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THE INFLUENCE OF IHF AND FIS ON THE ORDERED BINDING OF DnaA PROTEIN TO *oriC*, THE *E. coli* CHROMOSOMAL ORIGIN

By
Carla Eva Margulies

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Abstract

THE INFLUENCE OF IHF AND FIS ON THE ORDERED BINDING OF DnaA PROTEIN TO *oriC*, THE *E. coli* CHROMOSOMAL ORIGIN

By
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Initiation of *Escherichia coli* chromosomal replication from *oriC* is dependent on the sequence specific DNA binding protein, DnaA protein. Although DnaA protein is known to bind to four indirect repeats (called DnaA boxes R1, R2, R3, and R4) within *oriC*, little is known of whether DnaA protein binds these sequences in a random or ordered manner or whether other *oriC* binding proteins affect the binding of DnaA protein. Gel mobility shift, footprinting and *in vitro* replication assays, were used to study the binding of DnaA protein to *oriC* and the effect of two sequence specific DNA binding proteins, integration host factor (IHF) and factor for inversion stimulation (FIS), on the binding of DnaA protein. From these studies, DnaA protein was found to bind to *oriC* in an ordered manner in which DnaA boxes R1 and R4 are bound at low concentrations of DnaA protein, and the interior boxes, R2 and R3, are bound at higher concentrations. In addition replication of an *oriC* plasmid correlates with the binding of all four sites. Neither IHF nor FIS affects the binding of DnaA protein to a linear *oriC* fragment. However, IHF acts as a positive factor in *in vitro* replication, whereas FIS acts as a negative factor.

To my parents and brother

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ATPase	ATP synthase
bp	base pairs
Dam	deoxyadenosine methyltransferase
DnaA box	the 9-mer DNA consensus sequence recongized by DnaA protein
DTT	dithiothreitol
EDTA	(ethylenedinitrilo)tetraacetic acid
FIS	factor for inversion stimulation
HEPES	4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid
IHF	integration host factor
kDa	kilodalton
PVA	polyvinyl alcohol
rRNA	ribosomal RNA
tRNA	transfer RNA
SDS	sodium dodecyl sulfate
Tris	Tris(hydroxymethyl)aminomethane

Chapter I

Literature Review

Introduction

Cell division is essential for the survival of *Escherichia coli* or any organism. Cell division depends on the doubling of all the cell's components; the chromosome, the cytoplasm, the cell wall and the outer and cytoplasmic membranes. Without duplication and proper segregation of chromosomal material, daughter cells will not survive to pass on the instructions to allow the two new daughter cells to carry out their functions. Therefore, chromosomal replication must be tightly controlled and coordinated to growth of the cell. In 1963, Jacob proposed a model for the regulation of replication and the segregation of the bacterial chromosome (Jacob *et al.*, 1963). He envisioned that replication initiated from a unique site on the chromosome and the bacterial cell envelope served as a scaffold in much the same way that the eukaryotic spindle functions. As new material was incorporated into the growing membrane, new material would be inserted between the two new nucleoids.

The genetic material of *E. coli* is contained on a single circular chromosome of 4,720 kilobase-pairs (Kohara *et al.*, 1987). As Jacob speculated, replication of this chromosome occurs from a unique site (now referred to as *oriC*) located at approximately 85 minutes on the revised *E. coli* chromosome map (Louarn *et al.*, 1974; Marsh and Worcel, 1977). DNA synthesis then proceeds bidirectionally and terminates near the *trp* gene located at 25 minutes on the *E. coli* chromosome map (Bird *et al.*, 1972). In early studies, Messelson and Stahl determined that a newly replicated DNA molecule contained one newly synthesized strand and one old strand (Messelson and Stahl, 1958). This type of replication is referred to as semiconservative replication.

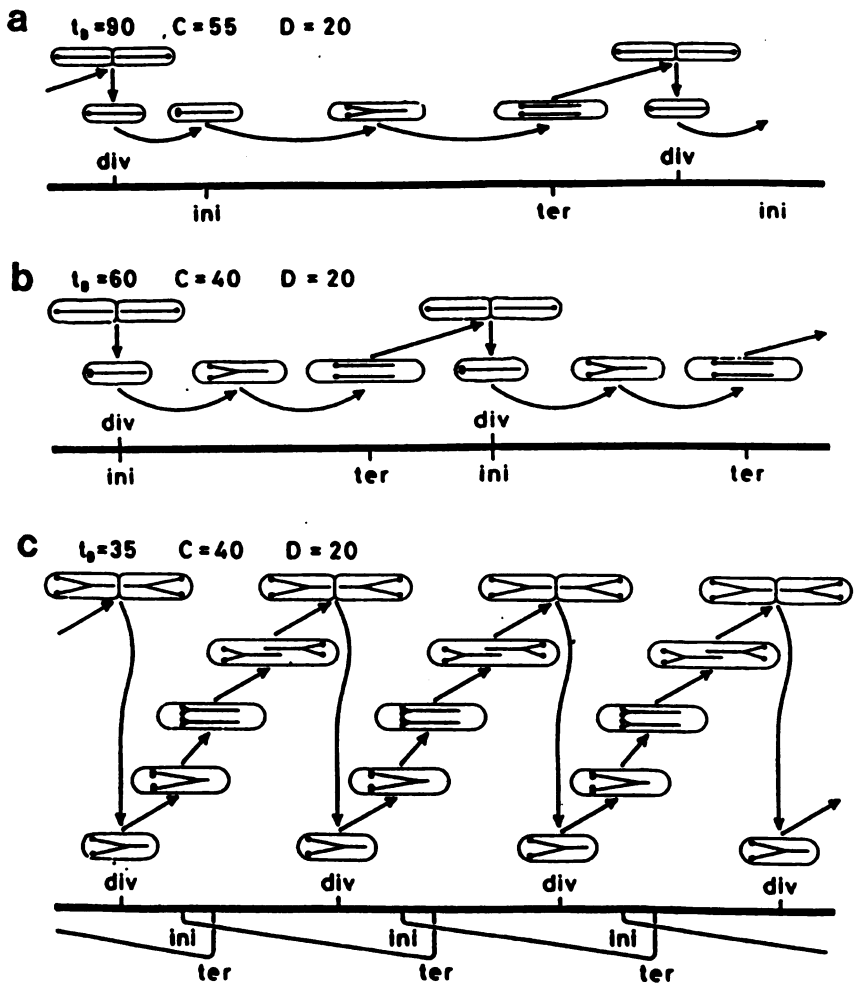
Physiological characteristics of chromosomal replication.

The frequency of initiation of replication and not the rate of elongation regulates chromosomal replication. This conclusion is based on observations from dividing the bacterial cell cycle into three periods and measuring the length of these periods during various growth rates (Cooper and Helmstetter, 1968; Helmstetter, 1968) (Figure 1). The minimum amount of time required to replicate the entire chromosome, period C, is approximately 45 minutes (Trueba *et al.*, 1982). Because period C and the time between the completion of chromosomal replication and cell division, period D, remains relativity constant regardless of variation in generation times from 22 to 70 minutes (Skarstad *et al.*, 1983), the time required to achieved the capacity to initiate chromosomal replication, period I, is concluded to be variable (Helmstetter, 1994).

The fact that the doubling time of *E. coli* can proceed twice as fast as the time required to replicate the chromosome seems to be contradictory. However, initiation of subsequent rounds of chromosomal replication can occur before previous replication cycles are completed (Figure 1). Thus, a fast growing cell contains multiple copies of the chromosome (Skarstad *et al.*, 1985). In rapidly growing cultures with multiple chromosomal origins, all *oriC* within an individual cell initiate at the same time (Skarstad *et al.*, 1986). This is called synchronous initiation. Mutations of factors involved in the regulation of initiation often result in asynchronous timing of initiation (Boye and Lobner–Olesen, 1990; Lu *et al.*,1994; Skarstad *et al.*, 1988).

Central to models of the regulation of initiation is the idea that the ratio of cell mass to the number of chromosomal origins within a cell triggers initiation

Figure 1. **Schematic presentation of the relationship between chromosomal replication and cell division** (von Meyenburg and Hansen, 1987). t_D represents the generation time. The bacterial cell cycle can be divided into distinct periods, C and D. C represents the time between initiation (ini) and termination (ter) of chromosomal replication. D represents the time between termination of chromosomal replication and cell division (div). The time of initiation varies as a function of t_D. **a)** When t_D is greater than the sum of the periods C and D, initiation occurs after the division event. **b)** When t_D is equal the sum of the periods C and D, initiation occurs at the time of the division event. **c)** When t_D is less than the time for C and D, initiation occurs before the prior cycle is complete. As a result a single cell will contain more than one *oriC*. All origins within a cell initiate simultaneously.


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

(Donachie, 1968). This ratio is called the initiation mass and remains constant irrespective of growth rate. Initiation of replication is dependent on *de novo* protein synthesis, as blocking protein synthesis with antibiotics allows the completion of replication but prevents additional initiation events (Lark and Renger, 1969; Messer, 1972). These observations led to the speculation that the accumulation of a specific protein may link initiation mass with initiation. DnaA protein, a protein involved in the first steps of initiation, is an obvious candidate for such a regulator of initiation.

Asynchronous initiation was mentioned as a common phenotype of initiation mutants. Another characteristic which differentiates genes involved in initiation of replication from those involved in chain elongation is slow and fast stop phenotypes (Kohiyama *et al.*, 1966). Temperature sensitive mutants defective in chain elongation result in an immediate stop in DNA replication at nonpermissive temperatures; they exhibit a fast stop phenotype. In contrast, mutants of genes involved in initiation, such as *dnaA* and *dnaC*, result in the completion of the round of replication but fail to initiate the next round of replication; they exhibit a slow stop phenotype (Wechsler, 1975; Zyskind *et al.*, 1977).

The origin of chromosomal replication, *oriC*

The origin of chromosomal replication (*oriC*) located between the *dnaA* and *bglA* genes was mapped by pulse labelling of synchronized cells (Louarn *et al.*, 1974; Marsh and Worcel, 1977). The minimal sequence of *oriC* required to confer autonomous replication on non replicating DNA is a 245 base pair

sequence (Oka *et al.*, 1980) (Figure 2). From mutagenesis (Oka *et al.*, 1984) and sequence comparison of *oriC* sequences from members of the *Enterobacteriaceae* family (Zyskind *et al.*, 1983), several key sequences are identified as important for replication (Figure 2): 1) an A-T rich region located at the left border of *oriC*, 2) four indirect repeats of the sequence 5'-TTAT(C/A)CA(C/A)A-3', called DnaA boxes, and 3) 8 Dam methylation sites (5'-GATC-3'). The AT-rich region is composed of three 13-mers, identified as left, middle and right (Figure 3). The four DnaA boxes are named, R1, R2, R3 and R4. Dam methyltransferase catalyzes the transfer of a methyl group to the N6 position of the adenine on both strands within its recognition sequence (Marinus and Morris, 1974). All three sequences will be discussed in more detail.

In addition to these sequences, several promoters are located within and flanking the minimal *oriC* (Figure 3) (Asai *et al.*, 1992; Braun and Wright, 1986; Lother and Messer, 1981; Schauzu *et al.*, 1987). Transcripts from the promoter P*ori*L originate from just right of DnaA box R2 within *oriC* and proceed toward the left end of *oriC* (Figure 3). This promoter appears to be negatively regulated by the binding of DnaA protein to boxes R2 and R4 (Asai *et al.*, 1992). Transcription from P*ori*R1, which begins near the 13-mers and proceeds rightward, appears to be positively regulated by DnaA protein (Asai *et al.*, 1992). An additional promotor, P*ori*R, is located just to the right of R4 and directs transcription to the right (Schauzu *et al.*, 1987). From a promoter located left of the AT-rich 13-mers, *gidA* (glucose inhibition of division) transcription is directed away from *oriC* (Kolling *et al.*, 1988). *mioC* (modulation of initiation at *oriC*) is located several hundred base-pairs to the right of *oriC* and is transcribed into

Figure 2. **Consensus sequence of the minimal chromosomal origin** (Zyskind *et al.*, 1983). The sequences from six bacterial chromosomal origins are compared. They were aligned to minimize the number of changes of the consensus sequence. The large box indicates the boundaries of the minimal *oriC* sequence required to confer autonomous replication. A large capital letter indicate that the residue is found in all six bacteria; a small capital letter indicates that the residue is found in five of the six origins; a lower case letter indicates that it is found in three or four origins; and n indicates that all four nucleotides are found at that position. Dashes indicate deletions. The bold letters indicate residues where single base substitutions produce a *oriC*- phenotype (Oka *et al.*, 1984). The AT-rich 13-mers are indicated with the arrows at the left border of *oriC*. The DnaA boxes and their orientations are indicated by arrows and are labelled R1, R2, R3, and R4. The Dam methylation sites (GATC) are underlined.

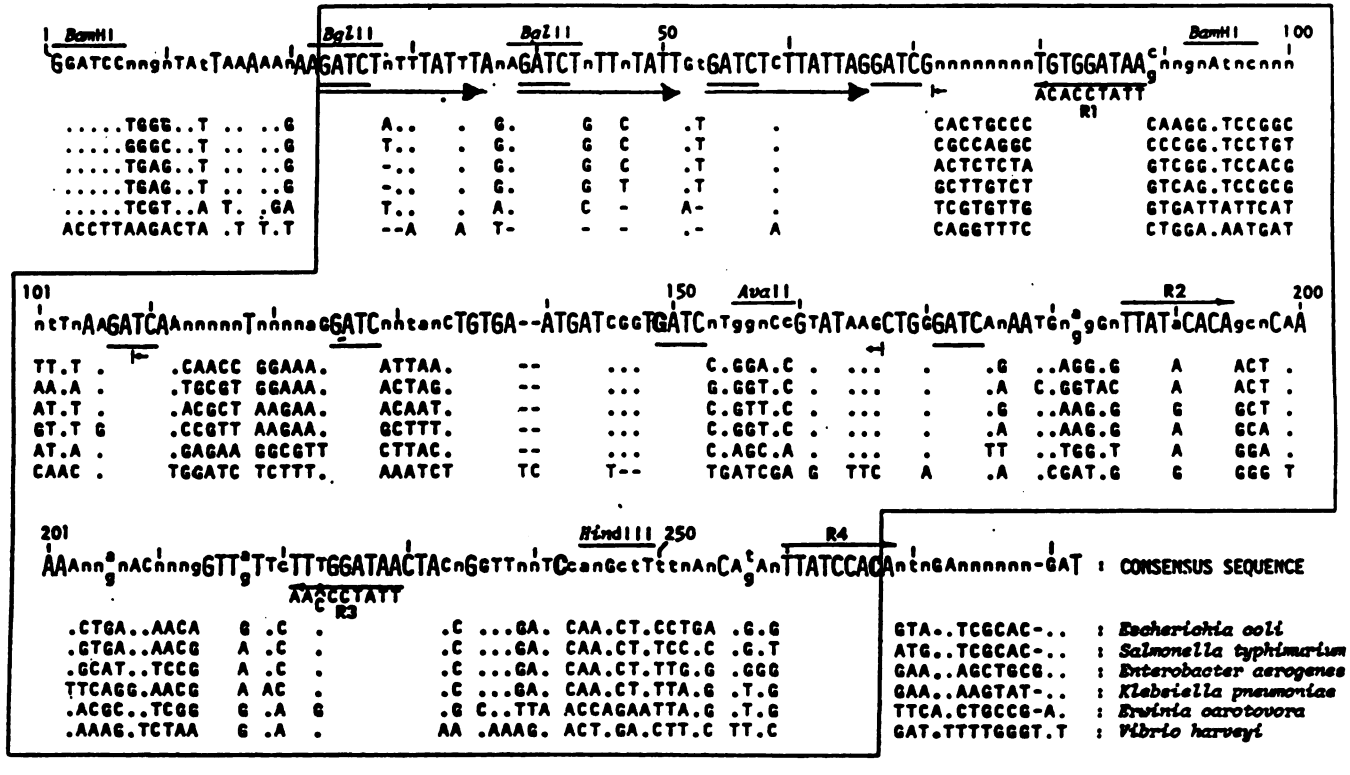
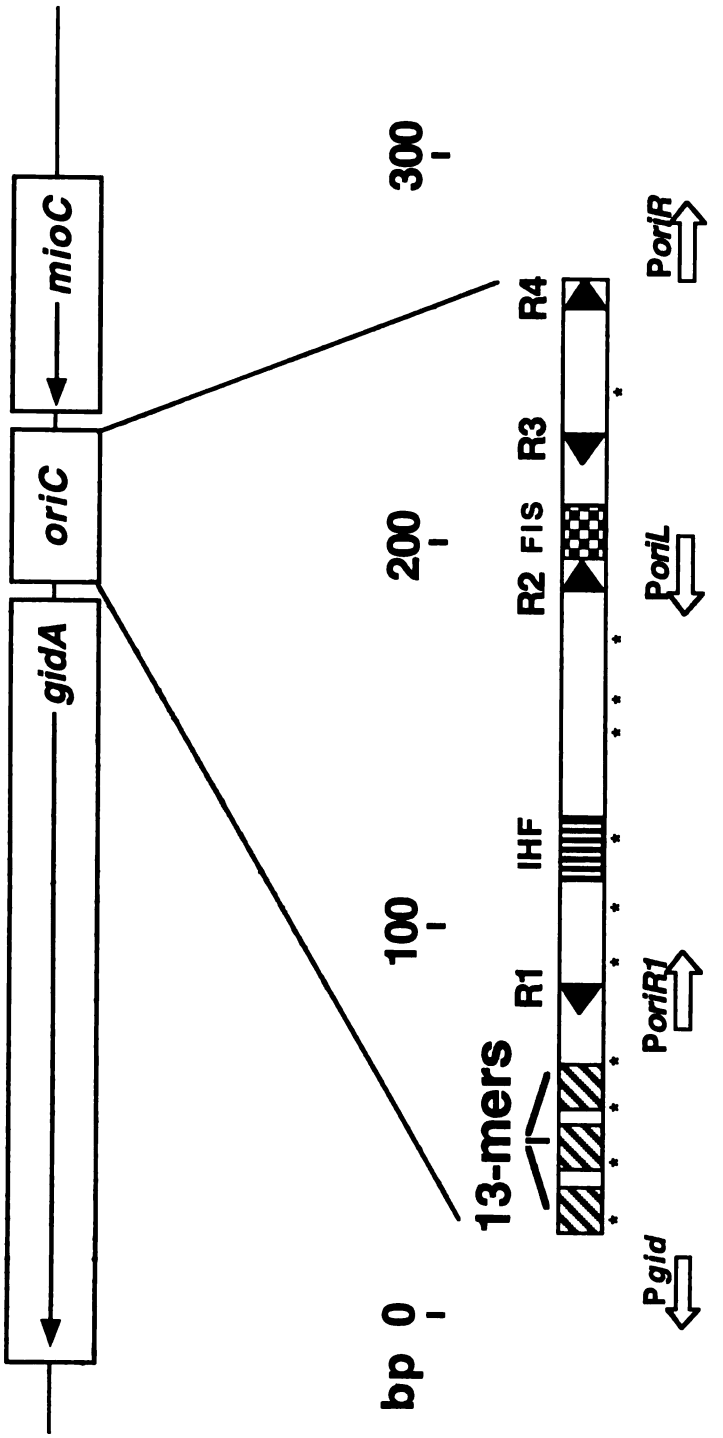

BamHI
BglII
BglII
50
BamHI
100
ACACCTATT
R1
101
150
AvaII
R2
200
R3
HindIII
250
R4
CONSENSUS SEQUENCE
Escheriahia coli
Salmonella typhimarium
Enterobacter aerogenes
Klebsiella pneumoniae
Erwinia carotovora
Vibrio harveyi

Figure 3. **A schematic representation of relevant sequences in and flanking *oriC*.** The top figure represents *oriC* between *mioC* and *gidA*. The directions of transcription of these genes are indicated by the arrows. The lower figure illustrates the location of important sequences involved in initiation. The promoters and their orientations are indicated by the open arrows; the three 13-mers by the slashed boxes; the DnaA boxes and their orientations by the filled triangles; an IHF binding site by the vertical lined box; and the FIS binding site by the checkered box. The astericks indicate the Dam methylation sites.


mioC
oriC
gidA
bp 0
100
200
300
13-mers
R1
IHF
R2
FIS
R3
R4
PgidA
PoriR1
PoriL
PoriR

without transcribing through *oriC*. The function of the gene products encoded by *gidA* and *mioC* are not known.

The difficulty of studying replication of the entire chromosome is overcome by the cloning of *oriC* on plasmids, called minichromosomes (Messer *et al.*, 1978). These plasmids are dependent on *oriC* for replication and retain many of the same characteristics as chromosomal replication including bidirectional fork movement (Meijer and Messer, 1980), synchronous initiation (Helmstetter and Leonard, 1987b; Leonard and Helmstetter, 1986), dependence on protein and RNA synthesis (Lark and Renger, 1969; Messer, 1972), and dependence on growth rate (Lobner-Olesen *et al.*, 1987).

Initiation of replication from *oriC*

The development of *in vitro oriC* dependent replication assays using crude enzyme extracts (Fuller *et al.*, 1981) or purified proteins (Kaguni and Kornberg, 1984), led to a model by which DNA replication is initiated from *oriC*. Like *in vivo*, *in vitro* replication from *oriC* proceeds bidirectionally (Kaguni *et al.*, 1982; Meijer and Messer, 1980), requires a negatively supercoiled template (Kowalski and Eddy, 1989; von Freiesleben and Rasmussen, 1992), and is dependent on DnaA protein (Carl, 1970; Wechsler and Gross, 1971), DnaB protein (Zyskind and Smith, 1977b), and DnaC protein (Wechsler, 1975).

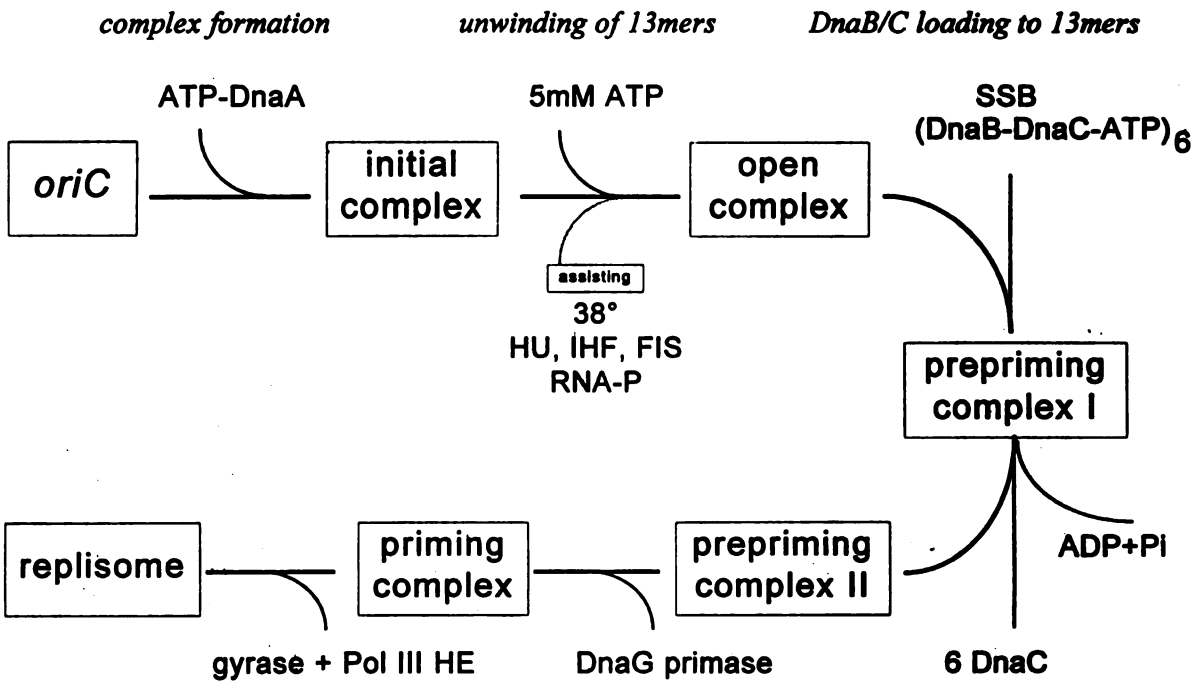
The molecular model of initiation of chromosomal replication

Biochemical studies have indicated that the first step in the replication of the *E. coli* chromosome involves recognition and topological alteration of the

oriC region by DnaA protein (Figure 4) (Fuller *et al.*, 1984). The initial binding of DnaA protein to the DnaA boxes is proposed to enhance the binding of additional DnaA molecules. The final DnaA-*oriC* complex that is thought to be active in promoting initiation of DNA replication contains approximately 30 to 40 DnaA monomers (Fuller *et al.*, 1984). *oriC* appears to be wrapped around a core of DnaA molecules. In the presence of HU or integration host factor (IHF) (Hwang and Kornberg, 1992a; Skarstad *et al.*, 1990), this nucleoprotein complex is proposed to distort the topology of *oriC* resulting in unwinding of the AT-rich 13-mers, forming the open complex (Bramhill and Kornberg, 1988a). In the absence of HU or IHF, unwinding of the 13-mers can be facilitated by RNA polymerase (Baker and Kornberg, 1988). Although DnaA protein binds to *oriC* equally well in the presence or absence of nucleotide (Bramhill and Kornberg, 1988a), unwinding of *oriC* requires the ATP-bound form of DnaA protein (Sekimizu *et al.*, 1988a; Yung *et al.*, 1990).

Following unwinding in the presence of ATP, six DnaC monomers form a complex with a hexameric DnaB helicase (termed DnaBC complex) (Kobori and Kornberg, 1982; Wickner and Hurwitz, 1975). DnaA protein is proposed to guide the DnaBC complex into the open complex thorough a direct DnaA-DnaB interaction to form the prepriming complex (Marszalek and Kaguni, 1994). DnaB helicase then is proposed to unwind the strands of DNA bidirectionally (Baker *et al.*, 1986), (Baker *et al.*, 1987). In this model, the presence of DnaC protein in the preinitiation complex inhibits DnaB helicase activity (Allen and Kornberg, 1991). DnaC protein is thought to dissociate from the complex of DnaA protein and DnaB protein bound to the origin sequence (Allen and Kornberg, 1993). Once

Figure 4. **Model of initiation from *oriC*** (Messer, 1996). Initiation occurs in 6 steps: 1) DnaA binds to *oriC* to form the initial complex; 2) DnaA unwinds the 13-mers to form the open complex; 3) DnaBC complex enters the unwound *oriC* to form the prepriming complex I; 4) DnaC dissociates from the prepriming complex; 5) primers are formed; and 6) DNA polymerase III holoenzyme uses these primers to initiate DNA synthesis.


complex formation
unwinding of 13mers
DnaB/C loading to 13mers
ATP-DnaA
5mM ATP
SSB
(DnaB-DnaC-ATP)6
oriC
initial complex
open complex
assisting
38°
HU, IHF, FIS
RNA-P
prepriming complex I
replisome
priming complex
prepriming complex II
ADP+Pi
gyrase + Pol III HE
DnaG primase
6 DnaC
replication fork assembly II
replication fork assembly I
DnaB positioning at start sites

DnaB helicase has separated a portion of the origin in both directions, primase enters the initiation complex to synthesize short RNAs (van der Ende *et al.*, 1985) which act as primers for DNA polymerase III holoenzyme (Maki and Kornberg, 1988). Elongation of these primers ultimately results in the formation of two daughter chromosomes.

Other factors required for oriC*-dependent replication*

In addition to DnaA protein, DnaB protein, DnaC protein, HU, primase and DNA polymerase III holoenzyme, several other proteins are required for *in vitro* replication. Several of these proteins, such as RNA polymerase, topoisomerase I and DNA gyrase are involved in changing the topology of DNA. Others factors, such as RNase H, are involved as *oriC* specificity factors (Kogoma *et al.*, 1985). Lastly, single stranded DNA binding protein (SSB), is involved in stabilizing single-stranded DNA (Sigal *et al.*, 1972) which is exposed during DNA replication. Though DnaA protein can unwind the origin in the absence of SSB (Bramhill and Kornberg, 1988a), SSB stimulates DnaB helicase activity (LeBowitz and McMacken, 1986) and the processivity of DNA polymerase III holoenzyme (Fay *et al.*, 1981; Fay *et al.*, 1982; LaDuca *et al.*, 1983).

Topoisomerase I and DNA gyrase are responsible for maintaining the negative superhelicity of the chromosome (Pruss, et al., 1982; Wang, 1996). Mutations in *gyrA* or *gyrB* which encode the A and B subunits of DNA gyrase, respectively, suppress deletion mutations in *topA*, encoding topoisomerase I (DiNardo *et al.*, 1982; Pruss *et al.*, 1982). Topoisomerase I and DNA gyrase have opposite effects; topoisomerase I relaxes negative supercoils (Pruss *et al.*, 1982),

whereas DNA gyrase introduces negative supercoils (Marians *et al.*, 1977).

DNA Gyrase plays an important role in maintaining the negative superhelicity required for initiation of chromosomal replication (Hiasa and Marians, 1994b; Kowalski and Eddy, 1989) and releasing positive supercoils that form ahead of the replication forks (Filutowicz and Jonczyk, 1983; Kreuzer and Cozzarelli, 1979). Both DNA gyrase and topoisomerase I, are involved in modulating the changes in supercoiling due to chain elongation by RNA polymerase (Liu and Wang, 1987). DNA Gyrase acts ahead of transcribing RNA polymerase removing positive supercoils whereas topoisomerase I acts behind RNA polymerase relaxing negative supercoils.

oriC-*independent replication*

Chromosomal replication can occur in the absence of *oriC* and *dnaA* via three different mechanisms. The first mechanism is by integrating a plasmid or phage origin into the chromosome (Koppes and Nordstrom, 1986). Replication can be also driven from other origins (collectively known as *oriK*) already located on the *E. coli* chromosome via induction of the SOS response (Magee *et al.*, 1992). This type of replication is termed damage-induced stable DNA replication (iSDR) and is thought to enable the cell to meet various environmental stresses (Hong, et al., 1995; Kogoma *et al.*, 1979). In addition, mutations in *rnhA* can suppress *oriC* and *dnaA* deletions (de Massy *et al.*, 1984; Kogoma *et al.*, 1985). However, these strains grow poorly. *rnhA* encodes RNase H which specifically degrades RNA-DNA hybrids (Arendes, et al., 1982; Miller *et al.*, 1973) which could potentially could act as primers for DNA polymerase III holoenzyme.

Mutations in *rnhA* are thought to allow RNA primers other than those located at *oriC* to serve as primers for DNA polymerase III (Horiuchi *et al.*, 1984). In support, *in vitro* replication remains dependent on *oriC* in the presence of RNA polymerase only with the addition of RNase H (Ogawa *et al.*, 1984).

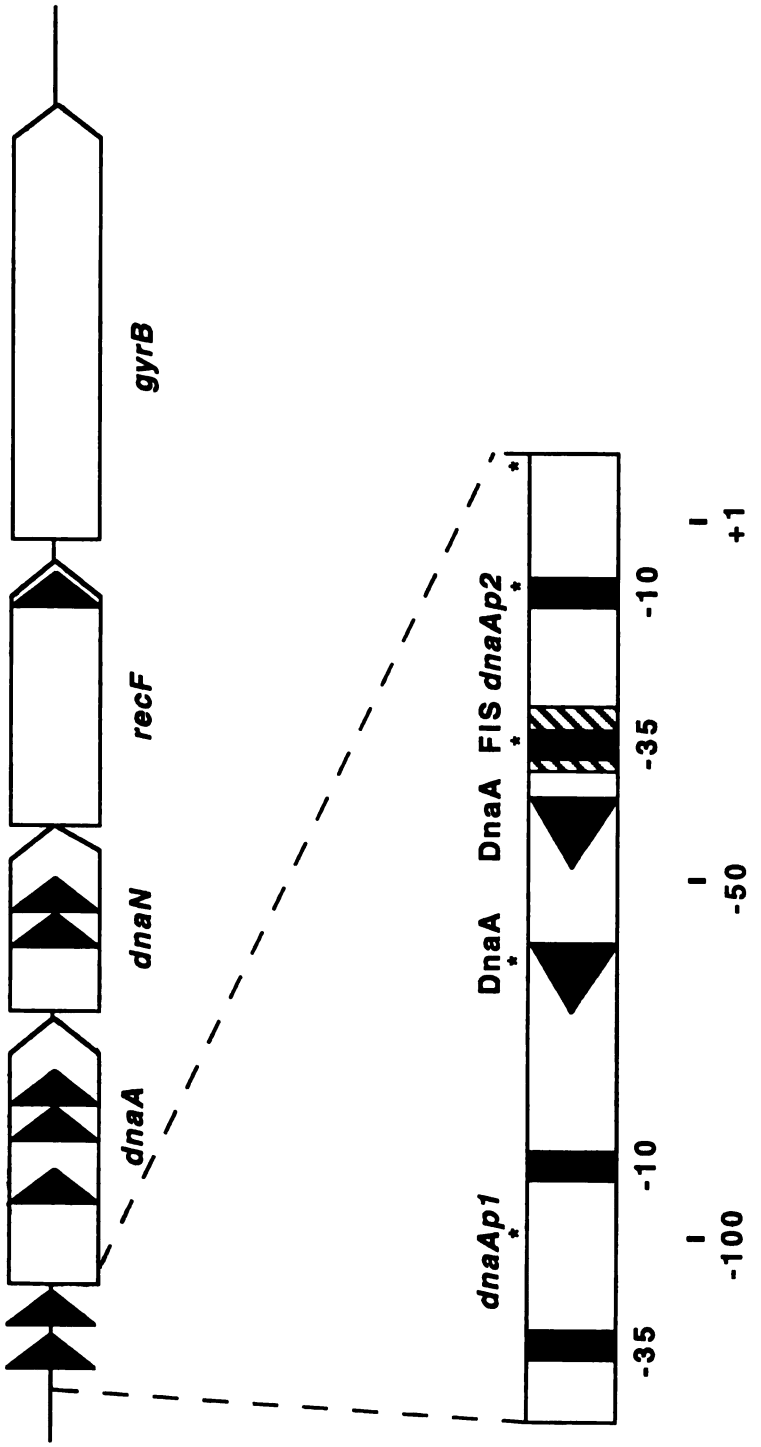
DnaA protein

The dnaA *Gene*

The *dnaA* gene was originally identified by the identification of temperature sensitive mutants that exhibited a slow stop phenotype at elevated temperatures (Kohiyama *et al.*, 1966). Employing a temperature sensitive mutant, the *dnaA* gene was mapped and cloned by complementation (Hansen and von Meyenburg, 1979; Miki *et al.*, 1979). The *E. coli dnaA* gene is located at 83 minutes on the revised *E. coli* genetic map, close to *oriC*. From the deduced amino acid sequence, the gene encodes a basic polypeptide of 467 amino acids with a calculated mass of 52.5 kDa (Ohmori *et al.*, 1984). The *dnaA* gene is the first gene in an operon containing *dnaN* (Sakakibara *et al.*, 1981), encoding the β sliding clamp of DNA polymerase III holoenzyme (Burgers *et al.*, 1981), *recF*, encoding a protein involved in recombination (Ream *et al.*, 1980) and *gyrB*, encoding the B subunit of DNA gyrase (Hansen and von Meyenburg, 1979) (Figure 5).

DnaA protein appears to be found in all bacteria from *E. coli* and *Bacillus subtilis* to *Mycoplasma* and cyanobacteria (reviewed in Messer, 1996). Both the conservation of the arrangement of the *dnaA* operon and the similarity of the DnaA amino acid sequence in a large variety of bacteria suggests that DnaA

Figure 5. **A schematic of the *dnaA* operon and its promoter region.** The genes and the direction of transcription are indicated by the open boxes. Promoters are indicated by the filled arrowheads. The *dnaA* promotor region is expanded below. The -10 and -35 sequences are indicated by the filled boxes, the DnaA boxes and their orientation by the shaded triangles; the FIS binding site with the slashed box; and the Dam methylation sites by the asterisks.


gyrB
recF
dnaN
dnaA
dnaAp1
DnaA
DnaA
FIS
dnaAp2
-35
-10
-100
-50
-35
-10
+1

protein shares a common function in these bacteria (Ogasawara and Yoshikawa, 1992; Yoshikawa and Ogasawara, 1991).

The promoter and regulation of the dnaA *gene*

The *dnaA* gene contains two promoters, *dnaAp1* and *dnaAp2*, located 250 and 160 nucleotides, respectively, from the translational start site (Hansen *et al.*, 1982) (Figure 5). A DnaA box identical in sequence to boxes R1 and R4 of *oriC* is located between the two promoters. Protection experiments reveal another potential DnaA box, also located between the promoters (Wang and Kaguni, 1987). This second site contains the sequence 5'-TTTTCCACA-3' which fits the more relaxed definition of the DnaA box (see below). Binding of DnaA protein to these sites inhibits transcription from both promoters (Braun *et al.*, 1985; Lee and Hwang, 1997; Wang and Kaguni, 1987). Like *oriC*, the promotor region also contains numerous Dam-methylation sites. Transcription from *dnaAp2* is more efficient when the promoter region is fully methylated (Braun and Wright, 1986; Kucherer *et al.*, 1986). Factor for inversion stimulation (FIS), a histone-like protein involved in regulating transcription from some promoters in relation to the rate of growth (Finkel and Johnson, 1992), binds to sequences that overlap the -35 box of *dnaAp2* (Froelich, 1996). FIS appears to negatively regulate *dnaA* expression as *fis* null mutants have a 2.5 fold increase in the level of DnaA protein (Froelich, 1996). The *dnaA2p* promoter, the DnaA box and several of the GATC sites are conserved between *E. coli*, *S. typhimurium* and *S. marcesces* suggesting that the *dnaA* gene in these different bacteria is regulated in a similar manner (Skovgaard and Hansen, 1987).

Biochemical properties of DnaA protein

DnaA protein was first purified by a DNA binding assay then by complementing crude extracts from a *dnaA*(ts) strain in *in vitro oriC* replication assays (Chakraborty *et al.*, 1982; Fuller and Kornberg, 1983). DnaA protein has several biochemical activities: DNA binding (Fuller *et al.*, 1984); ATP binding (Sekimizu *et al.*, 1987); ATP hydrolysis; cAMP binding (Hughes *et al.*, 1987); interaction with acidic phospholipids (Sekimizu *et al.*, 1988b), and self aggregation (Sekimizu *et al.*, 1988b).

DNase I protection and filter binding were used to demonstrate the ability of DnaA protein to bind the four DnaA boxes within *oriC* (Fuller *et al.*, 1984). DnaA protein binds to an oligonucleotide containing the sequence of *oriC* DnaA box R4 in its native context with a K_D of 1 nM (Schaper and Messer, 1995). Using gel mobility shift assays with 21-mer oligonucleotides, the DnaA protein recognition sequence is rigidly defined as 5'- TTATNCACA-3' (Schaper and Messer, 1995). However, flanking sequences adjacent to DnaA boxes are also important in determining the efficiency with which DnaA protein will bind as its affinity for a 21-base pair oligonucleotide containing the *oriC* DnaA box R4 is reduced 50 fold by changing the flanking sequences. Surprisingly, DnaA protein binds an oligonucleotide containing *oriC* DnaA box R3 with an affinity no greater than an oligonucleotide containing random sequences. The ability of DnaA protein to protect R3 contained within *oriC* is thought to be the result of cooperativity. The binding of highly divergent sequences in the presence of an adjacent strong consensus site is common for DnaA protein and suggests that protein-protein interactions facilitate the binding to weaker sites (Stenzel *et al.*,

1991; Wang and Kaguni, 1987). The combination of the effect of flanking sequences and cooperative binding may explain why DnaA protein appears to also recognize a more relaxed definition of the DnaA box, 5'-(T/C)(T/C)(A/T/C)T(A/C)C(A/G)(A/C/T)(A/C)-3' (Schaefer and Messer, 1991).

As expected from the conserved ATP binding motif, DnaA protein binds ATP and ADP with high affinity (K_D of 0.03 uM and 0.1 uM, respectively) (Sekimizu *et al.*, 1987). DnaA protein is speculated to have a second lower affinity site based on the requirement of 5 mM ATP to unwind the AT-rich 13-mers (Sekimizu *et al.*, 1987). Hydrolysis of ATP to ADP occurs slowly; 50% of bound ATP is hydrolysed in 15 minutes. This ATPase activity is dependent on DNA. ATP hydrolysis is not required for DnaA protein dependent unwinding of *oriC* as the nonhydrolyzable ATP analog, ATPγS, can substitute for ATP in unwinding *oriC*. Instead, the binding of ATP is thought to produce a conformational change which is important for unwinding (Yung and Kornberg, 1989).

DnaA protein also binds cAMP with high affinity (K_D 1 uM) (Hughes *et al.*, 1988). Although ATP inhibits cAMP binding, this inhibition is noncompetitive. Dissociation of ADP from DnaA protein in the presence of ATP is slow (Sekimizu *et al.*, 1987). cAMP increases the rate of dissociation of bound ADP but not of bound ATP (Hughes *et al.*, 1988).

Typically 50% of DnaA protein expressed from its natural promoter is found in the membrane fraction, suggesting a DnaA protein-membrane interaction (Sekimizu, et al., 1988b). Mutants of phospholipid metabolism which limit the levels of anionic phospholipids exhibit a slow stop phenotype (Heacock and

Dowhan, 1989; Xia and Dowhan, 1995). This phenotype is suppressed by mutations in *rnhA* (encodes RNase H) (Xia and Dowhan, 1995) which circumvents the requirement for *oriC* and *dnaA* (Ogawa *et al.*, 1984; von Meyenburg *et al.*, 1987). Acidic phospholipids inhibit the reassociation of DnaA protein with ATP and ADP. However, in the presence of *oriC,* DnaA protein can rebind both ATP and ADP, even in the presence of phospholipids (Castuma *et al.*, 1993; Crooke *et al.*, 1992; Sekimizu and Kornberg, 1988; Yung and Kornberg, 1988). This phospholipid effect does not discriminate between ATP and ADP; however, the cellular concentration of ATP is approximately 10 times higher than that of ADP (Mathis and Brown, 1976), suggesting a mechanism for regeneration of the ADP bound form of DnaA protein.

DnaA protein is isolated as a monomer and an aggregate (Hwang *et al.*, 1990). The aggregate is poorly characterized. This multimerization is thought to be important in DnaA protein function when bound at *oriC* (Fuller *et al.*, 1984) and even at promoters (Lee and Hwang, 1997). In electron micrographs, DnaA-*oriC* complexes appear to contain 20 to 30 monomers. Furthermore, a mutant of DnaA protein, which is defective in specific DNA binding and is inactive in *oriC* replication, is able to augment the limiting levels of wild type *in vitro oriC* replication (Sutton, 1996).

Structural organization of DnaA protein

Until recently, little was known about structure function relationships of DnaA protein. Only sequence comparisons gave any information of the DnaA protein structure (Figure 6). This comparison divided the protein into a Figure 6.

***E. coli* DnaA amino acid deduced sequence compared with the DnaA consensus sequence** (Messer, 1996). The deduced amino acid sequence of *E. coli* is indicated relative to the consensus sequence obtained from comparing fifteen different *dnaA* homologs (*Escherichia coli*, *Salmonella typhimurium*, *Serratia marcescens*, *Proteus mirabilis*, *Buchnera aphidicola*, *Pseudomonas putida*, *Bacillus subtilis*, *Streptomyces coelicolor*, *Micrococcu luteus*, *Caulobacter crescentus*, *Rhizobium meliloti*, *Synechocys sp.*, *Borrelia burgdorferi*, *Spiroplasma citri*, *Mycoplasma capricolum*). An underlined letter in the conserved sequence indicates a residue conserved in 12 or more of the 15 homologs. A letter represents a residue conserved in 9 or more. A dot (·) indicates a residue conserved (either identical residue or conservative replacement) in less than 9. See Messer, 1996 for a description of the method used for the sequence comparison.

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

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

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

        181                                                         240
E. coli   LLHAVGNGIMARKPNAKVVYMHSERFVQDMVKALQNNAIEEFKRYYRSVDALLIDDIQFF
consensus LLHAVGN..M...PNAKVVYM.SERFV.DMV.ALQ.N.IEEFK.YYRSVD.LLIDDIQFF

        241                                                         300
E. coli   ANKERSQEEFFHTFNALLEGNQQIILTSDRYPKEINGVEDRLKSRFGWGLTVAIEPPELE
consensus A.KE..QEEFTHTFNALLE...QIILTSDRYPKEI.GVEDRLKSRF.WGL.VAIEPPELE

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

        361                                                         420
E. coli   EALRDLLALQEKLVTIDNIQKTVAEYYKIKVADLLSKRRSRSVARPRQMAMALAKELTNH
consensus E.LRDLL...E.LVTIDNIQK.VAEYYKIKV.DLLSKRRSRSVARPROMAM.L.KELTNH

        421                                                 467
E. coli   SLPEIGDAFGGRDHTTVLHACRKIEQLREESHDIKEDFSNLIRTLSS
consensus SLPEIGD.FGGRDHTTVLHACRKIE.LR.E..DIK.DF..LIR.L..
```

conserved N-terminal segment, a less conserved linker segment and the larger conserved C-terminal segment which contains the conserved ATP-binding motif (G-X-X-G-X-G-K-T- X_5-V) between residues 172-185 (Fujita *et al.*, 1990; Ogasawara *et al.*, 1985; Skovgaard and Hansen, 1987; Yoshikawa and Ogasawara, 1991). There is little direct evidendence that ATP actually binds this site. Suggestive evidence is provided from a mutation of amino acid 184 from an alanine to a valine which results in a DnaA protein defective in binding ATP (Carr and Kaguni, 1996).

The C-terminal 94 amino acids of DnaA protein are responsible for DNA binding (Roth and Messer, 1995) (Figure 7). The region from residues 372-381 appears to be involved in phospholipid mediated turnover of bound nucleotide. A fragment containing residues 115 to 381 releases ATP in the presence of acidic phospholipids, whereas a peptide of residues 115 to 372 binds ATP, fails to release the nucleotide in the presence of phospholipids (Garner and Crooke, 1996).

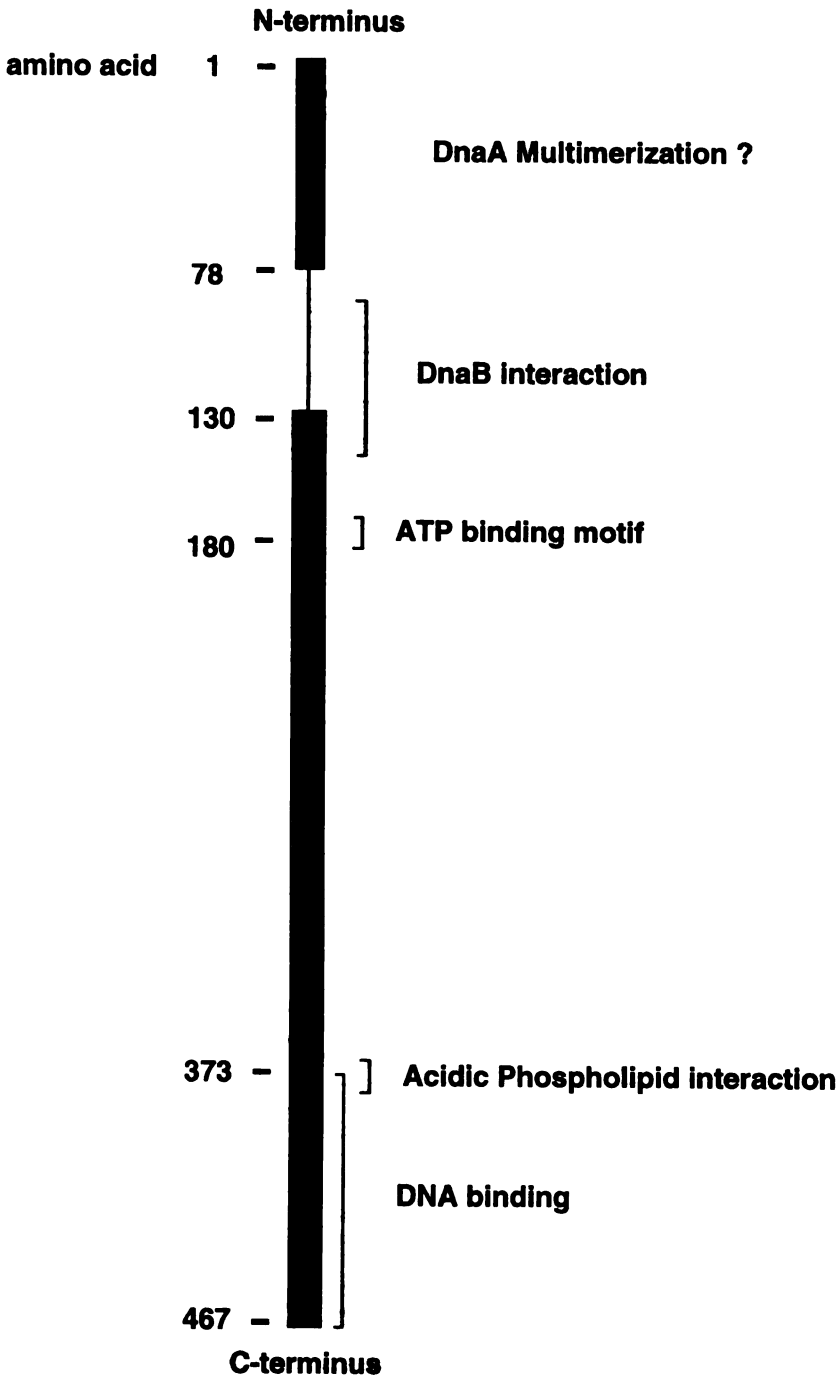
Sequences near the N-terminus are speculated to be involved in DnaA-DnaA interactions (Mark Sutton, personal communication). Residues 111-148 near the N-terminus are involved in the interaction with DnaB protein (Marszalek and Kaguni, 1994; Marszalek *et al.*, 1996).

Roles in the cell other than initiation of chromosomal replication

Replication of plasmids

DnaA protein is also involved in the replication of other replicons such as phage P1 (Mukhopadhyay *et al.*, 1993) and plasmids pSC101 (Murakami *et al.*,

Figure 7. **Structure-function of DnaA protein.** The amino acid sequences which are highly conserved among the dnaA homologs are indicated by the filled sequences. The less conserved residues are indicated by a line. The residue numbers are indicated to the left.


N-terminus
amino acid
1
78
130
180
373
467
C-terminus
DnaA Multimerization ?
DnaB interaction
ATP binding motif
Acidic Phospholipid interaction
DNA binding

1987), R1 (Masai and Arai, 1987), R6K (Wu *et al.*, 1992), and F (Hansen and Yarmolinsky, 1986). All replicons contain one or more DnaA boxes. We are only beginning to understand the role of DnaA protein in the initiation of these replicons.

These replicons have a structure similar to *oriC*. They contain several repeats, called interons, which are bound by a plasmid-encoded initiator protein, and an AT-rich region. Until recently, all *dnaA* mutants defective in *oriC* replication was found to be inactive for pSC101 replication (Felton and Wright, 1979; Frey *et al.*, 1979; Hasunuma and Sekiguchi, 1977). However, recently C-terminal point and deletion mutants that were active in pSC101 and inactive in *oriC* repication were isolated (Sutton, 1996). The other replicons (P1, F, RK2, R6K) can be maintained by various *dnaA*(ts) but could not be maintained in *dnaA* null mutants (Hansen and Yarmolinsky, 1986; Gaylo *et al.*, 1987; MacAllister, T. W. *et al.*, 1991; Wu, F. *et al.*, 1992). Although DnaA protein is required for R1 replication *in vitro* (Masai and Arai, 1987; Ortega *et al.*, 1986), R1 can be maintained *in vivo* though less efficiently without DnaA protein (Bernander *et al.*, 1991).

Although the role of DnaA protein at these origins is thought to be similar to that which it plays at *oriC*, primary recognition of the origin appears to be the role of a plasmid encoded initiator. DnaA protein is thought to be involved primarily in unwinding and/or loading of DnaB protein. However, evidence for DnaA protein actually unwinding or loading DnaB protein onto these origins is lacking except for the requirement of DnaA protein for unwinding the P1 origin (Mukhopadhyay *et al.*, 1993). In contrast to *in vitro* requirements, *in vitro*

replication of R1 and R6K plasmids proceeds independently of DnaA protein (Masai and Arai, 1987; Wu *et al.*, 1992).

Regulation of Transcription

DnaA protein regulates its own expression (Braun *et al.*, 1985; Wang and Kaguni, 1987) and the expression of other genes (Wang and Kaguni, 1987). Transcription of *mioC*, *rpoH* and *dnaA* is negatively regulated by the binding of DnaA protein to DnaA boxes located within promoters of these genes (Braun *et al.*, 1985; Hansen *et al.*, 1987; Lother, *et al.*, 1985; Schauzu *et al.*, 1987; Stuitje *et al.*, 1986; Wang and Kaguni, 1987; Wang and Kaguni, 1989). The binding of DnaA protein to a *dnaA* promoter fragment has been demonstrated to occlude binding of RNA polymerase (Lee and Hwang, 1997). Although many genes have been reported to contain DnaA boxes within their promoters, not all have been shown to be regulated by DnaA protein (Garrido *et al.*, 1993).

DnaA protein is also been reported to function as a transcriptional terminator (Gielow, et al., 1988; Schaefer and Messer, 1988). This termination activity is dependent upon the orientation of the DnaA box (Schaefer and Messer, 1989). Flanking sequences may also be required as not all DnaA boxes in the correct orientation are capable of terminating transcription (Wang and Kaguni, 1989).

In contrast to its more common role as a negative regulator of transcription, at least one operon, the *nrd* operon encoding the subunits of ribonucleoside diphosphate reductase, appears to be stimulated by the binding of DnaA protein (Augustin *et al.*, 1994). Though the mechanism of this stimulation is not known,

DnaA protein might change the topology of the promoter to make RNA polymerase binding more efficient.

Regulation of initiation at *oriC*

It is well documented that DNA replication *in vivo* is regulated at initiation and not at elongation or termination (Helmstetter, 1994). Little is known about the mechanism of its regulation. Models include the accumulation of the initiator protein (Donachie, 1968) or the dilution of repressor proteins (Pritchard, 1969). Any model must account for the physiological observations that the rate of initiation corresponds with the rate of cell growth, and that all origins are initiated synchronously.

Although DnaA protein is central to most models, there is a period of time in the cell cycle during which *oriC* is refractory to further initiation, even when activators, such as DnaA protein, are present in excess. This refractory period is now referred to as the eclipse period. For example, when some *dnaA*(ts) mutants are held at non-permissive temperature for several mass doublings and then returned to permissive temperatures, the first two rounds of replication are initiated 25 to 30 minutes apart, which defines the eclipse (Hanna and Carl, 1975; Helmstetter and Krajewski, 1982). The eclipse period presumably involves negative regulatory factors.

DnaA protein in regulation of chromosomal replication

Because DnaA protein acts in initiation of chromosomal replication and regulates the expression of a number of genes, it is an obvious candidate for

regulating the time of initiation. Both biochemical and genetic evidence indicates that DnaA protein plays a role in triggering initiation at the appropriate time. First, *dnaA* mutants result in a slow stop phenotype, indicating that DnaA protein plays a role in initiation (Kohiyama, et al., 1966; Wechsler and Gross, 1971). Second, mutants that reduce the ability of DnaA protein to bind ATP result in asynchronous initiation (Skarstad *et al.*, 1986; Skarstad *et al.*, 1988). Third, temperature sensitive mutants, *dnaA46* and *dnaA5,* retain the potential to initiate even when incubated at nonpermissive temperatures (Evans and Eberle, 1975; Hanna and Carl, 1975). When returned to the permissive temperatures the missed initiation events proceed. These initiations are independent of *de novo* protein synthesis but still dependent on RNA synthesis (Lark, 1972). Fourth, in a strain containing a temperature sensitive suppressor, initiation is rapidly inhibited when amber mutants of *dnaA* are shifted to the non-permissive temperature. This suggests that continuous synthesis of DnaA protein is required for initiation (Schaus *et al.*, 1981).

If the concentration of DnaA protein is important in determining the initiation mass, changes in the amount of DnaA protein expressed should change the initiation mass. In earlier studies, no change in DNA content is detected when wild type DnaA protein is overproduced (Churchward *et al.*, 1983). More recently, several groups using more sensitive techniques have observed that initiation is shifted to an earlier time in the cell cycle, when DnaA protein was overproduced (Atlung *et al.*, 1987; Lobner-Olesen *et al.*, 1989; Pierucci *et al.*, 1987; Skarstad *et al.*, 1989; Xu and Bremer, 1988). This led to the speculation that initiation was controlled by *de novo* DnaA protein synthesis and that the

concentration of DnaA protein would increase with the age of the cell. Although growth rate and cell cycle-dependent changes in the concentration of *dnaA* transcripts are observed (Campbell and Kleckner, 1990; Chiaramello and Zyskind, 1990; Polaczek and Wright, 1990), the concentration of DnaA protein does not appear to change (Hansen *et al.*, 1991; Sakakibara and Yuasa, 1982). This issue should be revisited using more sensitive techniques.

It may not be the concentration of DnaA protein but its activity in replication that is regulated (Hupp and Kaguni, 1993a). Several studies have led to suggestions that the levels of DnaA protein need not vary during the cell cycle but it may be modulated by interactions with nucleotides, acidic phospholipids, cAMP or possibly other proteins. The high affinity of DnaA protein for nucleotides may be significant for regulating DnaA protein *in vivo*. Tightly bound ADP can be released by DnaA protein interaction with phospholipids (Sekimizu and Kornberg, 1988) or cAMP (Hughes *et al.*, 1988). Another possible mechanism may involve a specific inactivator protein (Katayama and Crooke, 1995), or activators (Hupp and Kaguni, 1993b; Hwang *et al.*, 1990).

Other proteins which bind oriC

oriC binding proteins other than DnaA protein may also be involved in the regulation of initiation. These proteins could indirectly regulate DnaA protein activity by binding *oriC*, thereby stimulating or inhibiting the binding of DnaA protein or unwinding of *oriC*.

Inhibitor of chromosomal initiation (IciA)

IciA, originally identified for its ability to bind specifically to the AT-rich 13-mers of *oriC* (Hwang and Kornberg, 1990), is able to inhibit *oriC* dependent *in vitro* replication when added to the reaction prior to DnaA protein (Hwang and Kornberg, 1992b). IciA inhibits replication by blocking the DnaA-dependent unwinding of the AT-rich region (Hwang and Kornberg, 1990; Hwang and Kornberg, 1992b). However, no phenotype is observed in *iciA* null mutants, suggesting that it is not a key regulator of the timing of initiation *in vivo* (Thony *et al.*, 1991).

Histone-like proteins

Histone-like proteins are thought to organize the bacterial chromosome, absorbing superhelical density induced by the negative superhelical nature of the bacterial chromosome, or in some circumstances, increasing superhelical tension to facilitate the melting of DNA necessary for replication and transcription (Pettijohn, 1988; Tanaka *et al.*, 1995). Histone-like proteins HU, inversion host factor (IHF) and factor for inversion stimulation (FIS) act in DNA replication (Dixon and Kornberg, 1984; Filutowic, *et al.*, 1992; Skarstad *et al.*, 1990), site-specific recombination (Kano *et al.*, 1989; Mertens *et al.*, 1986; Nash and Robertson, 1981), and regulation of transcription (Kohno *et al.*, 1994; Molina *et al.*, 1994; Ninnemann *et al.*, 1992; Rouviere and Gros, 1975) by changing the topology of the DNA and acting as architectural elements by stabilizing protein-protein and protein-DNA interactions (Finkel and Johnson, 1992; Segall, 1994). At *oriC*, HU or IHF facilitate the activity of DnaA protein in initiation.

HU binds DNA with little sequence specificity but has a preference for dA tracts (Shimizu *et al.*, 1995), bent DNA (Tanaka, 1984), and kinked DNA (Pontiggia *et al.*, 1993). On the binding of several dimers of HU, HU bends DNA (Shimizu *et al.*, 1995). Furthermore, HU can induce supercoiling in relaxed plasmids in the presence of topoisomerase I (Rouviere *et al.*, 1979).

Unlike HU, both IHF and FIS bind to specific DNA sequences (Finkel and Johnson, 1992; Wang *et al.*, 1995) to induce a bend. IHF binds bent DNA with a higher affinity than unbent DNA (Bonnefoy and Rouviere, 1991). Although IHF can induce supercoiling in relaxed DNA similar to HU, this occurs only at concentrations 10 times that required by HU (Tanaka *et al.*, 1995). Both IHF and FIS have a binding site in *oriC* (Filutowicz and Roll, 1990; Gille, et al., 1991).

HU is required for efficient *in vitro* replication of an *oriC* minichromosome (Dixon and Kornberg, 1984). HU facilitates DnaA protein-dependent unwinding of the AT-rich sequences within *oriC* (Bramhill and Kornberg, 1988a). HU is thought to bend *oriC* by wrapping DNA around several heterodimers (Broyles and Pettijohn, 1986). This is thought to aid DnaA protein in inducing torsional stress that is required to separate the two strands at the AT-rich region. HU may bind specifically to a naturally bent sequence, or a sequence bent by the binding of other proteins, and further bends *oriC* in a manner similar to IHF. In support of this idea, HU appears to bind preferentially to *oriC* in prepriming complexes visualized in electron micrographs and probed with anti-HU antibodies (Funnell *et al.*, 1987). The bending introduced by HU may aid DnaA-DnaA and/or DnaA-*oriC* interactions. IHF can replace HU in *in vitro* replication assays and in facilitating DnaA protein dependent unwinding of the AT-rich sequences

(Skarstad *et al.*, 1990). Although IHF and HU have different DNA binding characteristics, the mechanism by which they aid DnaA protein in unwinding the origin may be similar.

Ample evidence exists for the role of IHF in *in vivo* replication. *oriC* contains an IHF site located between DnaA boxes R1 and R2 (Filutowicz and Roll, 1990). When several point mutations are introduced at this site, the mutant minichromosomes fail to replicate (Roth *et al.*, 1994). *In vivo*, footprinting in synchronized cells suggests that IHF binds just prior to initiation (Cassler, 1995). Mutations in *himA* or *hip*, encoding the α and the β IHF subunits, respectively, fail to replicate minichromosomes in a manner characteristic of *oriC* dependent replication (Filutowicz and Roll, 1990). In addition mutations in *him* result in asynchronous initiation (Boye *et al.*, 1993). IHF is not generally thought to be a candidate for the regulation of initiation as it is found at high cellular concentrations, 8,500 to 17,000 dimers per cell, levels well above the K_D for binding *oriC* (Ditto *et al.*, 1994). However, this conclusion appears to contradict the *in vivo* footprinting results that indicate that the IHF binding site in *oriC* is bound only just before initiation of replication (Cassler, 1995).

The role of FIS at *oriC* is not well understood. *fis* mutants have an asynchronous phenotype (Boye *et al.*, 1993), are inefficiently transformed by minichromosmes, and are unable to stably maintain *oriC* plasmids (Gille *et al.*, 1991). Point mutations in the FIS binding site, located in *oriC* between DnaA boxes R2 and R3, inactivate *oriC in vivo* (Roth *et al.*, 1994). *In vivo* footprinting studies suggest that FIS is bound to *oriC* until the time of initiation (Cassler, 1995). Binding of FIS to this site is proposed to occlude DnaA protein from

DnaA box R2 (Gille *et al.*, 1991). Dissociation of FIS immediately prior to initiation is thought to allow DnaA protein to bind. On the other hand, FIS has also been speculated to facilitate unwinding the AT-rich region (Messer, 1996).

Interestingly, FIS levels vary with growth phase (Ball *et al.*, 1992). In early log phase, its cellular concentration reaches a maximum of 33,000 dimers per cell then diminishes over 500-fold. A burst of FIS dependent transcription of rRNA and tRNA operons occurs upon resumption of growth from stationary-phase cultures. Nutritional shift-up to a faster growth rate is accompanied by an increase of FIS levels (Ball *et al.*, 1992; Xu and Johnson, 1995). Consequently, it is speculated to serve as an element involved in the coupling of the growth rate to nutritional and environmental conditions.

Dam Methylation

Proteins proposed to function in the control of replication during the eclipse period include Dam methylase, and a number of membrane bound proteins that bind hemimethylated DNA. Dam methylase is a methyltransferase that modifies adenosine residues of the sequence GATC. Eleven Dam methylation sites are located within *oriC* and are conserved throughout the enterobacterial family (Zyskind *et al.*, 1983). *dam* mutants are asynchronous (Boye *et al.*, 1988; Lobner-Olesen *et al.*, 1994). Overexpressing Dam causes random timing of initiation (Bakker and Smith, 1989; Boye and Lobner-Olesen, 1990). Finally, in contrast to pBR322, minichromosomes isolated from dam^+ strains have a lower transformation efficiency into dam^- strains than wild type strains (Landoulsi *et al.*, 1989; Messer *et al.*, 1985). Since DNA is replicated semiconservatively, newly

replicated DNA is hemimethylated. Hemimethylated *oriC* is speculated to be inactive for replication *in vivo*. In agreement with this hypotheses, Russel and Zinder found that hemimethylated DNA accumulated in a *dam* mutant when transformed with fully methylated DNA (Russell and Zinder, 1987).

Most Dam methylation sites on the *E. coli* chromosome are fully methylated within 3 minutes after passage of the replication fork. Methylation at *oriC* and at the *dnaA* promotor occurs more slowly, occurring approximately 13 minutes after replication (Campbell and Kleckner, 1990), suggesting that *oriC* is sequestered from Dam methylase following replication. In support of this hypothesis, overproducing Dam methylase shortens the time between initiation events (Smith *et al.*, 1985). Based on the observations that membrane fractions bind specifically to hemimethylated *oriC*, Ogden *et. al.* speculated that the cellular membrane is involved in sequestration (Ogden *et al.*, 1988). This model is supported by the observation that membrane fractions, which bind specifically to hemimethylated *oriC,* inhibit replication specifically from hemimethylated *oriC* (Boye, 1991; Landoulsi *et al.*, 1990).

A number of different proteins isolated from membrane fractions are implicated in *oriC*-membrane interactions (Chakraborti *et al.*, 1992; Herrick *et al.*, 1994; Jacq *et al.*, 1989; Jacq and Kohiyama, 1980; Jacq *et al.*, 1983; Landoulsi *et al.*, 1990). SeqA protein is the best studied of these and has characteristics suggesting its involvement in sequestration. The *seqA* gene was identified by its ability to restore the transformablity of a *dam*$^-$ strain with methylated plasmids (Lu *et al.*, 1994; von Freiesleben *et al.*, 1994). *seqA* mutants exhibit an asynchronous phenotype. In addition, the life time of hemimethylation of *oriC* is reduced in a

seqA mutant. SeqA binds hemimethylated DNA with an affinity 5 times greater than that for methylated *oriC* (Slater *et al.*, 1995). However, SeqA binds specifically to *oriC* only when it is fully methylated suggesting that there must be other factors that are involved in specifically sequestering hemimethylated *oriC*.

Transcription around oriC

In vivo, the RNA polymerase inhibitor, rifampicin, inhibits replication from *oriC* (Lark, 1972). This requirement for RNA synthesis is independent of subsequent protein synthesis. As a result, RNA polymerase was identified as a factor in initiation. Originally, RNA polymerase was thought to form the primer that DNA polymerase III holoenzyme extends from *oriC*. However, omission of primase and inclusion of RNA polymerase resulted in very inefficient *in vitro* replication (van der Ende *et al.*, 1985). In contrast, efficient *in vitro* replication occurs when primase is included in the reaction and RNA polymerase omitted, suggesting that primase fulfills the function of priming. Currently, RNA polymerase is thought to involve changing the topology of *oriC*. Efficient *in vitro* replication can be made dependent on RNA polymerase by non optimal levels of the histone-like HU protein, low temperatures, or high levels of topoisomerase I (Baker and Kornberg, 1988; Ogawa *et al.*, 1985).

Changes in DNA topology by RNA polymerase can have a negative or a positive effect on initiation. RNA polymerase forms positive supercoils ahead of the transcriptional complex, which inhibit unwinding of *oriC* and forms negative supercoils behind, which aid in unwinding (Liu and Wang, 1987). Transcription away from *oriC* stimulates initiation (Asai *et al.*, 1992), whereas transcription into

oriC inhibits initiation.

Sequestration of hemimethylated *oriC* appears to inhibit replication from occurring in the first portion of the eclipse period. As *oriC* is fully methylated 10 to 20 minutes after the previous initiation event (Campbell and Kleckner, 1990; Ogawa and Okazaki, 1994; von Freiesleben *et al.*, 1994), inhibition by sequestration is overcome one third through the eclipse period. However, initiation induced by DnaA protein does not occur for about another 15 minutes (Hanna and Carl, 1975; Helmstetter and Krajewski, 1982; Leonard *et al.*, 1982). Recently, transcription from promoters flanking *oriC* has received attention because of the proposal that transcription of *mioC* inhibits initiation after sequestration, and transcription from *gidA* stimulates initiation (Figure 3). However, transcription from these promoters is not required for replication of *oriC* containing plasmids either *in vivo* (von Meyenburg, 1980) or *in vitro* (Kaguni and Kornberg, 1984). However, several reports indicate that replication of minichromosomes is more efficient in the presence of these promoters (Lobner-Olesen *et al.*, 1987; Ogawa and Okazaki, 1991; Stuitje *et al.*, 1986; Stuitje and Meijer, 1983).

Both *mioC* and *gidA* promoters are stringently controlled by ppGpp (Ogawa and Okazaki, 1991; Rokeach and Zyskind, 1986). This signal molecule is the mediator of the stringent response, varying in concentration following shifts in growth rates or amino acid starvation (Svitil *et al.*, 1993). Transcription from these promoters is cell cycle dependent, indicating a link with the timing of initiation (Bogan and Helmstetter, 1996; Theisen *et al.*, 1993). In synchronized cells, at the time of initiation *mioC* transcription is down-regulated. Transcription

from an inducible promoter into *oriC* from the right strongly inhibited initiation, consistent with transcription of *mioC* being inhibitory (Tanaka and Hiraga, 1985). In contrast, transcription from *gidA* is highest at the time of initiation (Bogan and Helmstetter, 1996; Ogawa and Okazaki, 1994; Theisen *et al.*, 1993). This is consistent with the notion that the formation of negative superhelicity due to *gidA* transcription would enhance unwinding of the AT-rich 13-mers.

Other properties of the *mioC* promotor add support to the model. The promotor region contains a DnaA box and transcription is repressed by DnaA protein (Bogan and Helmstetter, 1996; Lother, et al., 1985; Nozaki, et al., 1988; Schauzu *et al.*, 1987) MGG 209,518). Maximal transcriptional activity requires that the promotor is fully methylated (Schauzu *et al.*, 1987). These observations suggest the importance of transcription from these promoters in the proper timing initiation from *oriC*. However, recent studies indicate that these promoters can be deleted with no apparent effect on the timing or the synchrony of initiation (Bogan and Helmstetter, 1996).

VI. Thesis overview

My thesis project involves investigating the detailed mechanism of binding of DnaA protein to *oriC*. This investigation, described in Chapter 2, resulted in the observation that DnaA protein binds to *oriC* in a sequential manner, in which DnaA protein recognizes the outer DnaA boxes with higher affinity than the interior boxes. In addition, the formation of a complex in which DnaA protein bound all four DnaA boxes correlated with intiation of DNA replication. Furthermore, *in vivo* observations supportes the notion that binding of the

interior boxes may be important for the regulation of initiation at *oriC* (Cassler *et al.*, 1995; Samitt *et al.*, 1989). Additional *in vivo* observations suggestes that other *oriC* binding proteins, IHF and FIS, may play a role in binding of DnaA protein to the interior boxes. As little is known of the role of FIS in replication and the mechanism by which IHF stimulates replication, we investigated the effect of both proteins on the binding of DnaA protein and on *oriC*-dependent replication which is described in Chapters 3 and 4.

Chapter II

Ordered and Sequential Binding of DnaA Protein to *oriC*, the Chromosomal Origin of *Escherichia coli*

Abstract

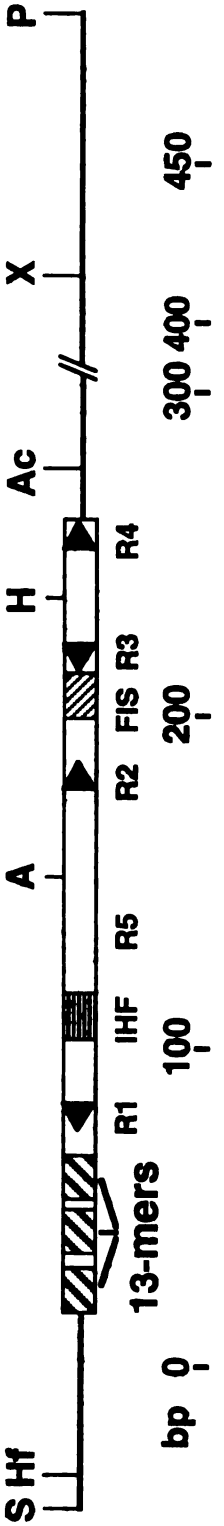
DnaA protein of *Escherichia coli* acts in initiation of chromosomal DNA replication by binding specific sequences, termed DnaA boxes in the chromosomal origin, *oriC*. On binding, it induces a localized unwinding to create a structure recognized by other replication proteins that act subsequently in the initiation process. In this report, we examined the binding of DnaA protein to each of the DnaA boxes in *oriC*. By gel mobility-shift assays, DnaA protein formed at least six discrete complexes. ATP or ADP included in the reaction mixture prior to electrophoresis was required. Chemical cleavage of isolated complexes with 1,10-phenanthroline-copper revealed that DnaA protein binds in an ordered manner to the DnaA boxes in *oriC*. Preferential binding to one DnaA box (R4) was confirmed by demonstration that a DNA fragment containing it was bound with greater affinity than another DnaA box sequence (R1). *In vitro* replication activity correlated with a complex formed at a ratio of 30 DnaA monomers per *oriC* in which all DnaA boxes are occupied. The last site bound is DnaA box R3. This event may be critical in promoting initiation from *oriC* as it correlates with *in vivo* observations that binding of DnaA protein to box R3 occurs at the time of initiation of chromosomal replication whereas other DnaA boxes are bound by DnaA protein throughout the cell cycle (M. R. Cassler, J. E. Grimwade, and A. C. Leonard (1995) *EMBO J.* **14**, 5833-5841).

Introduction

DnaA protein of *Escherichia coli* is a sequence-specific DNA binding protein, proposed to recognize 9-mer sequences termed DnaA boxes, present in 4 copies within the chromosomal origin, *oriC* (Figure 1) (Fuller *et al.*, 1984). At *oriC*, its binding promotes an ordered series of events to result in the initiation of chromosomal DNA replication (reviewed in Bramhill and Kornberg, 1988b). The binding of DnaA protein to *oriC* has been examined by a variety of methods. By electron microscopy (Crooke *et al.*, 1993; Funnell *et al.*, 1987) and DNase I footprinting (Fuller *et al.*, 1984), a large nucleoprotein structure containing 20-30 monomers of DnaA protein is formed at *oriC*. Experiments to examine its interaction with individual DnaA boxes led to the conclusion that it bound to the two centrally located DnaA boxes in *oriC* (R2 and R3) with greater affinity than to the flanking DnaA boxes (R1 and R4) (Schaefer and Messer, 1991). This was based on an indirect assay that assessed the activity of DnaA protein as a transcriptional terminator *in vivo*. Expression from the *lac* promoter, located upstream to the mutant DnaA box being examined, was measured.

By contrast, gel mobility shift assays with oligonucleotides of 21 base pairs containing various DnaA boxes with natural flanking sequences indicated that DnaA protein binds to DnaA boxes R1 and R4 of *oriC* with higher affinity than R2 (Schaper and Messer, 1995). DnaA box R3 was bound as poorly as non-specific oligonucleotides. In addition to these *in vitro* findings, *in vivo* footprinting of *oriC* plasmids with dimethylsulfate in exponentially growing cells revealed protection of DnaA boxes R1, R2, and R4 with little binding to R3

Figure 1. **The *oriC* region**. DnaA boxes R1-R4 are the 9-mers recognized by DnaA protein. DnaA box R5 (Matsui *et al.*, 1985) is also shown. Binding sites for IHF (Filutowicz and Roll, 1990; Polaczek, 1990), and Fis (Filutowicz *et al.*, 1992; Finkel and Johnson, 1992; Gille, et al., 1991), 13-mer motifs recognized by IciA protein (Hwang and Kornberg, 1990), and restriction enzyme sites (S, *Sma* I; Hf, *Hinf* 1; A, *Ava* II; H, *Hin*D III; Ac, *Acc* I; X, *Xho* I; and P, *Pst* I) of pBS*oriC* are indicated.


S
Hf
bp 0
13-mers
R1
IHF
R5
A
R2
FIS
R3
H
R4
Ac
300 400
X
450
P

(Cassler, 1995; Samitt *et al.*, 1989). These observations, suggesting that the binding of DnaA protein to R3 is critical for the initiation process, are supported by the observation that occupancy of R3 occurs at the time of initiation of DNA replication in synchronized cultures. Another study reported that mutations in single DnaA boxes (R1 and R4) of *oriC* reduced binding of DnaA protein to respective sites (Schaefer and Messer, 1991) but only reduced replication activity when both mutant sequences were present together (Holz *et al.*, 1992). The replication activity of *oriC* may tolerate an alteration of one of the binding sites (Holz *et al.*, 1992), perhaps by the speculated cooperative binding of DnaA protein, or occupancy of all four DnaA boxes is not required for replication.

Other proteins have been characterized to bind to specific regions of *oriC*. IciA protein was isolated by its ability to bind specifically to 13-mer motifs near the left boundary of *oriC* (Hwang and Kornberg, 1990) (Figure 1). Its binding inhibits the initiation process. IHF, and Fis binding sites in *oriC* have been described (Filutowicz and Roll, 1990; Filutowicz *et al.*, 1992; Finkel and Johnson, 1992; Gille *et al.*, 1991; Polaczek, 1990). Rob protein binds to a region near the right boundary of *oriC* but its significance is unknown (Skarstad *et al.*, 1993).

The studies summarized above do not provide a clear understanding of whether DnaA protein binds to sites in *oriC* randomly, or in an ordered and sequential manner in the process of initiation of chromosomal replication. If this event is ordered, the binding of other proteins to *oriC* may inhibit or augment the initiation process. In this report, we use gel mobility-shift and DNA footprinting techniques to characterize complexes of DnaA protein bound to *oriC*. Results indicate that DnaA protein binds to *oriC* in an ordered manner. DnaA box R4 is

bound first, then R1, and finally the two inner boxes. Formation of these discrete complexes was dependent on ATP or ADP. Replication activity correlates with binding to all four DnaA boxes.

Experimental Procedures

Reagents and chemicals--4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), Tris, and dithiothreitol (DTT) were obtained from Calbiochem-Behring. Cesium chloride was obtained from Gallar-Schlesinger Industries, Inc.; triton X-100 from Research Products International, Co.; agarose from Gibco BRL; acrylamide from Fisher Biotech; bisacrylamide from Boehringer Manheim; urea from Research Organics; (ethylenedinitrilo)tetracetic acid (EDTA) and $CuSO_4$ from JT Baker Chemical Co.; 1, 10-phenathroline and 2,9 dimethyl-phenanthroline were obtained from Aldrich; [α-^{32}P] dNTPs (3000 Ci/mmol) from DuPont-New England Nuclear Corp; [^{3}H]TTP obtained from ICN Radiochemicals.

Proteins and plasmids---*Sma*I was obtained from New England Biolabs: *Xho*l from United States Biochemicals; the large fragment of DNA polymerase I from Boehringer Manheim. All plasmids were purified by cesium chloride gradient.

Gel mobility-shift assays (Fried and Crothers, 1981; Garner and Revzin, 1981)--Unless noted, a *Sma* I-*Xho* l fragment containing *oriC*, gel-purified from pBS*oriC* (Baker and Kornberg, 1988) with a Qiaex DNA extraction kit (Qiagen) and quantified by absorbance at 260 nm, was 3' end-labelled with the large fragment of DNA polymerase I, and [α^{32}P-dTTP], then combined with the same unlabelled fragment to adjust its specific radioactivity to 4 x 10^3 cpm per 25 fmol of DNA. Reactions (10 µl) with the labelled *oriC* fragment (25 fmol) and

indicated amounts of DnaA protein were incubated in buffer containing 20 mM HEPES[1]-KOH pH 8.0, 5 mM magnesium acetate, 1 mM EDTA, 4 mM DTT, 0.2% Triton X-100, 5 mg/ml bovine serum albumin, and 0.5 μM ATP (unless noted otherwise) at 20 °C for 5 min (Parada and Marians, 1991). The samples were electrophoresed in a 4% polyacrylamide gel (60 parts acrylamide:1 part bis-acrylamide) (13.5 x 13.5 x 0.15 cm) in 45 mM Tris-borate, and 1 mM EDTA at 80 V for 3 to 4 hours. Gels were dried and autoradiographed with Hyperfilm MP (Amersham) at -70 °C using a Cronex Quanta III intensifying screen, or image-analyzed with a Molecular Dynamics PhosphorImager.

In situ cleavage with 1,10 phenanthroline-copper (Kuwabara and Sigman, 1987)--Gel mobility-shift assays were performed as above but scaled up 10-fold. After electrophoresis, the wet gel was immersed for 2.5 to 4.5 min in 200 ml of 42 mM Tris-HCl pH 8.0, 0.2 mM phenanthroline, 38 μM $CuSO_4$, and 5 mM 3-mercaptopropionic acid at room temperature. To quench the reaction, 2,9 dimethyl-phenanthroline was added to 2.3 mM followed by incubation for 2 min. The gel was quickly washed with water and autoradiographed for 1 h at room temperature. The developed film was used to guide excision of the complexes. DNA from the gel slices was eluted overnight at 37 °C in 500 μl of elution buffer (0.5 M ammonium acetate, 0.2% SDS, 1 mM EDTA, 10 μg/ml Proteinase K, and 100 μg/ml tRNA). After recovering the elution buffer, an additional 200 μl of elution buffer was used to wash the gel slices, and both were pooled. The eluted DNA was ethanol precipitated, washed with 70% ethanol, dried, and resuspended in 5 μl of 80% (v/v) formamide, 10 mM NaOH, 1 mM EDTA, 0.1% bromophenol

blue, and 0.1% xylene cyanol, heated to 95 °C for 2 min, and electrophoresed at 50 W on a pre-run 6% sequencing gel. After autoradiography, beta emission scanning was with a Molecular Dynamics PhosphorImager. Graphed with Excel (Microsoft), the radioactivity in each lane was normalized to the cleavage pattern of unbound DNA that was isolated from the gel.

DNA replication assays--Reactions (25 µl) were performed as described (Hwang and Kaguni, 1988) with a crude protein fraction deficient in DnaA protein activity, M13*oriC*26 DNA (25 fmol) as a template and the indicated amounts of DnaA protein. Incubation to measure DNA synthesis was at 30 °C for 20 min. Acid-insoluble incorporation of [^{3}H]TTP was quantified by liquid scintillation counting.

Results

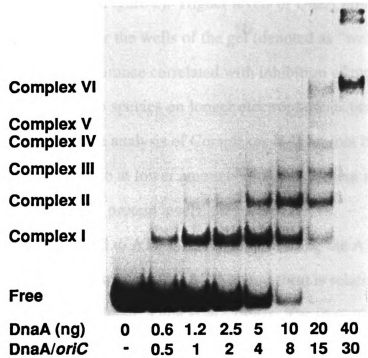
Six discrete complexes are formed on binding of DnaA protein to oriC--By footprint analysis, and electron microscopy, the nucleoprotein complex of DnaA protein bound to *oriC* is estimated to contain 20-30 monomers organized to occupy the four DnaA boxes (Crooke *et al.*, 1993; Fuller *et al.*, 1984; Funnell *et al.*, 1987). Sequence comparison of these DnaA boxes reveal that R1 and R4 are identical, whereas R2 and R3 differ at the fifth and seventh positions, respectively (Table 1). Inasmuch as other studies indicated that several nucleotide changes at each of the positions only marginally affected the ability of DnaA protein to bind to *oriC*, and that mutant DnaA boxes of *oriC* did not affect *in vivo* replication activity when singly present (Holz *et al.*, 1992), the binding of DnaA protein to *oriC* may not show a strong preference to one site relative to others. To investigate this, we examined the binding of DnaA protein to sites in *oriC* by use of a gel mobility-shift assay. With increasing amounts of DnaA protein, six discrete complexes were observed (Figure 2). Complex I was observed at the lowest ratio of DnaA protein to *oriC* fragment. Complexes of slower mobility were observed at higher ratios. Compared to other complexes, the minor abundance of Complexes IV, and V suggests their lesser stability. Complex VI was predominant at a ratio of 30 monomers of DnaA protein per DNA fragment, consistent with electron microscopic measurements (Fuller *et al.*, 1984). In this lane, other material near the well of the gel corresponds to that formed by a self-aggregate of DnaA protein (unpublished results).

Table 1. **Sequences of DnaA boxes in *oriC* and in the *dnaA* promoter region**

DNA	Sequence[a]			
oriC R1	CGGATCCTTG	TTATCCACA	GGGCAGTGCG	
oriC R2	GAATGAGGGG	TTATACACA	ACTCAAAAAC	
oriC R3	CAACCGGTAG	TTATCCAAA	GAACAACTGT	
oriC R4	CCTGACAGAG	TTATCCACA	GTAGATCGCA	
DnaA box in the *dnaA* promoter region	TTTTCCCGAT	TTATCCACA	GGACTTTCCA	
DnaA box in M13mp18	ATCACTCGAG	TTATCCACA	CTAGAGTCGA	
Position	-10 -1	123456789 core sequence	10	19

[a]The polarity of respective DnaA boxes is from left to right.

Figure 2. **DnaA protein bound to *oriC* forms six complexes that are resolved by native polyacrylamide gel electrophoresis.** Gel mobility-shift assays were performed as described in "Experimental Procedures" with the indicated amounts of DnaA protein and a ^{32}P-labelled DNA fragment containing *oriC*. Unbound DNA is noted as "Free."


Complex VI
Complex V
Complex IV
Complex III
Complex II
Complex I
Free
DnaA (ng) 0 0.6 1.2 2.5 5 10 20 40
DnaA/oriC - 0.5 1 2 4 8 15 30

Formation of Complex VI correlates with formation of an active replication complex--Quantitative analysis was done of complexes in an experiment comparable to that of Figure 2 but with a larger range of added protein (Figure 3). In parallel, the replication activity of DnaA protein was measured. Replication activity correlated with the level of DnaA protein that formed Complex VI. At this level, complexes of greater mobility were relatively minor in abundance (as in Figure 2). Higher levels of DnaA protein resulted in material that remained near the wells of the gel (denoted as "well" in Figure 3). This material, whose appearance correlated with inhibition of replication activity, could be resolved into two species on longer electrophoresis (data not shown). For simplicity, quantitative analysis of Complexes II-V has not been shown in Figure 3 as their abundance at lower amounts of DnaA protein are represented in Fig. 2, and at higher DnaA protein levels, they were rare.

DnaA protein bound to ATP is active in unwinding the AT-rich 13-mers located in *oriC* (Figure 1) whereas the ADP-bound form is relatively inert (Bramhill and Kornberg, 1988a; Yung *et al.*, 1990). The importance of the nucleotide-bound form led us to investigate the effect of ATP and ADP on complex formation. Formation of discrete complexes was dependent on inclusion of ATP or ADP in the reaction prior to electrophoresis (Figure 4), although with ADP, the amount of Complex VI was reduced. The minor species resolved between complexes V and VI in Figure 4 were rarely observed in other comparable experiments. In the absence of nucleotide, trace amounts of Complex I and VI were observed. Instead, the labelled DNA remained near the wells of the gel. The effect of nucleotide on formation of discrete complexes may relate to the

Figure 3. **Formation of Complex VI correlates with DNA replication activity**. A gel mobility-shift assay was performed with the indicated levels of DnaA protein. The relative amounts of Complex I, VI, (as well as Complexes II-V, not shown) and material near the well were quantified by beta emission scanning. In a parallel experiment, replication activity of DnaA protein was measured (see "Experimental Procedures").

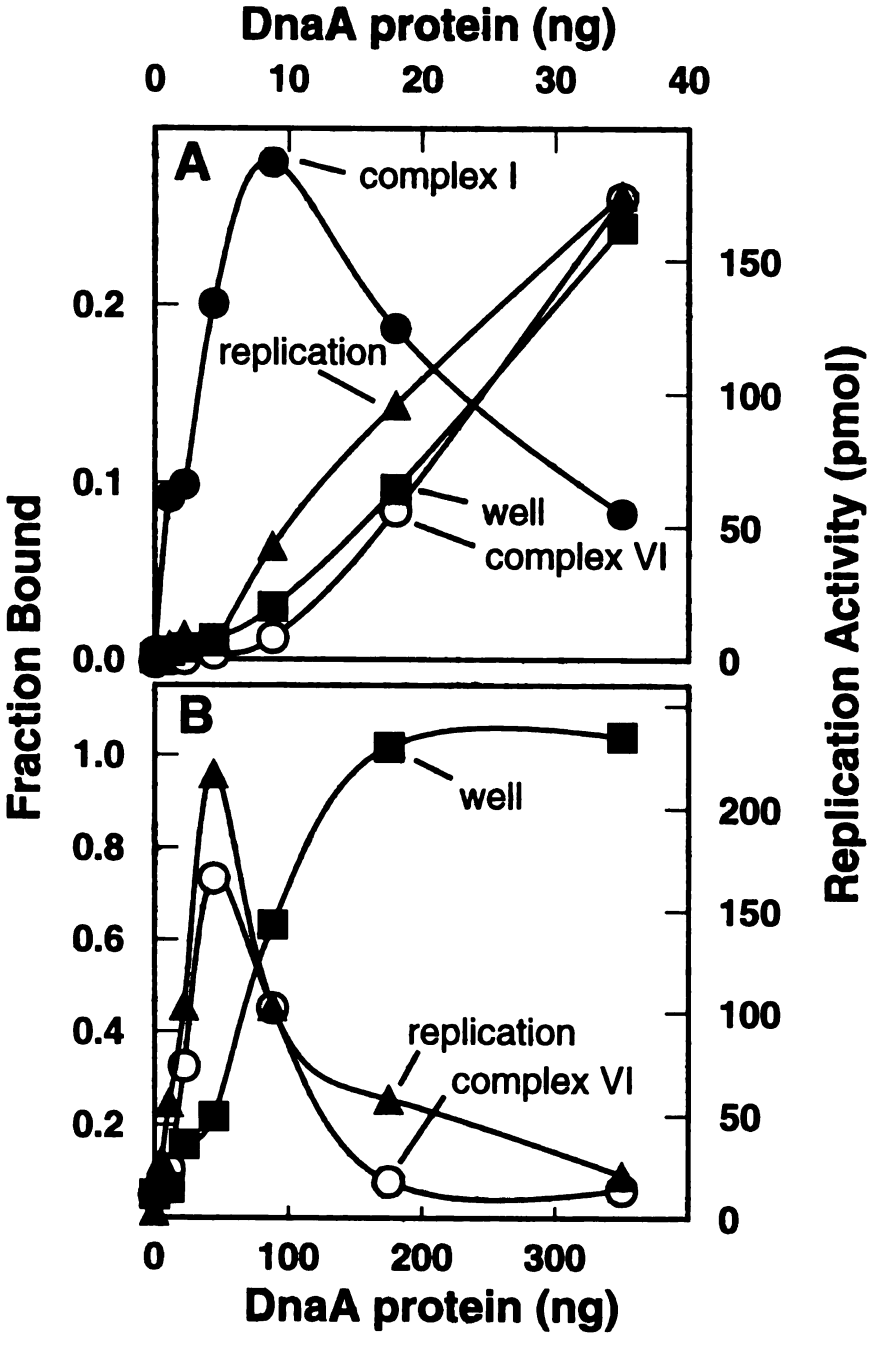
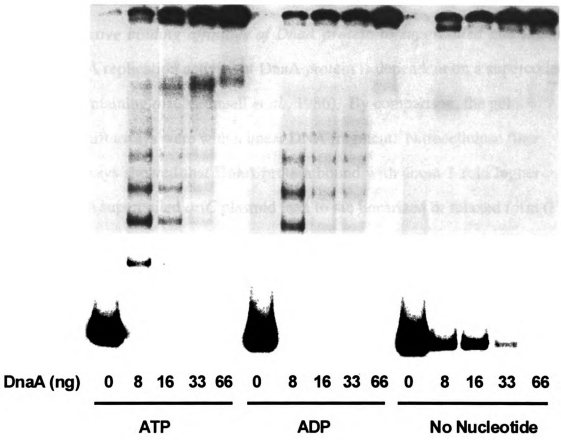

DnaA protein (ng)
0
10
20
30
40
A
complex I
replication
well
complex VI
0.2
0.1
0.0
150
100
50
0
B
well
replication
complex VI
1.0
0.8
0.6
0.4
0.2
200
150
100
50
0
0
100
200
300
DnaA protein (ng)
Fraction Bound
Replication Activity (pmol)

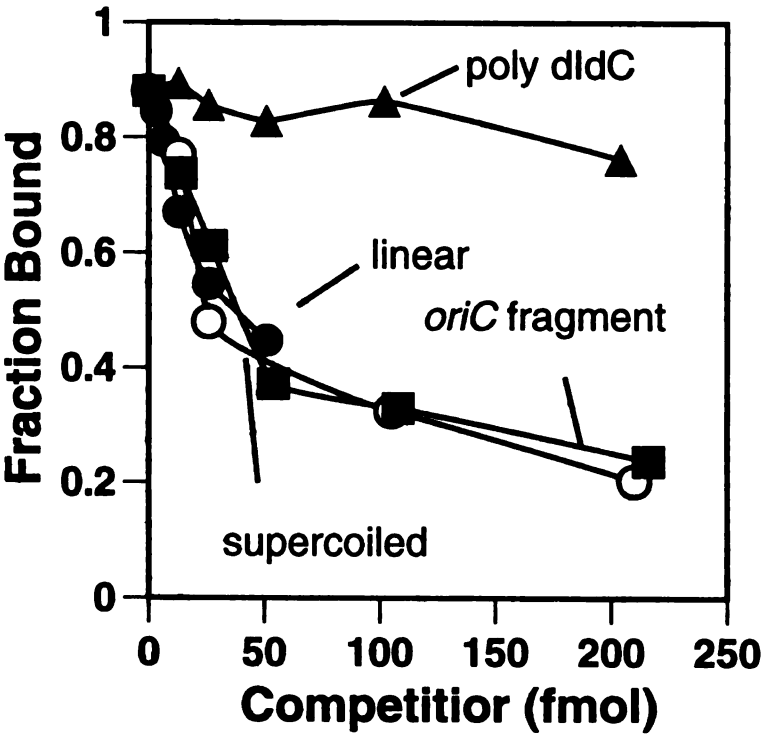
Figure 4. **Discrete complexes are formed by the ATP- or ADP-bound form of DnaA protein**. Gel mobility-shift assays were performed as described in "Experimental Procedures" with the indicated amounts of DnaA protein and a ^{32}P-labelled DNA fragment containing *oriC*. ATP or ADP (2 mM each) were included in the indicated reactions.


DnaA (ng) 0 8 16 33 66 0 8 16 33 66 0 8 16 33 66
ATP
ADP
No Nucleotide

observation that DNase I protection of DnaA boxes in *oriC* by the ATP-bound form of DnaA protein was localized to the DnaA boxes whereas binding by the nucleotide-free form was less specific (Hwang and Kornberg, 1992b) By electron microscopy (Crooke *et al.*, 1993), the classes of structures formed in the presence of either nucleotide may correlate to some of the complexes resolved here (Figures 2, & 4).

Relative binding affinities of DnaA protein to supercoiled and linear DNA--DNA replication activity of DnaA protein is dependent on a supercoiled plasmid containing *oriC* (Funnell *et al.*, 1986). By comparison, the gel mobility-shift assays were with a linear DNA fragment. Nitrocellulose filter binding assays showed that DnaA protein bound with about 3-fold higher affinity to a supercoiled *oriC* plasmid than to the linearized or relaxed form (Fuller and Kornberg, 1983). To examine this issue with the gel mobility-shift assay, unlabelled competitor DNA was added to reactions containing a fixed level of DnaA protein and radioactively labelled *oriC* fragment (Figure 5). Addition of either supercoiled (estimated to be contaminated by ~10% nicked DNA by resolution by agarose gel electrophoresis and quantitative densitometry), linearized *oriC* plasmid, or the same unlabelled restriction fragment resulted in a comparable reduction of DNA binding to the labelled DNA, measured by densitometric analysis of the autoradiogram. By contrast, poly dIdC was an ineffective competitor. These results indicate a comparable binding affinity of DnaA protein to supercoiled or linear DNAs containing *oriC*. The apparent discrepancy between these observations and the cited study may be due to the absence of ATP in the filter binding assays (Fuller and Kornberg, 1983) whereas it

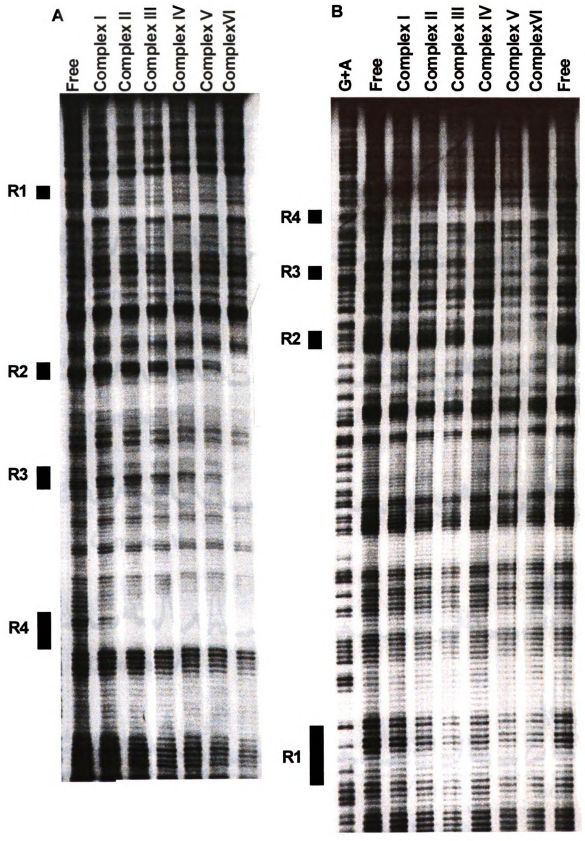
Figure 5. **Both supercoiled and linear *oriC* plasmids are comparable in reducing binding of DnaA protein to DnaA box R4.** The indicated amounts of unlabelled DNAs (supercoiled pBS*oriC*, it linearized with *Pst* I, or the *Sma* I-*Xho* I *oriC* fragment) as competitor were combined with a *Sma* I-*Xho* I *oriC*-containing fragment (25 fmol) radioactively labelled by 3' end-filling of the *Xho* I site with [α-^{32}P] TTP. DnaA protein (18 ng) was then added. Poly dIdC was added in equivalent mass to plasmid DNA. Other procedures were as described in "Experimental Procedures". The amount of free DNA in each lane was quantified by beta emission scanning to calculate the amount of DNA bound.


1
0.8
0.6
0.4
0.2
0
Fraction Bound
poly dIdC
linear
oriC fragment
supercoiled
0
50
100
150
200
250
Competitior (fmol)

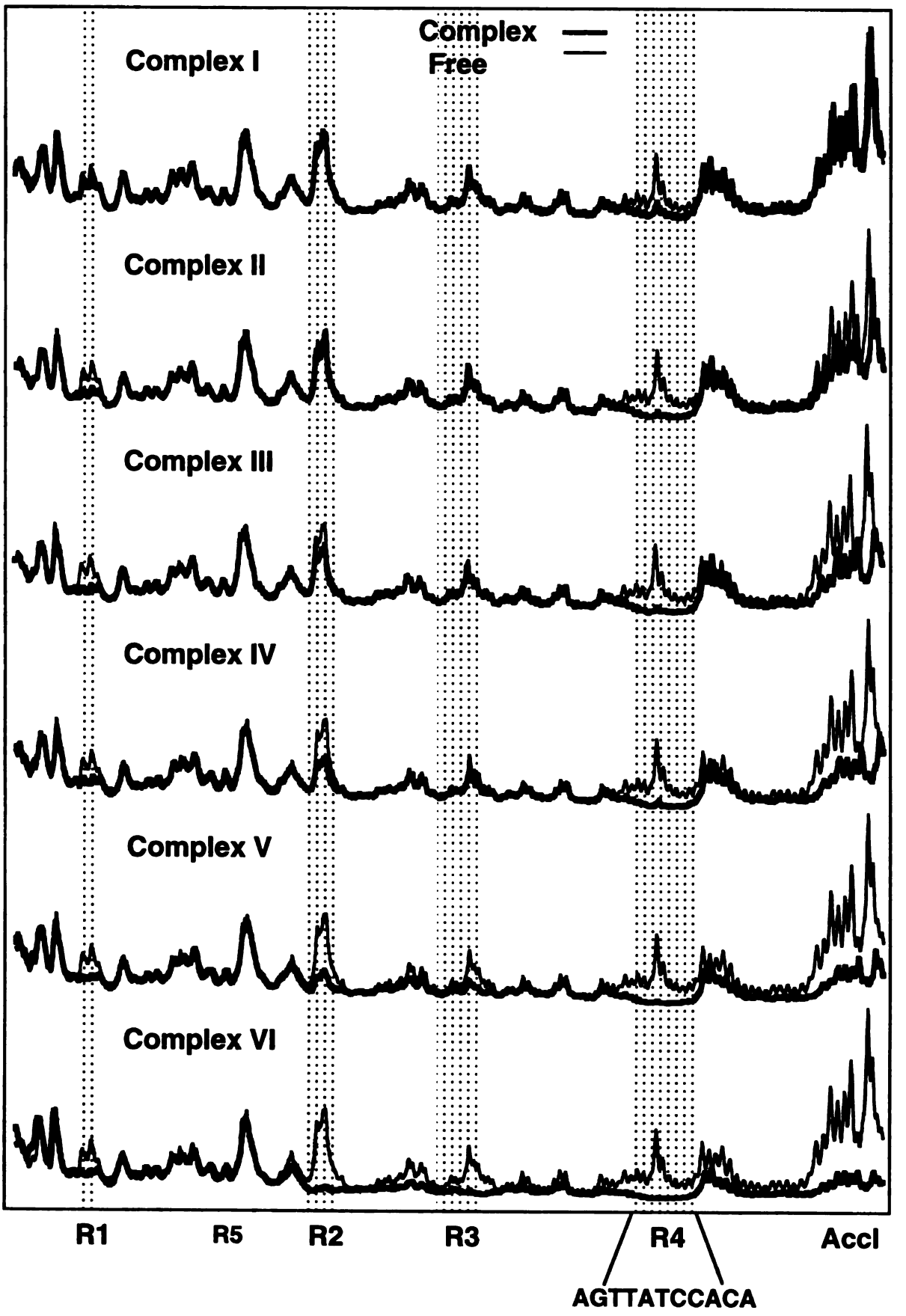
was present in the gel mobility-shift experiments (Figure 5). We have not examined the effect of ATP on binding affinity to different topological forms of *oriC*-containing plasmids nor compared the effect of supercoiled and linear *oriC*-containing DNAs by each assay method.

Ordered binding of DnaA protein to oriC--To determine the sites bound by DnaA protein in the separated complexes, *in situ* footprint analysis was performed with 1,10 phenanthroline-copper (Kuwabara and Sigman, 1987). Complexes formed with DNA labelled in the top or bottom strand were examined (Figure 6 A, B). Regions protected from chemical cleavage were identified by quantitative analysis of the resultant autoradiograms (Figure 6 C, D). Complex I consisted of DnaA protein bound to R4. R1 was additionally protected in Complex II. Complex III differed from Complex II by protection of R2 as well as sequences to the left of R4 in vicinity of the *Acc* I site. Binding to the region encompassing the *Acc* I site is likely not responsible for the electrophoretic position of this and more slowly migrating complexes (described below). Gel-mobility shift experiments with a DNA fragment lacking the *Acc* I site resulted in a similar number and proportion of complexes (data not shown). The scarcity of Complex IV and V relative to other complexes (Figure 2, lanes with 10-40 ng of DnaA protein) suggests that these may be of lesser stability. Nonetheless, greater protection of R2 was observed in Complex IV compared to Complex III (as well as protection of the region containing the *Acc* I site). Whereas some protection of R3 was seen in Complex IV (Figure 6 D), it was enhanced in Complexes V and VI. Complex VI consisted of protection of the 4 DnaA boxes as well as flanking sequences in the region from R2 to R4. The altered mobility of Complex IV and V

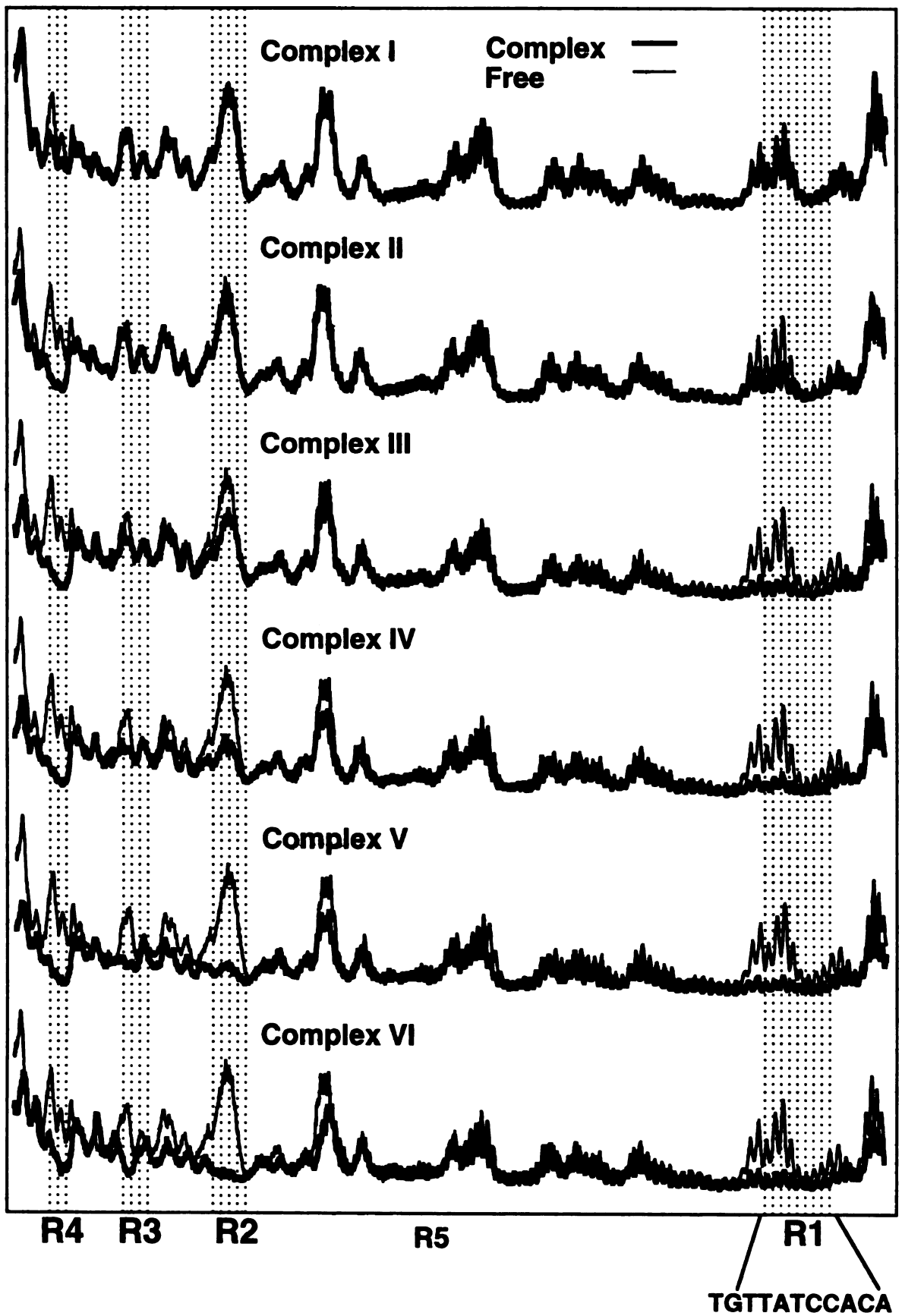
Figure 6. ***In situ* footprinting of complexes with 1,10-phenanthroline-copper.** *In situ* cleavage was performed as described in "Experimental Procedures" with a *Sma* I-*Xho* I fragment labelled at the *Xho* I site with the large fragment of DNA polymerase I and all 4 [α^{32}P] deoxyribonucleotides (top strand, panel **A**), or at the *Hin*f1 site of a *Hin*f1-*Pst* I fragment by incorporation of all 4 [α^{32}P] deoxyribonucleotides and subsequently cleaved with *Xho* I (bottom strand, panel **B**). The cleavage pattern of respective complexes was analyzed by beta emission scanning and compared to the cleavage pattern of the corresponding unbound fragment that was treated similarly (panels **C** and **D**). Maxam-Gilbert G and G+A reactions performed on the appropriate *oriC* fragment served as size markers. The sequence of R4 (panel **C**) and R1 (panel **D**) with sequences at -1 and -2 positions (Table 1) is presented at the bottom.


A
Free
Complex I
Complex II
Complex III
Complex IV
Complex V
ComplexVI
R1
R2
R3
R4
B
G+A
Free
Complex I
Complex II
Complex III
Complex IV
Complex V
ComplexVI
Free
R4
R3
R2
R1

C


Complex
Free
Complex I
Complex II
Complex III
Complex IV
Complex V
Complex VI
R1
R5
R2
R3
R4
AccI
AGTTATCCACA

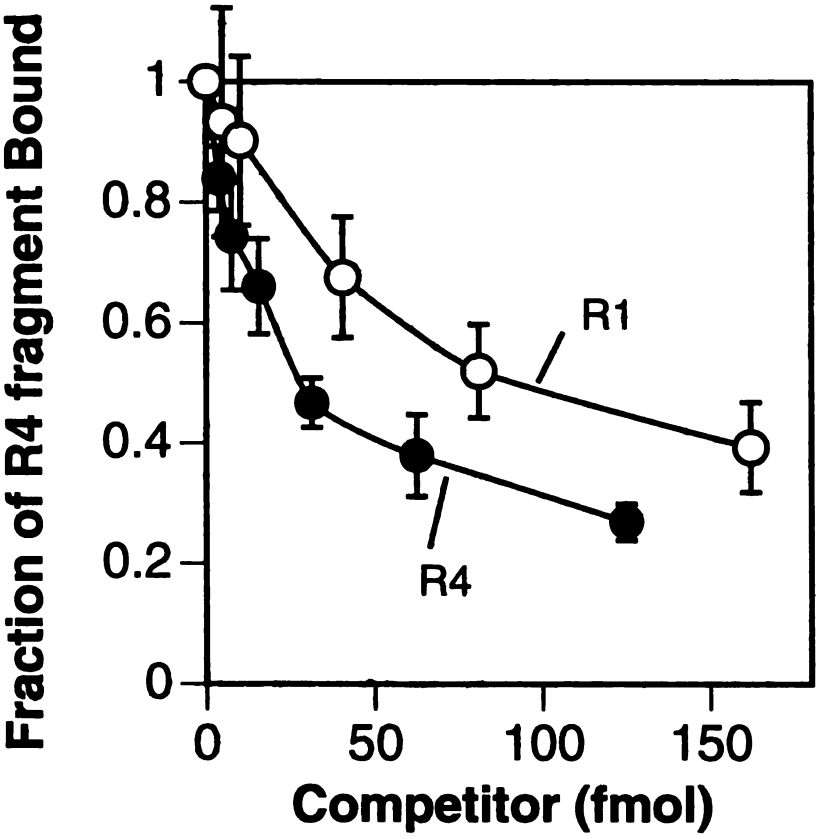
D


Complex I
Complex
Free
Complex II
Complex III
Complex IV
Complex V
Complex VI
R4
R3
R2
R5
R1
TGTTATCCACA

relative to III may be due to more stable binding of DnaA protein to R2, and R3, possibly by interaction among proteins bound to different DnaA boxes by bending or looping of the DNA. In summary, these findings indicate that DnaA protein binds, in order, to R4, R1, then R2 and R3. These methods do not distinguish the possible contribution that binding to one site may have on subsequent binding to another site due to cooperativity.

DnaA protein binds to DnaA box R4 with greater affinity than to R1--Results from gel retardation and footprinting experiments indicated that DnaA box R4 is bound with greater affinity than R1 by DnaA protein. To determine the relative affinity of DnaA protein to restriction fragments containing only box R1 or R4, gel shift assays were performed with unlabelled DNAs as competitors. Addition of increasing amounts of unlabelled fragment containing R4 resulted in proportional inhibition of binding to the labelled R4 fragment present at a constant level (Figure 7). By comparison, addition of unlabelled R1 fragment was less effective. These results, the average of 3 independent experiments, confirm that DnaA protein binds to R4 in *oriC* with greater affinity than R1. As the 9-mer sequence of R4 is identical to that of R1, sequences that flank the DnaA box appear to contribute to binding affinity. The protection of residues at -1 and -2 positions (Table 1) outside the 9-mer sequence of R4 in Complex I (Figure 6C) and of R1 in Complex II (Figure 6D) supports this conclusion. Also, the binding affinity to R2 and R3 apparently is less and may be due to sequence differences in respective 9-mers in addition to the influence of flanking sequences (Table 1).

Figure 7. **DnaA protein binds to DnaA box R4 with greater affinity than to R1.** An *Eco*O1091-*Pst* I restriction fragment (25 fmol) containing DnaA box R4 from pBS*R4*, radioactively labelled by end-filling at the *Eco*O1091 site with [α^{32}P] dGTP, was incubated with the indicated amounts of the same unlabelled fragment, or an unlabelled *Sma* I-*Ava* II restriction fragment containing DnaA box R1. pBS*R4* was constructed by insertion of the *Hin*d III-*Pst* I fragment (gel-purified) containing DnaA box R4 of *oriC* into corresponding sites of pBluescript II SK+ (Stratagene). DnaA protein (3 ng) was added and complexes resolved from free DNA as described in "Experimental Procedures." After autoradiography, the amount of free DNA remaining in each lane was quantified by beta emission scanning, then the amount of DNA bound was calculated. The amount of radiolabelled DNA bound in the absence of competitor was normalized to 1.


Fraction of R4 fragment Bound
1
0.8
0.6
0.4
0.2
0
0
50
100
150
Competitor (fmol)
R1
R4

Discussion

Ordered and sequential binding of DnaA protein to oriC--By use of a gel retardation assay in conjunction with protection from cleavage by 1,10 phenanthroline-copper, the binding of DnaA protein to sequences in *oriC* was found to be ordered and sequential. The 6 discrete complexes (and material near the wells seen at higher levels of DnaA protein) may correlate with the seven unique structures detected by electron microscopy (Crooke *et al.*, 1993). At higher levels of DnaA protein, the inhibition of replication activity correlated with formation of material that entered the gel poorly (Figure 3). Complex VI (Fig. 2) may correspond to structure 3 (Crooke *et al.*, 1993) as the formation of both correlated with optimal replication activity. Second, both structure 3 and complex VI formed more efficiently with ATP than with ADP in the reaction mixture. In the absence of nucleotide DnaA protein failed to form discrete complexes. This apparently is due to aggregation of DnaA protein that occurs on its incubation without ATP (J. Marszalek and J. M. Kaguni, unpublished results).

By comparison, similar experiments with a *dnaA* promoter fragment containing a DnaA box identical to R4 of *oriC* (Table 1) and a weak DnaA box have been performed (unpublished results). We observed two prominent complexes and two minor, more slowly migrating species.

A supercoiled template containing *oriC* is required for *in vitro* replication (Funnell *et al.*, 1986). Relating this requirement to structure 3, it was observed more frequently with supercoiled DNA than linear DNA (Crooke *et al.*, 1993). We have not determined whether complex VI forms more efficiently on

supercoiled DNA than linear or relaxed DNA, despite attempts to resolve complexes formed on supercoiled DNA in low percentage agarose gels. In addition, we have been unsuccessful in demonstrating ordered binding of DnaA protein on a supercoiled *oriC*-containing plasmid. The method used was quantitative footprint analysis with DNase I or 1,10 phenanthroline of complexes formed in solution, followed by primer extension. Footprinting in solution provides an averaged picture of complexes formed. At lower ratios of DnaA protein to *oriC* where we expected to see preferential binding to DnaA box R4 then to R1, we presume that the amount of free DNA masks the protection pattern resulting from ordered binding. Also, we presume that this reason explains why ordered binding was not observed in previous reports (Fuller *et al.*, 1984; Hwang and Kornberg, 1992b). Although solution footprinting on supercoiled DNA failed to detect ordered binding, competition experiments demonstrated that DnaA protein binds with a similar affinity to supercoiled or linear DNAs containing *oriC* (Figure 5). This suggests that DnaA protein binds to either topological form by a similar mechanism.

Despite the identical 9-mer sequences of R1 and R4 (Table 1), DnaA protein bound to R4 with about 3-fold higher affinity than to R1 (Figure 7). Presumably, sequences that flank the 9-mer in R4 contribute to its higher binding affinity. Indeed, the protection of flanking sequences at -1 and -2 positions of R4, and R1, clearly seen in Complexes I and II (Figure 6C & 6D), indicates that DnaA protein binds to residues outside of the core sequence. Whether the differences in sequences at the -2 position of R4 compared to R1 is responsible for the different binding affinities can be tested directly. Other evidence supports the

notion that flanking sequences contribute to binding affinity. With a nitrocellulose filter binding assay, we found that DnaA protein bound 4-fold greater to the DnaA box in a *dnaA* promoter-containing fragment than to a synthetic DnaA box (9-mer) inserted into the multiple cloning site of M13mp18 (Table 1) (Q. Wang and J. M. Kaguni, unpublished results). These observations are also supported by the 50-fold difference in binding affinity to a specific DnaA box when flanking sequences were varied (Schaper and Messer, 1995).

The observations described here are in contradiction to the conclusion that DnaA protein bound with higher affinity to central DnaA boxes (R2 and R3) relative to the flanking sites (R1 and R4) (Schaefer and Messer, 1991). This deduction was based on an indirect method that measured expression of *galK* dependent on transcription from the *lac* promoter. The DnaA box sequence being assessed was positioned between the promoter and the *galK* gene. The relative ability of DnaA protein to function as a transcriptional terminator to affect *galK* expression was the basis of the assay. It is possible that differences in mRNA stability and/or translational efficiency may have influenced the results obtained.

Four lines of evidence correlate the initiation of DNA replication to the binding of DnaA protein to DnaA box R3. First, dimethylsulfate treatment of an *oriC* plasmid carried in an exponentially growing strain revealed that DnaA box R3 was not bound whereas the remaining sites were (Samitt *et al.*, 1989). Such minichromosomes in which plasmid replication occurs from *oriC* are duplicated once per generation (Koppes and von Meyenburg, 1987), and synchronously with the bacterial chromosome (Helmstetter and Leonard, 1987a). Assuming that

replication fork movement is the same as that of the bacterial chromosome, *oriC* plasmid replication should be completed within a few seconds. As dimethylsulfate treatment was for 2 min, most plasmids should not be active in replication. These observations suggest 2 possibilities. One is that the level of DnaA protein throughout most of the cell cycle may be insufficient to bind to this site, and that a critical level must be attained to promote initiation. Alternatively, this site may be occluded (see below). Second, in synchronous cultures, initiation of *oriC* plasmid replication correlated with the binding of DnaA protein to R3 (Cassler, 1995). Third, elevated expression of DnaA protein stimulated initiation (Atlung *et al.*, 1987; Lobner-Olesen *et al.*, 1989; Skarstad *et al.*, 1989), possibly by increased occupancy of DnaA box R3. Fourth, formation of Complex VI in which DnaA box R3 is occupied last correlated with replication activity (Figure 3 & 6).

The method used here does not provide a clear picture of the structure of Complex VI except for additional protection of sequences flanking the DnaA boxes. The protected site between R1 and R2 (Figure 6D) contains the sequence TTATACGGT that resembles the DnaA box sequences (TTAT$^{A}/_{C}$CA$^{A}/_{C}$A) of *oriC* and presumably is bound for this reason. The protected region between R2 and R3 contains a Fis binding site (Figure 1) (Filutowicz *et al.*, 1992; Finkel and Johnson, 1992; Gille *et al.*, 1991). That *fis* null mutants maintain poorly *oriC*-dependent plasmids (Filutowicz *et al.*, 1992; Gille *et al.*, 1991), and are asynchronous in initiation (Boye *et al.*, 1993) suggests a positive role for Fis binding. However, certain *in vitro* conditions demonstrate that Fis is inhibitory to *oriC* plasmid replication (Hiasa and Marians, 1994a), a finding consistent with the

report that binding of DnaA protein to R3 is mutually exclusive to the binding of Fis at its respective site (Gille *et al.*, 1991). Indeed, footprinting studies of *oriC* minichromosomes in synchronous cultures suggest that Fis blocks the binding of DnaA protein to R3 (Cassler, 1995). At initiation, the protection pattern attributed to Fis was not observed. Instead, DnaA box R3 was protected. Shown here, occupancy of R3 by DnaA protein correlates with optimal replication activity (Fig. 3 & 6). DnaA protein and Fis may compete for binding with contrasting effects on replication activity.

HU protein or IHF act in initiation (Dixon and Kornberg, 1984; Skarstad *et al.*, 1990) to facilitate unwinding of the 13-mers near the left boundary of *oriC* (Hwang and Kornberg, 1992a). A preferred IHF binding site between R1 and R2 (Filutowicz and Roll, 1990; Polaczek, 1990) suggests the possibility that IHF may enhance one or more steps in formation of Complex VI as a prerequisite to unwinding. In synchronously growing cells, IHF occupies this site just before initiation (Cassler, 1995). IHF and DnaA protein were concluded to bind independently to *oriC*, based on the lack of effect of IHF on the DNase I protection pattern by DnaA protein (Fig. 5 of (Hwang and Kornberg, 1992a)). However, the level of DnaA protein examined was in excess of the level optimal for formation of Complex VI (Figure 2), and would have obscured detecting enhancement of DnaA protein binding.

In addition to the 4 DnaA boxes described above, a fifth site, R5, has been proposed as a site of binding of DnaA protein (Matsui *et al.*, 1985). Little, if any, protection was observed at this site. This may be partly due to the reduced ability of phenanthroline-copper to cleave in this region.

Except in Complex I and II, all other complexes revealed a protected region encompassing the *Acc* I site. DNase I footprinting with the nucleotide-free form of DnaA protein showed that it bound to this region (Hwang and Kornberg, 1992a). However, this region was not protected by DnaA protein bound to ATP, ADP, or ATPγS. Although the studies presented here involved incubation of DnaA protein with the *oriC*-containing restriction fragment and 0.5 μM ATP prior to electrophoresis, protection of the region containing the *Acc* I site suggests that dissociation of DnaA protein and rebinding of the nucleotide-free form occurs during electrophoresis. The significance of binding to this site is unclear as it is not part of the functional *oriC* sequences (Asai *et al.*, 1990).

Chapter III

The influence of IHF on the binding of DnaA protein to *oriC*

Abstract

The role of integration host factor (IHF) in initiation of DNA replication at the *E. coli* chromosomal origin (*oriC*) was investigated by studying the effect of IHF on the binding of DnaA protein to *oriC*. *In vitro*, IHF can replace HU for *oriC* dependent replication (K. Skarstad, T, A. Baker, A. Kornberg (1990) *EMBO J.* **9**, 2341-2348). HU or IHF is required in addition to DnaA protein for efficient unwinding of the AT-rich 13-mers found within *oriC* (D. S. Hwang, A. Kornberg (1992) *J Biol. Chem.* **276**, 23083-23086). For initiation, DnaA protein binds four sites, termed DnaA boxes, within *oriC*. Binding to the two outside DnaA boxes R1 and R4, is followed by the binding to the two inner boxes, R2 and R3. As IHF bends DNA on binding and as the IHF site is located between R1 and R2, IHF has been proposed to enhance DnaA-DnaA interactions and to facilitate the binding of DnaA protein to R2 and R3. Experiments described here indicate that IHF does not facilitate DnaA binding to linear *oriC*.

Introduction

HU and integration host factor (IHF) are abundant, small basic DNA binding proteins organize the bacterial nucleoid (Pettijohn, 1988). In addition, these proteins have been implicated in site specific recombination, transcription (Goosen and van de Putte, 1995), and the initiation of plasmid and chromosomal replication (Dixon and Kornberg, 1984; Funnell *et al.*, 1987; Gamas *et al.*, 1986; Skarstad *et al.*, 1990). HU and IHF are often interchangeable in many (Goodman *et al.*, 1992; Hwang and Kornberg, 1992a; Surette *et al.*, 1989) but for not all processes (Segall, 1994). These bacterial processes are dependent on highly ordered nucleoprotein complexes. HU and IHF are thought to play a role in stabilizing these complexes.

Both IHF and HU are composed of two subunits and share 50% amino acid similarity (Drlica and Rouviere, 1987). Based on mutational studies (Goshima *et al.*, 1992; Goshima *et al.*, 1990) and protein crystallographic analysis (Rice *et al.*, 1996; Tanaka, 1984), each subunit contains an anti parallel β-ribbon "arm" that contacts DNA. On binding DNA, the β-ribbon from each subunit inserts into the minor grove with one β-ribbon positioned on one side of the DNA strand and the other β-ribbon on the other side to encircle the DNA (Wang *et al.*, 1995; Yang and Nash, 1989).

Despite their homology, HU and IHF differ in their DNA binding properties. HU binds DNA that contains bends, cruciform structures or single stranded gaps with higher affinity, but it has little sequence specificity (Bonnefoy *et al.*, 1994; Castaing *et al.*, 1995; Pontiggia *et al.*, 1993; Shimizu *et al.*, 1995). In

contrast, IHF is a sequence specific DNA binding protein (Craig and Nash, 1984; Goodrich *et al.*, 1990). Second, IHF bends DNA on binding of a single heterodimer, whereas DNA bending by HU is thought to require the binding of 3 heterodimers (Tanaka, 1984). Furthermore, the models for their ability to bend DNA are different. Besides interacting with the residues on the β-ribbon, DNA interacts with the body of IHF to stabilize a bend. HU is proposed to introduce bends in DNA through protein-protein interactions with HU dimers bound to adjacent DNA sequences (Tanaka, 1984; Yang and Nash, 1989).

IHF is proposed to be a structural element in protein-DNA complexes by bending DNA to draw distantly located DNA elements into close proximity (Finkel and Johnson, 1992; Segall, 1994). In support of this model, lambda integration reactions were made independent of IHF by replacing the IHF binding site with either stably bent DNA or binding sites for other proteins that bend DNA (Giese *et al.*, 1992; Goodman and Nash, 1989; Goodman *et al.*, 1992). In the latter studies, site specific recombination became dependent on the sequence specific DNA binding protein, cyclic AMP activator protein (CAP) or lymphoid enhancer-binding protein (LEF-1), whose site replaced the IHF site (Giese *et al.*, 1992; Goodman and Nash, 1989).

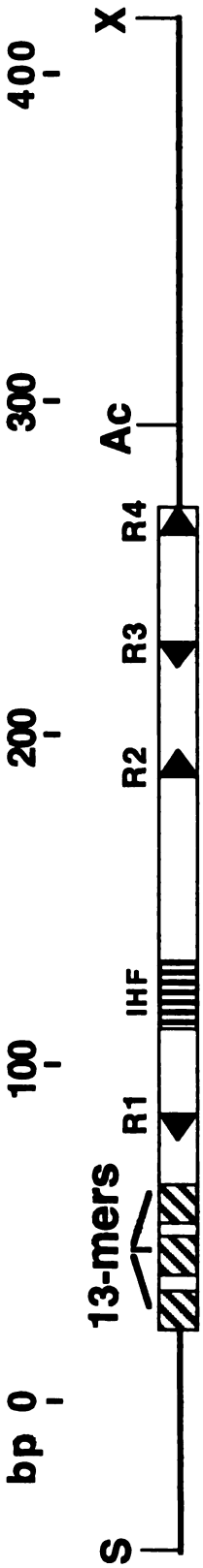
HU may facilitate the formation of nucleoprotein complexes via a general mechanism similar to IHF as HU can replace IHF in stimulating lambda excision (Goodman *et al.*, 1992), and Mu transposition (Surette *et al.*, 1989). In lambda excision, both proteins stabilize the binding of lambda recombinase, Int, to its DNA substrate (*attL*) which contains an IHF binding site between two binding sites for Int (Segall, 1994). As IHF inhibits HU binding to a fragment containing

this site (Segall, 1994), HU appears to bind to the IHF binding site when Int is bound to *attL*. HU may bind specifically to the sequences between Int binding sites because these sequences may be bent. Alternatively, the binding of Int may bend *attL* to stimulate HU binding.

As in site specific recombination, IHF facilitates replication of the *E. coli* plasmid pSC101 by stabilizing protein-DNA interactions. Replication of pSC101 requires IHF and DnaA protein as well as the plasmid encoded RepA protein (Gamas *et al.*, 1986). The pSC101 origin contains one consensus DnaA box, called $dnaA_s$ (Stenzel *et al.*, 1991) and other sites, $dnaA_w$, that do not rigorously match the consensus sequence and are bound weakly. In footprinting studies, little binding of $dnaA_w$ is observed when the adjacent $dnaA_s$ site was deleted (Stenzel *et al.*, 1991). Binding to these weak sites is probably mediated through protein-protein interactions by the bending of the intervening sequences. IHF binds to a site between the $dnaA_s$ and the two $dnaA_w$ sites. The binding of IHF enhances the binding of DnaA protein to both its sites, presumably by the ability of IHF to bend the intervening sequences (Stenzel *et al.*, 1991).

Replication from the *E. coli* chromosomal origin (*oriC*) also depends on DnaA protein and IHF (Kaguni and Kornberg, 1984; Skarstad *et al.*, 1990). *oriC* contains four DnaA boxes, R1, R2, R3, and R4 (Fuller *et al.*, 1984) (Figure 1). DnaA protein binds to R1 and R4 with higher affinity than to R2 and R3 (Schaper and Messer, 1995). As in the pSC101 origin, *oriC* contains an IHF binding site located between a strong site and a weak site; in the case of *oriC* the IHF binding site is located between R1 and R2 (Filutowicz and Roll, 1990; Polaczek, 1990). The binding of DnaA protein to all four DnaA boxes is thought

Figure 1. **The *oriC* region**. The 245 bp minimal *oriC* is indicated by the open rectangle. The IHF consensus sequence is indicated by the vertically striped box. DnaA boxes R1-R4 which are the 9-mers recognized by DnaA protein are indicated by the filled in triangles. The 13-mer motifs and restriction enzyme sites (S, *Sma* I; *Acc* I; and X, *Xho* I) of pBS*oriC* are indicated.


bp 0
100
200
300
400
S
13-mers
R1
IHF
R2
R3
R4
Ac
X

to be required for unwinding of the AT-rich 13-mers adjacent to the DnaA boxes (Margulies and Kaguni, 1996; Oka *et al.*., 1984). Either HU or IHF facilitates unwinding of the 13-mers (Hwang and Kornberg, 1992a). The mechanism by which IHF facilitates unwinding has not been determined. In a mechanism similar to the function of these proteins in replication of pSC101, IHF may act in early events at *oriC* by stimulating the binding of DnaA protein. Alternatively, it may act at the subsequent step of unwinding the AT-rich region. Here, we investigate whether IHF promotes the binding of DnaA protein to the DnaA boxes within *oriC*.

Experimental Procedures

Reagents and chemicals--3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) was obtained from United States Biochemical Corporation (USB); sodium pyrophosphate and trichloroacetic acid (TCA) from J.T. Baker

Proteins and enzymes--The monoclonal DnaA antibody, M43, was obtained as described in (Marszalek *et al.*, 1996). The polyclonal IHF antibody was obtain as a gift from Dr. Howard Nash at the National Cancer Institute, NIH. Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio Rad. Highly purified replication proteins were obtained as described (Hwang and Kaguni, 1988), DnaB protein (Fraction V, 6 x 10^5 units/mg); DnaC protein (Fraction VI, 6 x 10^5 units/mg); primase (Fraction V, 2 x 10^6 units/mg); single stranded DNA binding protein (SSB) (Fraction V, 4 x 10^5 units/mg); DNA polymerase III holoenzyme (Fraction V, 2 x 10^5 units/mg); DNA gyrase A subunit (Fraction III, 2 x 10^5 units/mg); DNA gyrase B subunits (Fraction V, 1 x 10^5 units/mg); RNA polymerase (4 mg/ml); RNase H (fraction IV, 8 x 10^5 units/mg), topoisomerase I (fraction IV, 5 x 10^4 units/mg). HU was purified as described (Dixon and Kornberg, 1984). IHF was a gift from Dr. Filutowiz at the University of Wisconsin, Madison. DnaA protein was purified as described (Sekimizu *et al.*, 1988b).

DNA replication assays--DNA replication reactions using purified enzymes were performed as previously described (Hwang and Kaguni, 1988). The reactions (25 μl) contained: HEPES-KOH, pH 7.8, 25 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)TTP (30 cpm/pmol) each at 100 μM; magnesium acetate, 11 mM; phosphocreatine, 2 mM; DTT, 5 mM; creatine kinase, 100 μg/ml; bovine serum albumin, 0.08 mg/ml; SSB, 160 ng; gyrase A subunit, 500 ng; gyrase B subunit, 600 ng; primase, 10 ng; DnaB protein, 50 ng; DnaC protein, 40 ng; DnaA protein, 100 ng; DNA polymerase III holoenzyme, 300 ng; pBS*oriC* supercoiled DNA, 25 fmol; and the indicated amounts of HU or IHF. The reactions were assembled at 0 °C. After incubating at 30 °C for 30 minutes, the reactions were stopped and the DNA precipitated with 1.0 ml 10% (wt/vol) trichloroacetic acid and 0.1 M sodium pyrophosphate. The incorporated (^{3}H)TTP was measured by collecting the precipitated DNA by filtration through glass-fiber filters (Whatman GF/C). The filters were dried and the radioactivity collected on the filters was quantified by liquid scintillation counting.

DNA binding assays--The gel mobility shift assays were performed as described (Chapter 2).

DNA protection assay--In situ cleavage with 1,10-phenanthroline-copper was performed as described (Chapter 2).

Probing complexes with antibodies--Gel mobility shift assays were performed as described in Chapter 2 except that the reactions were scaled up 5-fold. After electrophoresis, the complexes were transferred from the wet gel to a PVDF membrane (Schleicher & Schuell) in 9.9 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) pH 11, and 10% (vol/vol) methanol for 9 hours at 150 mAmp. The membrane was incubated with 2% (w/vol) milk then probed with anti-DnaA monoclonal antibody (M43) followed by a horseradish peroxidase-

conjugated goat anti-mouse IgG. Detection was by chemiluminescence (ECL, Amersham). The membrane was stripped with 0.2 M glycine (pH 2.8) and 1 mM EGTA, reblocked and reprobed for IHF with anti-IHF polyclonal antibody followed by horseradish peroxidase conjugated goat anti-rabbit IgG. Detection was as above. To locate the ^{32}P labelled *oriC* fragment, the blot was exposed to film after the ECL signal decayed.

Determination of the dissociation rate constant of the IHF Complex 1. A *Sma* I-*Xho* I *oriC* fragment 3'-end labelled at the *Xho* I site with [$\alpha^{32}P$] TTP was preincubated with IHF for 5 minutes under standard gel mobility shift conditions (Chapter 2) in a total of volume of 120 µl. After preincubation, a 20 µl aliquot was removed to a separate tube and 10 µl of this was loaded onto a native 4% polyacrylamide gel. A 100-fold molar excess of pBS*oriC* was added to the remaining reaction mixture and 10 µl was immediately loaded onto the gel electrophoresing at 90 V. Additional 10 µl aliquots of the mixture were then loaded onto the gel at various times after the addition of the competitor DNA. At the end of the time course the remaining, 10 µl of the mixture with IHF not containing the competitor and another 10 µl of the mixture containing just the labeled DNA were loaded. An additional control in which a 100 fold molar excess competitor was added before IHF was loaded onto the gel at the beginning of the time course. Electrophoresis continued for 4 hours at 90 V after the last sample was loaded. The amount of DNA bound at each time point was quantitated using β emission scanning. The data was plotted and the curve fitted according to the equation $Bound_t/Bound_o = e^{(-k_{off}t)}$, where $Bound_t$ is the

amount of DNA bound at time t, $Bound_o$ is the amount of DNA bound immediately prior to the addition of competitor, and k_{off} is the dissociation rate constant.

Results

IHF substitutes for HU in purified oriC*-dependent replication*--In the absence of RNA polymerase, HU or IHF is required for optimal replication (Figure 2A) (Skarstad *et al.*, 1990). *In vitro* replication can be made to be dependent on RNA polymerase by including topoisomerase I and RNase H. RNA polymerase is not involved in forming primers but is more likely involved in altering the topology of *oriC* making it more susceptible to unwinding. Either protein facilitates DNA replication in the presence and absence of RNA polymerase (Figure 2B). HU and IHF promote replication by altering the topology of *oriC* to facilitate DnaA protein unwinding the AT-rich 13-mer of *oriC* (Hwang and Kornberg, 1992a).

IHF forms a single major complex with oriC *whereas HU forms multiple complexes*--To investigate whether these proteins affected the initial binding of DnaA protein to the four DnaA boxes within *oriC*, gel mobility shift assays were performed. Although HU and IHF are similar at the amino acid and the structural level (Drlica and Rouviere, 1987), they are strikingly different in their DNA binding characteristics. These differences were also demonstrated on binding to *oriC* (Figure 3A and B). Three complexes were observed in proportion to the amount of IHF added (Figure 3A). A major and minor complex (Complex 1 and Complex 2, respectively) were formed at lower levels of IHF (0.6-10 ng). The third complex, Complex 3, with lower mobility was observed only when at least 10 ng of IHF was added.

In similar assays, HU formed as many as eight complexes with *oriC* (Figure

Figure 2. **IHF can replace HU in *in vitro oriC*-dependent replication in the presence or absence of RNA polymerase.** IHF (■) or HU (●) were titrated in reconstituted replication assays which were independent (A) or dependent (B) on RNA polymerase as described in "Experimental Procedures".

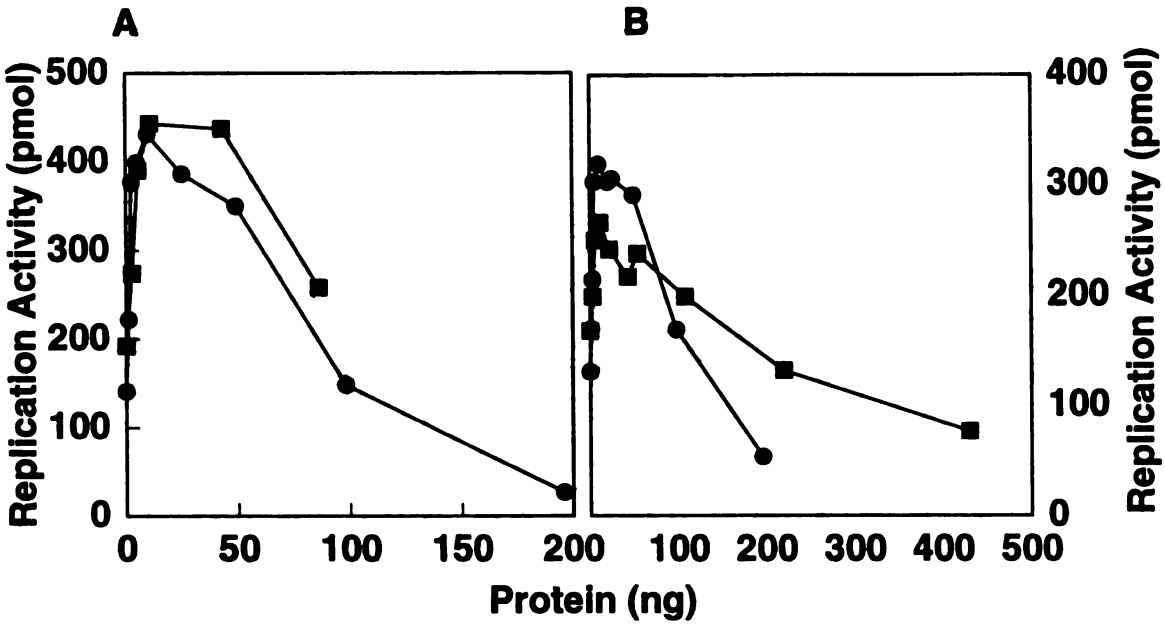
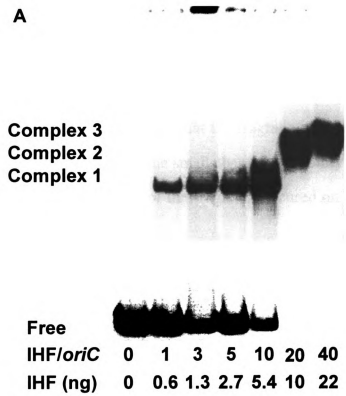
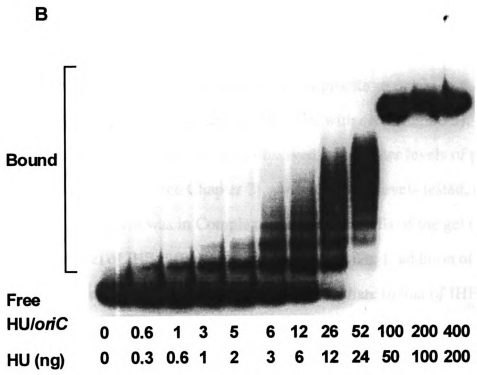

A
B
Replication Activity (pmol)
500
400
300
200
100
0
0
50
100
150
200
100
200
300
400
500
Protein (ng)
400
300
200
100
0
Replication Activity (pmol)

Figure 3. **Both IHF and HU bind *oriC*.** Gel mobility shift assays were performed as described in"Experimental Procedures" with the indicated amounts of IHF (A) and HU (B) protein and 25 fmol of a *Sma* I-*Xho* I *oriC* fragment labelled at the *Xho* I site with the large fragment of DNA polymerase I and [α^{32}P] TTP. Unbound DNA is noted as "Free".


A
Complex 3
Complex 2
Complex 1
Free
IHF/oriC 0 1 3 5 10 20 40
IHF (ng) 0 0.6 1.3 2.7 5.4 10 22


B
Bound
Free
HU/oriC 0 0.6 1 3 5 6 12 26 52 100 200 400
HU (ng) 0 0.3 0.6 1 2 3 6 12 24 50 100 200

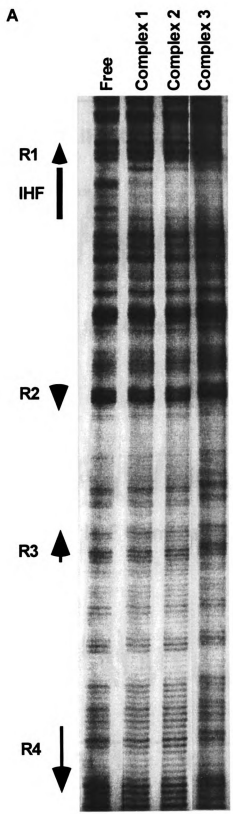
3B). The HU-*oriC* complexes decreased in mobility proportional to the amount of HU added. Only one complex was resolved when HU was added at levels above 24 ng (Figure 3B). As the IHF binding site was located near the center of the *oriC* fragment (Figure 1), the large retardation of the IHF complexes relative to the unbound DNA suggested that the fragment was highly bent by the binding of IHF. In contrast, the small change in mobility of the first HU complex suggested that less bending of the fragment occured on the binding of a single HU dimer.

IHF binds oriC *at a single site*--To investigate the DNA sequences bound, the IHF complexes were footprinted using an *in situ* method with phenanthroline-copper. In each of the complexes, the IHF binding site between DnaA boxes R1 and R2 was protected (Figure 4). Complex 2 included additional protection of flanking sequences compared with Complexes 1 and 3.

Novel complexes with oriC *form in the presence of IHF and DnaA protein*--As the binding of IHF to *oriC* resulted primarily in a single complex when IHF was added at lower amounts, gel mobility shift assays were used to investigate the effect of IHF on binding of DnaA protein to *oriC*. In the absence of IHF, DnaA protein formed at least 6 complexes with *oriC* (Figure 5). Complexes with slower mobilities were observed with higher levels of protein added (Figure 5 lanes 2-7, see Chapter 2). At the highest levels tested, the majority of the fragment was in Complex VI and at the wells of the gel (lane 7).

With a level of IHF sufficient to form IHF Complex 1, addition of DnaA protein resulted in complexes with mobilities intermediate to that of IHF Complex 1 (lane 8) and that of DnaA Complex VI (lane 7). Based on the relative mobilities

Figure 4. ***In situ* footprinting of IHF complexes with 1,10-phenanthroline-copper**. *In situ* cleavage was performed as described ("Experimental Procedures") with IHF and a *Sma* I-*Xho* I fragment labelled at the *Xho* I site with the large fragment of DNA polymerase I and all 4 [α^{32}P] deoxyribonucleotides. The IHF complexes were resolved on a standard native gel. The entire gel was immersed in a phenanthroline-copper solution. The reaction was stopped. Film was exposed to the gel and was used to guide the excision of the complexes. The DNA was eluted, precipitated, resuspended in formamide buffer then loaded on a standard 6% sequencing gel. The resulting cleavage patterns are shown in panel A. The gel was quantified by β emission scanning. The quantified cleavage pattern of each complex was compared with the cleavage of unbound and shown in panel B.


A
Free
Complex 1
Complex 2
Complex 3
R1
IHF
R2
R3
R4

B

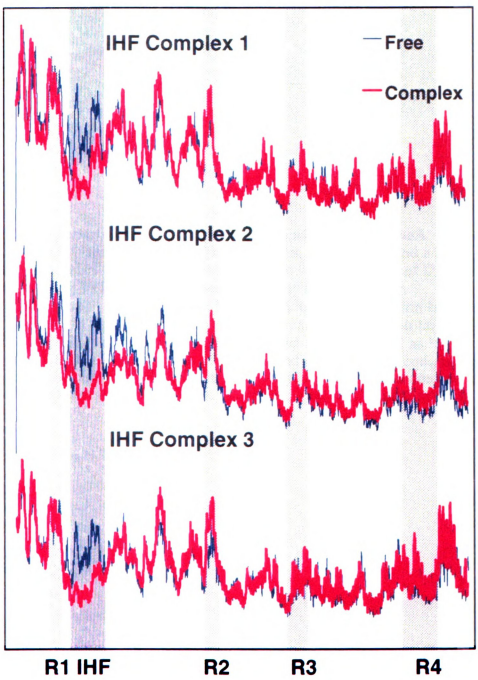
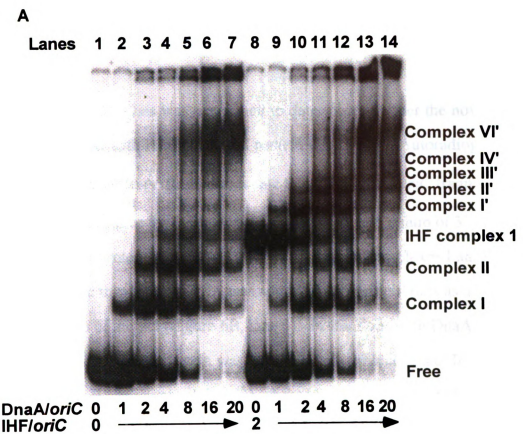
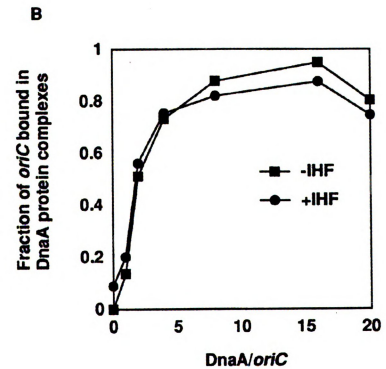

IHF Complex 1
Free
Complex
IHF Complex 2
IHF Complex 3
R1 IHF
R2
R3
R4

Figure 5. **Novel complexes form in the presence of both IHF and DnaA protein.** A) Gel mobility-shift assays with DnaA protein, IHF or both and a *Sma* I-*Xho* I *oriC* fragment labelled at the *Xho* I site with the large fragment of DNA polymerase I and [α^{32}P] TTP were performed essentially as described in "Experimental Procedures" of Chapter 2. IHF was added to the reaction before DnaA protein. B) The gel was quantified by β emission scanning. The total amount bound in each lane (1 though 7) was quantified and expressed as the fraction bound of *oriC* (■). The fraction of *oriC* bound in complexes containing DnaA protein in lanes 8 through 14 was determined by subtracting the amount of *oriC* bound in the position of IHF Complex 1 from the total *oriC* bound in each lane (●).

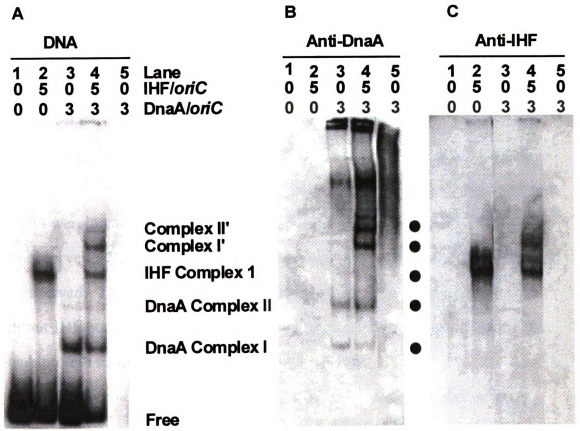

A
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Complex VI'
Complex IV'
Complex III'
Complex II'
Complex I'
IHF complex 1
Complex II
Complex I
Free
DnaA/oriC 0 1 2 4 8 16 20 0 1 2 4 8 16 20
IHF/oriC 0 2


B
Fraction of oriC bound in DnaA protein complexes
1
0.8
0.6
0.4
0.2
0
0 5 10 15 20
-IHF
+IHF
DnaA/oriC

of these complexes (Complex I' through Complex VI') to the mobility of IHF complex, they presumably were the result of binding of DnaA protein to IHF-bound *oriC*.

Western blot analysis was performed to determine whether the novel complexes contained both IHF and DnaA protein (Figure 6). Autoradiography of the membrane was also performed to locate the ^{32}P-labelled *oriC* fragment (Figure 6 A). To minimize the complexity of this experiment, a ratio of 3 molecules of DnaA protein to *oriC* was used so that only Complexes I and II formed. In the lane in which no DNA was added, DnaA protein runs as a large smear from the top of the gel (Figure 6B, lane 5). In reactions with DnaA protein and an *oriC* fragment, DnaA protein was detected in Complexes I and II, as well as Complex I' and II', as expected (lane 3). Likewise, IHF was located in IHF Complex 1 formed with IHF alone and Complex I' (Figure 6C). Complex II' also cross reacts with anti-IHF antibody (data not shown). These observations supported the conclusion that Complexes I' through VI' each contain both DnaA protein and IHF bound to *oriC*. The ratio of DnaA protein to *oriC* appears higher in Complex I' than in Complex I (compare lanes 3 and 4 in Panel A and B) suggesting that IHF facilitated the binding of DnaA protein to *oriC*. However, the total amount of *oriC* fragment bound in DnaA-*oriC* complexes was similar in the presence and absence of IHF (Figure 5 B) indicating that IHF did not alter the affinity of DnaA protein for an *oriC* fragment. Results may indicate that IHF increased DnaA-DnaA interactions.

Figure 6. **Novel complexes contain both DnaA protein and IHF.** Gel mobility shift assays were performed as in Figure 5 ("Experimental Procedures" of Chapter 2) except that reactions were scaled-up 5-fold. In all reactions containing IHF and DnaA protein, IHF was added first. The complexes were electrophoretically transferred to a membrane then probed for DnaA protein and IHF as described in "Experimental Procedures". The ^{32}P-labelled *oriC* fragment was detected by autoradiography. No *oriC* was added to lane 5. The position of unbound *oriC* is noted as "Free". The positions of the various complexes are marked.

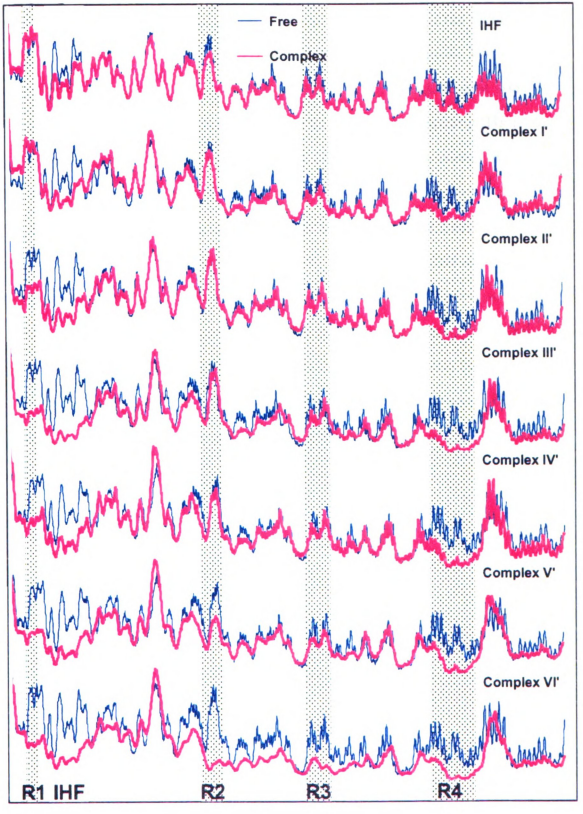

A
DNA
1 2 3 4 5 Lane
0 5 0 5 0 IHF/oriC
0 0 3 3 3 DnaA/oriC
Complex II'
Complex I'
IHF Complex 1
DnaA Complex II
DnaA Complex I
Free
B
Anti-DnaA
1 2 3 4 5
0 5 0 5 0
0 0 3 3 3
C
Anti-IHF
1 2 3 4 5
0 5 0 5 0
0 0 3 3 3

IHF does not aid the binding of DnaA protein to the weaker inside DnaA boxes within oriC--DnaA protein bound to *oriC* in an ordered manner as demonstrated by *in situ* footprinting (Chapter 2). At lower levels, DnaA protein formed Complex I and II (Figure 5A, lanes 2 and 3) by binding to the outside DnaA boxes, R1 and R4 (Margulies and Kaguni, 1996). At higher concentrations, DnaA protein formed higher ordered complexes by the additional binding to the interior boxes, R2 and R3.

More DnaA protein appeared to bind in Complex I' than Complex I indicating additional sites in *oriC* may be bound by DnaA protein in the presence of IHF (Figure 6A and B lane 4). To investigate this possibility, *in situ* phenanthroline-copper footprinting was performed (Figure 7). As expected, *oriC* was protected from cleavage at the IHF binding site in the complex which had the same mobility as IHF Complex 1. In Complex I', both the IHF site and DnaA box R4 were protected. In Complex II, additional protection of DnaA box R1 was observed. Only in the lowest mobility complexes was protection of R2 and R3 observed. The same order of binding was observed in complexes with or without IHF bound. This suggests that IHF does not facilitate the binding of DnaA protein to sequences within an *oriC* fragment.

IHF does not stabilize DnaA-oriC *complexes*--Although the order of binding of DnaA protein was not altered by binding of IHF, the kinetics of binding of DnaA protein to *oriC* might be altered in the presence of IHF. Competition gel shifts were used to investigate whether IHF might stabilize DnaA protein binding by decreasing the dissociation rate of DnaA protein bound to a radiolabelled *oriC* fragment. These assays were performed by incubating the

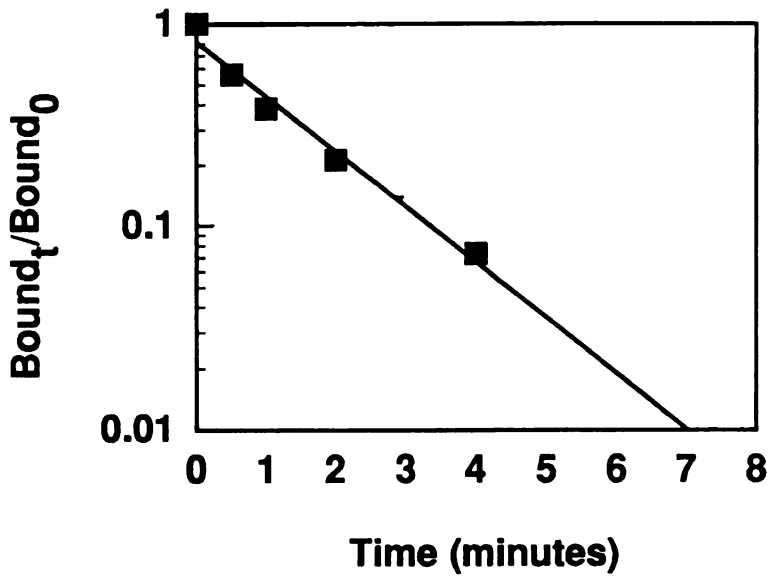
Figure 7. **The order of DnaA protein binding to *oriC* is not altered by IHF.** Gel mobility shift assays were performed as described ("Experimental Procedures" of Chapter 2) except that reactions were scaled-up 10-fold. In all reactions containing IHF and DnaA protein, IHF was added first. *In situ* cleavage with 1,10-phenanthroline-copper was performed as described ("Experimental Procedures" Chapter 2) with a *Sma* I-*Xho* I fragment labelled at the *Xho* I site with the large fragment of DNA polymerase I and all 4 [α^{32}P] deoxyribonucleotides. The cleavage pattern of respective complexes was analyzed by β emission scanning and compared to the cleavage pattern of the corresponding unbound fragment that was treated similarly.


Free
Complex
IHF
Complex I'
Complex II'
Complex III'
Complex IV'
Complex V'
Complex VI'
R1 IHF
R2
R3
R4

appropriate proteins with an *oriC* fragment under standard conditions as described (see"Experimental Procedures") except that the complexes were challenged with unlabelled supercoiled *oriC* plasmid as a competitor. The half-life of IHF Complex 1 was approximately 1 minute on the addition of 100-fold excess of supercoiled *oriC* plasmid (Figure 8). To avoid competing IHF from *oriC*, a 20 base pair oligonucleotide containing one DnaA box sequence was used as a competitor to investigate whether IHF affected the dissociation of DnaA protein form *oriC*. On the addition of the competitor, DnaA-*oriC* Complexes I and II dissociated quickly (Figure 9). When DnaA-IHF-*oriC* complexes were challenged with an excess of the oligonucleotide containing a DnaA box, Complexes I and II have comparable half-lives as Complexes I' and II'. These results suggested that IHF did not stabilize DnaA protein bound to *oriC*. DnaA protein complexes of lesser mobility formed with or without IHF dissociated at similar rates on the addition of competitor DNA (data not shown, Chapter 4).

Figure 8. **Determination of the half-life of the IHF Complex 1.** Dissociation of IHF/*oriC* Complex 1 was measured by preincubating IHF with an end-labelled *Sma I-Xho I oriC* fragment under gel mobility shift assay conditions for 5 minutes at 20 °C (described in "Experimental Procedures"), then 100-fold molar excess of supercoiled pBS*oriC* to the labelled *oriC* fragment was added. At various times aliquots were removed and electrophoresed on a native 4% polyacrylamide gel. A) This graph represents the data derived from the experiment below. $Bound_o$ represents the amount of DNA bound by IHF prior to the addition of unlabeled DNA. $Bound_t$ represents the amount of DNA in complex with IHF at time t. The resulting line is described by the equation $f(t) = 0.91\ e^{-0.63t}$. The first order dissociation rate constant, k_{off}, is 0.63 min^{-1} and corresponds to a half-life of 1 minute for Complex 1. B) Autoradiograph of the gel mobility shift assay. The asterisks indicate that the challenge DNA was added before the protein. Time 0 has no competitor DNA added.

A


1
0.1
0.01
Bound$_t$/Bound$_0$
0 1 2 3 4 5 6 7 8
Time (minutes)

B

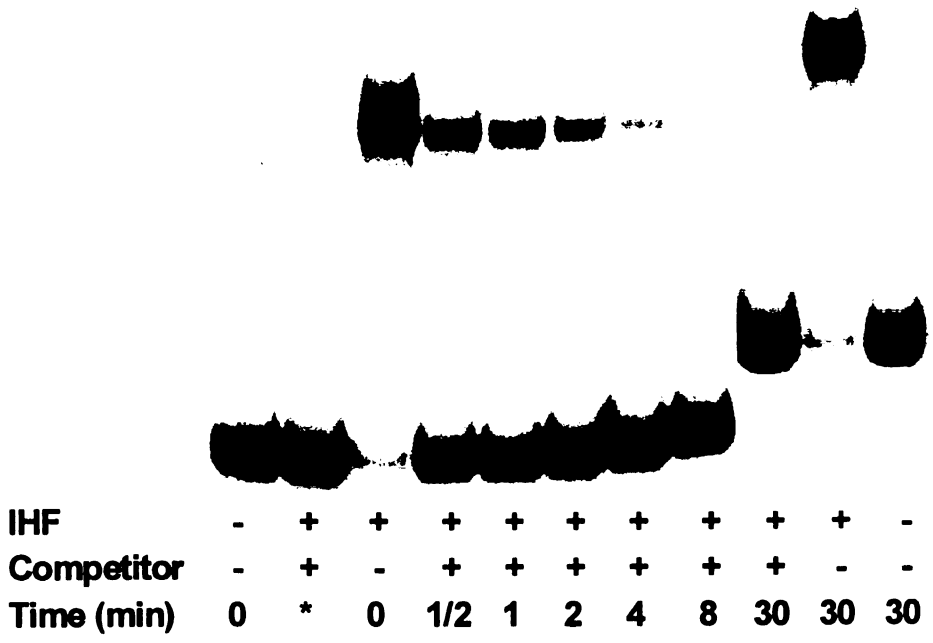
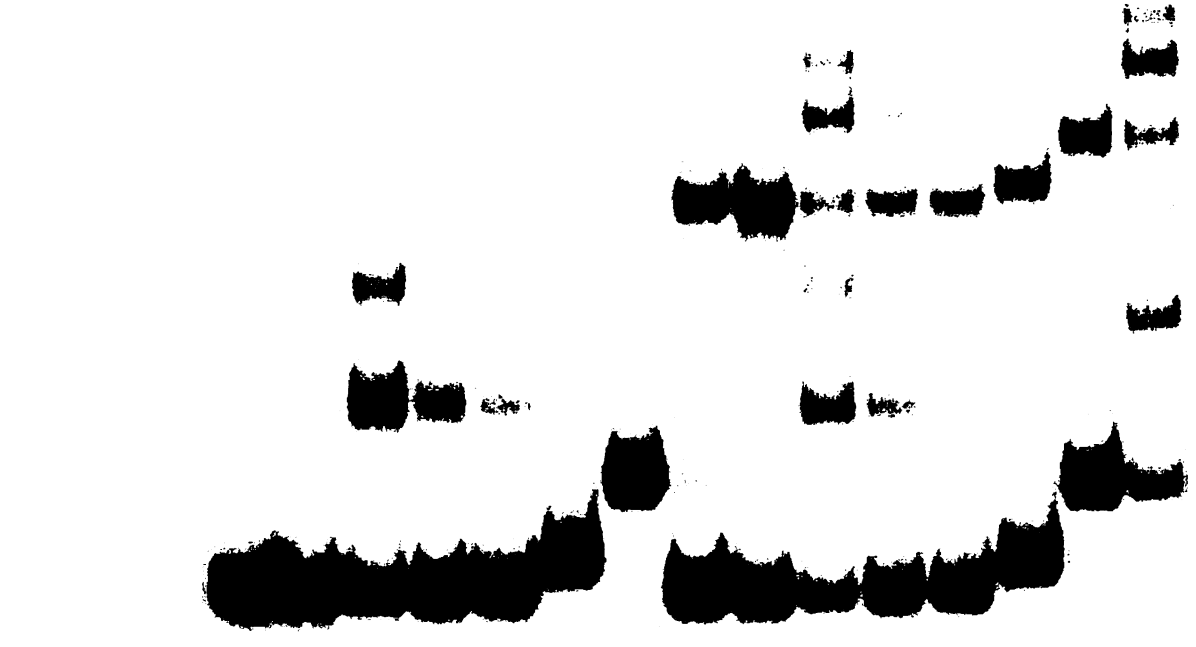

IHF - + + + + + + + + + -
Competitor - + - + + + + + + - -
Time (min) 0 * 0 1/2 1 2 4 8 30 30 30

Figure 9. **The dissociation of DnaA protein from *oriC* was not altered by IHF.** DnaA protein and/or IHF as noted was incubated with an end-labelled *Sma* I-*Xho* I *oriC* fragment in a scaled-up gel mobility shift assay. The complexes were challenged with a 1000-fold molar excess of an unlabelled double stranded oligonucleotide containing a DnaA box. At various times aliquiots were removed and separated on a 4% polyacrylamide gel. The asterisks indicate that the challenge DNA was added before the protein.



DnaA	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
IHF	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
Competitor	-	+	-	+	+	+	+	+	-	+	+	+	+	+	-
Time (min)		*	0	1/2	2	5	10	*		0	1/2	2	5	10	10

Discussion

Although IHF and HU bind to oriC *differently, they can replace each other in* oriC *replication*--Eight complexes were formed on the 464 base pair *oriC* fragment on binding of HU, suggesting that at least 8 HU dimers could bind a single *oriC* fragment. However, Bonnefoy *et al.* (Bonnefoy and Rouviere, 1991) found that two complexes were formed with 21- and 29-mer double stranded oligonucleotides, and four complexes with a 42-mer oligonucleotide, suggesting that a HU dimer could bind approximately every 11 bp. Based on one dimer every 11 bp, as many as 40 dimers may bind to the *oriC* fragment used. At the highest level of HU tested (a ratio of 100 dimers per *oriC* fragment), the complex formed was much more resistant to phenanthroline-copper cleavage than free DNA or the complexes with greater electrophoretic mobility (data not shown) suggesting that *oriC* in this complex was coated with a large amount of protein. As expected for a DNA binding protein with little sequence specificity, no distinct protection pattern of *oriC* was seen in any of the HU complexes (data not shown). Because of the large number of complexes formed, gel shift assays investigating the effect of HU on DnaA protein binding was not pursued.

Addition of IHF to the *oriC* fragment resulted in three complexes by gel mobility shift analysis. IHF is thought to bind as a heterodimer composed of IHF-α and IHF-β subunits (Nash and Robertson, 1981). However, the homodimer IHF-β was reported to bind specifically to the same IHF site as the heterodimer, albeit with lower affinity (Zulianello *et al.*, 1994). Cu-phenanthroline footprint analysis of the IHF complexes with *oriC* revealed protection patterns that were essentially identical. As crosslinking studies suggested that IHF forms

homodimers and trimers and tetramers (Bonnefoy and Rouviere, 1991). A possible explanation for the differing mobilities of these complexes is the binding of homodimers or possibly the binding of trimers or tetramers to *oriC*.

As HU can replace IHF in some processes (Goodman *et al.*, 1992; Surette *et al.*, 1989), HU has been speculated to bind specifically to DNA sequences involved in forming nucleoprotein complexes. Results from immunoelectron microscopy localizing HU to *oriC* in replicative complexes with minichromosomes (Funnell *et al.*, 1987) and footprinting of HU when bound in a Mu invertasome complex, (Lavoie and Chaconas, 1993; Lavoie and Chaconas, 1994) indicated that HU could be targeted by the binding of other proteins.

HU can replace IHF in *oriC*-dependent replication (Figure 1). Both act early in initiation to facilitate DnaA protein-dependent unwinding of *oriC*. They have been speculated to produce a bend in *oriC* thereby facilitating the cooperative binding of DnaA protein. Although HU has no sequence-specific DNA binding activity, it might bind specifically to some bent or kinked DNA to facilitate DnaA protein function at *oriC*. This model was supported by the observations that a relatively low ratio of HU to DNA was required for stimulation of replication (Figure 2) and that HU has been localized by immunoelectron microscopy to a complex involved in initiation at *oriC* (Funnell *et al.*, 1987).

IHF does not affect the affinity of DnaA protein for DnaA boxes within oriC--Based on crude estimates by electron microscopy, 20-40 monomers of DnaA protein were bound to *oriC* to form an active complex (Fuller, et al., 1984). Because the spatial arrangement of individual DnaA boxes in *oriC* is important (Oka *et al.*, 1984; Zyskind *et al.*, 1983), it is likely that this nucleoprotein complex

has a specific structure.

Results by Fuller *et al.* (Fuller *et al.*, 1984) suggested that the binding of DnaA protein to *oriC* is cooperative, possibly involving interactions among DnaA protein monomers. The high affinity DnaA boxes, R1 and R4, probably could be filled without any protein-protein interactions, as a single monomer binds to an oligonucleotide containing these DnaA box sequences (Schaper and Messer, 1995). Binding to the other two sites, R2 and R3, was weaker (Schaper and Messer, 1995) and may be stabilized by protein-protein interactions. Whereas the severe bend introduced by IHF between DnaA boxes R1 and R2 (Figure 3A) might aid in the binding of DnaA protein to R2 and R3, the studies presented here indicated that IHF did not aid in the formation or stabilization of DnaA complexes with a linear *oriC* fragment. One interpretation of these results is that the bent structure induced by IHF is not required to form the DnaA-*oriC* complex that is active in replication. Alternatively, the proposed interaction among DnaA protein monomers may be sufficient in formation of a large nucleoprotein complex. A third possibility is that a supercoiled *oriC* plasmid is required to observe an effect of IHF on DnaA protein binding. The studies described here were with a linear DNA containing *oriC*.

Chapter IV

The Influence of FIS on the Binding of DnaA Protein to *oriC* and on Replication from *oriC*

Abstract

A model of factor for inversion stimulation (FIS) protein function in initiation of replication from *oriC* is based largely on observations from *in vivo* footprinting of *oriC* in synchronized cells (M. R. Cassler, J.E. Grimwade, and A. C. Leonard (1995) *EMBO J.* **14**, 5833-5841). From these experiments, the FIS protein binding site appears to be occupied throughout much of the cell cycle but is vacant just prior to the time of initiation. Furthermore, dissociation of FIS is coincident with binding of DnaA protein to DnaA box R3. As binding of DnaA protein to R3 correlates with initiation of chromosomal replication *in vivo* and *in vitro*, this event appears to be a key step in the initiation process. Because FIS may antagonize the binding of DnaA protein to DnaA box R3 due to their overlapping binding sites (H. Gille, J. B. Egan, A. Roth, W. Messer (1991) *Nucleic Acids Res.* **15**, 4167), we investigated the effect of FIS in reconstituted *in vitro* replication, gel mobility shift and footprinting assays. Contrary to our expectations, our results suggest that FIS does not block the binding of DnaA protein to the two DnaA boxes R2 and R3; nor does it inhibit *in vitro* replication. In light of these results, other possible mechanisms in which FIS might play a role in initiation of replication are discussed.

Introduction

The factor for inversion stimulation (FIS), a small basic histone-like protein, is a member of a group of DNA-binding proteins that are proposed as major constituents of nucleoid (Pettijohn, 1988). FIS is a homodimer composed of 11.5 kDa subunits. X-ray diffration studies indicate that each subunit contains four α-helices (Kostrewa *et al.*, 1991; Kostrewa *et al.*, 1992) with the two N-terminal helices of each subunit interdigitated to form the homodimer. The C-terminal helices form a helix-turn-helix DNA binding motif. The N-terminal 26 amino acids are disordered in the crystal structure and are thought to be involved protein-protein interactions (Heichman and Johnson, 1990; Koch *et al.*, 1991; Osuna, et al., 1991). FIS is thought to interact physically with proteins, such as σ70, and Hin recombinase (Muskhelishvili *et al.*, 1995; Newlands *et al.*, 1992; Osuna *et al.*, 1991; Ross *et al.*, 1990).

FIS was originally identified by virtue of its ability to stimulate DNA inversion by a family of site specific recombinases, Hin, Gin and Cin (Haffter and Bickle, 1987; Johnson *et al.*, 1988; Koch and Kahmann, 1986). Hin controls flagellar phase variation in *Salmonella typhimurium*; Gin and Cin control tail-fiber expression for bacteria phage Mu and P1, respectively. FIS also stimulates lambda excision (Thompson *et al.*, 1987) and integration (Ball and Johnson, 1991). Finally, FIS acts both as a positive and a negative transcriptional regulator. FIS operates via several different mechanisms in these processes. In some processes, DNA binding of FIS sterically inhibits the binding of another protein to an overlapping DNA sequence (Ball and Johnson, 1991; Ball *et al.*,

1992; Froelich, 1996; Ninnemann *et al.*, 1992; Numrych *et al.*, 1991; Thompson and Landy, 1988). In other processes, FIS stabilizes nucleoprotein complexes through bending DNA and possibly via protein-protein interactions (Koch *et al.*, 1991; Muskhelishvili, et al., 1995; Numrych, et al., 1992; Osuna, et al., 1991; Ross, et al., 1990).

Although FIS is considered a sequence specific DNA binding protein, recognizing the highly degenerate sequence (G/T)NNN(A/G)NN(T/A)NNTNNN(C/A), DNA topology is as important as sequence in determining FIS affinity (Bailly *et al.*, 1995; Finkel and Johnson, 1992). Like the other histone-like proteins, HU and integration host factor (IHF), FIS bends DNA upon binding. Bent angles of 40^{o} to 90^{o} have been measured for different FIS-DNA complexes (Gille *et al.*, 1991; Thompson and Landy, 1988).

Expression of FIS protein is growth-rate dependent (Ball *et al.*, 1992; Thompson *et al.*, 1987). The expression of FIS is greatest in early log phase. FIS concentrations increase rapidly when cells move from stationary to log phase growth and decrease through mid to late log phase. Consequently, it is speculated to serve as an element involved in the coupling of the growth rate to nutritional and environmental conditions.

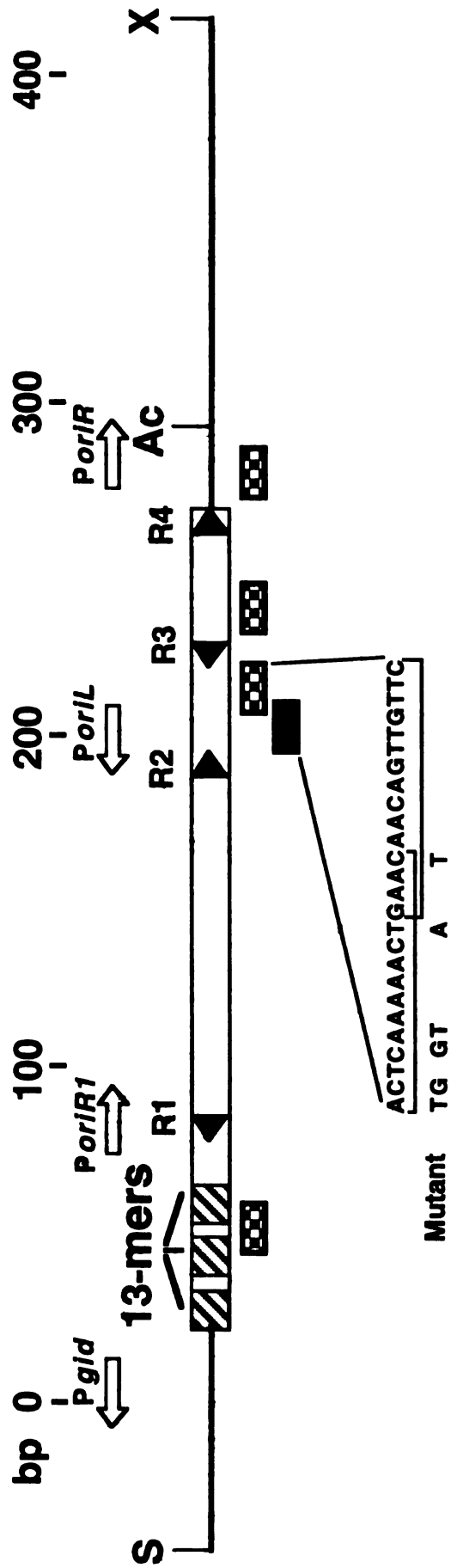
Initiation at *oriC*, the *E. coli* chromosomal origin, is dependent on the binding of DnaA protein to four DnaA boxes (Fuller *et al.*, 1984)(referred to as R1, R2, R3 and R4; see Figure 1) within *oriC*. On binding its four sites in *oriC*, DnaA protein is proposed to unwind the AT-rich 13-mers allowing other replication proteins access to single stranded DNA to form primers which are then elongated by DNA polymerase III holoenzyme (Bramhill and Kornberg, 1988a;

Bramhill and Kornberg, 1988b). Although the general mechanism for initiation of replication is well understood, its regulation is not.

The binding of DnaA protein to DnaA boxes within *oriC* is cell-cycle dependent (Cassler, 1995; Samitt *et al.*, 1989). This could be due to either an increase of DnaA protein concentration or its activity. However, there is little evidence for variations in DnaA protein concentrations as a function of cell cycle (Hansen *et al.*, 1991; Sakakibara and Yuasa, 1982). Other models for the regulation of chromosomal replication involve the regulation of DnaA protein activity by its interaction with ATP (Sekimizu *et al.*, 1988a), acidic phospholipids (Sekimizu and Kornberg, 1988) or possibly other proteins (Hupp and Kaguni, 1993b; Katayama and Crooke, 1995). Another model involves other *oriC* binding proteins that either facilitate or inhibit DnaA binding to *oriC*. It has been suggested previously that FIS is one of these factors (Gille *et al.*, 1991).

Four lines of evidence suggest that FIS is involved in initiation of chromosomal replication at *oriC*: 1) mutants of FIS are asynchronous in initiation from *oriC* (Boye *et al.*, 1993); 2) *fis* mutants inefficiently maintain plasmids dependent on *oriC* for replication (Filutowicz *et al.*, 1992); 3) mutations (Figure 1) in the FIS binding site within *oriC* result in an inactive origin ; and 4) *in vivo* footprinting of *oriC* suggests that FIS is bound throughout the cell cycle until just prior to the time of initiation (Cassler, 1995). Based primarily on the latter observation, a model was proposed in which the binding of FIS to a site overlapping with DnaA box R3 occludes the binding of DnaA protein to this site (Gille *et al.*, 1991). Under this model, dissociation of FIS is required for the binding of DnaA protein to box R3. Previously, we determined that DnaA

Figure 1. **The *oriC* region**. The 245 bp minimal *oriC* is indicated by the open rectangle. The arrows indicate promoters and their direction of transcription. DnaA boxes R1-R4, the 9-mers recognized by DnaA protein, are indicated by filled triangles also indicating their orientation. The AT-rich 13-mer motifs are indicated by cross-hatched squares. Restriction enzyme sites (S, *Sma* I; *Acc* I; and X, *Xho* I) of *oriC* are indicated. The sequences that match the FIS consensus sequence (Finkel and Johnson, 1993) are indicated by the checkered boxes. The sequences that diverge from the consensus sequence are indicated by the shaded box. The sequence of the putative FIS binding sites between DnaA boxes R2 and R3 are indicated. A set of point mutations that destroy binding of FIS between R2 and R3 and inactivate *oriC* for *in vivo* replication is indicated.


X
400
300
200
100
bp 0
S
PgId
13-mers
PoriR1
R1
PoriL
R2
R3
PoriR
R4
Ac
ACTCAAAAACTGAACAACAGTTGTTC
Mutant TG GT A T

protein binds to DnaA boxes in *oriC* in an ordered manner (Margulies and Kaguni, 1996). Its binding to DnaA boxes R2 and R3 correlates with optimal replication activity. Because of the possibility that FIS binding may modulate the initiation process, we investigated the effect of FIS on *oriC* replication using *in vitro* assays, gel mobility shift, footprinting and reconstituted *oriC* dependent replication. Our results indicate that FIS does not occlude binding of DnaA protein to box R3 within *oriC*.

Experimental Procedures

Proteins and plasmids--FIS protein was a generous gift of Reid Johnson (Department of Biological Chemistry , University of California, Los Angeles).

DNA binding assays-- The procedure used for the binding reactions was described in "Experimental Procedures" in Chapter 2.

In situ *Cleavage with 1, 10-Phenanthroline -Copper*--The procedure used for the protection experiments was described in "Experimental Procedures" in Chapter 2.

Dissociation of protein-oriC *complexes*--The procedure used for comparing the dissociation of DnaA-*oriC* complexes and FIS-*oriC* complexes were performed as described in "Experimental Procedures" in Chapter 3.

Replication assays--The reconstituted replication assays were performed as described in "Experimental Procedures" in Chapter 3.

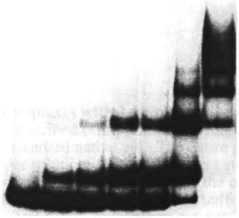
Supercoiling assay--Reactions (20 µl) containing 90 fmol of supercoiled pBS*oriC*, the indicated amounts of FIS and 20 mM Tris-HCl (pH 7.5) 10 mM $MgCl_2$, 2 mM DDT, 5% glycerol and 5 mM NaCl were preincubated for 15 minutes, at 30 °C. *E. coli* topoisomerase I (15 U) was added. Reactions were incubated for 30 minutes at 30 °C and then quenched with 5 µl 30% glycerol, 10 mM EDTA, 6% SDS, and heated to 85 °C for 3 minutes prior to electrophoreses through a 0.8% alkaline agarose gel containing 1 µg/mL chloroquine.

Results

Fis forms multiple complexes with a DNA fragment containing oriC--The consensus DNA binding site recognized by FIS is highly degenerate [(G/T)NNN(A/G)NN(T/A)NNTNNN(C/A)] (Finkel and Johnson, 1992). Although *oriC* contains several exact and close matches to the FIS consensus sequence (Figure 1), the consensus is a poor indicator of FIS binding. FIS binding is in a large part dependent on DNA topology (Bailly *et al.*, 1995). To determine whether FIS bound specifically to a DNA fragment containing *oriC*, gel mobility shift assays were performed (Figure 2). Multiple complexes were resolved by the binding of FIS to *oriC*. At levels as low as 0.1 ng, FIS formed a single complex (Complex 1). At higher levels (0.4 ng), Complex 1, as well as a second complex (Complex 2) were observed. At levels greater than 0.8 ng of FIS, complexes with slower electrophoretic mobilities were observed. The resolution of multiple complexes suggested the binding of FIS to several sites in *oriC*.

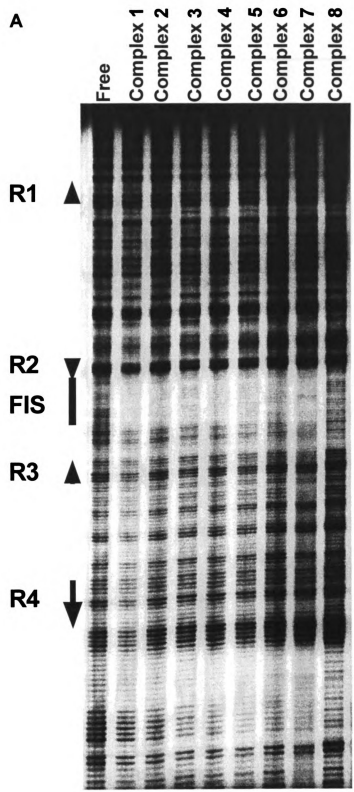
Fis binds several sites within oriC. To determine which sites were bound in each complex, FIS-*oriC* complexes were footprinted by cleaving the DNA *in situ* with phenanthroline-copper and analyzed quantitatively. The only site protected in Complex 1 was located between DnaA boxes R2 and R3 (Figure 3A and 3B). Two FIS sites are predicted between R2 and R3, with the site bound as the more degenerate of the two (Figure 1). Protection of sequences between R2 and R3 was seen in all FIS-*oriC* complexes. In Complex 2 and the other complexes of lower mobility, additional sequences to the right of DnaA box R4 were protected. The protection pattern of Complexes 3, 4 and 5 was similar to

Figure 2. **FIS bound to *oriC* forms multiple complexes that are resolved by native polyacrylamide gel electrophoresis.** Gel mobility shift assays were performed as described in "Experimental Procedures" with the indicated amounts of FIS protein and 25 fmol of a ^{32}P labelled *Sma* I-*Xho* I *oriC* fragment. Unbound DNA is noted as "Free."



FIS (ng)	0	0.1	0.2	0.4	0.8	1.6	3.2
FIS/*oriC*	0	0.2	0.3	0.7	1.4	2.8	6

Figure 3. ***In situ* footprinting of complexes with 1,10-phenanthroline-copper.** *In situ* cleavage was performed as described ("Experimental Procedures"). The FIS complexes were resolved on a standard native gel. The entire gel was immersed in a phenanthroline-copper solution. The reaction was stopped. Film was exposed to the gel and was used to guide the excision of the complexes. The DNA was eluted, precipitated, resuspended in formamide buffer and loaded on a standard 6% sequencing gel. The resulting cleavage patterns are shown in panel A. The gel in panel A was quantified by β emission scanning. The quantified cleavage pattern of each complex was compared with the cleavage of free DNA in panel B.


A
Free
Complex 1
Complex 2
Complex 3
Complex 4
Complex 5
Complex 6
Complex 7
Complex 8
R1
R2
FIS
R3
R4

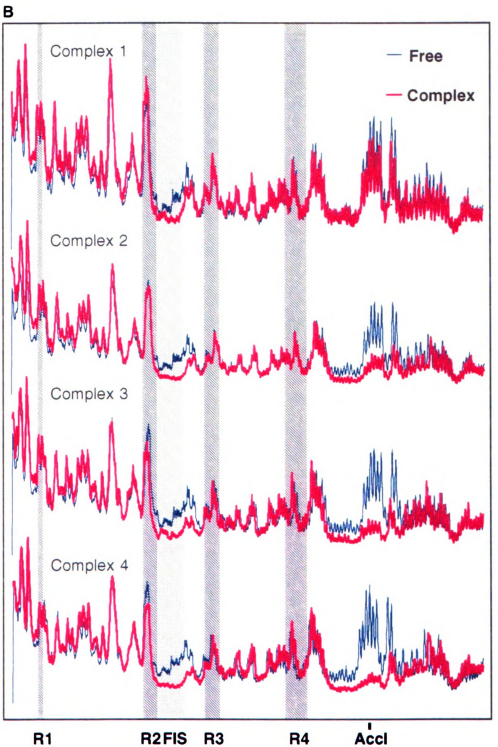
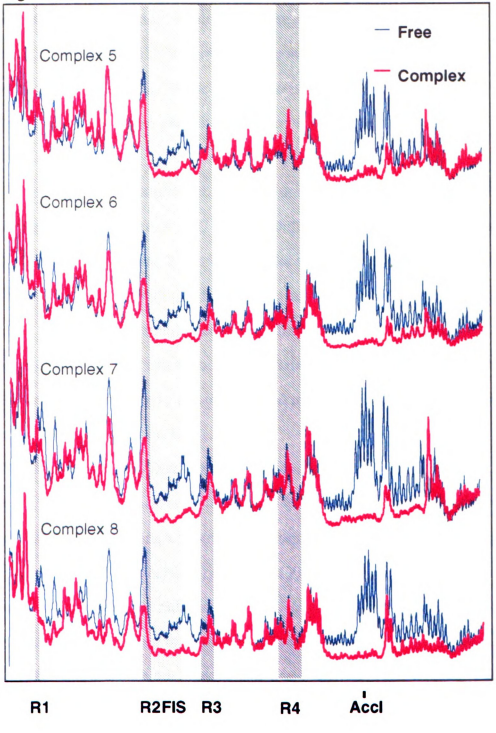

B
Complex 1
Free
Complex
Complex 2
Complex 3
Complex 4
R1
R2
FIS
R3
R4
Accl

Figure 3B continued



that of Complex 2 except that protection between R2 and R3 extended into R2. In Complexes 6 and 7, the protected sequences between R2 and R3 extend into boxes R2 and R3. In addition, sequences to the right of R1 were protected. In Complex 8, protection by FIS became more pronounced and was seen within DnaA boxes R1, R2, and R3. These observations suggest that FIS at high levels may inhibit binding of DnaA protein to *oriC* to inhibit the initiation of DNA replication.

FIS dissociates from oriC *more slowly than DnaA protein.* DnaA protein formed several complexes with *oriC* in gel mobility shift assays (Margulies and Kaguni, 1996) (Figure 5, lane 2 and 9). In order to compare the dissociation rates of DnaA-*oriC* complexes with the dissociation rate of FIS-*oriC* complexes, the appropriate protein was preincubated with radiolabelled *oriC*, and then challenged with a 100-fold molar excess of unlabelled *oriC* plasmid. At the indicated times, reactions were electrophoresed in native polyacrylamide gels to separate bound from unbound DNA.

Without competitor, DnaA protein (at a molar ratio of protein to DNA equal to 30) formed a specific complex termed Complex VI (Figure 4A) that involveed the binding of DnaA protein to all four DnaA boxes in *oriC* (Margulies and Kaguni, 1996). At the earliest time point (30 seconds) after addition of the unlabelled *oriC*, most of the radiolabelled *oriC* fragment migrated to the position of "free" fragment indicating the half-life of Complex VI was less than 30 seconds. The complex with the greatest half-life, Complex I, had a half-life of approximately 30 seconds (data not shown, see Chapter 3, Figure 9).

At a molar ratio of 1.5 of FIS to *oriC* fragment that resulted in the

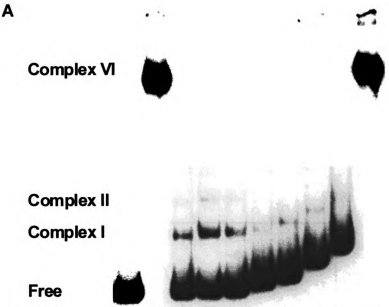
formation of three discrete complexes, the addition of the unlabelled competitor did not result in the dissociation of FIS from the radiolabelled *oriC* fragment until 5-10 minutes had elapsed (Figure 4B, C). These results suggest that FIS dissociates from its respective binding sites at a rate slower than DnaA protein (Figure 4C).

Novel oriC *complexes form in the presence of both FIS and DnaA protein.* To determine whether FIS-DnaA-*oriC* complexes could be resolved from DnaA-*oriC* and FIS-*oriC* complexes, binding reactions with both proteins were compared to reactions with the individual proteins. At a fixed level of FIS and the lowest level of DnaA protein a complex (labelled A) was resolved with an intermediate mobility to DnaA Complexes II and III (Figure 5). Two other novel complexes, labelled B and C, were resolved at higher levels of DnaA protein. These intermediates complexes suggested that both FIS and DnaA protein were bound in a single complex.

Binding of FIS and DnaA protein are not mutually exclusive--We were interested in confirming the report that the binding of DnaA protein to DnaA box R3, and that of FIS to the site between R2 and R3 were mutually exclusive (Gille, et al., 1991). Gel mobility shift assays similar to that shown in Figure 5 were performed, except that the level of FIS used was sufficient for forming only FIS Complex 1 in which FIS was bound only to the site between R2 and R3. To determine the sites bound in these novel complexes, *in situ* footprinting with Cu-phenanthroline was performed. Complex A contained protection at DnaA box R1 and R4, and the FIS site between R2 and R3 (Figure 6). Complex C was protected at all four DnaA boxes and the FIS site between R2 and R3. These

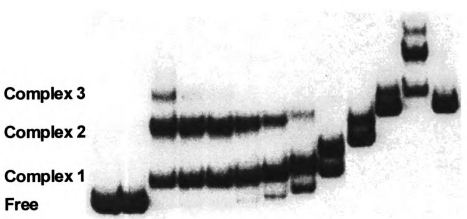
Figure 4. **The dissociation of DnaA-*oriC* and FIS-*oriC* complexes.** The gel mobility shift assay as described in "Materials and Methods"was scaled-up 10-fold. After incubating the appropriate protein with a ^{32}P-labelled DNA fragment containing *oriC*, pBS*oriC* (100-fold molar excess over the *oriC* fragment) was added. Aliquots of the binding reactions were loaded on an already electrophoresing nondenaturing polyacrylamide gel at the indicated times following addition of the challenge DNA. A) DnaA protein was added (at a protein to *oriC* fragment ratio of 30) and incubated for 5 minutes at 20 °C prior to adding the challenge DNA. B) FIS was added (at a protein to *oriC* fragment ratio of 1.5) and incubated for 90 minutes at 20 °C prior to adding the challenge DNA. The asterisks in lane 2 indicates that the challenge DNA was added prior to FIS. C) The resulting gels in panels A and B were quantified by β emission scanning. The total *oriC* bound in each lane was expressed as a fraction of the amount bound prior to the addition of challenge DNA.

A



DnaA	-	+	+	+	+	+	+	+	+	+
Competitor	-	-	+	+	+	+	+	+	+	-
Time (min)	0	0	1/2	1	2	5	10	20	30	30
Lane	1	2	3	4	5	6	7	8	9	10

B



FIS	-	+	+	+	+	+	+	+	+	+	+	+	-
Competitor	-	+	-	+	+	+	+	+	+	+	+	-	-
Time (min)	0	*	0	0.5	1	2.5	5	10	20	40	60	60	60
Lane	1	2	3	4	5	6	7	8	9	10	11	12	13

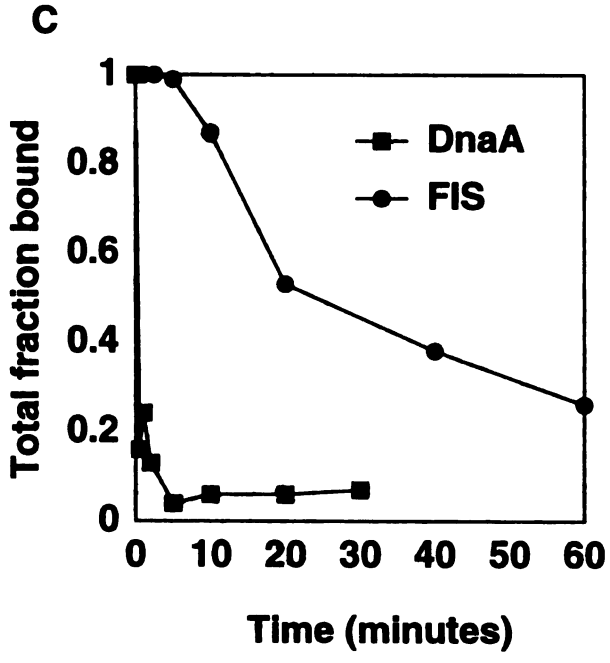
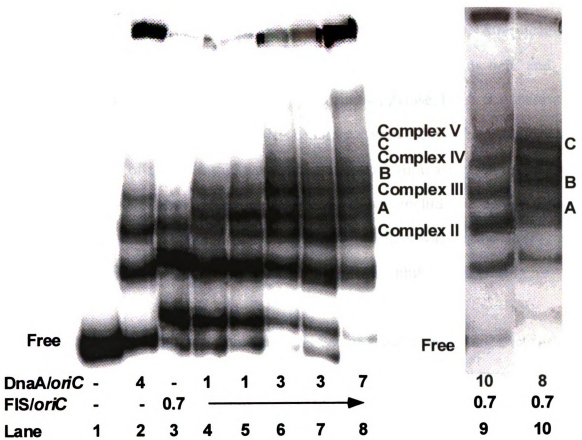

C
Total fraction bound
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
Time (minutes)
DnaA
FIS

Figure 5. **Novel complexes with *oriC* form in the presence of both DnaA protein and FIS.** Examples of two gel mobility shift experiments with DnaA protein or FIS or both proteins were performed essentially as described in "Materials and Methods" except that reactions were incubated for 30 minutes before loading on a polyacrylamide gel. Lanes 1 through 8 represent results from one experiment and lanes 9 and 10 from another.


Complex V
C
Complex IV
B
Complex III
A
Complex II
Free
C
B
A
Free
DnaA/oriC - 4 - 1 1 3 3 7 10 8
FIS/oriC - - 0.7 0.7 0.7
Lane 1 2 3 4 5 6 7 8 9 10

results indicate that FIS bound to the site between R2 and R3 does not apparently block the binding of DnaA protein to these respective sites.

FIS does not affect DNA replication on binding oriC--The influence of FIS on *oriC* plasmid replication was examined in a reconstituted DNA replication assay. This assay is dependent on a supercoiled template containing *oriC* and purified DnaA, DnaB, and DnaC proteins, HU, primase, gyrase, DNA polymerase III holoenzyme, and single stranded DNA binding protein (SSB). The same molar amount (25 fmol) of *oriC* plasmid was used in the replication assays as in the gel shift assays described above in Figure 1. When FIS was included at levels (0.1 to 3 ng) tested in gel mobility shift assays (Figure 2), its effect was negligible (Figure 7A). Only at elevated levels (greater than 100 ng) was inhibition seen (Figure 7B).

To investigate the possibility that the vector sequences included on the *oriC* plasmid were competing FIS from binding the *oriC* sequences in replication assays, unlabelled supercoiled plasmid containing only the vector sequences were included in of gel mobility shifts with a labelled *oriC* fragment (Figure 8). Even at the lowest level tested (0.5 ng), FIS bound *oriC* in the presence of the vector sequences. At levels (160 ng) which were inhibitory for replication, a complex with low mobility formed. These results verified that levels of FIS required to bind sequences within *oriC* did not inhibit replication.

We investigated the biochemical basis of this inhibition, suspecting that these high levels of FIS may result in overwinding of the plasmid, as HU does when added at inhibitory levels for *oriC* replication (Skarstad *et al.*, 1990). The topological alteration in *oriC* plasmids bound by FIS could be fixed with *E. coli*

Figure 6. ***In situ* footprinting of the DnaA-FIS-*oriC* complexes with phenanthroline-copper.** *In situ* cleavage was performed as described in "Experimental Procedures" with a *Sma* I-*Xho* I fragment labelled at the *Xho* I site with the large fragment of DNA polymerase I and all 4 [α^{32}P] deoxyribonucleotides. The cleavage pattern of respective complexes was analyzed by β emission scanning and compared to the cleavage pattern of the corresponding unbound fragment that was treated similarly.

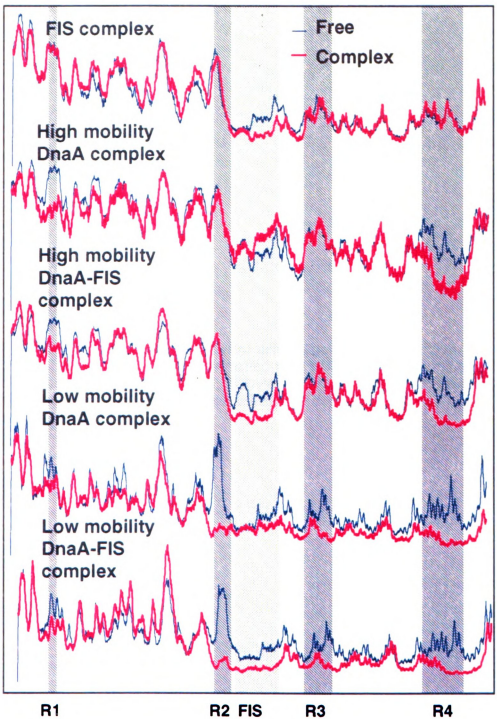

FIS complex
Free
Complex
High mobility
DnaA complex
High mobility
DnaA-FIS
complex
Low mobility
DnaA complex
Low mobility
DnaA-FIS
complex
R1
R2
FIS
R3
R4

Figure 7. **At high levels, FIS inhibits *in vitro oriC* dependent replication.** Reconstituted replication assays were performed with pBS*oriC* as a template as described in "Experimental Procedures". FIS was added prior to DnaA protein. The FIS/*oriC* molar ratio is indicated in parenthesis for the different levels of FIS added to each reaction.

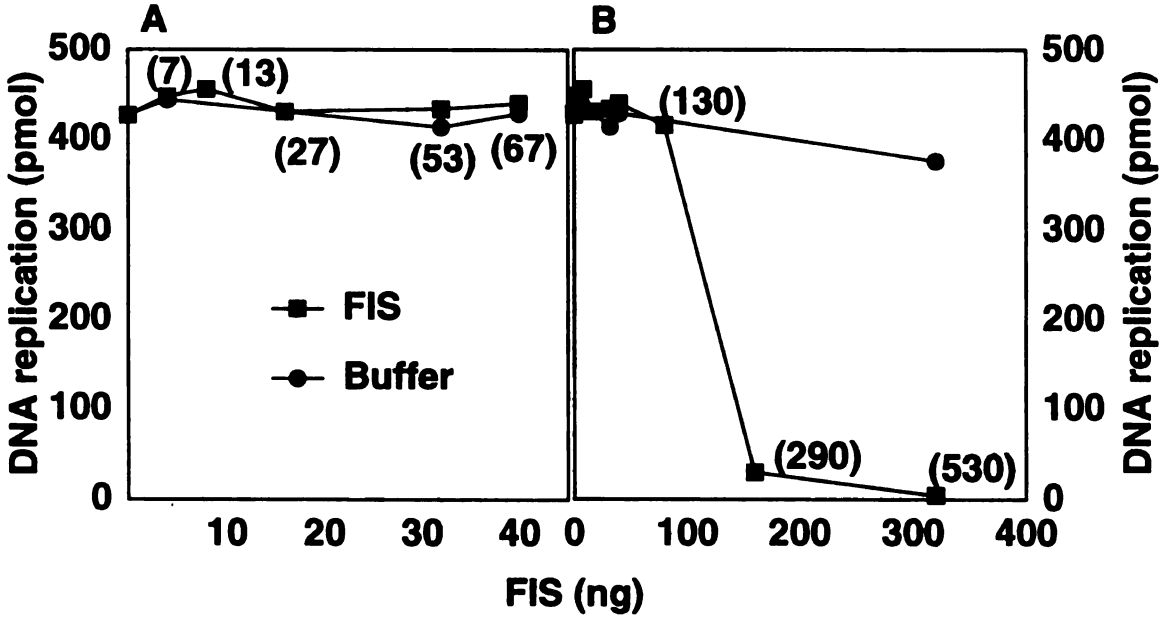
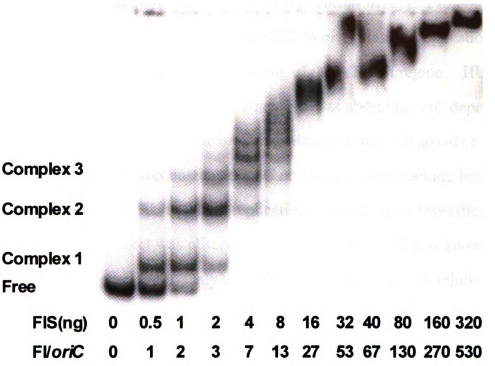

A
B
500
400
300
200
100
0
(7)
(13)
(27)
(53)
(67)
(130)
(290)
(530)
FIS
Buffer
DNA replication (pmol)
10
20
30
40
0
100
200
300
400
FIS (ng)

Figure 8. **FIS binds *oriC* in the presence of supercoiled vector DNA.** Gel mobility shift assays were performed as described in "Experimental Procedures" with the indicated amounts of FIS protein and 25 fmols of a ^{32}P labelled *Sma* I-*Xho* I *oriC* fragment and 25 fmols of unlabelled supercoiled pBluescript. Unbound DNA is noted as "Free".


Complex 3
Complex 2
Complex 1
Free
FIS(ng) 0 0.5 1 2 4 8 16 32 40 80 160 320
FI/oriC 0 1 2 3 7 13 27 53 67 130 270 530

topoisomerase I and measured by electrophoresing the deproteinated plasmid on an agarose gel containing chloroquine (Figure 9). Levels of FIS that are inhibitory for replication form a highly negatively supercoiled topoisomer of the *oriC* plasmid.

FIS can not substitute for HU in in vitro *replication assays.* FIS could potentially functionally substitute for HU and IHF in *oriC* plasmid replication. Both proteins aid in DnaA-dependent unwinding of the AT-rich region. HU and IHF appear to perform similar functions. When HU was absent in *oriC* dependent replication assays, IHF could stimulate *oriC* plasmid replication (Skarstad *et al.*, 1990). FIS was tested in the reconstituted *oriC* replication assay lacking both HU and IHF. In the absence of HU and IHF, replication occured, albeit less efficiently (Figure 10). Again, no effect was observed at levels at which FIS was known to bind to linear *oriC* (Figure 10A). Only at an 800-fold excess was FIS inhibitory (Figure 10B).

The effect of FIS at non-optimal levels of HU was examined to explore the possibility that FIS might be stimulatory. At various levels of HU, no effect of FIS was seen when added at levels known to bind linear *oriC* (Figure 10A). At higher levels, FIS was able to inhibit (Figure 10B). Similar experiments were performed in which non-optimal levels of IHF replaced HU with comparable results (data not shown). These experiments indicated that FIS cannot substitute for HU or IHF nor stimulated when the latter proteins were present at non optimal levels in *oriC* plasmid DNA replication.

Binding of FIS to oriC *does not affect initiation from* oriC *in RNA polymerase dependent initiation*--The sequences flanking and within *oriC*

Figure 9. **Inhibitory levels of FIS induce a highly negatively supercoiled topoisomer of pBS*oriC*.** *E. coli* topoisomerase I was add after FIS was preincubated with the supercoiled template DNA (lanes 1-9). The position of nicked and relaxed plasmid DNA migrates is indicated. Negatively supercoiled pBS*oriC* which is active for replication migrates to positions intermediate to the nicked and relaxed DNA (lane 10).

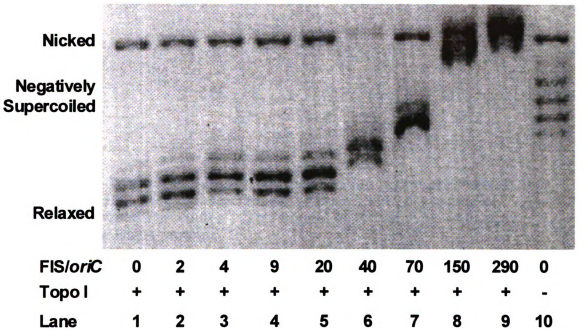
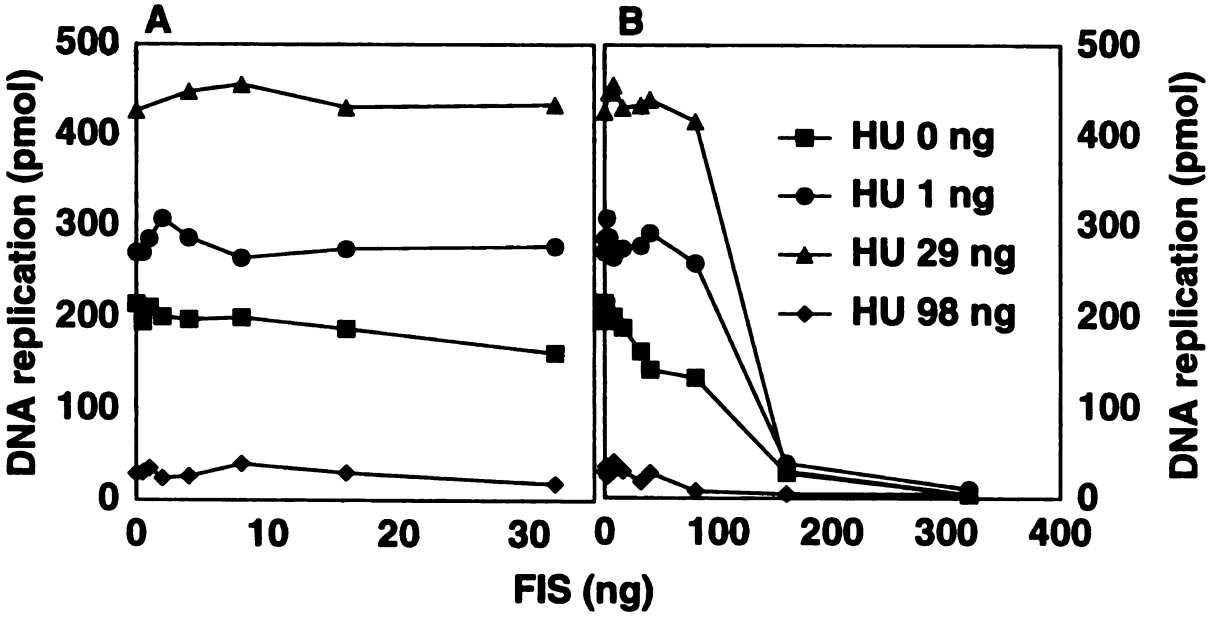
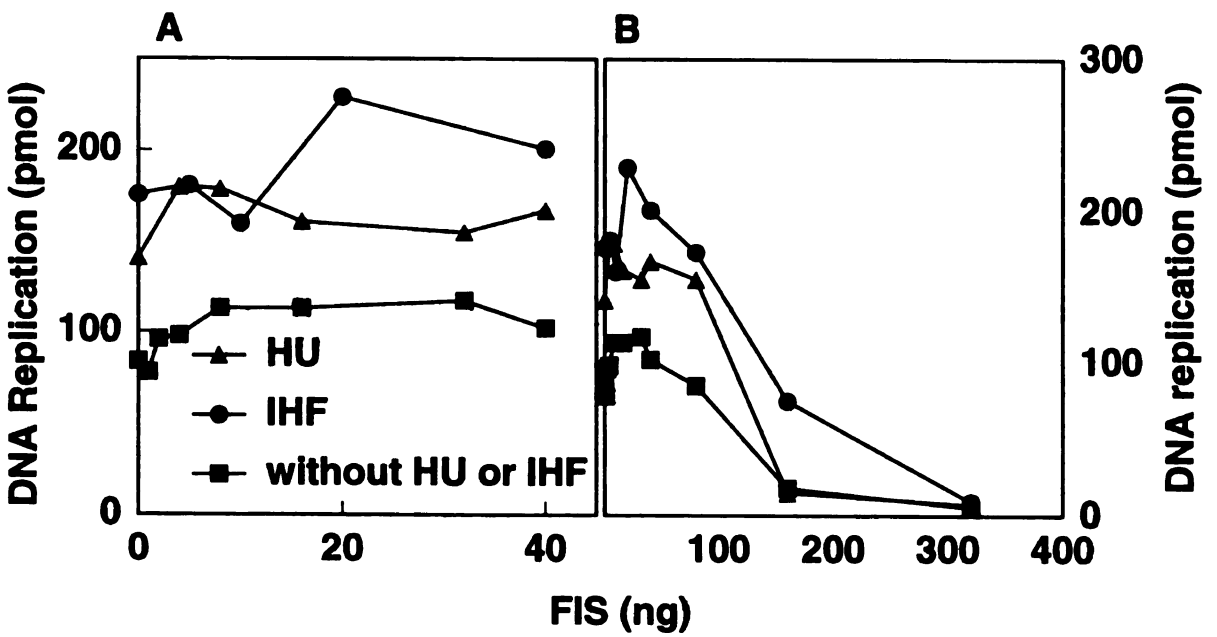

Nicked
Negatively Supercoiled
Relaxed
FIS/oriC 0 2 4 9 20 40 70 150 290 0
Topo I + + + + + + + + + -
Lane 1 2 3 4 5 6 7 8 9 10

Figure 10. **Only high levels of FIS inhibit *oriC* replication in the presence of various amounts of HU.** Reconstituted replication assays were performed as described in "Experimental Procedures". The amount of HU added is indicated. FIS was added prior to DnaA protein.


A
B
500
400
300
200
100
0
DNA replication (pmol)
0
10
20
30
100
200
300
400
FIS (ng)
HU 0 ng
HU 1 ng
HU 29 ng
HU 98 ng

contain several promoters (Figure 1). Transcription from promoters in and around *oriC* may influence, positively or negatively, replication initiation events that occur at this site (Asai *et al.*, 1992). As FIS acts as a transcription factor (reviewed in (Finkel and Johnson, 1992)), FIS could modify transcription from promoters within or flanking *oriC*, thereby affecting replication. To investigate the effect of FIS in a reconstituted system containing RNA polymerase, it was titrated in assays in the presence and absence of either HU or IHF (Figure 11). As in the other replication experiments (Figures 7, and 10) FIS had little effect at low levels and was inhibitory at much higher levels. These findings suggest that FIS bound to *oriC* has no stimulatory effect under these *in vitro* conditions.

Figure 11. **The binding of FIS to *oriC* does not affect initiation in RNA polymerase dependent initiation.** Reconstituted replication assays were performed as described in "Experimental Procedures". IHF (11 ng) or HU (10 ng) were added as indicated. FIS was added prior to DnaA protein.


A
B
DNA Replication (pmol)
DNA replication (pmol)
200
100
0
300
200
100
0
HU
IHF
without HU or IHF
0
20
40
100
200
300
400
FIS (ng)

Discussion

FIS binds specifically to oriC. Here we showed that FIS bound to sequences near the right boundary of DnaA box R2, and did not bind to a proposed site (Gille *et al.*, 1991) located nearer to R3 that more closely resembles the FIS consensus sequence (Figure 3). This finding confirmed those of Filutowicz *et al.* (Filutowicz *et al.*, 1992). In complexes of greater mobility, protection by FIS extending into DnaA boxes R2 or R3 was not observed, as previously reported (Filutowicz *et al.*, 1992; Gille *et al.*, 1991). The discrepancies may be due to the procedures used. The previous footprinting studies used DNaseI, whereas we used phenanthroline-copper as a cleavage agent which is subject to less steric hindrance. Another procedural difference was that the DNaseI protection experiments were performed in solution with a mixture of FIS-*oriC* complexes, whereas we used an *in situ* method that analyzes separated complexes. Indeed, weak protection of the R2 and R3 boxes was observed in complexes of lower mobility. This protection may be due to FIS binding elsewhere on the *oriC* fragment and stabilizing weaker interactions to flanking sequences by the FIS dimer bound at the site between R2 and R3.

Specific binding of FIS to oriC *has no affect on initiation of chromosomal replication* in vitro. The binding of DnaA protein to DnaA box R3 was reported to be mutually exclusive to the binding of FIS (Gille *et al.*, 1991). Not only was FIS able to inhibit the binding of DnaA protein when FIS was bound first, DnaA protein was able to inhibit FIS binding when DnaA protein was bound first. This observation is unexpected in light of the additive protection

pattern seen in the presence of DnaA protein and FIS (Figure 6), and the relative dissociation rates of DnaA protein and FIS when bound to *oriC* (Figure 4). The rapid dissociation of DnaA protein from *oriC* would allow the binding of FIS. A plausible explanation for these discrepancies is that DNaseI footprinting was performed when binding had not reached equilibrium, as FIS has a slow rate of association relative to DnaA protein (data not shown).

When added at a level 100-fold over that sufficient to fill the highest affinity site in *oriC*, FIS neither inhibited nor stimulated DNA replication from *oriC* (Figure 7). Inclusion of FIS did not alter replication in the presence of HU or IHF, or replication that was dependent on RNA polymerase (Figures 10 and 11). Only at high concentrations (greater than 80 ng) was FIS inhibitory. The conclusion that the inhibitory effect of FIS was not dependent on FIS binding to the site between R2 and R3 was supported by the observation that a mutant *oriC* plasmid (Mutant Figure 1) deficient in FIS binding to the site between R2 and R3 was equally inhibited by FIS as wild type *oriC in vitro* replication (Wold *et al.*, 1996). Instead, inhibition correlated with an alteration in superhelical density of the DNA template (Figure 9). The *in vitro* inhibition of replication by high concentrations of FIS may be relevant *in vivo* as in early log phase the cellular concentrations of FIS are approximently 100-fold greater than required to inhibit *in vitro* replication.

Roles for FIS in initiation at oriC. The results presented here do not support a model in which FIS plays a direct role in initiation at *oriC*. However, as we used an *in vitro* system, some component critical for FIS function may be missing. For example, the cellular membrane has been speculated to play a role in

regulation of initiation by associating with *oriC*. *In vitro* replication is inhibited in the presence of cellular membrane fractions (Landoulsi *et al.*, 1990). Several membrane proteins that bind *oriC* are speculated to play role in this inhibition (Jacq *et al.*, 1983; Lu *et al.*, 1994). The role of FIS may be to alter the binding of an inhibitor of initiation which was not included in these reconstituted replication assays.

Alternatively, FIS may not have a specific role in initiation. *In vivo* observations with *fis* mutants may be attributable to pleiotropic effects on supercoiling and/or transcriptional regulation of initiation factors. FIS is proposed to help organize the bacterial chromosome as *fis* mutants have aberrant nucleoid structure. *fis* mutants may have an altered chromosome that affects chromosome replication (Filutowicz *et al.*, 1992).

Furthermore, as FIS is also a transcription factor, it may indirectly affect initiation by changing the expression of replication factors. Recently, FIS was reported to change the expression of DnaA protein (Froelich, 1996). In *fis* null mutants, the level of DnaA protein increased approximately 2- to 3-fold relative to a wild type *fis* strain. A 2-fold increase in the level of DnaA protein was sufficient to cause asynchronous initiation (Atlung and Hansen, 1993; Lobner-Olesen *et al.*, 1989). Lastly, FIS may only play a role under specific growth conditions, such as at elevated temperatures or at the transition from stationary phase cell to exponential growth (Ball *et al.*, 1992; Filutowicz *et al.*, 1992).

Chapter V

Summary and Perspectives

This thesis demonstrates that DnaA protein binds to the DnaA boxes within *oriC* in an ordered manner, and that the binding to all four DnaA boxes correlates with replication of an *oriC* plasmid. These findings not only reveal more detail into the mechanism of initiation but also provide insight into how initiation from *oriC* may be regulated. The binding of the interior boxes at higher concentrations of DnaA protein after the outer boxes are bound suggests that binding of DnaA protein to the interior boxes triggers initiation. This model is supported by *in vivo* footprinting which suggests that DnaA boxes R1, R2 and R4 are bound throughout the cell cycle and that only at the time of initiation is the R3 bound. Recently, mutational analysis of *oriC* plasmids, in which exchanging R3 and R4 sequences reduces *oriC* activity *in vivo*, also supports the model (Langer *et al.*, 1996). It would be of interest to determine the order of binding of DnaA protein to this mutant and whether initiation is synchronous from this at other mutant origins which alter the binding of DnaA protein.

As little evidence exists for changes in DnaA protein concentration in a cell-cycle dependent manner, other modes of DnaA protein regulation have received attention. These include the regulation of DnaA protein activity through its ability to bind ATP or through its ability to bind *oriC* (Katayama and Crooke, 1995). The latter mechanism involves proteins which bind *oriC* and therefore altering the binding of DnaA protein to *oriC*. FIS has been speculated to be a regulatory factor as *fis* mutants have phenotypes that are consistent with this function (Boye *et al.*, 1993, Gille *et al.*, 1991) and mutations in the FIS

binding site inactivate *oriC in vivo* (Roth *et al.*, 1994). In addition, binding of its site in *oriC* appears to be altered immediately prior to initiation (Cassler *et al.*, 1995). Furthermore, FIS cellular concentrations vary with different growth conditions as might be expected for a regulatory protein (Ball *et al.*, 1992). However, *in vitro* studies presented here do not support the model in which the binding of FIS to the site between R2 and R3 plays a direct role in initiation. Binding of FIS to its site between boxes R2 and R3 does not inhibit the binding of DnaA protein to the interior boxes as originally speculated.

These results do not exclude the possibility of FIS playing a role in initiation. The *in vitro* inhibition of replication by high concentrations of FIS may be relevant *in vivo* as in early log phase the cellular concentrations of FIS are approximately 100-fold greater than required to inhibit *in vitro* replication. It would be of interest to measure the rate and the synchrony of initiation and footprint *oriC in vivo* when FIS is present at various cellular concentrations. The conditions for *in vitro* replication vary from those *in vivo*. As these experiments were performed with highly purified proteins, factors important in FIS function may be lacking. Experiments adding inhibitors of *oriC* replication (i.e. membrane fractions, or possibly SeqA) to the purified system or using extracts deficient in FIS may reveal a direct effect of FIS on replication. However, FIS may not play a direct role in initiation. Initiation defective phenotype of *fis* mutants may be due to increased levels of DnaA protein in *fis* mutants rather than any direct interaction at *oriC*. Studies altering the levels of DnaA protein in a *fis* background may reveal that the *fis* mutant phenotype is the result of pleiotropic effects.

Another *oriC* binding protein which could affect DnaA protein activity at *oriC* is IHF. This protein is not thought to be a regulator of initiation as it is found at high cellular concentrations (Ditto *et al.*, 1994). Although IHF can stimulate replication from *oriC* in the absence of HU, no effect of IHF on the binding of DnaA protein to a linear *oriC* fragment was observed. Because the DNA binding experiments were performed with linear DNA, and DnaA protein dependent unwinding of *oriC* only occurs on a supercoiled template, we can not exclude the possibility that IHF aids initial binding of DnaA protein only on a supercoiled template. Additional footprinting experiments using a supercoiled template may show that IHF stimulates the binding of DnaA protein to supercoiled *oriC* as was seen for the binding of Int protein to lambda *att*P involved in lambda integration (Richet *et al.*, 1986). However, the results presented here suggest that IHF facilitates DnaA protein-dependent unwinding by promoting steps subsequent to the initial binding of DnaA protein to *oriC*.

Though my thesis has concentrated on three proteins involved in initiation, other factors (i.e. Dam methylase and SeqA) have been demonstrated to play a role in regulating replication, and other factors probably have yet to be identified. SeqA may remain bound to *oriC* even when fully methylated, inhibiting DnaA protein from interacting with *oriC*. As of now SeqA has not been tested *in vitro* for its ability to inhibit replication of hemimethylated or fully methylated *oriC*. Finally, genetic approaches have been useful in identifying factors involved in initiation in the past and may prove useful in identifying other factors that are important in regulating initiation. Hyperactive initiation mutants may be identified by selecting for an increased copy number of an *oriC*

minichoromosome carrying an antibiotic resistance marker.

Bibilography

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