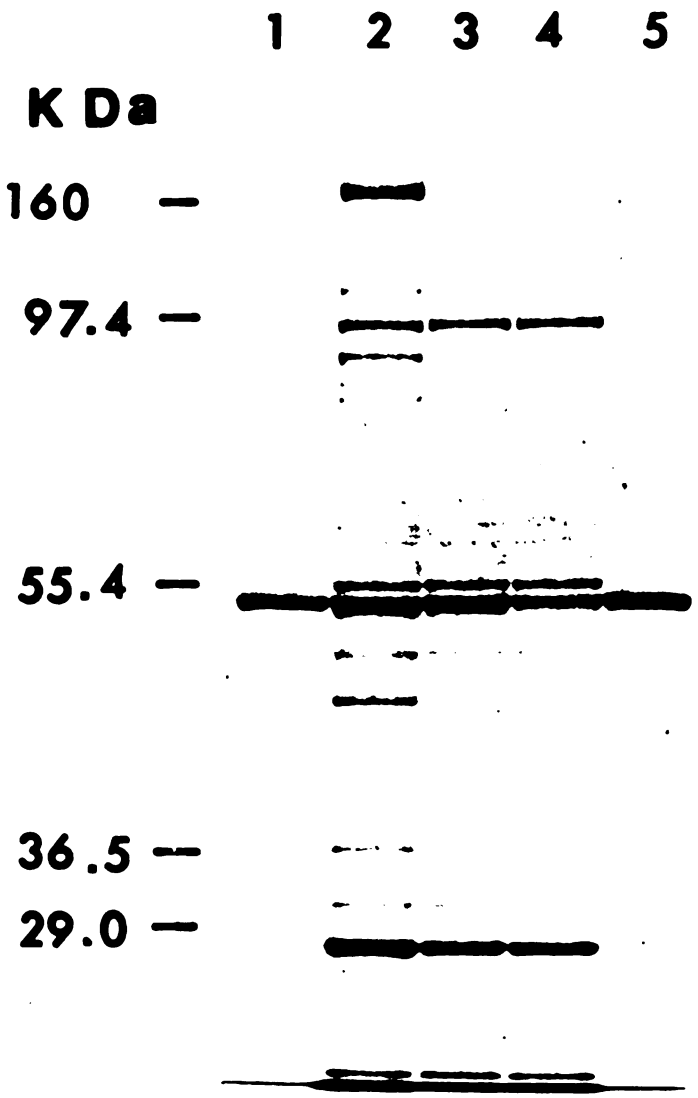

Specific Activity (10^{-3} U/mg)(o—o)
Activity (%) (●--●)
80
60
40
20
100
80
60
40
20
0.1
0.2
0.3
0.4
KCl concentration (M)

centrifugation (Figure 6). Ionic strengths below 0.35 M KCl resulted in a proportional loss of soluble activity upon centrifugation. Conditions for resolubilization of dnaA46 protein activity with reasonable yield were also determined (Figure 6). At very low salt concentrations, contaminants present in the dialysed sample coprecipitated to result in a decrease in specific activity of solubilized dnaA46 protein (Figure 6, data not shown). SDS-polyacrylamide gel electrophoresis of the resuspended precipitate indicated that at concentrations between 0.12 and 0.35 M KCl, dnaA46 protein selectively precipitated by this method was greater than 95% pure by scanning densitometry of Coomassie-blue stained, SDS-polyacrylamide gels (Figure 7, lane 5).

Part of fraction IV (2 ml) was thawed and dialysed against 500 ml of Buffer C containing 0.1 M KCl to a conductivity equivalent to Buffer B containing 0.2 M KCl. The precipitate was collected by centrifugation for 10 min in a microcentrifuge (Brinkman). The precipitate was resuspended on an oscillating rocker overnight in 0.5 ml of Buffer B containing 1 M $(NH_4)_2SO_4$. Insoluble material was removed by centrifugation for 5 min in a microcentrifuge. The activity of the resuspended sample (fraction V) stored in liquid nitrogen was stable for over six months. The relative amount of dnaA46 protein in fraction IV (18%) was determined by densitometric analysis of the Coomassie-blue stained, SDS-polyacrylamide gel (Figure 7, lane 5). This figure and the concentration of dnaA46 protein activity indicate a specific activity of 65×10^3 units/mg in fraction IV. This value was similar to the specific activity of dnaA46 protein purified by selective precipitation (68×10^3 units/mg, Table 3).

Figure 7. SDS-polyacrylamide gel electrophoresis of dnaA46 protein.

Samples were denatured and electrophoresed in a 10% SDS-polyacrylamide slab gel (39). Protein was detected by staining with Coomassie brilliant blue. Lane 1, 2 ug of $dnaA^+$ protein; 2, fraction III (100 ul); 3, fraction IV (5 ul, 140 units); 4, supernatant (5 ul, 60 units) after dialysis and centrifugation of fraction IV; 5, 2 ug (140 units) of dnaA46 protein (fraction V). Molecular weight standards (KDa) were E. coli RNA polymerase beta and beta' subunits, 160; phosphorylase B, 97.4; glutamate dehydrogenase, 55.4; lactate dehydrogenase, 36.5; and carbonic anhydrase, 29.


1 2 3 4 5
K Da
160
97.4
55.4
36.5
29.0

A modification of this procedure was used to purify dnaA$^+$ protein. MC1061 containing pDS596 (dnaA$^+$) was grown, induced with arabinose, and lysed under conditions similiar to those described above. The supernatant from centrifugation of the whole cell lysate was concentrated by ammonium sulfate precipitation, and applied to a heparin-Sepharose 4B column. Activity was retained on the column and was eluted in a single peak with a linear salt gradient of 0.22 M to 1.5 M KCl in Buffer A . Active fractions were pooled and concentrated by hydroxylapatite chromatography as described above. The active fractions were precipitated by addition of 0.32 g of ammonium sulfate per ml of the fraction. The precipitate was collected by centrifugation for 30 min at 30,000 rpm in a Beckman Ti 45 rotor. The pellet was resuspended by addition of Buffer A. Part of this fraction was chromatographed on a Superose 12 gel permeation column (Pharmacia) equilibriated in Buffer A containing 50mM ammonium sulfate. Active fractions at the position expected for monomeric dnaA protein were pooled and stored in liquid nitrogen. A 50-fold purification of dnaA$^+$ protein which contains a specific activity of 200×10^3 units per mg protein was obtained with this overproducing strain (data not shown) with a purity of 95% by densitometric analysis of Coomassie-blue stained, SDS-polyacrylamide gels (Figure 7, lane 1).

Replication by dnaA46 Protein Depends on the oriC Sequence

DNA replication with either dnaA$^+$ protein (37) or dnaA46 protein required plasmid DNAs containing the oriC sequence (Table 3). M13oriC26

Table 3. Template Specificity of dnaA$^+$ and dnaA46 Protein in DNA Replication

Template	DNA Synthesis (pmol) dnaA$^+$ protein	dnaA46 protein
M13oriC26 RF	269	286
M13oriC2LB5 RF	190	175
M13ΔE101 RF (oriC)	3.3	3.9

Reactions were as described in Experimental Procedures with 200 ng (600 pmol in nucleotide) of supercoiled DNAs and 100 ng of dnaA$^+$ or dnaA46 protein. DNA synthesis in the absence of dnaA$^+$ or dnaA46 protein with M13oriC26 RF was 5 pmol. With M13oriC2LB5 RF as template, this value was 10 pmol.

is a recombinant containing the E. coli chromosomal origin inserted into an M13 vector (30). M13oriC2LB5 contains the minimal oriC sequence (54) inserted into M13ΔE101, an M13 derivative lacking the M13 complementary strand origin (31-32). Both M13oriC26 DNA and M13oriC2LB5 are active templates for replication dependent on $dnaA^+$ protein or dnaA46 protein. The vector M13 E101 lacking the oriC sequence was inert as a template.

DNA Replication by dnaA46 Protein is Preceded by a Pronounced Lag

A lag of 3-5 min was observed in a time course for DNA synthesis with $dnaA^+$ protein at either saturating or subsaturating amounts (Figure 8, Reference 53,55). In contrast, DNA synthesis with comparable amounts of dnaA46 protein was preceded by a lag of about 15-18 min. DNA synthesis with dnaA46 protein after 40 min of incubation was similar to that observed with a similar amount of $dnaA^+$ protein in 20 min (Figures 8,9). Addition of excess dnaA46 protein did not stimulate the amount of DNA synthesis observed in 20 min (Figure 9). Addition of equal amounts of dnaA46 protein to reactions containing $dnaA^+$ protein did not markedly stimulate nor inhibit DNA synthesis.

DISCUSSION

The dnaA46, dnaA5, dnaA204 and $dnaA^+$ alleles were cloned into a protein overproducing vector which facilitated the purification of the $dnaA^+$ and dnaA46 gene products active in DNA replication on oriC

Figure 8. Time course of DNA synthesis by dnaA$^+$ and dnaA46 protein.

DNA replication assays were performed as described in Experimental Procedures with the indicated amounts of dnaA$^+$ or dnaA46 protein. At the indicated times, reactions were stopped and acid-insoluble radioactivity was determined.

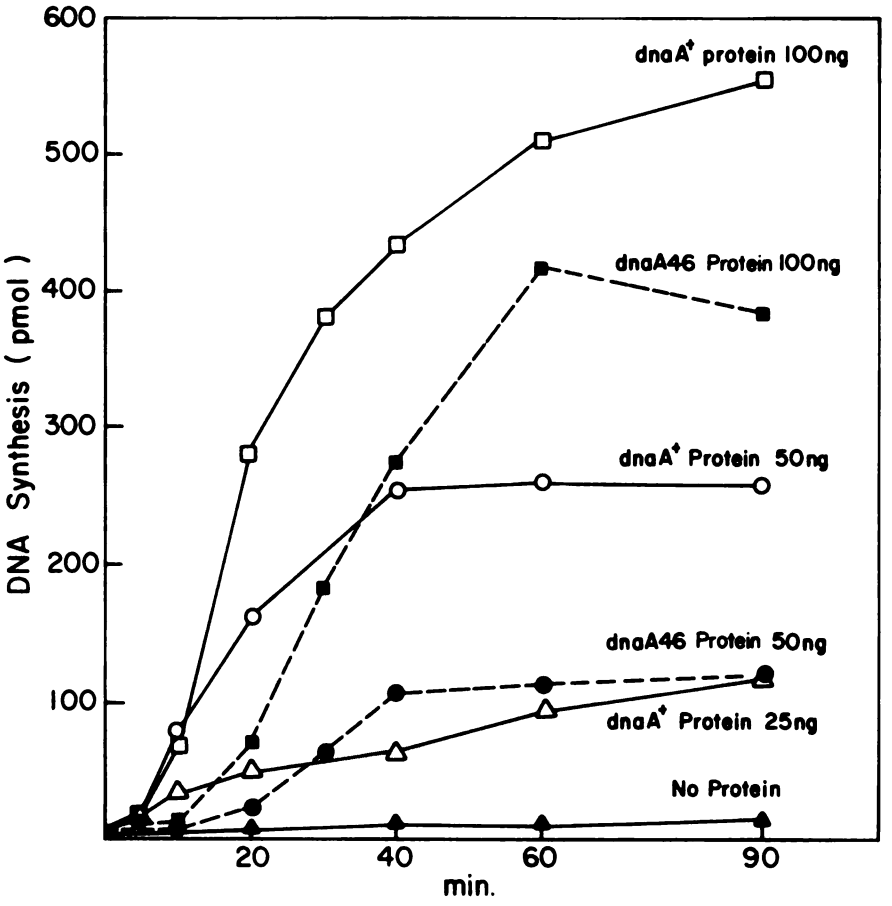
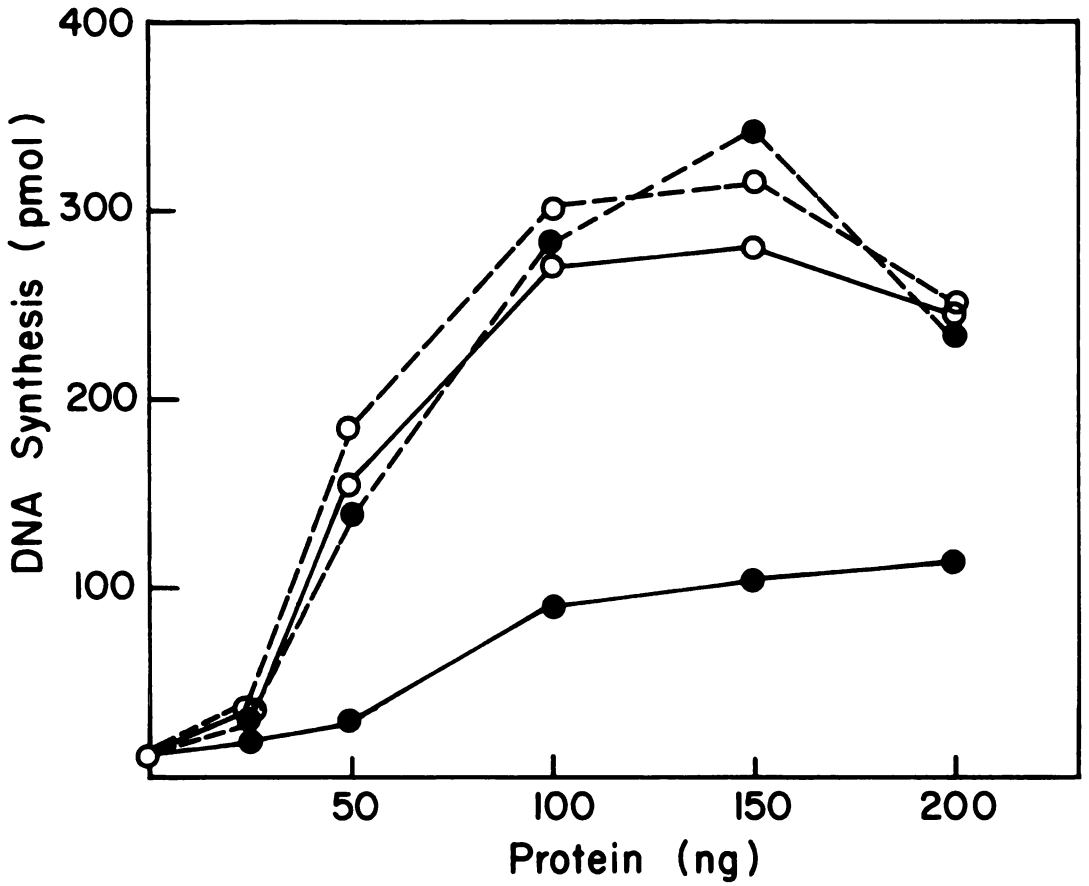

600
500
400
300
200
100
DNA Synthesis (pmol)
20
40
60
90
min.
dnaA+ protein 100ng
dnaA46 Protein 100ng
dnaA+ Protein 50ng
dnaA46 Protein 50ng
dnaA+ Protein 25ng
No Protein

Figure 9. Titration of dnaA$^+$ and dnaA46 protein in DNA replication reactions.

DNA replication assays were performed as described in Experimental Procedures with the indicated amounts of dnaA$^+$ and dnaA46 protein. Incubations were for 20 min with dnaA$^+$ protein (o—o), dnaA46 protein (●—●), both combined at the indicated amounts of each (o- -o), and for 40 min with dnaA46 protein (●- -●).


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

plasmids. Expression of dnaA$^+$ protein from the araB promoter in induced cultures was comparable to levels observed when the dnaA gene is transcribed from the lambda P_L promoter (53, data not shown). Replication activity of dnaA protein was examined from induced cultures harboring recombinant plasmids containing the different dnaA alleles. Based on the similar levels of dnaA46 and dnaA5 protein in maxicell experiments, this result indicated that dnaA46 protein was more active than dnaA5 protein. dnaA204 protein appeared inactive for replication. Replication activity observed from these plasmid-containing strains was dependent on induction by the addition of arabinose (data not shown). As dnaA$^+$ protein activity was not observed in extracts from uninduced cultures of MC1061 containing pDS596 (dnaA$^+$), the contribution of dnaA$^+$ protein activity encoded by the chromosomal dnaA$^+$ gene was minimal.

dnaA46 protein was purified from induced cultures of TD3 containing pDS215 (dnaA46). This host strain containing the dnaA46 gene ensures that plasmid-encoded dnaA46 protein and the analogous chromosomally encoded gene product are identical. The level of expression of dnaA46 protein in MC1061 appeared to be greater than in TD3 (Table 1). This difference is not understood.

Initial steps in purification of dnaA46 protein were based on conditions determined for purification of its wild type counterpart. Attempts to further purify dnaA46 protein (fraction IV) by chromatography were not successful. This included chromatography on Biorex-70 (Biorad), single-stranded DNA cellulose, Cibacron Blue A agarose, DEAE cellulose (DE-52, Whatman), and octyl sepharose (Pharmacia). With these chromatographic resins, replication activity

adsorbed to the column matrix but was eluted broadly with poor yield (data not shown). dnaA46 protein was not retained on ATP-agarose (type IV, Sigma) under conditions where the wild type protein was retained and eluted with reasonable yield (data not shown). Chromatography of dnaA46 protein (fraction IV) on Superose 6 or Superose 12 (Pharmacia) gel permeation columns, or of this fraction on Mono S or Mono Q (Pharmacia) columns under conditions suitable for adsorption of $dnaA^+$ protein resulted in a dramatic increase in resistance to solvent flow. These observations suggested that dnaA46 protein may self-aggregate to cause either the broad elution with conventional chromatographic resins, or resistance to solvent flow in columns with microscopic particle bead sizes.

This apparent property of aggregation was used as a means for its purification. It appeared that this protein had a greater tendency to aggregate or multimerize than $dnaA^+$ protein. This aggregated form could be recovered as a precipitate by centrifugation and resolubilized with retention of its replication activity. $dnaA^+$ protein was unable to be precipitated under conditions appropriate for dnaA46 protein. Sekimizu and Kornberg (56) have used a method for purification of $dnaA^+$ protein similar to that described here, except that precipitated $dnaA^+$ protein was collected by high speed centrifugation and solubilized by use of guanidinium hydrochloride followed by dialysis. This involved dialysis of the sample to a conductivity equivalent to Buffer B containing 0.2 M KCl and centrifugation in a microfuge. This suggests that higher centrifugal force is required for collection of $dnaA^+$ protein as a precipitate.

The pronounced lag in DNA synthesis observed with dnaA46 protein may represent the relative inefficiency of this mutant protein in some aspect of the initiation process. It may be noteworthy that the timing of initiation of DNA replication in wild type cells is tightly coordinated with the cell cycle. In dnaA46 mutants, this coordination appears to be interrupted such that initiation of chromosomal replication occurs randomly throughout the cell cycle (52). Of the many activities of dnaA protein, the influence of the dnaA46 mutation on this multifunctional protein can be addressed biochemically.

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CHAPTER III

Characterization of dnaA46 Protein

INTRODUCTION

dnaA46 protein, a mutant form of dnaA protein, was purified under conditions where its replication activity was preserved (Chapter II). *In vitro* DNA replication sustained by dnaA46 protein depended upon the *oriC* sequence. The protein exhibited a reduced rate of *oriC* plasmid replication compared to the rate of $dnaA^+$ protein. The reduced rate of DNA replication by dnaA46 protein appears to result from a pronounced lag before DNA synthesis, suggesting that the mutation affects an early step in initiation of DNA replication *in vitro*.

To understand an altered function(s) of dnaA46 protein in replication of *oriC* plasmid DNA, this chapter describes the biochemical characterization of dnaA46 protein in comparison with $dnaA^+$ protein.

EXPERIMENTAL PRCEDURES

Reagents

Phages, plasmids, commercial enzymes, chemicals and radiochemicals were as described in the Chapter II. E. coli tRNA was from Sigma. [alpha ^{32}P]ATP (3000 Ci/mmol) was from New England Nuclear Corp. Plasmid pTSO182, a gift from Dr. Mituru Takanami, contains oriC and adjacent chromosomal DNA in a HaeIII fragment inserted into a pBR322 derivative (1).

Enzymes

Highly purified DNA replication proteins were: dnaA$^+$ protein (fraction VI, $2x10^5$ unit/mg) (Chapter II); dnaA46 protein (fraction V, $6.8x10^4$ unit/mg) (Chapter II); dnaB protein (fraction V, $6.8x10^5$ unit/mg) (as described (2) except that hydroxylapatite chromatography was replaced by gel permeation chromatography on a TSK 3000 SW column (Altex); dnaC protein (fraction VI, $3x10^6$ unit/mg) (as described (3) except that hydroxylapatite chromatography was replaced by Mono S chromatography (Pharmacia-PL); primase (fraction V, $2x10^6$ unit/mg) (D. Siemieniak and J. Kaguni, unpublished procedure from an overproducing strain) (4); single strand DNA binding protein (SSB) (fraction IV, $4x10^4$ unit/mg) (5-6); DNA polymerase III holoenzyme (fraction V, $2x10^5$ unit/mg) (7); DNA gyrase A (fraction III, $2x10^5$ unit/mg), and DNA gyrase B subunits (fraction V, $1.5x10^5$ unit/mg) (unpublished procedures from overproducing strains) (8). RNA polymerase (fraction V, 250 munit/mg) was prepared as described (9-10) except that Biogel A5m chromatography

(Biorad) was replaced by chromatography on a TSK 3000 SW column (Altex). RNase H (fraction IV, $8x10^5$ unit/mg) (11), protein HU (fraction IV, $5x10^5$ unit/mg) (12), and topoisomerase I (fraction V, $5x10^4$ unit/mg) (13) were gifts from Dr. A. Kornberg. Except where noted, one unit (1 pmol of acid-insoluble deoxynucleotide incorporated per min at 30°C) corresponds to activity in oriC plasmid replication reactions with crude enzyme fractions prepared from the corresponding mutant strain (14). Replication activity of these inactive enzyme fractions was complemented for by addition of the corresponding active gene product.

DNA Replication Assays

Replication assays to measure $dnaA^+$ or dnaA46 protein activity by addition to reactions containing a crude protein fraction deficient in dnaA protein were as described (Chapter II, Experimental procedures). Incubations with $dnaA^+$ or dnaA46 protein were at 30°C for 20 min unless noted. DNA replication reactions with purified replication proteins (25 ul) contained HEPES-KOH (pH 7.6), 40 mM; Tris-HCl (pH 7.5), 20 mM; sucrose, 4% (w/v); ATP, 2 mM; CTP, GTP, and UTP, each at 0.5 mM; dATP, dCTP, dGTP, and [^{3}H]dTTP (15-45 cpm/pmol), each at 100 uM; magnesium acetate, 11 mM; phosphocreatine, 6 mM; dithiothreitol (DTT), 5 mM; creatine kinase, 100 ug/ml; bovine serum albumin, 0.08 mg/ml; SSB, 160 ng; HU, 25 ng; topoisomerase I, 2.5 units where noted; RNase H, 0.3 ng where noted; gyrase A subunit, 470 ng; gyrase B subunit, 600 ng; primase, 10 ng; dnaB protein, 50 ng; dnaC protein, 40 ng; RNA polymerase, 900 ng where noted; DNA polymerase III holoenzyme, 270 ng;

M13oriC26 supercoiled DNA, 200 ng (600 pmol of nucleotide); and the indicated amounts of $dnaA^+$ or dnaA46 protein (15-17). Reactions were assembled at 0°C. DNA synthesis was initiated by incubation at 30°C for 30 min. Total nucleotide incorporation was measured in a liquid scintillation counter after trichloroacetic acid precipitation onto glass-fiber filters (Whatman GF/C).

oriC Binding Assays

Fragment retention assays (18) were performed by incubation of the indicated amounts of $dnaA^+$ or dnaA46 protein with 0.028 pmol (in plasmid DNA) of TaqI-digested pTS0182 containing oriC, and end-labeled with the large fragment of DNA polymerase I and [alpha ^{32}P]dCTP (19), in 25 ul of binding buffer containing 40 mM HEPES-KOH (pH 7.6), 5 mM magnesium acetate, 2 mM DTT, and 100 mM KCl. Reactions, incubated for 10 min at 30°C, were immediately filtered with gentle suction through nitrocellulose filters and washed with 250 ul of binding buffer. Filters (Millipore HAWP, 0.45 uM, 24mm diameter) were boiled for 5 min, stored in H_2O at 4°C, and equilibrated in binding buffer at room temperature prior to use. DNA retained on the filters was eluted by incubation at 65°C for 20 min in buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.5 M sodium acetate, and 0.5% sodium dodecyl sulfate. The eluted DNA, and DNA which flowed through the filters were concentrated by ethanol precipitation with 10 ug of E. coli tRNA as carrier. The precipitates (resuspended in 5% glycerol, 0.1% bromophenol blue, 10 mM Tris-HCl (pH 8), and 1 mM EDTA) were electrophoresed in 6% polyacrylamide gels in 80 mM Tris-borate (pH 8.3), and 1 mM EDTA. After

electrophoresis, the gels were dried onto Whatman DE81 paper and autoradiographed at -70°C with Kodak XAR-5 film and Cronex Quanta III intensifying screens.

Alternatively, pTSO182 was cleaved with SalI and XhoI restriction enzymes. The 459 base pair (bp) fragment containing the oriC sequence was purified by electroelution (18), ^{32}P-labelled with [alpha ^{32}P]dTTP, [alpha ^{32}P]dCTP, and DNA polymerase I (large fragment) (19) and filtered through Sephadex G-50 (Pharmacia) to remove unincorporated deoxynucleotides. 7.5 ng (0.025 pmol in DNA) of the fragment was used per reaction as described above. Where noted, the indicated amount of nonradioactive pBR322 DNA digested with HinfI was included in the reaction mixture. Filters were dried before determination of retained radioactivity by liquid scintillation counting.

ATP-Binding Assay

ATP-dnaA protein complex formation was measured as described (20). Reactions (25 ul) contained 50 mM Tricine-KOH (pH 8.3), 0.5 mM magnesium acetate, 0.3 mM EDTA, 20% (v/v) glycerol, 0.007% Triton X-100, 7 mM DTT, 1 pmol (50 ng) of dnaA^{+} or dnaA46 protein, 10 uCi of [alpha ^{32}P]ATP, and ATP at the indicated concentrations ($5x10^{4}$ cpm/pmol at 0.5 uM to 10^{7} cpm/pmol at 2.5 nM). After incubation at 0°C for 15 min, the reaction mixtures were filtered through nitrocellulose filters (Millipore HAWP, 0.45 um, 24 mm diam.) equilibrated in buffer containing 50 mM Tricine-KOH (pH 8.3), 0.5 mM magnesium acetate, 0.3 mM EDTA, 17% (v/v) glycerol, 0.005% Triton X-100, and 5 mM DTT, and washed with 1 ml of

this ice-cold buffer. Filters were dried before determination of retained radioactivity by liquid scintillation counting.

RESULTS

Thermal Stability of dnaA46 Protein

Due to the temperature-sensitive phenotype of dnaA46 mutants, in vitro experiments were performed to determine whether the activity of dnaA46 protein was thermolabile. $dnaA^+$ and dnaA46 proteins were incubated at 20°C, 30°C, and 40°C for various lengths of time, then added to reactions incubated at 30°C to measure DNA synthesis activity (Figure 1). Incubation of dnaA46 protein at 40°C prior to its addition to DNA synthesis reactions did not markedly reduce its activity compared to $dnaA^+$ protein or to its relative activity at 20°C or 30°C (Figure 1).

These results contrast with results of others indicating that $dnaA^+$ protein, in the absence of ATP, was unstable at 38°C with a half-life of less than 2 min (20). At ATP concentrations of 1 uM or less, this inactivation was prevented. Possible explanations for this difference could relate to incubation conditions or to the method of measuring activity. In the published report (20), activity of $dnaA^+$ protein was measured in a purified enzyme system. In this study, activity of $dnaA^+$ and dnaA46 protein was measured in a crude enzyme system.

Figure 1. Thermal stability of $dnaA^+$ and dnaA46 protein.

$dnaA^+$ and dnaA46 protein in Buffer A containing 0.2 M KCl were incubated at the indicated temperatures and times. Samples containing 100 ng of $dnaA^+$ or dnaA46 protein after this prior incubation were added to DNA replication reactions (Experimental Procedures) to measure activity. Relative activity is expressed (in percent) as the ratio of activity of the sample treated by a prior incubation in comparison to the untreated control (180, 206, and 151 pmol with $dnaA^+$ protein, and 120, 151, and 134 pmol with dnaA46 protein in the untreated controls for the 40°C, 30°C, and 20°C incubations).

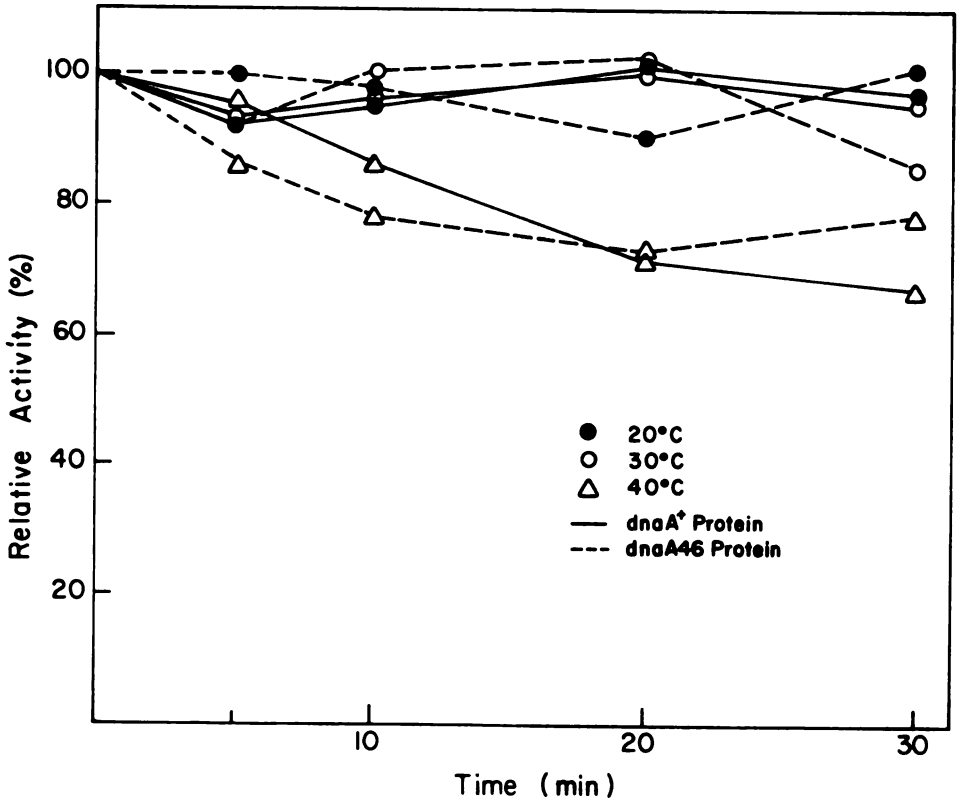
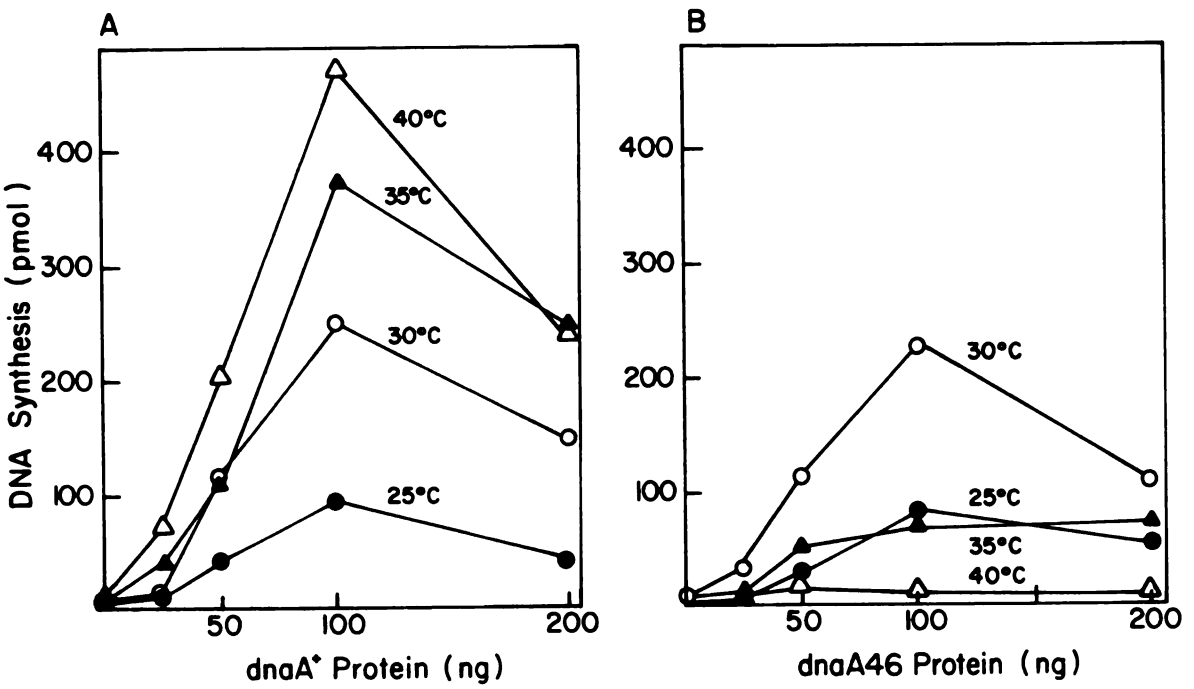

100
80
60
40
20
Relative Activity (%)
10
20
30
Time (min)
20°C
30°C
40°C
dnaA+ Protein
dnaA46 Protein

Figure 2. Thermal inactivation of dnaA46 protein in DNA replication.

The indicated amounts of $dnaA^+$ (A) and dnaA46 protein (B) were assayed in DNA replication reactions as described in Experimental Procedures except that incubations were at the indicated temperatures.


A
B
DNA Synthesis (pmol)
400
300
200
100
50
100
200
40°C
35°C
30°C
25°C
dnaA* Protein (ng)
dnaA46 Protein (ng)

Thermal Inactivation of dnaA46 Protein Under Conditions of Coupled DNA Synthesis

Incubation of DNA replication reactions at temperatures from 25°C to 40°C stimulated DNA replication dependent on $dnaA^+$ protein in a proportional fashion (Figure 2). Under comparable conditions, DNA replication dependent on dnaA46 protein was detectable at 25°C and greatest at 30°C. Incubation at 35°C resulted in comparatively less incorporation with almost no detectable DNA synthesis occurring at 40°C. The thermal stability of dnaA46 protein activity when incubated at elevated temperatures prior to its addition to DNA synthesis reactions, and its reduced activity when measured concomitant with DNA synthesis at elevated temperatures are consistent with the reversible thermolability of the dnaA46 gene product in chromosomal DNA replication in vivo (21-23).

dnaA46 protein is defective in oriC binding activity

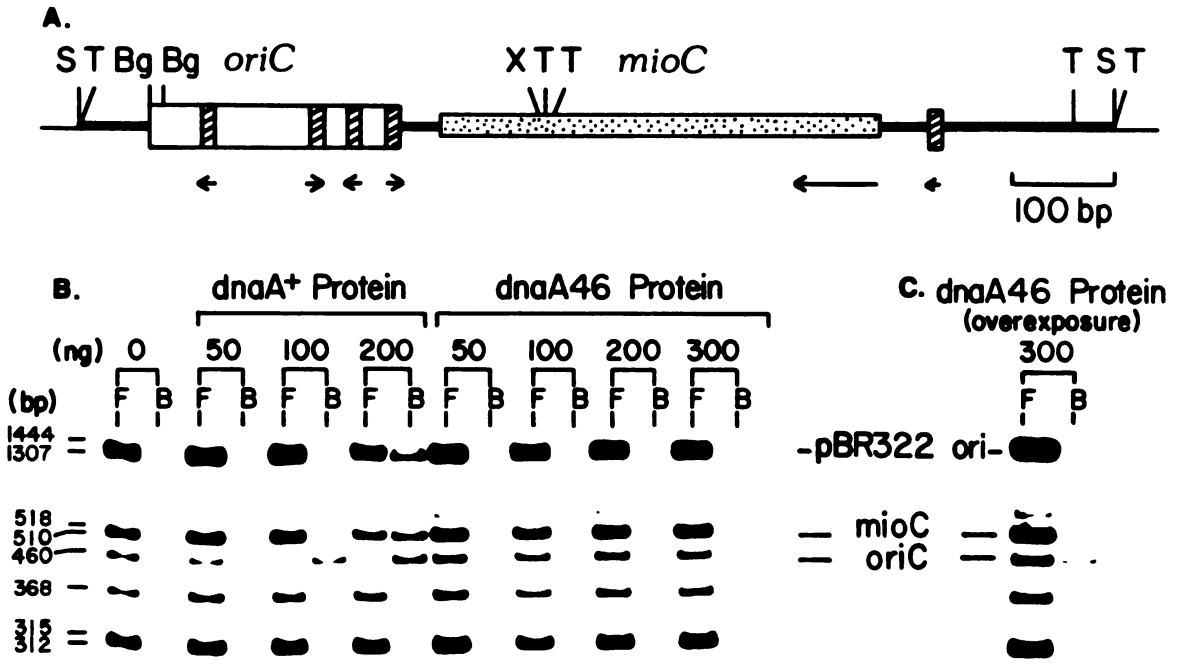
Upon complementing crude enzyme fractions deficient in dnaA protein activity, purified dnaA46 protein was about 3-fold reduced compared to $dnaA^+$ protein in its replication activity of oriC plasmids (Chapter II). The replication activity of $dnaA^+$ protein may involve one or more of its other biochemical activities. We were interested in correlating the reduced replication activity of dnaA46 protein to one or more of these biochemical functions.

Purified dnaA46 protein was compared to $dnaA^+$ protein in binding to DNA fragments containing dnaA protein recognition sequences. The plasmid pTSO182 (Figure 3A) contains dnaA protein binding sites within

Figure 3. Specific DNA binding activity of $dnaA^+$ and dnaA46 protein.

A. Physical map of the oriC region. The chromosomal HaeIII fragment containing the oriC region was inserted into the SalI site of a pBR322 derivative to form pTS0182 (1). Restriction sites: S, SalI; T, TaqI; Bg, BglII; X, XhoI. The open box indicates the oriC sequence; cross-hatched boxes indicate dnaA box, which is dnaA protein recognition sequences, with arrows beneath to indicate polarity. The stippled box indicates the open reading frame of mioC. The direction of transcription is indicated by the arrow beneath.

B and C. Fragment retention assays were performed as described in Experimental Procedures with the indicated amounts of $dnaA^+$ or dnaA46 protein and 100 ng (0.028 pmol in plasmid DNA) of ^{32}P-labeled, TaqI-digested pTS0182 DNA. Fragments containing oriC, the mioC promoter, and the pBR322 origin are indicated. F, fragments which flowed through the filter, B, bound fragments eluted from the filter. Autoradiography was for 4 hours (B), or 12 hours (C).

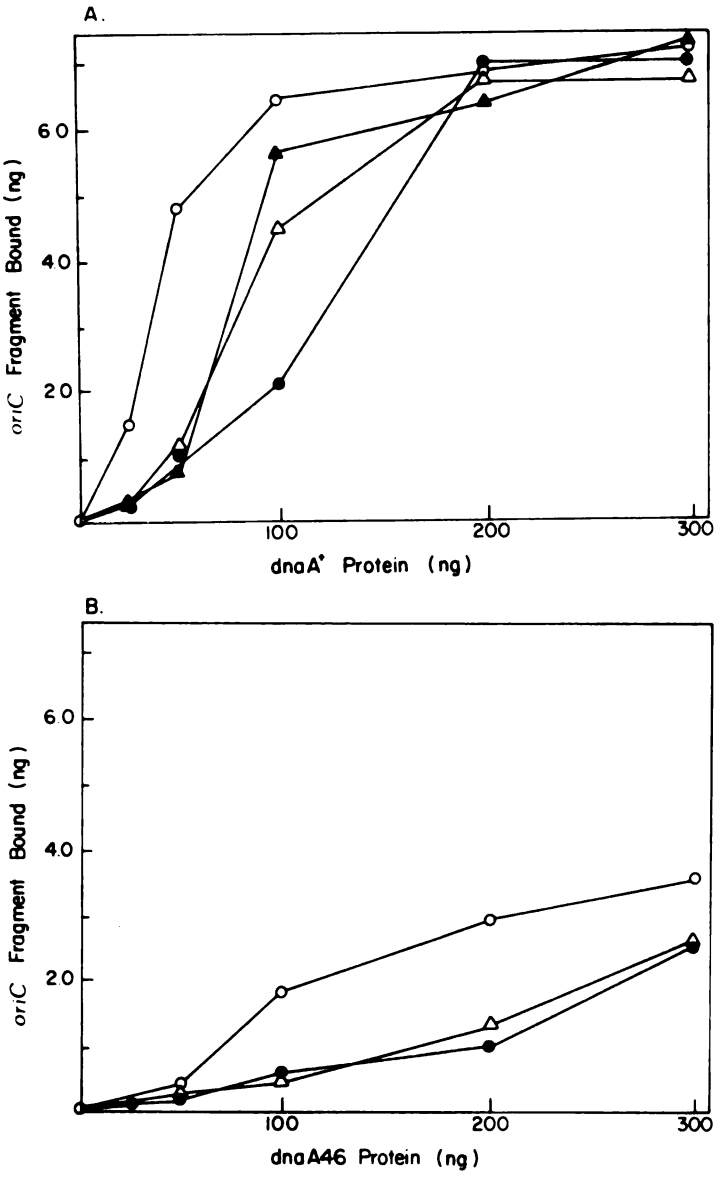

A.
S T Bg Bg oriC
X T T mioC
T S T
100 bp
B.
dnaA+ Protein
dnaA46 Protein
(ng) 0 50 100 200 50 100 200 300
(bp) F B F B F B F B F B F B F B F B
1444
1307
518
510
460
368
315
312
C. dnaA46 Protein
(overexposure)
300
F B
-pBR322 ori-
mioC
oriC

oriC, the mioC promoter region involved in transcription of this gene adjacent to oriC (24), and a site near the pBR322 origin. These sites are bound cooperatively and with different affinities by $dnaA^+$ protein (25). This plasmid was digested with TaqI restriction enzyme to separate these different binding domains. DNA fragments were end-labeled with DNA polymerase I (large fragment) and [alpha ^{32}P]dCTP and incubated with various amounts of $dnaA^+$ protein or dnaA46 protein at 30°C, the optimal temperature for replication activity of dnaA46 protein. Fragments which were retained, or flowed through nitrocellulose filters were electrophoresed on a polyacrylamide gel and visualized by autoradiography (Figure 3B). At intermediate levels of $dnaA^+$ protein, the fragment containing oriC was preferentially bound. At the highest level of added protein, three fragments were retained. As observed previously (25), the fragment containing oriC was more tightly bound by $dnaA^+$ protein than either the fragment containing the mioC promoter or the fragment containing the pBR322 origin.

By comparison, dnaA46 protein bound to the oriC-containing fragment with much less affinity than its wild type counterpart (Figure 3B). Even upon prolonged autoradiography, preferential binding was not observed to DNA fragments containing the mioC promotor or the pBR322 origin (Figure 3C). The SalI-XhoI restriction fragment from pTS0182 was purified by electroelution from a restriction enzyme digest of this DNA. The purified fragment containing oriC was end-labeled with [alpha ^{32}P]dCTP, [alpha ^{32}P]dTTP, and DNA polymerase I (large fragment) and used in filter binding assays with the indicated amounts of $dnaA^+$ or dnaA46 protein (Figure 4). This radioactively-labeled fragment was used

Figure 4. DNA binding activity of $dnaA^+$ and dnaA46 protein to a restriction fragment containing oriC.

Fragment retention assays (Experimental Procedures) were performed with the indicated amounts of $dnaA^+$ (A) or dnaA46 protein (B) and 7.5 ng (0.025 pmol in DNA) of a 459 bp SalI-XhoI restriction fragment containing oriC and radioactively labeled. Symbols are: (o), oriC fragment; (Δ), oriC fragment and 100 ng (0.035 pmol in plasmid DNA) of nonradioactive, HinfI-digested pBR322 DNA; (●), oriC fragment and 200 ng (0.07 pmol) of nonradioactive, HinfI-digested pBR322 DNA; (▲), oriC fragment and 100 ng (0.035 pmol) of nonradioactive, HinfI-digested pBR322 DNA with $dnaA^+$ and dnaA46 protein combined at the indicated amounts of each.


A.
oriC Fragment Bound (ng)
60
40
20
100
200
300
dnaA° Protein (ng)
B.
oriC Fragment Bound (ng)
6.0
4.0
2.0
100
200
300
dnaA46 Protein (ng)

to quantitate the affinity of binding without the influence of competitor DNA fragments present in a restriction enzyme digest, and without the error introduced from manipulations of ethanol precipitation, electrophoresis, and autoradiography. As in Figure 4, 100 ng of $dnaA^+$ protein was sufficient to retain most of the oriC fragment (Figure 4A). A HinfI restriction site (nucleotides 2450 to 2454) is present next to the consensus recognition sequence for $dnaA^+$ protein (nucleotides 2441 to 2449) in the pBR322 origin region (25-26). HinfI digestion of pBR322 results in the inability of $dnaA^+$ protein to bind preferentially to fragments from the pBR322 origin region (data not shown). In the presence of 100 ng of unlabeled pBR322 DNA digested with HinfI, 100 to 200 ng of $dnaA^+$ protein was sufficient to retain most of the oriC fragment. This result is comparable to published observations of $dnaA^+$ protein binding to a restriction enzyme digest of an oriC plasmid (25,27). With 100 ng of $dnaA^+$ protein and less, the addition of HinfI-digested pBR322 DNA to these assays competed in a proportional way with the binding of $dnaA^+$ protein to the oriC-containing DNA fragment. This suggests that $dnaA^+$ protein is able to bind with less affinity to DNA fragments lacking the dnaA protein consensus sequence. The nonlinear binding at subsaturating levels of $dnaA^+$ protein suggests its cooperativity in binding.

In comparison, dnaA46 protein bound to the oriC-containing fragment with less affinity than $dnaA^+$ protein (Figure 4B). The addition of HinfI-digested pBR322 DNA reduced the binding of dnaA46 protein to the oriC fragment. 200 ng of the competor DNA (HinfI-digested pBR322 DNA) was used to quantitate binding of dnaA46 protein to the oriC, in

which the ratio of the oriC fragment to competor DNA is similiar to the molar concentration of the oriC fragment in the plamid template used for DNA replication assay containing 200ng of oriC plasmid DNA. Under this condition, 100 ng of dnaA46 protein which amount was near-saturuation in the DNA replication assay (Chapter II, Figure 10) bound 20-30% of the oriC fragment bound by the same amount of $dnaA^+$ protein (Figure 4). A prolonged incubation did not increase binding of dnaA46 protein to the fragment (data not shown), suggesting that the pronounced lag in DNA replication with dnaA46 protein is not caused by a reduced rate of binding.

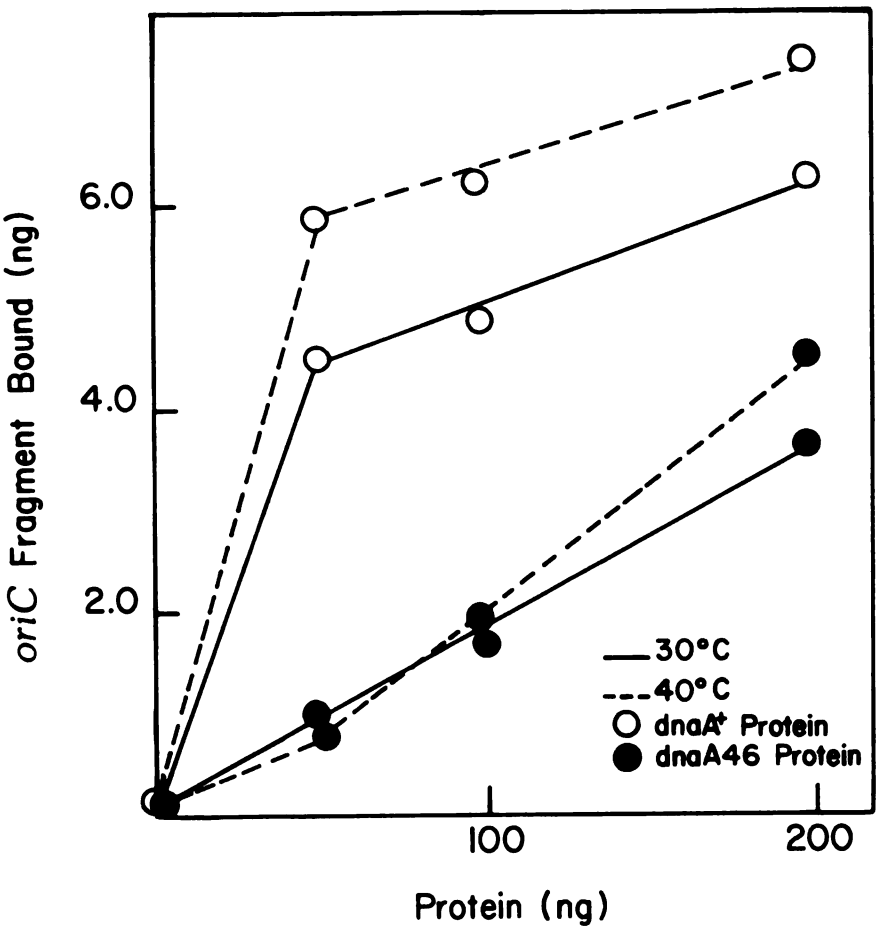
The addition of equal amounts of dnaA46 protein and $dnaA^+$ protein did not inhibit retention of the oriC fragment by $dnaA^+$ protein on these filters. These results indicate that the ability of dnaA46 protein to bind to oriC is reduced compared to $dnaA^+$ protein under these experimental conditions. The lack of a pronounced additive or inhibitory effect when both $dnaA^+$ and dnaA46 proteins were included also suggests that the mutant and wild type proteins do not form mixed complexes. The binding of $dnaA^+$ protein at 40°C was slightly increased compared to binding at 30°C (Figure 5). Comparable experiments with dnaA46 protein indicated that binding to the oriC fragment was not diminished at the higher temperature. These results indicate that the DNA binding activity of dnaA46 protein is not thermolabile.

dnaA46 protein is defective in ATP binding

$dnaA^+$ protein binds ATP (K_D 0.03 uM) and other adenine nucleotides with high affinity (20). Whereas $dnaA^+$ protein binds to oriC with equal

Figure 5. DNA binding activity of $dnaA^+$ and dnaA46 protein is not thermolabile.

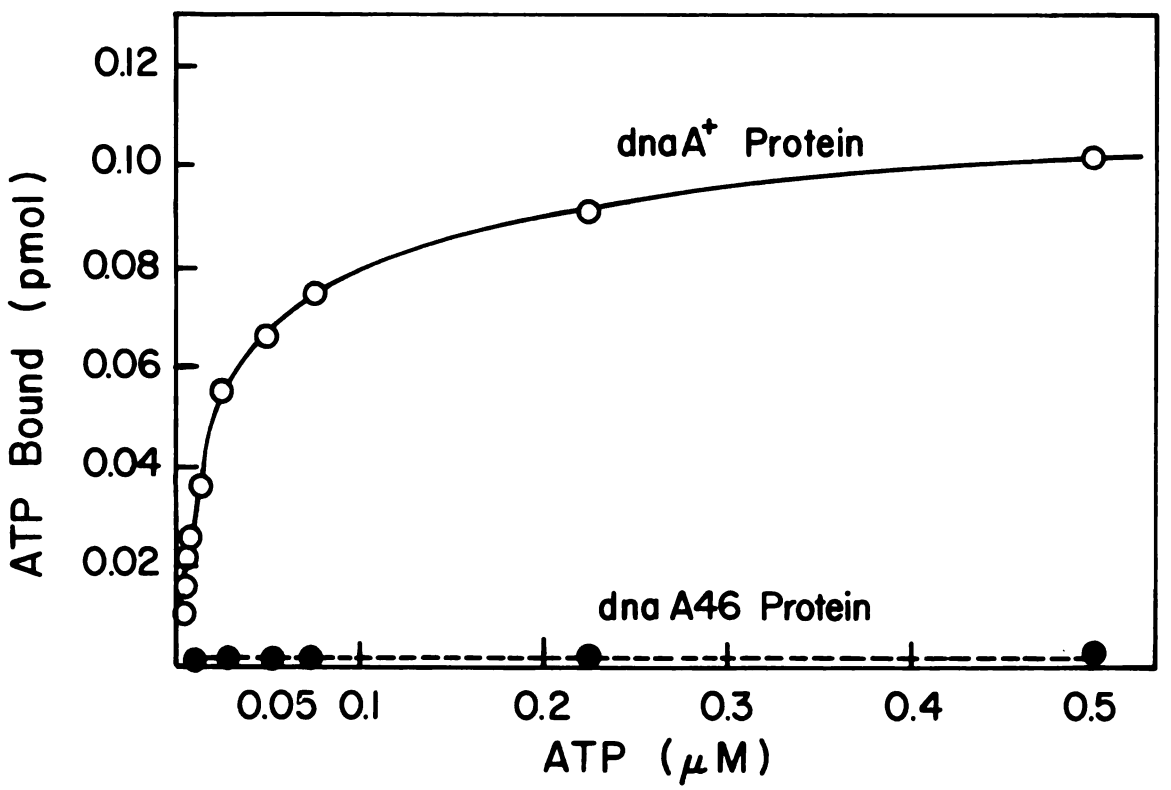
Fragment retention assays (Experimental Procedures) were performed with the indicated amounts of $dnaA^+$ or dnaA46 protein and 7.5 ng (0.025 pmol) of the 459 bp, oriC containing SalI-XhoI restriction fragment end-labeled with [alpha ^{32}P]ATP and polynucleotide kinase. Incubations were performed at the indicated temperatures and filters were washed with binding buffer equilibrated at the temperature of incubation.


oriC Fragment Bound (ng)
6.0
4.0
2.0
100
200
Protein (ng)
30°C
40°C
dnaA+ Protein
dnaA46 Protein

affinity in the presence or absence of ATP, the dnaA protein-ATP complex is the active form for oriC replication in a purified enzyme system. Experiments were performed under the reported conditions to determine the ability of dnaA46 protein to bind ATP in comparison with $dnaA^+$ protein (Figure 6). Based on binding of [alpha ^{32}P]ATP to $dnaA^+$ protein on nitrocellulose membranes, a KD value of 0.023 uM was calculated, with a stoichiometric ratio of 0.1 ATP bound per monomer by the method of Scatchard (28). Experiments with other preparations of $dnaA^+$ protein also resulted in stoichiometric ratios near 0.1. Under similar conditions, no detectable binding of [alpha ^{32}P]ATP to dnaA46 protein was observed. Even under conditions where the ATP concentration was increased to 100 uM (1400 cpm per pmol of ATP), [alpha ^{32}P]ATP binding to dnaA46 protein could not be detected (data not shown). A systematic study of the effects of pH, temperature, ionic strength, and time of incubation on ATP binding have not been performed. However, ATP binding of dnaA46 protein was not observed in nitrocellulose filter binding assays under several different reaction conditions or times of incubation (data not shown). UV-crosslinking experiments of ATP to $dnaA^+$ or dnaA46 protein at 10 J/m^2 were performed under reaction conditions for ATP binding and containing 1 uM [alpha ^{32}P] ATP. Samples were removed from the irradiated reaction mixture at various times, electrophoresed on a 10% SDS-polyacrylamide gel, stained with Coomassie blue to identify the position of dnaA protein, then subjected to autoradiography after drying the gel. Densitometric analysis of autoradiograms indicated that crosslinking of [alpha ^{32}P]-ATP to dnaA46 protein was more than 100-fold reduced compared to $dnaA^+$ protein (data

Figure 6. Binding of ATP to dnaA$^+$ and dnaA46 protein.

One pmol of dnaA$^+$ (o) or dnaA46 protein (●) was incubated with the indicated amounts of [alpha ^{32}P]ATP and ATP binding was measured as described in Experimental Procedures.


0.12
0.10
0.08
0.06
0.04
0.02
ATP Bound (pmol)
dnaA+ Protein
dnaA46 Protein
0.05
0.1
0.2
0.3
0.4
0.5
ATP (μM)

not shown). This reduced ATP binding activity by dnaA46 protein is also consistent with the inability of dnaA46 protein to adsorb to ATP agarose (type IV, Sigma) under conditions where $dnaA^+$ protein was retained (data not shown). The ability of dnaA46 protein to bind ADP has not been examined.

dnaA46 protein is inactive in purified enzyme systems which replicate oriC plasmid

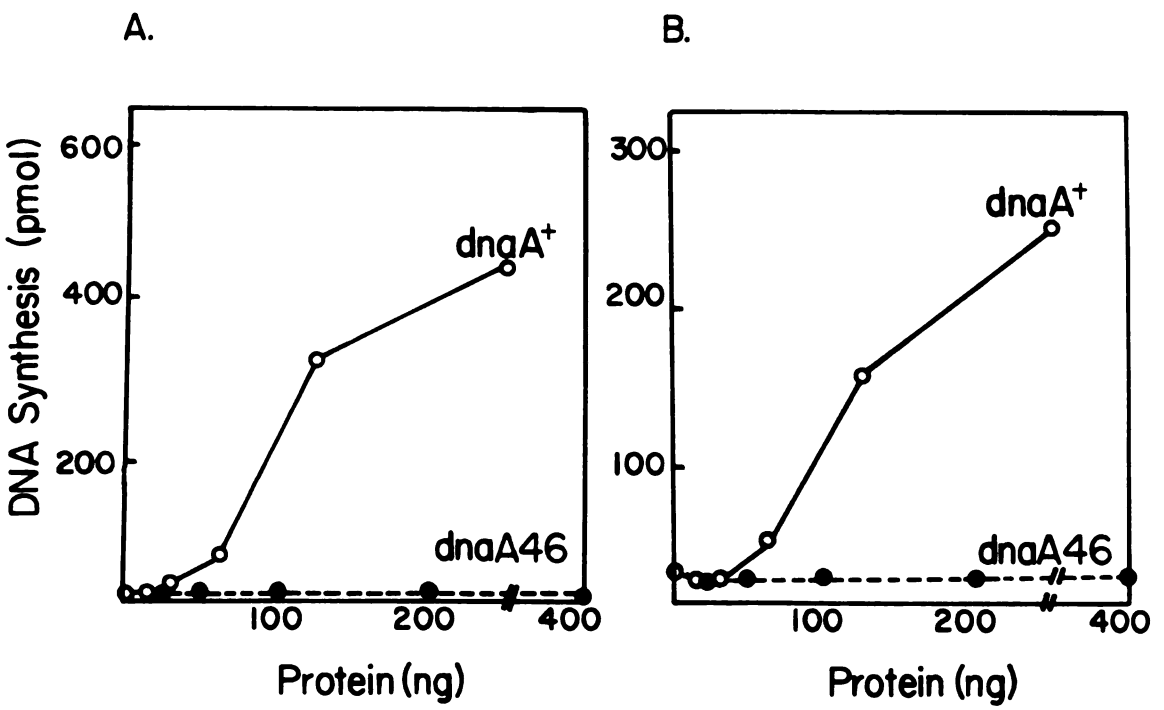
Soluble protein fractions prepared from dnaA mutants of E. coli are not capable of sustaining replication of oriC-containing plasmids unless active dnaA protein is added. Based on such complementation assays (14), $dnaA^+$ and dnaA46 protein have been purified (Chapter II). Enzyme systems composed of purified replication proteins have been developed which depend on $dnaA^+$ protein (15-17). One system utilizes primase as the priming enzyme for DNA replication. The other system includes RNA polymerase, and primase for priming, and topoisomerase I, and RNase H, to maintain template specificity. In either purified enzyme system, dnaA46 protein was inactive for DNA replication (Figure 7). Under similar reaction conditions, DNA synthesis occurred upon addition of $dnaA^+$ protein.

DISCUSSION

This Chapter characterizes the biochemical properties of a mutant form of dnaA protein encoded by the dnaA46 allele. Contrasting its

Figure 7. Replication activity of $dnaA^+$ and dnaA46 protein in purified enzyme systems.

DNA replication reactions with primase (A) or with primase, RNA polymerase, and auxiliary proteins topoisomerase I and RNase H (B) were performed (Experimental Procedures) with the indicated amounts of $dnaA^+$ or dnaA46 protein.


A.
B.
DNA Synthesis (pmol)
600
400
200
300
200
100
dnaA+
dnaA46
100
200
400
Protein (ng)

properties to wild type dnaA protein, this mutant polypeptide is 1) less active in DNA replication at temperatures elevated above 30°C, 2)reduced in its binding affinity to oriC and other restriction fragments bound by $dnaA^+$ protein, 3) defective in ATP binding, and 4) inactive for replication in purified enzyme systems. Despite the relative inactivity of dnaA46 protein in these biochemical assays, this mutant protein is active for DNA replication dependent on a crude enzyme system.

Among the dnaA alleles which have been isolated, the dnaA46 gene product is among the most extensively studied physiologically. It and the dnaA5 gene product appear to be reversible in its thermolability (21-25). In this study, dnaA46 protein did not appear thermolabile when incubated at elevated temperatures prior to its addition to DNA synthesis reactions. When incubated in DNA synthesis reactions at temperatures greater than 30°C, its activity in DNA synthesis was reduced. Under similar conditions, the activity of wild type dnaA protein was stimulated. These results are consistent with the reversible thermolability of dnaA46 protein in vivo.

Binding of dnaA46 protein to a restriction fragment containing oriC was feeble in comparison with $dnaA^+$ protein. Prolonged incubation of dnaA46 protein with radioactively-labeled oriC DNA did not result in an increased binding to this DNA fragment (data not shown). In the absence of competitor DNA, the weak binding to the oriC-containing fragment at 30°C, the optimal temperature for its replication activity, was not diminished at 40°C. At the higher temperature, the replication activity of dnaA46 protein was severely reduced. If it is assumed that oriC binding activity also represents binding activity to other specific

sites including the dnaA promoter region, the observation that the binding of dnaA46 protein was not thermolabile contrasts with in vivo studies (29-31). These studies suggested that dnaA46 protein represses transcription of the dnaA gene at the permissive temperature by binding to sequences in the dnaA promoter region and that this binding is thermolabile.

Wild type dnaA protein was observed to bind ATP with high affinity (20). This chapter has confirmed these findings and that of less than stoichiometric binding of [alpha ^{32}P]ATP to dnaA^{+} protein. Although the reaction conditions for ATP binding to dnaA^{+} protein were as described (20), experiments with several preparations of dnaA^{+} protein resulted in ratios of [alpha ^{32}P]ATP to dnaA^{+} protein near 0.1. This discrepancy between the published value of 0.48 and that determined from our experiments has not been resolved. Under similar conditions, [alpha ^{32}P]ATP binding to dnaA46 protein could not be detected. UV-crosslinking experiments also suggested that dnaA46 protein was more than 100-fold reduced compared to dnaA^{+} protein in its ability to bind [alpha ^{32}P]ATP (data not shown).

The nucleotide substitution of the dnaA46 mutation (nucleotide 783) (32-33) resides in a coding region similar to a consensus amino acid sequence (A-type) found in proteins which bind adenine nucleotides (Table 1, 34-35). The missense mutation of dnaA46 predicts the replacement of an alanine residue with valine (amino acid 184) in the postulated ATP-binding domain (20). Although this alanine residue does not correspond in position to a conserved amino acid in the consensus sequence, the result that dnaA46 protein is defective in ATP binding

Table 1. Amino acid sequence comparison between a putative ATP binding domain of dnaA protein and other adenine nucleotide binding proteins.

Consensus Sequence	I/V/L-X-A/G-X-X-X-X-G-K-T-X-X-X-X-X-X-I/V
$dnaA^+$ protein	L-Y- G -G-T-G-L-G-K-T-H-L-L-H-A-V-G
dnaA46 protein	L-Y- G -G-T-G-L-G-K-T-H-L-L-H-V-V-G

Sequences of amino acid homology between $dnaA^+$ protein (residues 170 to 186), and the consensus sequence of adenine nucleotide binding protein (35) are underlined. Amino acid sequences were deduced from the nucleotide sequence (32-33) which predicts the replacement of alanine with valine (downward arrow) at residue 184 for the dnaA46 mutation.

indicates that this residue in dnaA$^+$ protein participates in ATP binding. The complex of dnaA protein with ATP has been reported to beits active form for DNA replication (20). Formation of a dnaA protein-ADP complex correlated with its inactivity in DNA replication. The ATP- or ADP- form of the dnaA protein complex, as well as free dnaA protein, bound with similar affinities to restriction fragments containing oriC. The observation shown here that dnaA46 protein was markedly reduced in binding to oriC can be attributed to the mutational alteration in the polypeptide and not due to its inability to bind ATP.

The addition of dnaA46 protein to reactions containing dnaA$^+$ protein did not interfere with DNA replication in a crude enzyme system (Chapter II, Figure 10), with oriC binding, or with ATP binding activity (at 0.5 uM [alpha ^{32}P]ATP) (data not shown). These results indicate that the relative inactivity of dnaA46 protein is not due to the presence of an inhibitor. The cooperative binding of dnaA$^+$ protein to oriC involving 20 to 30 molecules of dnaA protein is thought to be a key event in initiation of DNA replication (25,36-37). This binding in the presence of 5 mM ATP is postulated to locally unwind duplex DNA in the oriC region to allow binding of dnaB and dnaC proteins (9). The apparent lack of an additive or inhibitory effect on oriC binding in the presence of both dnaA$^+$ and dnaA46 protein suggests that mixed complexes do not form.

The activity of dnaA46 protein in DNA replication dependent on other replication proteins in a crude enzyme fraction contrasts with its inactivity in purified enzyme systems. This inactivity was also observed with partially purified fractions of dnaA46 protein (data not

shown). These results and the prolonged lag observed with dnaA46 protein prior to DNA synthesis led to consider whether a factor in the crude enzyme fraction was required for dnaA46 protein activity.

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CHAPTER IV

Interaction of dnaA46 Protein with dnaK Protein is Required prior to the Initiation of oriC Plasmid DNA Replication

INTRODUCTION

dnaA protein is required for *in vitro* replication of plasmids containing the *oriC* sequence in both crude and purified enzyme systems (Chapter II, Chapter III, 1-6). In the presence of ATP, preferential and cooperative binding of dnaA protein to *oriC* is followed by unwinding of of the AT rich region of *oriC* (7-10). This process has been shown to initiate *oriC* plasmid DNA replication *in vitro*. Despite the fact that the dnaA46 protein, purified as in Chapter II, exhibits a reduced binding affinity to *oriC*, no measurable ATP binding, and inactivity in a purified enzyme system for *oriC* plasmid replication, the mutant protein is active in a crude enzyme system. A pronounced lag was observed prior to DNA synthesis. This pronounced lag suggests a relative inefficiency of the mutant protein in some aspect of the initiation process of DNA replication. In this chapter, it is shown that the dnaA46 protein interacts with dnaK protein in a crude enzyme system for *oriC* plasmid replication.

The *dnaK* gene product is a protein with a molecular weight 72 KDa (11). Its cellular abundance is 1% of the total protein. dnaK protein has been suggested to be involved in *in vivo* chromosomal DNA replication, RNA metabolism, and protein synthesis (12-13). The pleiotrophic effect of mutations in *dnaK* hampered study of the role of dnaK protein in *in vivo* chromosomal DNA replication. Recently, a temperature-sensitive mutant, *dnaK111*, was isolated by mutagenesis (14).

The mutant is unable to initiate a new round of chromosomal DNA replication at nonpermissive temperature, but the mutation does not affect protein synthesis in rich medium. The temperature-sensitivity in the initiation of chromosomal DNA replication is phenotypically suppresed by a mutation in the rnh gene, which allows initiation to occur at non-oriC sites on chromosomal DNA (15-17). The temperature-sensitivity and the suppression by a mutation in rnh indicate that the dnaK gene product plays a role in initiation of chromosomal DNA replication at oriC.

dnaK protein is essential for initiation of bacteriophage lambda DNA replication in vivo and in vitro (11, 18-22). Electron microscopic studies of the initiation intermediate of in vitro lambda DNA replication suggested that dnaK protein and dnaJ protein allow dnaB helicase to unwind the replication origin bound by lambda O and lambda P proteins in the presence of SSB and ATP (21-22).

dnaK protein is a heat shock protein whose expression increases at elevated temperature (23). The protein participates in negative modulation of heat shock response. dnaK protein appears to phosphorylate itself in vitro (24). In vivo, dnaK protein and dnaJ protein appear to be involved in phosphorylation of glutamyl-tRNA synthetase and threonyl-tRNA synthetase (25).

This chapter describes the interaction of dnaA46 protein with dnaK protein and another factor(s). This interaction stimulates dnaA46 protein activity in oriC plasmid replication by reducing the pronounced lag before DNA synthesis. The reduction of the pronounced lag by the stimulation reaction and the thermolability of the interaction suggests

that the dnaA46 mutation results in a mutant protein which interacts inefficiently with dnaK protein or the unidentified factor(s). This may correlate with the asynchrony of the dnaA46 mutant in in vivo chromosomal DNA replication (26-27).

EXPERIMENTAL PROCEDURES

Reagents

Plasmids, commercial enzymes, chemicals and radiochemicals were as described in the Chapter II. N-ethylmaleimide (NEM), chloramphenicol, bovine pancreas trypsin (TPCK treated), soybean trypsin inhibitor, beta-galactosidase, glyceraldehyde 3-phosphate dehydrogenase and trypsinogen were from Sigma.

Bacterial strains

E. coli WM433 (relevant genotype, *dnaA204* and *dnaK*$^+$) is described in Chapter II. RLM483 *dnaK756*, *thr*$^-$, *thyA*, *lac*$^-$, *tonA*, *thy*$^-$, *supE44* (19); RLM896 (isogenic with RLM483 except *dnaK7*) (28); and RLM893 containing the dnaK gene cloned into a runaway plasmid pMOB45 (11) were obtained from Dr. Roger McMacken, Johns Hopkins University, Baltimore.

Enzymes

Highly purified DNA replication proteins were: dnaA$^+$ protein (Chapter II, $2x10^5$ unit/mg); dnaA46 protein (Chapter II, $6.8x10^4$ unit/mg) (Chapter II), and dnaK protein (fraction IV of this Chapter, or obtained from Dr. Roger McMacken, Johns Hopkins University, Baltimore)

Buffers

Buffer D contains 25 mM HEPES-KOH (pH 7.8), 0.1 mM EDTA and 2 mM DTT.

Partial purification of a protein which stimulates the activity of dnaA46 protein.

E. coli WM433 (relevant genotype, dnaA204) was grown to an OD at 595 of 0.6 to 0.8, lysed, and fractionated with ammonium sulfate as described (1). The resuspended precipitate (fraction II) was dialysed against 100 volumes of buffer containing 25 mM HEPES-KOH (pH 7.6), 0.1 mM EDTA, and 2 mM DTT to a conductivity equivalent to 0.4 M KCl, frozen in liquid nitrogen, and stored at -70°C. Fraction II was thawed on ice, diluted 2-fold in the above buffer, incubated at 55°C for 7.5 min and chilled on ice. Insoluble protein was removed by centrifugation at 15,000 rpm for 10 min at 4°C in a Sorvall SS-34 rotor. The supernatant was incubated at 55°C, chilled, and centrifuged as described above. This resultant supernatant was stimulatory protein fraction III.

Stimulatory protein fraction IIIs of RLM896 (relevant genotype, dnaK7), RLM483 (relevant genotype, dnaK756) and RLM893 (dnaK protein overproducing strain), grown in the presence of chloramphenicol (10 ug/ml) were prepared as described above. To induce production of dnaK protein at high temperature, the dnaK protein overproducing strain, RLM893, was grown in the presence of 10 ug/ml of chloramphenicol to an OD at 595 of 0.35 at 30°C, mixed with a same volume of the medium prewarmed at 50°C, then continued to grow for 3 hr at 40°C. Stimulatory protein fraction III of the induced RLM893 was prepared as described in the above.

Stimulation of dnaA46 protein by a prior incubation.

Assay method A. Stimulation of dnaA46 protein by an incubation prior to measuring its replication activity was in a 10 ul reaction volume containing 40 mM HEPES-KOH (pH 7.6), 11 mM MgSO4, 2 mM DTT, and unless indicated, 2 mM ATP, 7% (w/v) polyvinyl alcohol (PVA), 40 mM phosphocreatine, 1 ug of creatine kinase, 0.5 ug of dnaA46 protein, and 200 ug of fraction II or 20 ug of stimulatory protein fraction III from WM433. Reaction mixtures were assembled at 0°C, incubated at 30°C for 30 min unless indicated, and portions were added to DNA replication reactions to measure activity. Unless noted, 200 ng of dnaA46 protein incubated as described above (in 4 ul) was assayed in DNA replication reactions dependent on a crude enzyme fraction as described in Chapter II. This assay was used for the experiments performed in Figures 1-4, and Tables 1-2.

Assay method B. To quantitate the stimulatory activity in fraction III, the above stimulation and replication assays were modified as follows and used for the experiments in Figures 5-8, and Tables 3-4. Stimulation reaction mixtures (7.5 ul) containing 40 mM HEPES (pH7.6), 11 mM magnesium acetate, 2 mM DTT, 2 mM ATP, 40 mM phosphocreatine, 0.75 ug of creatine kinase, 7% (w/v) polyvinyl alcohol, 0.1 ug of dnaA46 protein, and 8 ug of stimulatory protein fraction III, unless indicated, were assembled in ice, incubated at 30°C for 30 min, then placed in ice. To measure DNA replication activity, reaction mixtures (17.5 ul) containing 40 mM HEPES-KOH (pH 7.6), 11 mM magnesium acetate, 2mM ATP, 0.714 mM each of CTP, UTP and GTP, 143 uM each of dATP, dCTP, dGTP and [^{3}H] dTTP (30 cpm/pmol), 7% (w/v) polyvinyl alcohol, 40mM phosphocreatine, 1.75 ug of creatine kinase, 200 ng supercoiled

M13oriC26 DNA and 200 to 250 ug of enzyme fraction II from WM433 were assembled at 0°C and added to the incubated stimulation mixture (7.5 ul). DNA synthesis was measured by incubation at 30°C for 20 min and the total nucleotide incorporation was measured as described in Chapter II.

RESULTS

Identification of a protein which stimulates dnaA46 protein

The activity of dnaA46 protein in the crude enzyme system, and its inactivity in either of the purified enzyme systems suggested that a factor(s) necessary for dnaA46 protein activity might be absent in the purified enzyme systems. Assays with crude enzyme fractions (an ammonium sulfate precipitate of a cell lysate) dependent on $dnaA^+$ protein indicated a lag of 3 to 5 min prior to the incorporation of deoxynucleotides (2-3, Figure 1). By comparison, DNA synthesis with dnaA46 protein was preceded by a prolonged lag of about 15-18 min. This lag may indicate the time interval required for interaction of dnaA46 protein with this factor present in the crude enzyme fraction. Based on this premise, experiments were performed to determine if a prior incubation with components of the crude enzyme system could reduce or abolish this prolonged lag. As indicated, prior incubation of dnaA46 protein with an enzyme fraction, PVA, ATP, and an ATP regenerating system, but in the absence of deoxynucleotides and an oriC plasmid, reduced the prolonged lag (Figure 1) and stimulated its activity about

Figure 1. The pronounced lag in DNA synthesis by dnaA46 protein is reduced by a prior incubation.

Assays were performed (Experimental Procedures) with 100 ng of $dnaA^+$ (o——o), dnaA46 (●——●), or dnaA46 protein treated by a prior incubation (Experimental Procedures) with an ammonium sulfate precipitate of a cell lysate, PVA, ATP, phosphocreatine, and creatine kinase (o- -o). DNA synthesis dependent on a crude enzyme fraction was initiated by incubation at 30°C. Reactions (25 ul) were stopped at the indicated times and acid insoluble radioactivity was determined.

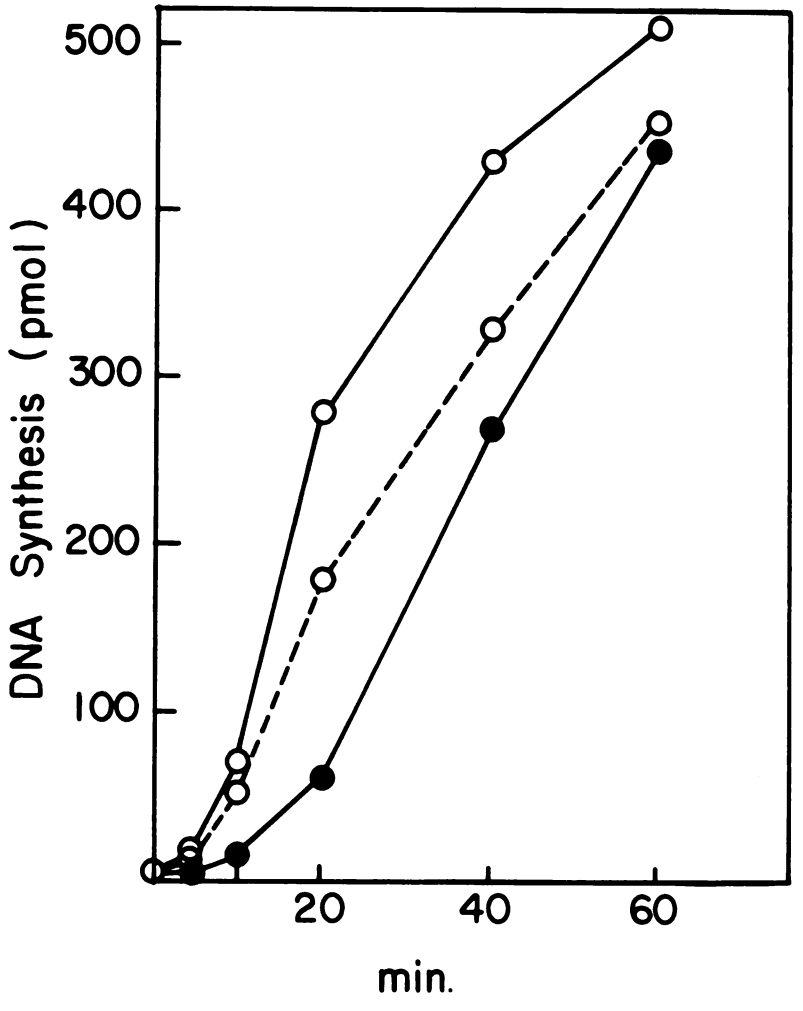
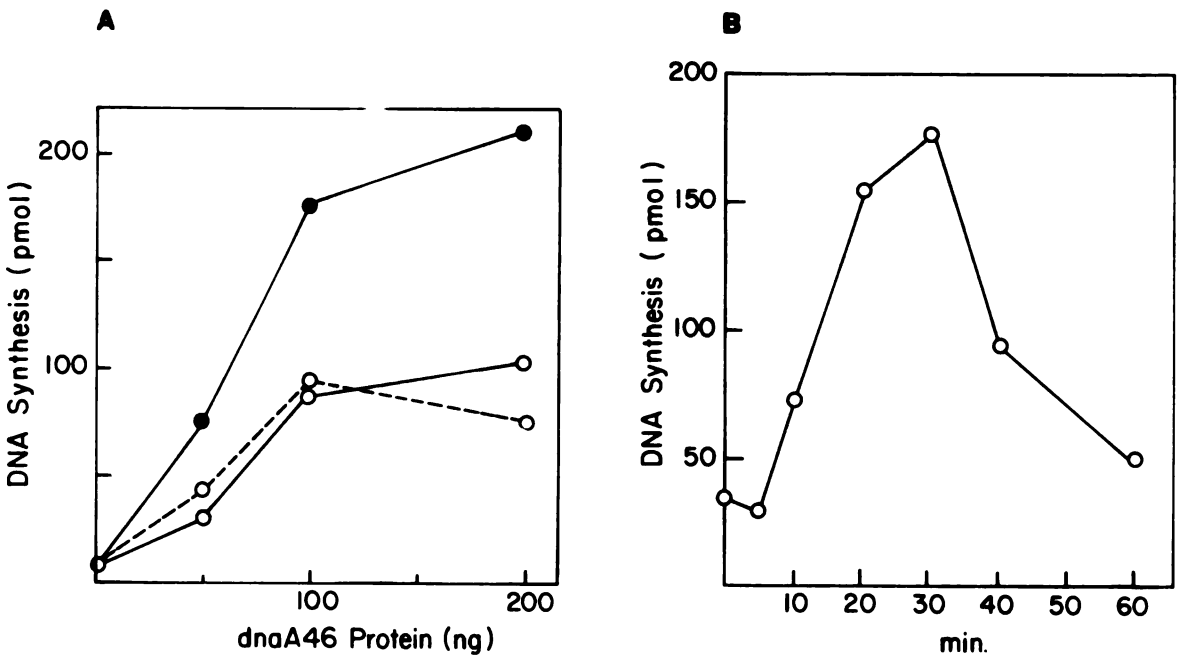

500
400
300
200
100
DNA Synthesis (pmol)
20
40
60
min.

Figure 2. Stimulation of dnaA46 protein activity requires a prior incubation with a crude protein fraction.

A. Assays were performed as described in Experimental Procedures with the indicated amounts of dnaA46 protein not treated (o——o), or treated by a prior incubation with PVA, ATP, phosphocreatine, and creatine kinase with (●——●) or without (o- - -o) the stimulatory protein fraction (fraction II). DNA synthesis was measured with a crude enzyme fraction deficient in dnaA protein activity.

B. Time-dependent stimulation of dnaA46 protein activity by a prior incubation. dnaA46 protein was incubated in 10 ul as described in Experimental Procedures with the stimulatory protein fraction (fraction II) for the times indicated and placed on ice. To measure DNA synthesis activity, 2 ul of the incubation mixture containing 100 ng of dnaA46 protein was added to DNA synthesis reactions containing a crude enzyme fraction deficient in dnaA protein activity.


A
DNA Synthesis (pmol)
200
100
100
200
dnaA46 Protein (ng)
B
DNA Synthesis (pmol)
200
150
100
50
10
20
30
40
50
60
min.

2-fold (Figure 2A). The initial rate of DNA synthesis with dnaA46 protein treated by a prior incubation was comparable to that observed with $dnaA^+$ protein (Figure 1).

Reaction components required to stimulate dnaA46 protein activity during this prior incubation were examined (Table 1). Components required for stimulation of dnaA46 protein included the enzyme fraction (fraction II), polyvinyl alcohol, ATP, and an ATP regenerating system provided by creatine kinase and phosphocreatine. In these experiments, the omitted component was included in the subsequent incubation of DNA synthesis. The inhibitory effect observed by omission of ATP or an ATP regenerating system has not been investigated. This stimulatory effect required dnaA46 protein. Comparable studies with $dnaA^+$ protein indicated that its activity was not stimulated by a prior incubation.

The optimum time required for stimulation of dnaA46 protein by this prior incubation was between 20 and 30 min (Figure 2B). This time roughly coincides with the prolonged lag period observed when dnaA46 protein was not treated by a prior incubation before its addition in DNA synthesis reactions. Longer periods of incubation resulted in a decrease in this stimulatory effect.

This factor which stimulates dnaA46 protein activity appears to be a protein. Experiments (Table 2) indicated that this stimulatory factor remains active upon incubation at 55°C for 15 min (Experimental Procedures, data not shown) but is inactivated by incubation at 70°C for 15 min. 20 ug of the stimulatory protein fraction III (treated at 55°C) replaced a requirement of 200 ug of the enzyme fraction II (Table II), indicating a 10-fold purification of this protein by heat treatment.

Table 1. Requirements for stimulation of dnaA46 protein by a prior incubation in the absence of DNA synthesis.

Component Omitted	DNA Synthesis (pmol)
None	139
Fraction II	21
PVA[a]	62
ATP	8
Phosphocreatine and creatine kinase	8
ATP, phosphocreatine, and creatine kinase	12
Fraction II, ATP, creatine kinase, and phosphocreatine	27
Fraction II, ATP, phosphocreatine, creatine kinase, and PVA	46

Prior incubation of dnaA46 protein was performed as described in Experimental Procedures. The indicated components were omitted from this prior incubation and included in DNA replication reactions dependent on a crude enzyme fraction deficient in dnaA protein activity.

a 7% PVA (final concentration) was replaced by 10% glycerol (final concentration). Incubations of DNA synthesis were for 20 min.

Table 2. A heat-labile, N-ethylmaleimide-sensitive protein is required for stimulation of dnaA46 protein activity.

Where indicated, incubation of dnaA46 protein prior to DNA synthesis was performed as described in Experimental Procedures with or without a protein fraction (fraction III) untreated or treated as described below. To measure DNA synthesis activity of dnaA46 protein after this prior incubation, 2 ul of the incubation mixture containing 100 ng of dnaA46 protein was added to DNA synthesis reactions containing a crude enzyme fraction as the source of other replication proteins (Experimental Procedures). Treatment of the protein fraction containing stimulatory activity included incubation at 70°C for 7.5 min. NEM sensitivity was determined by its addition to 5 mM, incubation at 30°C for 15 min, followed by addition of DTT to 50 mM to inactivate the NEM. As a control, DTT was added to 50 mM followed by addition of NEM to 5 mM and incubation at 30°C for 15 min. Trypsin sensitivity was determined by addition of trypsin to 0.25 mg/ml incubation at 0°C for 30 min, followed by addition of trypsin inhibitor to 1 mg/ml. As a control, trypsin inhibitor was added to 1 mg/ml followed by addition of trypsin to 0.25 mg/ml and incubation at 0°C for 30 min.

Table 2

Treatment of Protein Fraction (FrIII) in Prior Incubation	DNA Synthesis (pmol)
no prior incubation	90
FrIII absent	78
untreated	287
70°C for 7.5 min	87
NEM followed by DTT	87
DTT followed by NEM	266
trypsin followed by trypsin inhibitor	119
trypsin inhibitor followed by trypsin	272

Treatment of this partially purified fraction with N-ethylmaleimide(NEM) indicated that this factor is a protein with a free sulfhydryl group (Table 2). As a control, addition of dithiothreitol to the protein fraction prior to the addition of NEM protected against inactivation by this alkylating agent. This factor also appeared trypsin-sensitive. The addition of soybean trypsin inhibitor prior to trypsin treatment counteracted the inhibitory effect of trypsin.

Stimulation of dnaA46 protein activity is thermolabile

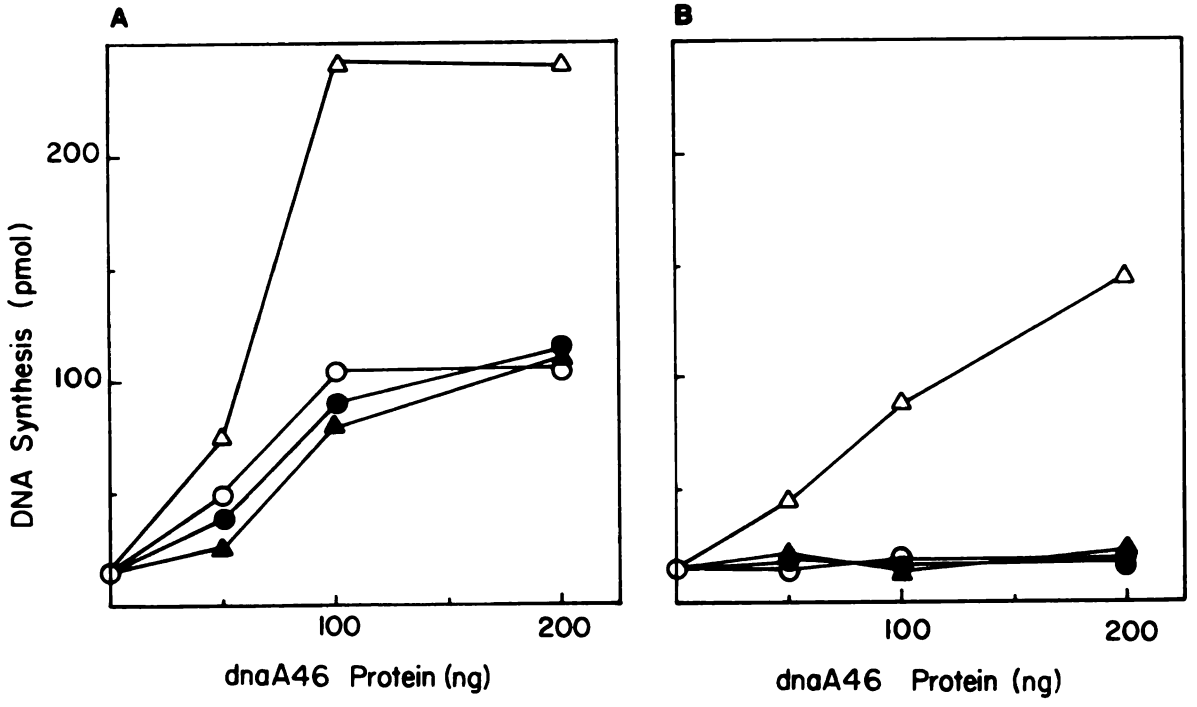
Incubation of dnaA46 protein at various temperatures prior to its addition to DNA replication reactions incubated at 30°C indicated that its activity was not dramatically affected by the temperature of this prior incubation (Chapter III). This result is in contrast with its thermolabile replication activity when added to DNA replication reactions incubated at elevated temperatures (Chapter III).

Experiments were performed to determine whether the stimulation of dnaA46 protein by a prior incubation was thermolabile (Figure 3). dnaA46 protein treated by a prior incubation at 30°C in the presence of required components was stimulated in its replication activity (Figure 3A). In contrast, dnaA46 protein not treated by a prior incubation or incubated in the absence of the partially purified protein fraction was not stimulated. The stimulatory effect was not observed by prior incubation of dnaA46 protein at 40°C. The activity remaining after this 40°C incubation is consistent with the reversible thermolability of the dnaA46 gene product observed in vivo (29-32).

Experiments were conducted in parallel in which the incubation

Figure 3. Stimulation of dnaA46 protein by a prior incubation is thermolabile.

dnaA46 protein was not treated (o), or treated by a prior incubation at 30°C (Δ) or at 40°C (▲) with the stimulatory protein fraction (fraction III). As a control, dnaA46 protein was incubated at 30°C without the stimulatory protein fraction (●). Incubations at this step were for 20 min. The indicated amounts of dnaA46 protein, treated as described above or untreated, were added to DNA synthesis reactions containing a crude protein fraction deficient in dnaA protein activity. DNA replication reactions were incubated at 30°C (A) or 40°C (B).


A
B
200
100
DNA Synthesis (pmol)
100
200
dnaA46 Protein (ng)
100
200
dnaA46 Protein (ng)

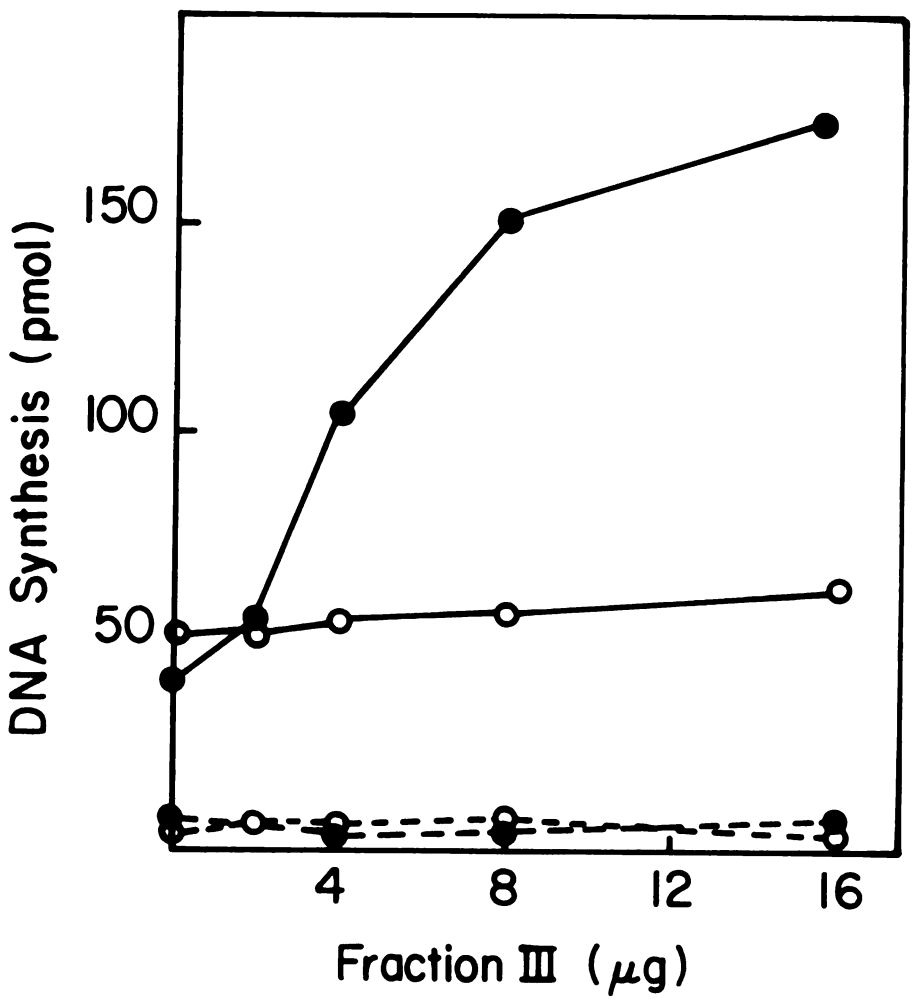
temperature for DNA synthesis was at 40°C (Figure 3B). As observed previously (Chapter III), little, if any, DNA synthesis occurred upon addition of dnaA46 protein not treated by a prior incubation. Treatment of dnaA46 protein by a prior incubation at 40°C, or at 30°C without the stimulatory protein fraction also resulted in background levels of DNA synthesis. However, a prior incubation of dnaA46 protein at 30°C with necessary components before its addition to DNA replication reactions incubated at 40°C resulted in measurable levels of DNA synthesis. Taken together, these results suggest that a thermolabile defect of the dnaA46 mutation resides at the step of stimulation of its activity.

dnaK protein is required for the stimulation of dnaA46 protein

In order to purify the stimulatory factor(s) in the protein fraction, a standard assay was set up for its purification (Experimental Procedure, assay method B). Addition of the stimulatory protein (fraction III) to the stimulation reaction containing dnaA46 protein increased DNA synthesis, indicating that the increase in DNA synthesis is dependent upon the stimulatory protein(s) in the fraction III (Figure 4). Prior incubation of the fraction in the absence of dnaA46 protein and its subsequent addition with dnaA46 protein to the DNA replication reaction was not stimulatory. In the absence of dnaA46 protein, no DNA synthesis was measurable. M13ΔE101 RF DNA which lacks the oriC sequence was inactive as a template in contrast to M13oriC26 RF DNA containing the oriC region (9,33-34). These results indicate that DNA synthesis is dependent on dnaA46 protein and the oriC sequence.

Figure 4. Stimulation of dnaA46 protein by fraction III.

Stimulation and DNA replication assays were performed as described in assay method B (Experimental Procedure). The indicated amounts of the stimulatory protein (fraction III) were added in the stimulation assay. 100 ng of dnaA46 protein was added to the stimulation assay (●——●), or the DNA replication assay (o——o). No dnaA46 protein was added (●- - -●). 100 ng of dnaA46 was added to the stimulation assay and M13oriC26 DNA in the replication assay was replaced by M13ΔE101 DNA (o- - -o).


DNA Synthesis (pmol)
150
100
50
4
8
12
16
Fraction III (μg)

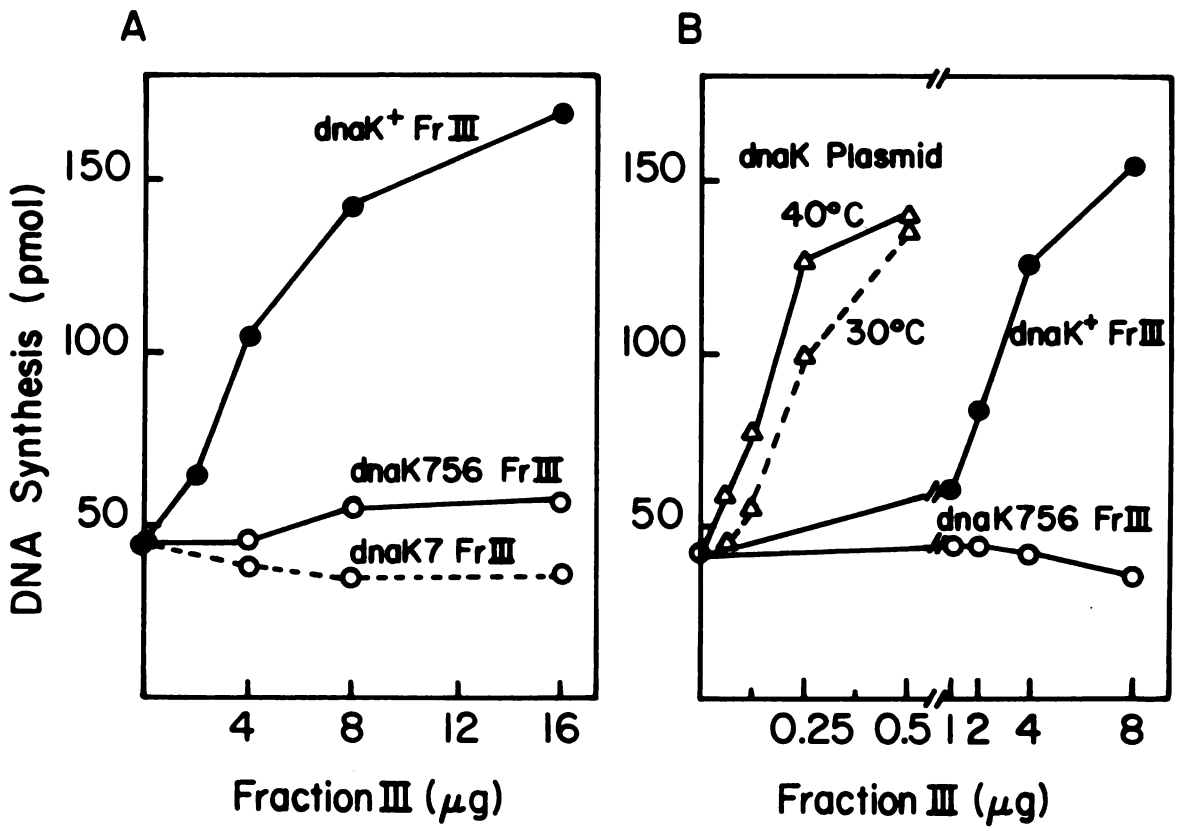
The assay of stimulation of dnaA46 protein in replication was used to purify the stimulatory protein(s). During the purification of the stimulatory protein from extracts of WM433, it was found that at least two factors are required for this stimulation. One of the factors seemed to be dnaK protein (11) based on 1) chromatographic properties, 2) molecular weight in SDS-polyacrylamide gel electrophoresis, and 3) cellular abundance (1%) of the protein (data not shown).

The possibility that dnaK protein is the stimulatory factor was confirmed by use of protein fractions prepared from dnaK mutants and a dnaK protein overproducing strain (Figure 5). The dnaK mutants, dnaK7 and dnaK756, exhibit phenotypes of temperature-sensitivity in growth and a deficiency in replication of bacteriophage lambda (19,28). Whereas the stimulatory protein fraction III prepared from WM433 (relevant genotype, $dnaK^+$) was active in stimulation of dnaA46 protein (Figure 4, Figure 5A), the fraction IIIs from dnaK mutants, RLM896 (relevant genotype, dnaK7) and RLM483 (relevant genotype, dnaK756), were unable to stimulate dnaA46 protein (Figure 5A).

The stimulation reactions in Figure 5B contain an enzyme fraction (fraction III) from the dnaK7 mutant which itself can not stimulate dnaA46 protein in DNA replication (Figure 5A) but provides another unidentified factor(s) necessary for the stimulation of dnaA46 protein (refer the next section). The inability of this protein fraction III from the dnaK7 mutant to stimulate dnaA46 protein in DNA replication is efficiently complemented by 20 to 30 fold less protein (fraction III) prepared from a dnaK protein overproducing strain, RLM893, than (fraction III) of stimulatory protein fraction III from a non-

Figure 5. Function of dnaK is required for stimulation of dnaA46 protein.

Stimulation and DNA replication assay was performed as described in assay method B (Experimental procedure). A. Stimulation assay was performed with the indicated amount of stimulatory protein (fraction III) prepared from: WM433 (relevant genotype, dnaA204 and $dnaK^+$), ●——●; RLM483 (relevant genotype, $dnaA^+$ and dnaK7), o- - -o; or, RLM896 (relevant genotype, $dnaA^+$ and dnaK756), o——o. B. In the presence of 8 ug of the stimulatory protein (fraction III) of RLM483, the stimulation assay was performed with the indicated amount of stimulatory protein (fraction III) prepared from WM433 (●——●), RLM896 (o- - -o), RLM893 (dnaK protein overproducing strain) grown at 30°C (Δ- - -Δ), or RLM893 induced at 40°C (Δ——Δ) for 3 hr as described in Experimental Procedures.


A
B
DNA Synthesis (pmol)
150
100
50
dnaK+ FrIII
dnaK756 FrIII
dnaK7 FrIII
4
8
12
16
Fraction III (μg)
dnaK Plasmid
40°C
30°C
dnaK+ FrIII
dnaK756 FrIII
0.25
0.5
1
2
4
8
Fraction III (μg)

overproducing dnaK$^+$ strain (WM433) (Figure 5B). The dnaK protein overproducing strain, RLM893, harbors a multicopy plasmid containing the dnaK gene in a runaway vector pMOB45 (11). The copy number of the plasmid in cell increases at elevated temperature, resulting in higher levels of protein by a gene dosage effect. The enzyme fraction prepared from the dnaK protein overproducing strain induced at 42°C sustained the same level of the stimulation with a lower amount of the fraction compared to the fraction prepared from the same strain grown at 32°C. Under comparable conditions, the protein fraction from the dnaK756 mutant was unable to stimulate dnaA46 protein when added to protein fraction IIIs from the dnaK7 mutant.

A stimulatory activity was purified from the dnaK protein overproducing strain induced at 42°C. The assay to detect activity required the protein fraction III from the dnaK7 mutant, dnaA46 protein, and the stimulatory protein to be assayed in the stimulation mixture (Table 3). The purified protein obtained in the final step by Mono Q chromatography was near homogeneous with a molecular weight of 72 KDa in a SDS-polyacrylamide gel electrophoresis (Figure 6). Its mobility was identical with the dnaK protein obtained from another laboratory (Figure 6). Addition of the purified 72 KDa protein in a stimulation reaction allowed the fraction III from the dnaK7 mutant to be active in stimulation of dnaA46 protein (Figure 7A). Authentic dnaK protein (Figure 6) exhibited an identical activity with the 72 KDa protein (data not shown). Thus, it was concluded that the 72 KDa protein is dnaK protein and is required for the stimulation of dnaA46 protein in *in vitro* oriC plasmid DNA replication. In addition, the procedure in Table

Table 3. Purification of dnaK protein.

dnaK protein overproducing strain, RLM893, were grown at 32°C in 350 ml of LB medium containing 10 ug/ml chloramphenicol to an OD at 595 nm of 0.35, mixed with a same volume of the medium prewarmed at 50°C, continued to be grown for 3 hr at 42°C, and harvested by centrifugation for 15 min in a Sorvall GSA rotor at 6,000 rpm at 2°C. The cell paste was resuspended in buffer containing 25 mM HEPES-KOH (pH 7.8), 1 mM EDTA, and 2 mM DTT to an OD at 595 nm of 150-200, frozen in liquid nitrogen and stored at -80°C. Protein fraction II and III were prepared as described in Experimental Procedures. The fraction III was diluted with Buffer A (Chapter I) to a conductivity equivalent to Buffer A containing 0.05 M KCl, and applied to a Mono Q HR 5/5 column (Pharmacia) equilibriated in Buffer A containing 0.05 M KCl. The column was washed with 3 ml of Buffer A containing 0.05 M KCl, and proteins were eluted with a linear gradient (20 ml) of 0.05 M to 0.6 M KCl. The stimulatory activity eluted at about 0.4 M KCl, and a profile of the activity was coincident with that of 72 KDa protein in SDS-polyacrylamide gel electrophoresis (data not shown).

Table 3. Purification of dnaK protein.

		Volume (ml)	Protein (mg)	Activity (10^{-3}U)	Specific Activity (10^{-6}U/mg)	Yield (%)
II	$(NH_4)_2SO_4$	0.1	11.2	114.2	10.2	100
III	55°C	0.2	5.6	94.0	16.8	82.3
IV	Mono Q	1.0	2.5	68.0	27.2	59.6

Stimulation and DNA replication assays were performed as described in assay method B (Experimental Procedure). Stimulation assay contained 8 ug of stimulatory protein (fraction III) of RLM483. One unit of activity was defined as one pmol of DNA synthesis increased by dnaK protein.

Figure 6. SDS-polyacrylamide gel electrophoresis of dnaK protein.

Samples were denatured and electrophoresed in a 10% SDS-polyacrylamide slab gel. Proteins were detected by staining with coomassie brilliant blue. Lane 1, 4 ug of the dnaK protein obtained from Dr. Roger McMacken; 2, fraction II (109 units); 3, fraction III (109 units); and 4, 4 ug (109 units) of dnaK protein (fraction IV). Molecular standards (KDa) were beta-galactosidase, 116; bovine serum albumin, 68; dnaA protein, 52; ovalbumin, 45; glyceraldehyde 3-phosphate dehydrogenase, 36; and trypsinogen, 24.

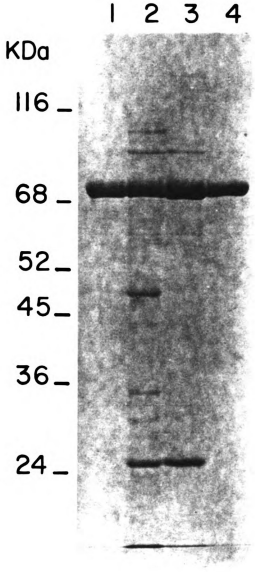
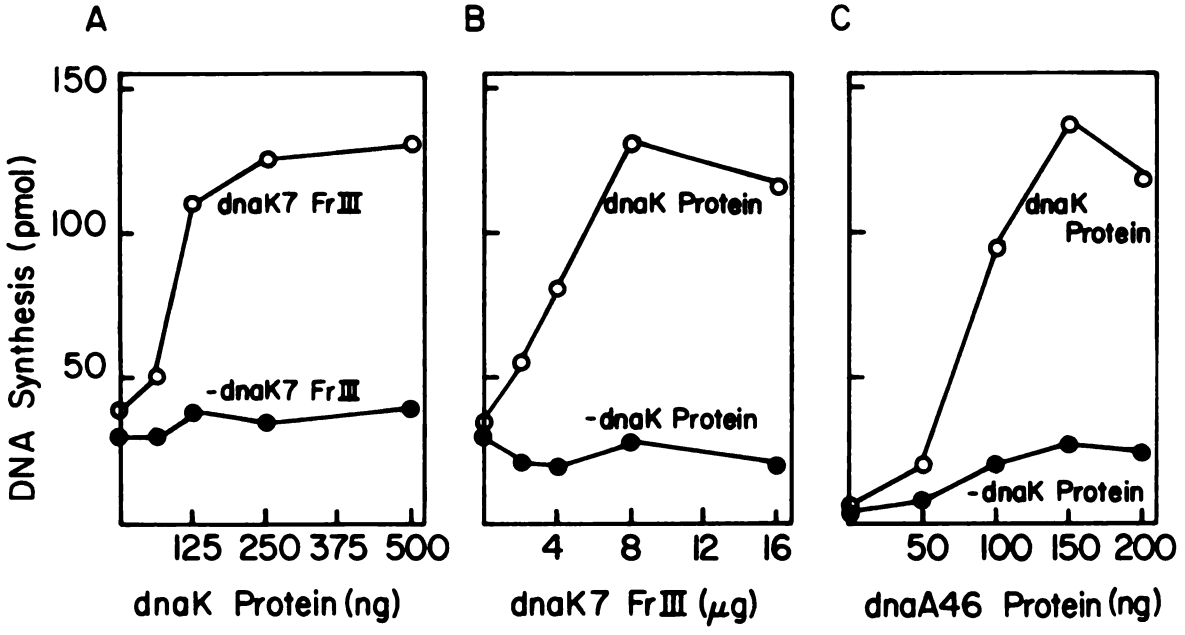

1 2 3 4
KDa
116
68
52
45
36
24

Figure 7. Requirements for stimulation of dnaA46 protein.

Stimulation and DNA replication assays were performed as described in assay method B (Experimental Procedure). The stimulation assay contained 100 ng of dnaA46 protein, 250 ng of dnaK protein (fraction IV in Table III) and 8 ug of stimulatory protein (fraction III) of RLM483 unless indicated. A, dnaK protein was titrated in the stimulation assay with 8 ug of stimulatory protein (fraction III) of RLM483 (o——o) or without (●——●). B, stimulatory protein (fraction III) of RLM483 was titrated in the stimulation assay with 250 ng of dnaK protein (o——o) or without (●——●). C, dnaA46 protein was titrated in the stimulation assay with 250 ng of dnaK protein (o—o) or without dnaK protein (●—●).


A
B
C
150
100
50
DNA Synthesis (pmol)
dnaK7 FrIII
-dnaK7 FrIII
dnaK Protein
-dnaK Protein
dnaK
Protein
-dnaK Protein
125 250 375 500
dnaK Protein (ng)
4 8 12 16
dnaK7 FrIII (μg)
50 100 150 200
dnaA46 Protein (ng)

III optimizes the purification of dnaK protein compared to the reported procedure which requires 7 steps of the purification from the same strain (11).

Interaction of dnaA46 protein with dnaK protein and another factor(s)

Addition of dnaK protein to a stimulation reaction containing 100 ng of dnaA46 protein and 8 ug of the fraction III from the dnaK7 mutant increased DNA synthesis in DNA replication reaction in contrast to no enhancement of DNA synthesis in its absence (Figure 7A). In the presence of the stimulatory fraction, 125 to 250 ng (1.7 to 3.5 pmol) of dnaK protein (fraction IV) exhibited a maximal stimulation with 100 to 150ng (1.9 to 2.9 pmol) of dnaA46 protein, suggesting a possible stoichiometric interaction of dnaA46 protein with dnaK protein (Figure 7A,C). Addition of the stimulatory fraction to a stimulation reaction containing dnaA46 protein increased DNA synthesis and was dependent on dnaK protein (Figure 7B). dnaK protein alone was insufficient in stimulation of dnaA46 protein. These results indicate the requirement for a factor(s) provided by the stimulatory fraction III which is not dnaK protein.

Requirements for the stimulation of dnaA46 protein was determined by omission of purified dnaK protein, the protein fraction III from the dnaK7 mutant, or dnaA46 protein in the stimulation assay, and by subsequent addition of the omitted component to reactions of DNA replication (Table 4). Components omitted in stimulation reaction were replaced by bovine serum albumin in the same buffer as that for the omitted component. Stimulation reactions were incubated at 30°C for 30

min, then added to DNA replication reactions to measure the level of stimulation (Table 4). Maximal DNA synthesis was obtained with dnaA46 protein, dnaK protein and the protein fraction from the dnaK7 mutant. These results indicate that the interaction of dnaA46 protein with the stimulatory factor(s) occurs prior to initiation of DNA replication as described in Figure 3 of Chapter I. This is consistent with the observations shown in (Figure 4, Figure 5, and Figure 7 of this chapter). Residual activity of 21 to 44 pmol shown in the absence of the stimulatory factors is dependent upon dnaA46 protein (Table 4) and the oriC sequence (Figure 4). This may be due to interaction of dnaA46 protein with the corresponding components in a crude enzyme fraction of E. coli WM433 during the stage of DNA replication (see Experimental Procedure).

dnaK protein stimulates oriC replication dependent on a crude enzyme from a dnaK mutant

DNA synthesis with a crude enzyme fraction from WM433 (relevant genotype, dnaA204, $dnaK^+$) is dependent upon addition of purified dnaA protein (Figure 8, Chapter II). Addition of purified dnaK protein did not affect DNA synthesis in the presence or absence of the purified $dnaA^+$ protein. If dnaK protein is required for oriC plasmid replication, DNA synthesis with extracts prepared from a dnaK mutant might show a dependence for dnaK protein. Enzyme fraction was prepared from RLM483 (relevant genotype, $dnaA^+$, dnaK756) and used for oriC plasmid replication (Figure 8). 35 pmol of residual DNA synthesis was observed. Addition of purified dnaK protein increased DNA synthesis

Table 4. Interaction of dna46 protein with dnaK protein and another unidentified factor(s) occurs prior to oriC replication.

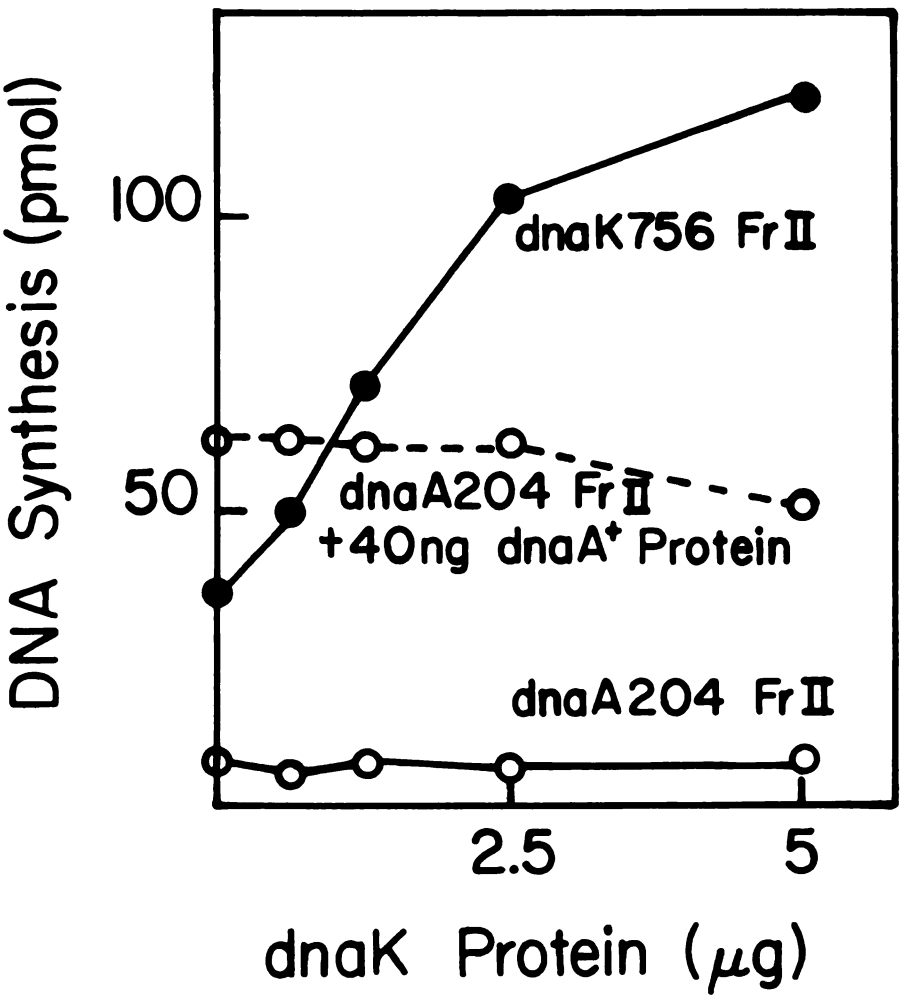
Component Omitted	DNA Synthesis (pmol)
None	126
Fraction III of dnaK7	21
dnaK protein	36
dnaA46 protein	44
dnaK protein, and fraction III of dnaK7	26
dnaA46 protein, dnaK protein, and fraction III of dnaK7	40
dnaA46 protein, dnaK protein, and fraction III of dnaK7[a]	4.6

Stimulation and DNA replication assays were performed as described in Experimental Procedure. The omitted components from the stimulation assay were replaced by the same amount of bovine serum albumin, and the omitted components were included into the DNA replication assay.

a The omitted components were not included into DNA replication assay.

Figure 8. dnaK protein is required for oriC replication with the crude enzyme fraction II of dnaK756 mutant.

Preparation of soluble enzyme fraction II from RLM896 and crude enzyme system of DNA replication assay were performed as described in Experimental Procedure of Chapter II. The indicated amount of dnaK protein was included into the crude enzyme system of oriC replication with crude enzyme fraction II: of RLM893 (●——●); and, of WM433 in the presence of $dnaA^+$ protein (40 ng) (o- - -o), or in the absence of $dnaA^+$ protein (o——o).


DNA Synthesis (pmol)
100
50
dnaK756 FrII
dnaA204 FrII
+40ng dnaA+ Protein
dnaA204 FrII
2.5
5
dnaK Protein (μg)

proportionally (Figure 8). DNA synthesis in the presence or absence of the purified dnaK protein was dependent upon the oriC sequence (data notshown). The residual synthesis may be due to weak residual activity of the dnaK756 mutant protein in the crude enzyme fraction. This requirement for dnaK protein in oriC plasmid replication dependent on an crude enzyme fraction of a dnaK mutant suggests that dnaK protein is required for oriC replication.

DISCUSSION

dnaA46 protein, whose function is temperature-sensitive in in vivo chromosomal DNA replication, has been purified and characterized in comparison with $dnaA^+$ protein. This mutant form of dnaA protein which has a reduced affinity for oriC and no observable binding to ATP is active in the crude enzyme system for oriC plasmid replication with a prolonged lag before incorporation of dNTPs. This contrasts with its inactivity in the purified enzyme system. This led to the consideration of whether a factor(s) in the crude enzyme fraction was required for dnaA46 protein activity in the purified system.

Fractions II and III have been shown to reduce the prolonged lag in DNA synthesis dependent on dnaA46 protein when dnaA46 protein was incubated with these stimulatory protein fractions prior to its function in DNA synthesis. The incubation time optimal for stimulation of dnaA46 protein coincided approximately with the prolonged lag period in DNA synthesis observed with untreated dnaA46 protein. This time interval is

apparently required for interaction of dnaA46 protein with these stimulatory factors.

The stimulation reaction requires dnaA46 protein, polyvinyl alcohol, ATP, and an ATP generating system provided by creatine phosphate and creatine kinase in addition to the stimulatory protein factors. The ATP requirement in stimulation suggests that the interaction of dnaA46 protein with the stimulatory factors is mediated by binding of ATP or by hydrolysis of ATP.

An increase in incubation temperature of DNA replication reactions stimulated the replication activity of $dnaA^+$ protein (Chapter III). In contrast, temperatures greater than 30°C reduced DNA synthesis activity of dnaA46 protein to near background levels. The effect of temperature on the interaction between these stimulatory factors and dnaA46 protein was examined. A prior incubation of dnaA46 protein with necessary components at 30°C, followed by its addition to DNA replication reactions at 40°C resulted in substantial DNA synthesis. In contrast, prior incubation at 40°C followed by addition to DNA replication reactions at 30°C resulted in no stimulation of dnaA46 protein activity. These results suggest that an event prior to initiation of DNA replication by dnaA46 protein is thermolabile.

The factors which stimulate dnaA46 protein in oriC replication have been identified from a crude enzyme fraction. Several lines of evidence have demonstrated that one of the factors is dnaK protein: 1) protein fractions prepared from dnaK mutants were inactive in stimulation of dnaA46 protein; 2) protein fractions from a dnaK protein overproducing strain contain 20 to 30 fold more stimulatory activity

than that of a nonoverproducing strain; 3) mobility of the protein purified by the stimulation assay as 72 KDa in SDS-polyacrylamide gel electrophoresis is identical with that of the dnaK protein obtained from another laboratory; and, 4) dnaK protein obtained from another laboratory contains an identical stimulatory activity with the stimulatory protein.

Also, it has been observed that another factor(s) which is present in partially purified protein fractions and distinct from dnaK protein is required for stimulation. This requirement was deduced from the observation that a constant amount of stimulatory protein fraction from a dnaK mutant was required even with an excess amount of dnaK protein (2.5 ug) which is 10 fold more than the saturating level (data not shown). The stimulation reaction was separated from the components which sustain DNA replication which include oriC plasmid DNA; CTP, GTP and UTP for primer synthesis; and, dNTPs for DNA polymerization. This indicates that the interaction of dnaA46 protein with dnaK protein and the unidentified factor(s) occurs prior to binding of dnaA46 protein to the oriC sequence. This binding has been shown to be an early step in the initiation process of in vitro oriC replication (7-10).

At this point, the in vitro effect of dnaK protein on $dnaA^+$ protein has only been demonstrated in the observation that the addition of the purified dnaK protein increases oriC replication dependent on an crude enzyme fraction prepared from a dnaK756 mutant. Under the stimulation conditions for dnaA46 protein, prior incubation of $dnaA^+$ protein failed to stimulate oriC replication in the crude enzyme system or in the purified enzyme system (data not shown). Also, addition of

dnaK protein and fraction III from dnaK mutants to the purified enzyme system did not affect oriC replication with $dnaA^+$ protein (data not shown). Prior incubation of dnaA46 protein with required components followed by its addition to a purified enzyme system which sustains oriC replication resulted in only 20 to 30 pmol of DNA synthesis (data not shown). This contrasts with an extent of 300 to 400 pmol of DNA synthesis upon addition of $dnaA^+$ protein (Chapter III). This result suggests that another component(s) is required for oriC replication by dnaA46 protein in the purified enzyme system.

The enzyme system which sustains bacteriophage lambda DNA replication shares many similarities with the enzyme system for oriC replication (11, 20-22). Exceptions include the requirements for lambda O and P proteins, dnaK and dnaJ protein, and a plasmid DNA containing the origin of lambda DNA replication. Lambda replication is independent of dnaA protein. dnaJ protein (20-22) and grpE protein (35) appear to interact with dnaK protein in the initiation of lambda DNA replication. Replacement of fraction III from the dnaK7 mutant in the stimulation assay with dnaJ or grpE protein resulted in the inability to stimulate DNA synthesis by dnaA46 protein (data not shown). However, the possibility can not be eliminated that another factor in addition to dnaJ or grpE protein is required for the stimulation. With regard to the requirement for dnaK protein, about 3 ug of the purified dnaK protein is required for lambda DNA synthesis (11). This is similiar to levels required to saturate oriC replication (Figure 8). This amount is also consistent with the amount of $dnaK^+$ protein present in the crude enzyme fraction from WM433 (relevant genotype, dnaA204 and $dnaK^+$) which

is used for oriC replication (data not shown). In contrast, about 0.25 ug of dnaK protein is required to saturate the stimulation assay containing 100 ng of dnaA46 protein (Figure 7A). Separation of stimulation from DNA replication may result in a reduced requirement for dnaK protein. While these results indicate a requirement for dnaK protein at an early stage of oriC replication, it may also participate at a later stage.

At the permissive temperature, the dnaA46 mutant exhibits different phenotypes compared to $dnaA^+$ strain. These include asynchrony of chromosomal DNA replication (26-27) and reduction in a ratio of DNA content to cell mass (36-37), suggesting that the coordination between DNA replication and cell growth is aberrant. This may relate to the observation that in vitro oriC plasmid replication dependent on dnaA46 protein exhibits a pronounced lag before DNA synthesis. The pronounced lag and the reduction of the lag by prior incubation of dnaA46 protein with dnaK protein and another unidentified factor(s) suggest that the alteration in dnaA46 protein results in an inefficient interaction with dnaK protein and the unidentified factor(s).

PERSPECTIVES

dnaA protein is essential for E. coli chromosomal DNA replication in vivo (38-40) and appears to be involved in the timing of initiation of chromosomal DNA replication during the cell cycle (26-27). Also, dnaA protein plays a key role in the initiation process in vitro (1-10).

Abundance and continuous synthesis of dnaA protein during stages of cell growth (41-42) may suggest a requirement for modulation of its activity to initiate replication at a specific time during the cell cycle. Based on studies presented here, it is speculated that the influence of dnaK protein and a stimulatory factor(s) on dnaA46 protein also applies to $dnaA^+$ protein. This interaction of dnaA46 protein with dnaK protein and another stimulatory factor(s) occurs prior to its action in DNA synthesis. If this represents the mode of action of $dnaA^+$ protein, this modulation occurs prior to its function in initiation. The regulation could occur at the step of interaction of dnaA protein with dnaK protein and the unidentified factor(s), although interaction of $dnaA^+$ protein with the stimulatory factors remains to be demonstrated. Identification of the unidentified factor(s) will provide insights in the mechanism of the interaction with dnaA46 protein.

Identification of the unidentified factor(s)

A stimulation assay containing dnaA46 protein and dnaK protein will be used for purification of the unidentified factor(s). Addition of the chromatographic fractions containing the factor stimulates oriC replication in the crude enzyme system as shown in Figure 8B. Chromatographic properties, molecular weight in SDS-polyacrylamide gel electrophoresis, partial amino acid sequence of the purified protein and biochemical characterization may result in its correlation to a known protein.

Postulated mechanisms for the interaction of dnaA46 protein with its stimulatory factors

1. Covalent modification of dnaA protein.

It has been demonstrated that phosphorylation regulates the function of proteins in prokaryotes and eukaryotes. dnaK protein exhibits autophosphorylation activity *in vitro* (24). dnaK and dnaJ proteins participate in the *in vivo* phosphorylation of glutamyl-tRNA synthetase and threonyl-tRNA synthetase (25). It is possible that dnaK protein modifies the activity of dnaA46 protein by phosphorylation. Experiments to radioactively label $dnaA^+$ protein with [gamma ^{32}P] ATP and dnaK protein *in vitro*, or to detect $dnaA^+$ protein as a phosphoprotein from overproducing strains grown in $^{32}PO_4$-media have not been successful (data not shown). Despite these negative results, suggestive evidence of a modification may be obtained by comparison of $dnaA^+$ protein activity before and after alkaline phosphatase treatment. A comparison of $dnaA^+$ protein activity isolated from *dnaK* wild type and mutant strains is in progress (Carr, K.M. and Kaguni, J.M.).

2. Formation of a protein complex.

Some *E. coli* replicative proteins form a complex with other proteins in order to function in DNA replication. For example, dnaB protein forms a complex with dnaC protein in the presence of ATP. This complex functions in the replication of *oriC* plasmids and SSB-coated ϕX174 ss DNA (43-45). The binding of n' protein to the origin of SSB-coated ϕX174 ss DNA is mediated by the proteins, n and n'' (46-48).

Possible stoichiometric interaction (1:1 molar ratio) of dnaA46 protein with dnaK protein (Figure 7A) suggests the formation of a complex between the two proteins.

Experiments can be designed to examine the following: 1) that the complex may be separated by glycerol gradient sedimentation or gel permeation chromatography; 2) that binding domains of the complex may be protected from proteases capable of cleaving accessible peptide bonds which are occluded by complex formation.

3. Requirement for interaction with stimulatory factors is restricted to dnaA46 protein.

The purified enzyme system for oriC plamid replication does not depend on $dnaK^+$ protein, and $dnaA^+$ protein is active in this system (Chapter III, 4-6). Addition of dnaK protein, or $dnaA^+$ protein which was incubated under the stimulation conditions for dnaA46 protein did not result in a stimulation of DNA synthesis in the purified enzyme system when added (data not shown). This may suggest that the interaction with the stimulatory factors is required only for dnaA46 protein in order to restore the defect in DNA replication. However, several observations suggest an interaction of $dnaA^+$ protein with dnaK protein: 1) the dnaK111 mutant harboring $dnaA^+$ has a defect in initiation of chromosomal DNA replication (14); 2) a crude enzyme fraction prepared from a dnaK756 mutant requires the addition of purified dnaK protein for active oriC plasmid replication (Figure 8); 3) by analogy to the requirement of E. coli dnaK protein for the initiation of lambda DNA replication (11,18-22), a role for dnaK protein in

initiation of *E. coli* chromosomal DNA replication is suggested; 4) the purified system requires different levels of proteins or addition of certain proteins under the condition of reaction (5-6), and the proteins which have been suggested to function in *in vivo* chromosomal DNA replication do not affect DNA synthesis in the system.

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SUMMARY

The alleles of dnaA5, dnaA46, and dnaA204 from the E. coli chromosome have been cloned into a protein overproducing vector. Under conditions of induction, similiar levels of these mutant gene products were observed in maxi-cell experiments. The activities of the dnaA mutant proteins were measured from ammonium sulfate precipitated soluble extracts of induced strains containing plasmids pDS105 (dnaA5), pDS215 (dnaA46), and pDS319 (dnaA204). Assays using a crude enzyme system for oriC plasmid replication which is specific for dnaA protein demonstrated low but detectable dnaA protein activity from dnaA5 and dnaA46 protein overproducing clones. No activity was detected from strains which overproduced the dnaA204 protein.

dnaA46 protein was purified from an overproducing strain based on its replication activity in a crude enzyme system. Steps in purification from a soluble extract included ammonium sulfate precipitation, heparin-sepharose 4B chromatography, hydroxylapatite chromatography, and selective precipitation by reducing the ionic strength of the hydroxylapatite fraction. Replication activity of dnaA46 protein depended upon plasmids containing the oriC sequence. Compared to the wild type protein, DNA synthesis dependent upon dnaA46 protein showed a pronounced lag before incorporation of deoxynucleotides.

The activity of dnaA46 protein did not appear to be thermolabile upon preincubation at various temperatures. However, thermolability was observed under conditions of coupled DNA synthesis. This result is consistent with reversible inactivation of dnaA46 activity *in vivo*. dnaA46 protein exhibited reduced binding affinity to DNA fragments containing the *oriC* sequence compared to $dnaA^+$ protein. This binding by dnaA46 protein was not decreased at 40°C. Binding of ATP to dnaA46 protein was not measurable. Whereas the mutant protein activity could be measured in a crude enzyme system for *oriC* plasmid replication, it was inactive in purified enzyme systems. This suggests that a factor(s) required for dnaA46 activity in the crude enzyme system is absent in the purified enzyme systems.

It was discovered that prior incubation of dnaA46 protein in the presence of a crude protein fraction, ATP, phosphocreatine, creatine kinase and polyvinyl alcohol stimulated DNA replication in the crude enzyme system by reducing the prolonged lag. Whereas dnaA46 protein was inactive in DNA synthesis at 40°C, a prior incubation of dnaA46 protein at 30°C with necessary components resulted in measurable DNA synthesis at 40°C, suggesting that a thermolabile defect of the dnaA46 protein resides at the step of stimulation of its activity.

Several observations have indicated that one of the stimulatory factors is dnaK protein, which has been suggested to function in *in vivo* chromosomal DNA replication and is essential for the initiation of bacteriophage lambda DNA replication. The evidence is as follows: 1) protein fractions from a dnaK protein overproducing strain contain 20 to 30 fold more stimulatory activity than those from a non-overproducing

strain; 2) protein fractions from dnaK mutants were inactive in the stimulation; 3) mobility of the protein purified by the stimulation assay was identical in SDS-polyacrylamide gel electrophoresis with that of dnaK protein; and 4) the purified 72 KDa protein exhibits an identical stimulatory activity as dnaK protein obtained from an independent source. dnaK protein is required in a 1:1 molar ratio with dnaA46 protein, suggesting a stoichiometric interaction. The requirement for another unidentified factor(s) in addition to the dnaK protein was deduced from the observation that a constant amount of protein fraction from a dnaK mutant was necessary for the stimulation even with a 10-fold excess amount of the dnaK protein.

The interaction of dnaA46 protein with dnaK protein and another factor(s) does not require the presence of oriC plasmid DNA, UTP, CTP, GTP and dNTPs, indicating that the interaction occurs prior to the known processes of in vitro oriC plasmid replication including binding of dnaA protein to oriC, priming, and DNA elongation. Although the interaction of $dnaA^+$ protein with the stimulatory factors remains to be demonstrated, this presumed interaction may be involved in regulation of $dnaA^+$ activity for the initiation of in vivo chromosomal DNA replication during the cell cycle.