IDENTIFICATION OF GENES REGULATED BY dnaA PROTEIN IN *Escherichia coli*

By

Qingping Wang

A DISSERTATION

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1989

ABSTRACT

IDENTIFICATION OF GENES REGULATED BY dnaA PROTEIN IN Escherichia coli

By

Qingping Wang

dnaA protein is an essential protein for initiation of DNA replication from the Escherichia coli chromosomal origin, oriC. It binds to the oriC region by recognizing a nine base-pair sequence TTAT(A/C)CA(A/C) A. The binding induces localized strand melting which promotes subsequent events in the initiation process.

The dnaA protein binding sequence is also present in promoter regions of several genes. This project was undertaken to test the possibility of dnaA protein as a regulatory protein of gene expression. It was observed that dnaA protein binds to promoter regions of genes dnaA, rpoH, polA, uvrB, nrd, and a gene located near oriC encoding a 16 KDa protein whose function is unknown (referred to as 16 KDa gene). It also binds to some unidentified chromosomal fragments. The binding resulted in transcriptional repression of dnaA, rpoH, and the 16 KDa gene. The effect of binding at other sites was not conclusively determined. A model was proposed to explain the regulatory function of dnaA protein.

In addition, a new sigma factor was discovered while working with the rpoH gene. It has a molecular weight of 24 KDa and promotes transcription from one of the three rpoH promoters.

To

My Parents and Husband

ACKNOWLEDGMENT

I would like to express my appreciation to Dr. Jon Kaguni for his guidance and encouragement through the years. I also owe thanks to Dr. Laurie Kaguni for her suggestions and advice. I wish to thank members of my guidance committee, Drs. Susan Conrad, Jerry Dodgson, Arnold Revzin and John Wang for their advice and invaluable time.

I would like to acknowledge the support of my coworkers, Dr. Deog Su Hwang, Dr. Cathy Wernette, Ted Hupp, Kevin Carr, Shelly Houston, David Siemieniak, Matthew Olson, David Lewis, Rhod Elder, Philis Yang, and Zhun Lu. I am also grateful to my Chinese friends here who made the years more enjoyable.

I wish to thank my parents for their support and encouragement along the way. Finally, my appreciation goes to my husband for his help, understanding and support.

TABLE OF CONTENTS

LIST OF TABLES

page

Chapter V

LIST OF FIGURES

ABBREVIATIONS

bp	base pair
dNTP	deoxyribonucleotide
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
HEPES	4-(2-hydroxethyl)-1-piperizineethane sulfonic acid
kb	kilo-base pair
KDa	kilo-dalton
oriC plasmid	plasmid containing the oriC sequence
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
SDS	sodium dodecyl sulfate
SSB	single-stranded DNA binding protein
$E\sigma^{70}$	RNA polymerase with sigma70
$E\sigma^{32}$	RNA polymerase with sigma32

INTRODUCTION

Escherichia coli is probably the best studied organism. DNA replication of its chromosome has been a major subject of study for over thirty years. E. coli has a single circular chromosome of about 4,000 kilo-base pairs (kb) (1-3). Replication of the chromosome starts at a specific site, the chromosomal origin or oriC, proceeds bidirectionally with equal velocity, and terminates at the opposite position on the circle (4-6).

dnaA, the first genetic locus identified whose function is involved in initiation of DNA replication (7), encodes a protein that specifically binds to the replication origin (8). The binding promotes subsequent events in the initiation process to occur (9). Physiological studies indicate that initiation of DNA replication is tightly coupled to cell growth. It occurs at a critical DNA/cell mass ratio. dnaA protein plays an key role in determining the initiation mass.

dnaA protein also binds to promoter regions of its own gene and the gene encoding a 16 KDa protein of unknown function which is located adjacent to oriC (8). In vivo data indicate that dnaA protein represses transcription from the dnaA promoters and from the 16 KDa promoter (10-12). DNA fragments bound by dnaA protein share a nine base-pair consensus sequence TTAT(A/C)CA(A/C)A (8). This sequence is also found in promoter regions of other genes including rpoH, polA, uvrB and nrd (13-16). Based on an assumption that dnaA protein recognizes the above consensus sequence and

binds to it, and that binding of a protein at the promoter region of a gene will influence its expression, this project was undertaken to investigate the possibility of dnaA protein regulating expression of other genes. This regulation by dnaA protein may play an important role in coordination of DNA replication to cell growth.

At the time the project was started, in vivo data suggested that dnaA protein represses expression of its own gene and of the 16 KDa gene (10-12). However, these in vivo experiments do not address whether dnaA protein alone is sufficient for the repression. In addition, an inherent problem of in vivo experiments is that one cannot distinguish a primary effect of dnaA protein from a secondary effect. Since these are very critical questions to the project, in vitro experiments were performed to establish that dnaA protein repressed expression of the dnaA and the 16 KDa genes with purified enzymes. Transcripts from the dnaA promoters and from the promoter of the 16 KDa gene were observed in run-off transcription assays as expected. Addition of purified dnaA protein resulted in specific inhibition of these promoters. The degree of repression was well correlated with the extent of binding of dnaA protein to the promoter regions in filter binding assays. These results demonstrated that binding of dnaA protein directly inhibits transcription from the dnaA promoters and from the 16 KDa promoter. This work was published in MOLECULAR and GENERAL GENETICS (1987) 209, 518-525.

Two copies of the dnaA protein recognition sequence are present in the promoter region of the rpoH gene (13). The rpoH gene encodes a sigma factor

of RNA polymerase which recognizes promoters of heat shock genes. Heat shock proteins of E. coli are involved in many macromolecular processes including DNA replication, RNA synthesis, and protein processing. The mechanism of action remains to be revealed. Using filter binding assays and DNaseI protection assays, dnaA protein was observed to bind to the two nine base-pair consensus sequences in the rpoH promoter region. The binding resulted in transcriptional repression from two of the three promoters of the rpoH gene both in vivo and in vitro. Regulation of the rpoH gene by dnaA protein suggests that synthesis of heat shock proteins which are required for normal cell growth is coordinated with initiation of DNA replication. This work was published in THE JOURNAL OF BIOLOGICAL CHEMISTRY (1989) 264, 7338-7344.

In examining transcription of the rpoH promoters, it was observed that several sigma70 RNA polymerase preparations were able to transcribe from two of the three rpoH promoters. Interestingly, one preparation resulted additionally in transcription from the third promoter rpoH3P. Reports from others indicate that this promoter is not recognized either by sigma70 or sigma32 (17). Transcription from this promoter must require a positive regulatory protein or a new form RNA polymerase. In pursuing this matter, a new sigma factor was discovered which is required for transcription from rpoH3P. This work is in press in the JOURNAL OF BACTERIOLOGY (1989).

The presence of dnaA protein recognition sequences in promoter regions of polA, uvrB, and nrd resulted in binding of dnaA protein to these sites. polA, uvrB, and nrd encode DNA polymerase I, uvrB protein, and

ribonucleotide reductase, respectively. The former two are involved in DNA repair and the later is required for the conversion of ribonucleotides to deoxyribonucleotides. The effect of binding on transcription from promoters of these genes was inconclusive.

In addition to these sites, dnaA protein was also observed to preferentially bind to unidentified chromosomal fragments from E. coli.

This thesis is composed of five chapters. The first chapter is a review of the literature on studies of DNA replication and heat shock response in E. coli. The following chapters are derived from publications in MOLECULAR and GENERAL GENETICS (1987) 209, 518-525 (chapter II); THE JOURNAL OF BIOLOGICAL CHEMISTRY (1989) 264, 7338-7344 (chapter III); and the JOURNAL OF BACTERIOLOGY (in press) (chapter IV). Chapter V summarizes the unpublished studies on the binding affinity of dnaA protein to promoter regions of polA, uvrB, nrd, and an unidentified chromosomal fragment.

REFERENCES

1. Jacob, F., and Wollman, E. L. (1961) Sexuality and the genetics of bacteria. Academic Press, New York.

2. Cairns, J. (1963) J. Mol. Biol. 6, 208-213.

3. Lewin, B. (1983) Gene. John Wiley & Sons.

4. Prescott, D. M., and Kuempel, P. L. (1972) Proc. Nat. Acad. Sci. USA 69, 2842-2845.

5. Louarn, J., Funderburgh, M., and Bird, R. (1974) J. Bacteriol. 120, 1-5.

6. Marsh, R. C., and Worcel, A. (1977) Proc. Nalt. Acad. Sci. USA 74, 2720-2724.

7. Hirota, Y., Mordoh, J., and Jacob, F. (1970) J. Mol. Biol. 53, 369-387.

8. Fuller, R. S., Funnell, B. E., and Kornberg, A. (1984) Cell 38. 889-900.

9. Bramhill, D., and Kornberg, A. (1988) Cell 52, 743-755.

10. Braun, R. E., O'Day, K., and Wright, A. (1985) Cell 40, 159-169.

11. Atlung, T., Clausen, E. S., and Hansen, F. G. (1985) Mol. Gen. Genet. 200, 442-450.

12. Lother, H., Kolling, R., Kucherer, C. and Schauzu, M. (1985) EMBO J. 4, 555-560.

13. Landick, R., Vaughn, V., Lau, E. T., VanBogelen, R. A., Erickson,. J. W., and Neidhardt, F. C. (1984) Cell 38, 175-182.

14. Joyce, C. M., Kelley, W. S., and Grindley, N. D. F. (1982) J. Biol. Chem. 257, 1958-1964.

15. Sancar, G. B., Sancar, A., Little, J. W., and Rupp, W. D. (1982) Cell 28, 523-530.

16. Carlson, J., Fuchs, J. A., and Messing, J. (1984) Proc. Natl. Acad. Sci. USA 81, 4294-4297.

17. Erickson, J. W., Vaughn, V., Walter, W. A., Neidhardt, F. C., and Cross, C. A. (1987) Genes & Develop. 1, 419-432.

Chapter I

LITERATURE REVIEW

LITERATURE REVIEW

I. dnaA Protein and Replication of the E. coli Chromosome

Ever since DNA was proven to carry genetic information four decades ago (1), replication of DNA has received a lot of attention. In 1953, Watson and Crick proposed the double stranded DNA model based on X-ray diffraction data (2). The model predicted that DNA is replicated in a semiconservative fashion, which was confirmed experimentally by Meselson and Stahl in 1958 (3). Since these discoveries, a considerable amount of research effort has been focused on DNA replication in E. coli. Early genetic studies indicate that the E. coli chromosome is a closed circular duplex DNA (4). This is supported by autoradiographic studies performed by Cairns in 1963, who discovered that the chromosome exists as a closed circle of about 1000-1400 μm in length by labeling the chromosome with tritiated thymidine (5). This length was determined later to be about 4000 kb (6). Cairns also observed only one replication bubble on each chromosome suggesting that the replication is initiated at only one point (5). Later studies indicate that DNA replication starts at a fixed position on the chromosome, oriC, proceeds bidirectionally with equal velocity, and terminates at a point, terC, opposite from oriC on the circular chromosome (6-8).

Jacob and coworkers, based on limited and circumstantial evidence of DNA replication of E. coli chromosome, F factor, and of phages, proposed a

replicon model in 1963 to explain regulation of DNA replication (9). The model proposed the following. 1. Any independent replicating DNA consists of a replicon, which contains a operator of replication, or replicator. The replicator is a specific element on the chromosome which allows an initiator protein to recognize and to promote DNA replication. 2. An initiator protein is required for initiation of DNA replication from a replicator of a specific replicon. The synthesis of the initiator is controlled so that it is accumulated to allow the replication to occur in a precisely timed manner. This model, formulated similarly to the repressor-operator model in the regulation of transcription, is demonstrated to be largely correct after 25 years of study.

A. Genetic and Physiological Studies of DNA Replication and dnaA Protein

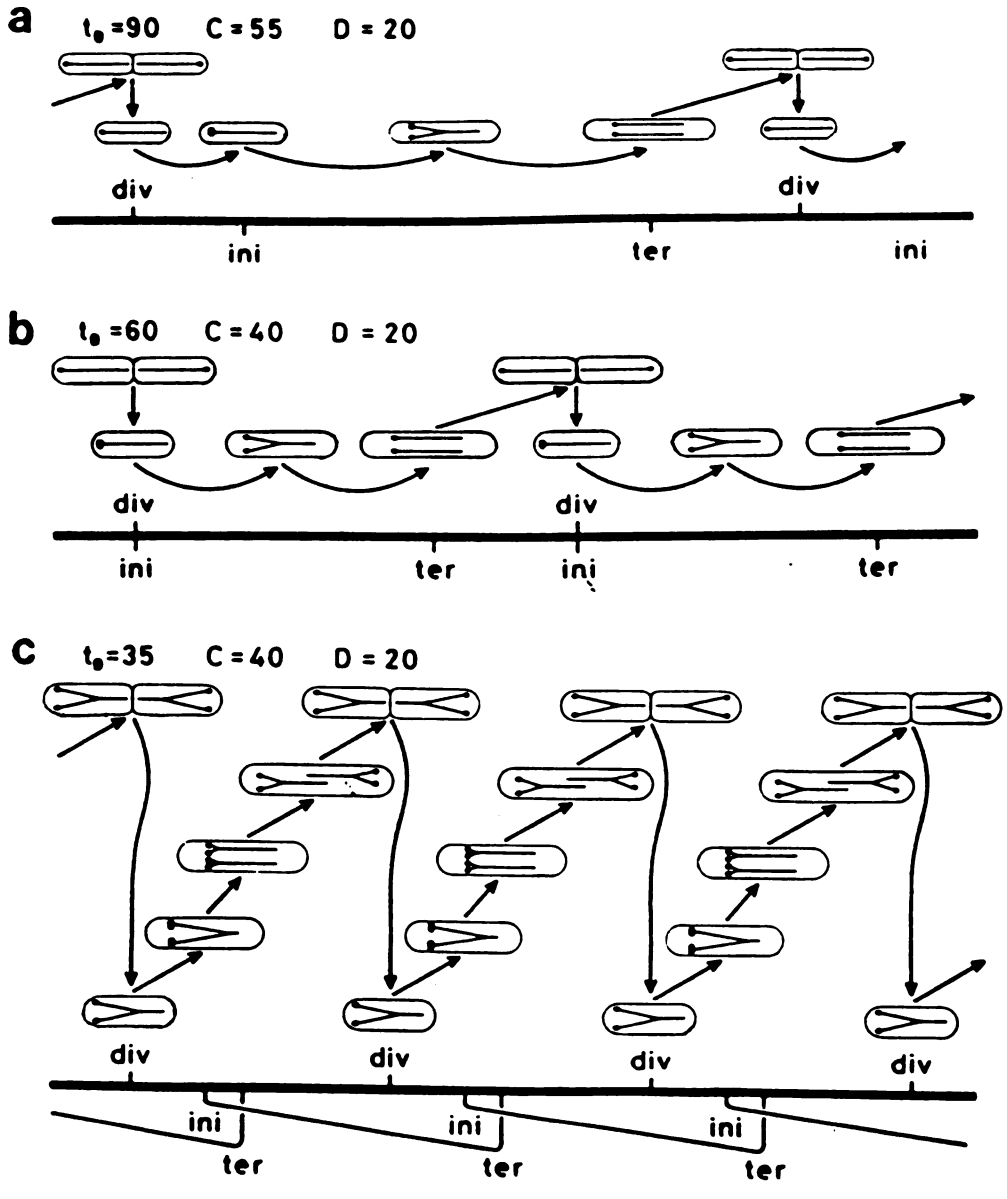
1. DNA Replication and Cell Growth

The doubling time of E. coli varies from 20 minutes to 150 minutes depending on nutrient conditions (10). The size of fast growing cells are larger than those growing slower. Small cells usually contain one to two genome equivalents of DNA, while large, fast growing cells can have more than four genome equivalents of DNA. DNA content per cell changes as the cell size varies resulting in a relatively unbiased ratio of DNA to cell mass independent of growth rate (10).

Regulation of DNA replication in relation to the cell division cycle was studied in E. coli by Helmstetter and Cooper (11, 12) (Figure 1). DNA synthesis was measured by pulse labeling an undisturbed population of

Figure 1. Schematic presentation of the cell division and chromosome replication cycle (170).

The doubling time is assumed to be 90 minutes (a), 60 minutes (b), and 35 minutes (c). t_D, doubling time; C, DNA synthesis period from initiation (ini) to termination (ter) of chromosomal replication; D, time between termination and cell division (div).


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

exponentially growing cells. The amount of label was later correlated to cells at different stages in the division cycle by a filter elution technique. The rate of DNA synthesis in cells at different stages was deduced from the amount of label incorporated. Assuming that the rate of DNA synthesis is constant at each replication fork, an increase in the rate was explained as an increase in the number of replication forks, that is initiation of a new round of replication. A decrease in the rate was interpreted as a termination of existing replication forks. By measuring initiation and termination in closely timed cell cycles, the following was concluded. 1. The time for a complete round of replication is constant and requires about 41 minutes. The time between the termination of replication and cell division is also a constant of about 20 minutes. 2. New rounds of replication start at a time independent of the cell age, but at a constant time before a given division occurs. 3. In rapidly growing cells, new rounds of replication begin before the previous round has completed. Individual chromosome in such cells contains multiple forks. Further refined studies indicate that initiation begins at a fixed ratio of cell mass to replication origin (13) (Figure 1).

2. Regulation of Initiation and Function of dnaA Protein

a. Proteins Required for Initiation from oriC

Since DNA replication is regulated by the frequency of initiation, proteins involved in initiation became the focus of study. Jacob's initiator protein was the apparent target to start with (9). A genetic approach was used to identify

genes whose products function in initiation of DNA replication. Since initiator proteins are required for viability, mutations in initiator genes were selected in temperature sensitive conditional mutants. These mutants were able to synthesize DNA at permissive temperature. But at nonpermissive temperature, such mutants could only finish the on-going replication forks and were unable to initiate new rounds of replication. The first of such mutants, CRT46, was isolated and characterized by Hirota et al (14). This locus was named dnaA, and was located at 83 minutes on the 100 minute genetic map of the chromosome. The mutation was named dnaA46. Soon after the discovery of dnaA46, other initiation mutants, with similar phenotypes but located at different positions on the chromosome, were isolated. These loci were named dnaB, dnaC, dnaI, and dnaP (15-18). Mutants of dnaB and dnaC were also found with a phenotype characteristic of genes involved in elongation rather than in initiation (15, 104, 169). Mutations in dnaI and dnaP were not extensively studied. Physiological studies indicated that protein and RNA synthesis are also required for initiation (19-22). The products of dnaA, dnaB, and dnaC will be referred to as dnaA, dnaB, and dnaC proteins, respectively.

dnaA protein functions in an early stage of initiation. The finding by Zyskind et al in 1977 that rifampin had a different effect on dnaA and dnaC mutants suggested an order of action between these two gene products (23). Mutants of dnaA or dnaC held at nonpermissive temperature (42°C) for 1.5 generation time can express the initiation potential accumulated at 42°C when shifted back to permissive temperature (30°C). Rifampin blocks RNA synthesis

by inhibiting RNA polymerase. Addition of rifampin 10 minutes before returning the culture back to 30°C completely abolished the potential to initiate DNA synthesis in a dnaA mutant. No effect was observed in a dnaC mutant. These results indicated that the interaction between dnaA protein and RNA polymerase occurs before the function of dnaC.

Kung and Glaser arrived at the same conclusion with a different approach (24). They constructed a double mutant carrying a heat-sensitive dnaA allele and a cold-sensitive dnaC mutation. DNA synthesis proceeds normally at 32°C but initiation is inhibited both at 20°C and 42°C. When a culture growing at 32°C was first shifted to 20°C for some time before shifting to 42°C, DNA synthesis stopped at 20°C slowly due to the defect in dnaC protein; it resumed at 42°C for one round of replication even in the presence of defective dnaA protein. This suggested that an initiation intermediate formed by dnaA protein at 20°C can be extended by dnaC protein at 42°C. When the culture was first shifted to 42°C and then to 20°C, DNA synthesis did not resume at 20°C. These experiments indicated that the functions of dnaA and dnaC protein are independent and that dnaA protein acts before dnaC protein.

Extragenic suppressor studies confirmed the involvement of dnaA protein in initiation of replication. According to the replicon model, an initiator protein is required for a specific replicator (replication origin). If dnaA protein is the initiator for oriC, substituting oriC with other replication origins should suppress the replication defect in dnaA temperature sensitive mutants. Soon after dnaA46 was isolated, Nishimura and Caro showed that integration of

the E. coli F factor into the chromosome suppressed the temperature sensitivity of a dnaA46 strain (25). Similar results were obtained by Lindahl et al. that a strain of dnaA46 under control of prophage P2 became temperature resistant (26). However, the integration sites of F factor or phage P2 were shown to be crucial (27).

Another suppressor of dnaA dependent initiation was found in rnh, the gene encoding RNaseH which degrades RNA in a DNA-RNA hybrid. Initiation of DNA replication from oriC dependent on the dnaA gene product requires protein synthesis (19-21). Mutant of rnh gene can sustain continued DNA replication in the absence of protein synthesis suggesting that an alternate pathway of replication was utilized (28, 29). In 1983, Kogoma and von Meyenburg showed that a rnh mutation suppressed both oriC deletion and dnaA mutations (30). Their results were confirmed by studies from other laboratories (31-34).

b. dnaA Protein and the Frequency of Initiation

Initiation potential accumulates in dnaA mutants shifted to nonpermissive temperature (42°C). In an experiment performed by Tippe-Schinder in 1978, a dnaA5 strain was grown at 30°C and shifted to 42°C for various times (35). Initiation potential was assayed by DNA synthesis at 30°C in the presence of chloramphenicol, which blocks synthesis of dnaA protein. DNA synthesis at 30°C was observed to be directly related to the time that the culture was held at 42°C. Further studies showed that this potential is dependent on protein synthesis at 42°C and can only be expressed in

reversible mutants (36). These results indicate that dnaA protein synthesized at 42°C, though not active at 42°C, can support initiation when returned to 30°C. Consistent with this conclusion is the observation that more than two round of initiation occurred in cells shifted to 30°C after being held at 42°C for 1.5 generations (37-40).

In contrast to heat sensitive mutants, a cold sensitive dnaA mutant overinitiates DNA synthesis at nonpermissive temperature (30°C) (41). This mutant grows normally at 42°C but cannot form colonies at 30°C. Measurements of DNA and protein synthesis indicated that mutant cells had a normal DNA content at 42°C. Upon shifting to 30°C, the ratio of DNA to protein increased up to 8 fold compared to the value at 42°C as a result of increased DNA synthesis. The ratio did not change significantly in a wild type control strain. Apparently, the temperature sensitivity of this mutant was due to overinitiation. Similar lethal effects were also observed in other strains that overinitiate DNA replication (42).

If dnaA protein controls the initiation frequency, overproduction of dnaA protein from a plasmid carrying the dnaA gene should result in increased initiation. Initial observations on the ratio of DNA to cell mass indicated that the ratio was not significantly influenced by overproduction of dnaA protein (43-45). This apparent dilemma was resolved when Atlung et al. observed that genetic markers near oriC were present at a higher frequency than the rest of the genome as determined by hybridization to different marker DNA probes (46). These results suggest that replication forks produced by overinitiation were halted soon after leaving oriC resulting in no substantial change in DNA

content. The presence of excessive replication forks at elevated levels of dnaA protein was confirmed by measurement of DNA accumulation in the presence of rifampin, which blocks initiation but allows existing replication forks to proceed. A two- to three-fold increase in DNA accumulation was observed in a dnaA protein overproducing strain (47). These results indicate that dnaA protein is an important factor in regulation of initiation of DNA replication from oriC.

c. Other Factors Involved in the Control of DNA Replication

Factors other than dnaA protein seem to be involved in the control of DNA replication. Multiple initiations occurred after a reversible heat sensitive dnaA mutant held at 42°C and returned to 30°C were separated by about 30 minutes (37-40). This separation time was independent of the mutations used and is likely determined by some other limiting factors. One such factor could be the state of methylation of the oriC sequence. The oriC region contains an extraordinarily high number of the sequence, GATC, which is recognized and methylated by deoxyadenosine methylase (48, 49). E. coli DNA is normally fully methylated. It is only hemimethylated for a short time following replication. The state of methylation is widely known to influence biological properties of DNA (50). Studies of the oriC sequence indicate that hemimethylated DNA cannot support initiation (51-53). Methylation of oriC takes about 10 minutes after initiation (54). During this period, the oriC region remains attached to cell membrane and is later released (54). Subsequent events might need to take place before another initiation can be

engaged in.

Another line of evidence indicating other factors are involved in the control of initiation is based on studies of dnaA mutants at intermediate temperatures (25°C-35°C) (55). Hansen et al. noticed that the replication activity of a dnaA46 strain was significantly reduced at temperatures above 35°C presumably due to inactivation of dnaA46 protein. At temperatures between 25°C and 35°C, where inactivation of dnaA46 was not apparent, the total initiation events per cell still decreased with increasing temperature. Simultaneously, an increasing initiation potential was accumulated linearly with temperature. This potential can be expressed at the same growth temperature in the absence of protein synthesis. These results suggest that an inhibitor of initiation, which is rapidly degraded in the absence of protein synthesis, is produced at increasing levels between 25°C and 35°C. Similar results were also observed by others (56).

Consistent with this finding is the following observation. In cultures of dnaA46 and dnaA5 grown at intermediate temperatures, addition of chloramphenicol increased the rate DNA of synthesis (57, 35, 39). The amount of increase was dependent on the growth temperature. In contrast to the mutant cells, no increase in the rate of DNA synthesis was observed in the wild type control indicating that the inhibitor was not normally present at a significant level. Since dnaA protein is temperature sensitive in these strains, and dnaA protein is currently known to regulate gene expression, it is highly possible that dnaA protein represses the expression of this inhibitor, which normally under tight control is overexpressed in dnaA mutants even at

intermediate temperatures. Unfortunately, this subject was not further studied.

B. Biochemical Studies on oriC Replication

Biochemical studies were made possible by two lines of research: the cloning of the oriC sequence; and the identification of replication proteins. Using plasmid DNA carrying the oriC sequence, in vitro oriC replication reactions were reconstituted with purified enzymes. The function of individual proteins was then studied in detail.

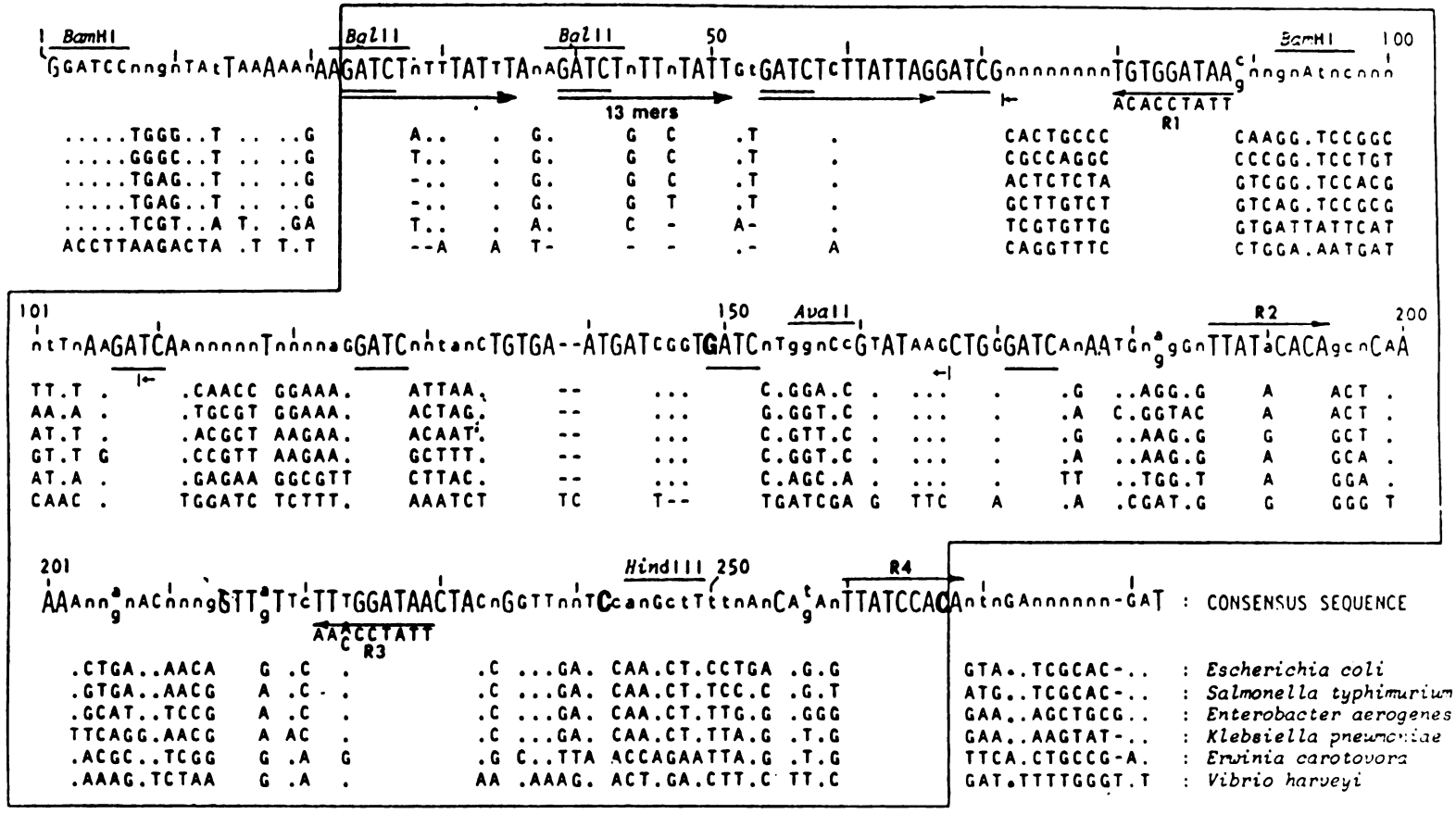
1. Cloning of the oriC Region

Genetic studies located oriC at about 84 minutes on the 100 minute E. coli chromosome between genetic markers asnA and bglB (58). These latter genes encode proteins involved in arginine synthesis and glucose metabolism, respectively (58). The origin region was first isolated in a 9 kb EcoRI fragment (59, 60). Subcloning of this fragment localized the origin to a 422 base pairs (bp) region and this region was sequenced (61, 62). By deletion analysis of the 422 bp region, the origin function was located within a minimum of 245 bp region (48).

Comparison of oriC sequences in six related bacteria reveals several significant features (Figure 2) (49). 1. The sequence GATC, the site for deoxyadenosine methylase, is highly concentrated in the oriC region. Only two sites are expected in the 245 bp minimum region if GATC is present at

Figure 2. Consensus sequence of the minimal origin of the bacterial chromosome (49).

The consensus sequence is derived from six bacterial origin sequence, those of E. coli, S. typhimurium, Enterobacter aerogenes, K. pneumoniae, Erwinia carotovora, and V. harveyi. The alignment of the six sequences is such that the least number of changes are introduced into the consensus sequence. In the consensus sequence, a large capital letter means that the same nucleotide is found in all six origins; a small capital letter means the nucleotide is present in five of the six sequences; a lowercase letter is used when that nucleotide is present in three or four of the six bacterial origins but only two different nucleotides are found at that site; and where three or four of the four possible nucleotides, or two different nucleotides plus a deletion, are found at a site, the letter n is used. Bold large capital letters locate at positions 149, 242, and 267, where single-base substitutions produce an oriC⁻ phenotype in E. coli. GATC sites are underlined in the consensus sequence and certain E. coli restriction sites are noted. The minimal origin of E. coli is enclosed within the box. The numbering of the nucleotide positions is that used for E. coli, and the upper left end is the 5' end. The four dnaA binding sequence are indicated as R1, R2, R3, and R4. The 13-mer repeats are also indicated on the upper left corner.


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

random. Eleven methylation sites are present in the E. coli origin and eight of them are conserved. The significance of these methylation sites may relate to its involvement in initiation of DNA replication as discussed above. 2. Four copies of a nine base pair sequence TTAT(A/C)CA(A/C)A are also conserved. This sequence is recognized and bound by dnaA protein. 3. The left side of the minimum sequence contains three 13 base pair repeats which are also highly conserved. The 13 base pair sequences are involved in early steps in the initiation process. This and the function of the dnaA protein binding sequence will be discussed later.

Initiation of oriC plasmids is under a control similar to that of initiation of the chromosome. The products of dnaA, dnaC, and other genes required for the chromosomal replication are also needed for the plasmid replication (63). However, the kinetics of oriC plasmid replication seems to be different from that of the chromosome. No coupling to initiation of chromosomal replication was observed (64). Furthermore, unidirectional replication of some oriC plasmids was observed, in contrast to the bidirectional replication of the chromosome (65-67). The difference between replication of the chromosome and oriC plasmids suggests that sequences outside the minimum region are required for accessory functions.

2. DNA Replication Proteins

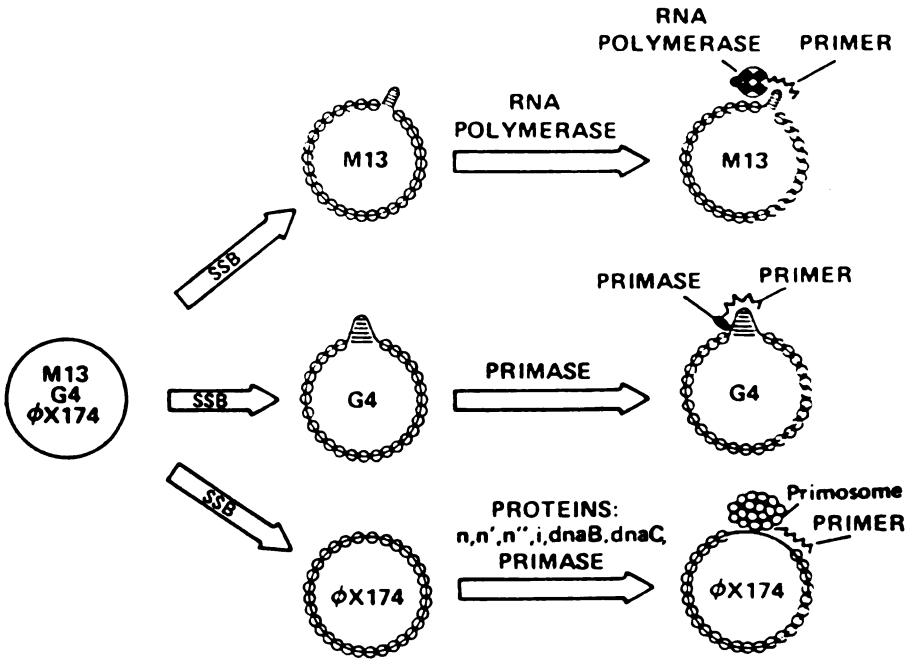
Three DNA polymerases, pol I, pol II, and pol III, exist in E. coli (68). The first effort in searching for enzymes involved in DNA replication resulted in the discovery of pol I in the late fifties (69). Because of the assay system

used in pol I purification, the activity of pol II and pol III was not detected. The fact that DNA replication in vivo was hardly affected by some mutations in polA, the gene coding for pol I, indicated that other DNA replication enzymes were present in E. coli. By altering assay conditions, DNA replication activity was found in polA mutant extracts. This replication activity was resolved into two distinct peaks in subsequent chromatography. The two peaks were designated pol II and pol III. Pol III has a very high processivity on primed single-stranded DNA template covered with single-stranded DNA binding protein (SSB). pol III is responsible for chromosomal replication. The function of pol I has been considered as gap filling because of its low processivity. Much less is known about pol II. This enzyme seems to be involved in DNA repair. All DNA polymerases require a primer complementary to the template to provide a 3'-OH terminus for subsequent elongation.

Other than DNA polymerases, many accessary proteins are involved in DNA replication, mostly in primer synthesis. The major body of knowledge about these proteins came from studies of replication of single-stranded DNA phages M13, G4, and ϕX174 (Figure 3) (68). Genomes of these phages are circular single-stranded DNAs of several kilo-bases (68). The priming and replication process were expected to be relatively simple, since no topological constrains are involved.

Replication of M13 is initiated by synthesis of a RNA primer by RNA polymerase at a specific hairpin structure on the circular DNA (70, 71). Polymerase III functions to extend the primer to full length. Pol I will remove

Figure 3. Specific priming systems for M13, G4, ϕX174 DNAs coated with SSB (68).


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

the RNA primer and fill the small gap left by pol III. The ends of the newly synthesized strand are then joint by DNA ligase. G4 replication uses a different enzyme, primase, for primer synthesis (72-74). Primase can use both ribonucleotides and deoxynucleotides as substrate (75-77). In a similar manner, primase also recognizes a specific hairpin structure. The DNA synthesis is the same as that of M13.

Replication of ϕX174 is much more complicated than that of M13 and G4. No single enzyme is able to prime DNA synthesis. A multi-protein complex, the primosome, containing at least seven proteins, is required for primer synthesis (78-80). The assembly of the primosome is initiated by binding of a sequence-specific DNA binding protein, n', to the assembly site (81, 82). Other primosomal proteins, n, n", i, dnaC, dnaB, and primase, are then successively assembled on n' (80). When priming is uncoupled from DNA synthesis, 5-10 primers are synthesized on the circle (83), yet the primosome is still tightly associated with the template (84), indicating the primosome has moved. Detailed studies revealed that the primosome moves in the direction opposite of primer synthesis (82). It was proposed that the primosome functions in primer formation on the lagging strand during replication of a double stranded DNA--the primosome moves along with the replication fork, while primer formation and DNA synthesis are in the opposite direction (82). The n' protein seems to provide energy for propelling the translocation of the primosome by hydrolysis of ATP (85). dnaB is proposed to induce a conformational change in the DNA so that it can be used as a template by primase (86). dnaC, in a complex with dnaB in the presence of ATP, is

pictured at the present time to deliver dnaB to the primosomal complex (87, 88). The functions of protein n", n, and i are still unknown.

3. dnaA Protein

Because no readily assayable functions are associated with dnaA protein, its purification was delayed until the successful establishment of the following two things: 1) cloning of the dnaA structural gene under control of an inducible promoter in a high copy number plasmid; 2) development of a crude enzyme system dependent on dnaA protein that can initiate DNA replication from the cloned oriC in an oriC plasmid.

The coding sequence of the dnaA gene was not revealed until 1982 (89-91). The DNA sequence indicated that dnaA encodes a 52.5 KDa protein. At about the same time, a crude enzyme system that replicates oriC plasmids was developed (92). Although lysates from wild type cells of E. coli were inactive in supporting replication from oriC, active protein fractions were obtained by fractionation of the crude lysate by selective ammonium sulphate precipitation. These enzyme fractions sustained bidirectional replication of oriC plasmids from the cloned oriC (93). In contrast to the protein fractions from wild type cells, those from two dnaA mutant strains were inactive. Addition of a crude lysate from a dnaA protein overproducing strain complemented the inactive fractions in oriC plasmid replication. These inactive protein fractions from dnaA mutants provided an assay for dnaA protein. This assay will be referred to as the complementation assay.

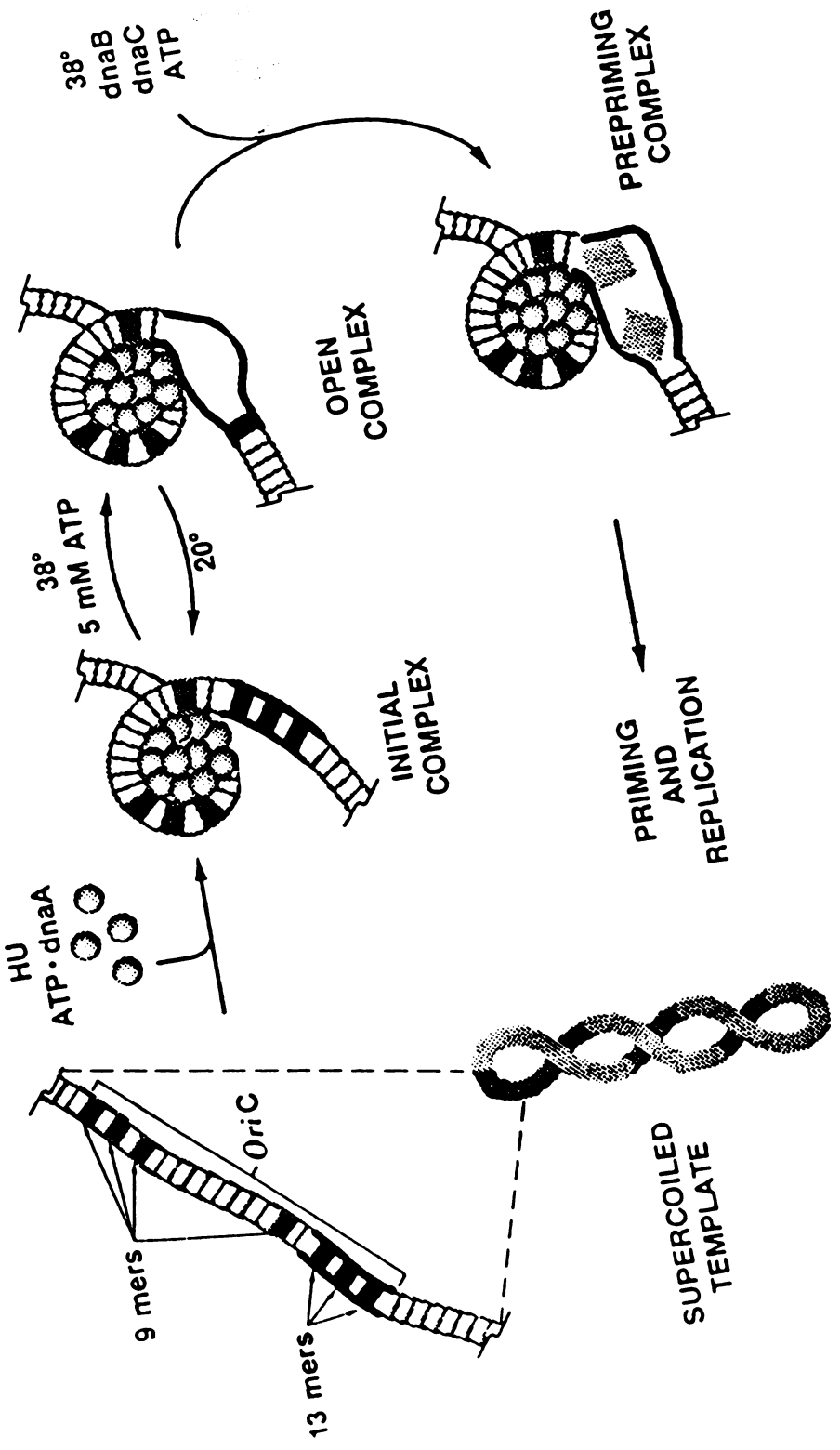
dnaA protein was then purified in large quantity from an overproducing strain (94). Two forms of dnaA protein were observed: an aggregated form eluted in the void volume of a gel permeation column, and a monomeric form. The reason for the presence of two forms is unknown. Studies to be discussed are with the monomeric form. dnaA protein was observed to bind to the oriC minimum sequence (95, 96). It also binds to several other sites which will be discussed later. The binding sites share a consensus sequence TTAT(A/C)CA(A/C)A, which is present four times in the oriC region (95, 96).

4. Mechanism of Initiation from oriC

Experiments to address the biochemical mechanism of oriC plasmid replication were made possible by development of a replication system dependent on purified enzymes (97). Twelve enzymes were included in the assay to fulfil the need for several functions in replication: RNA polymerase, dnaA, DNA gyrase for initiation; DNA polymerase III, primosomal proteins, and SSB for chain elongation; and topoisomerase I, RNaseH, and protein HU (a histone like protein) for specificity control. The assay sustained extensive DNA synthesis of oriC plasmids. Replication was dependent on dnaA, dnaB, dnaC protein, DNA gyrase, and oriC. The dependence on RNA polymerase was more pronounced than that on primase.

Upon varying the amount of topoisomerase I and protein HU, an assay system completely dependent on primase was established (98, 99). Unlike the RNA polymerase dependent assay, the specificity of oriC-dependent DNA synthesis does not rely on the presence of RNaseH, indicating that the

Figure 4. A model for initiation of replication from oriC (106).


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

priming was oriC specific. To study the initiation process, priming was uncoupled from DNA replication by omitting DNA polymerase III and dNTPs from the assay. The priming process which requires dnaA, dnaB, dnaC protein, DNA gyrase, and primase occurs at an optimum temperature of 36°C and cannot happen below 24°C. DNA synthesis, on the other hand, can occur even at 15°C to relatively the same extent as at 30°C. Further dissection of the priming process indicated that it is not priming itself, but a pre-priming step which is temperature dependent. The prepriming only requires dnaA, dnaB, dnaC and DNA gyrase. The presence of SSB also stimulates the process.

Detailed studies have examined the function of each individual proteins involved in the prepriming process (Figure 4). dnaA, a sequence-specific DNA binding protein, recognizes oriC and binds to it. Binding induces localized strand melting at the 13-mer repeat region on the left side of the minimum sequence (100). This localized unwinding is temperature dependent and requires the presence of ATP. The involvement of ATP in strand melting is rather complicated (100, 101). It seems to function as an allosteric factor as well as an energy source (100). dnaA protein binds ADP as well as ATP. The ADP·dnaA is inactive in strand melting and subsequent replication (100, 101). However, binding of dnaA protein to oriC is not affected by the presence or absence of either ATP or ADP (101).

Further opening of the oriC region requires the combined action of dnaB and DNA gyrase (102, 103). dnaB functions as a helicase to separate the double-stranded DNA by hydrolyzing ATP. DNA Gyrase releases the localized

positive supercoils created by dnaB, which also requires ATP as an energy source. SSB stabilizes the single-stranded region generated by dnaB protein and DNA gyrase.

The function of dnaC protein has been considered as delivering dnaB protein to the dnaA protein-oriC complex (87, 88). dnaC protein forms a very stable complex with dnaB in the presence of ATP. So far no specific functions of dnaC have been observed with the prepriming complex. Electron microscopic studies using gold labeled antibodies failed to locate dnaC protein in the prepriming complex, although both dnaA and dnaB have been localized with the same method (105).

Transcription by RNA polymerase seems to assist in strand separation rather than in primer formation for DNA replication (106). Promoters placed within 200 bp from the border of the oriC sequence but directing transcription away from the oriC also activate DNA replication. However, this does not exclude the possibility that RNA polymerase primes DNA synthesis, especially in the absence of primase.

In summary, dnaA protein is required for recognizing oriC and for the initial unwinding of duplex DNA. Other proteins function in successive steps of priming and replication (Figure 4).

C. Oth

1. Inv

T

or nea

The re

Replica

temper

once th

these p

phenot

indicat

deletio

role of

dnaA p

purifie

dnaC,

lagging

2. Repr

As

initiato

express

a const

C. Other Functions of dnaA Protein

1. Involvement in Replication of Plasmids pSC101, R1, R100, F, P1 and ColE1

The dnaA protein recognition sequence TTAT(A/C)CA(A/C)A is present at or near replication origins of plasmids pSC101, R1, R100, F, P1, and ColE1. The requirement for dnaA protein is absolute for replication of pSC101. Replication is completely blocked in a dnaA46 strain at nonpermissive temperature (107-109). By contrast, replication of R1, R100, F, and P1 were once thought not to require dnaA protein for replication since integration of these plasmids into the chromosome suppressed the temperature sensitive phenotype of a dnaA46 strain. Studies under more stringent conditions indicate that these plasmids cannot be maintained in dnaA insertion or deletion mutants where dnaA protein was completely absent (110-115). The role of dnaA protein in replication of these plasmids has not been resolved. dnaA protein is also involved in replication of ColE1 plasmid. Studies with purified enzymes indicate that dnaA protein directs the assembly of dnaB, dnaC, and primase, to the replication origin, which is presumably required for lagging strand synthesis (116, 117).

2. Repressor Function in Gene Expression

As early as 1973, Sompayrac and Maaloe proposed that expression of the initiator protein is controlled by a repressor which also represses the expression of its own gene (118). This simple feedback control system provides a constant concentration of the repressor and the initiator. The rate of

initiator accumulation should be proportional to the rate of increase in cell volume. Assuming initiation begins when a certain amount of initiator protein has accumulated, the frequency of initiation then could be coupled to the rate of growth.

dnaA protein has been shown to autoregulate its own expression transcriptionally (119, 120). Two transcriptional promoters separated by 82 bp were mapped in the dnaA regulatory region (90). One dnaA protein recognition sequence is present between the two promoters, 51 bp upstream from the mRNA start site of the proximal promoter, and 23 bp downstream from the distant promoter. Inactivation of dnaA46 at nonpermissive temperature resulted in elevated levels of β-galactosidase whose expression is under control of the two dnaA promoters. High levels of dnaA protein inhibited expression of β-galactosidase. Detailed examination indicated that the dnaA protein recognition sequence is required for the repression. Both promoters seem to be repressed by dnaA protein (121). Autoregulation of dnaA protein, the initiator, circumvented the need for an additional repressor in the Sompayrac and Maaloe model.

Other than regulating expression of its own gene, dnaA protein was observed to repress expression of a gene encoding a 16 KDa protein in vivo (122, 123). This gene is located to the right of oriC. The function of this protein is still unknown. One dnaA binding site was found about 40 bp upstream of the mRNA start site. Transcription from the 16 KDa promoter seems to be involved in the control of initiation (124-126).

II. The Heat-shock Response in E. coli

E. coli can maintain balanced growth over a temperature range from 10°C to 42°C, with optimum temperature at 37°C (127). Temperatures higher than 42°C result in restricted growth (128). When cultures are shifted to elevated temperatures, synthesis of more than seventeen proteins, named heat-shock proteins, is observed to increase transiently (128, 129). This phenomenon is termed the heat-shock response. Expression of heat-shock proteins is under control of a regulatory protein, the product of rpoH gene (130, 131). The rpoH gene encodes a sigma factor of 32 KDa which confers upon RNA polymerase the ability to initiate transcription from promoters of heat-shock genes (132, 133). Stress conditions other than heat, such as ethanol in the culture media, UV irradiation, and chemicals that block DNA synthesis or change DNA structure, are also known to trigger the heat-shock response (128).

A. Heat-shock Proteins

Eight heat-shock proteins have been identified since the discovery of the heat-shock response in E. coli in 1978 (129). These proteins are involved in diverse cellular processes from DNA replication, RNA synthesis, to protein processing.

Some heat-shock proteins are among the most abundant proteins of E. coli. The product of groEL gene is one of them, which constitutes about 1-2% of the total cellular protein at 37°C (128). The 65 KDa groEL protein has

been purified and it exists as homopolymeric particles (134, 135). It functionally interacts with another heat-shock protein, the 15 KDa groES protein (136, 137). The groE genes were first defined by mutations affecting the morphogenesis of several bacterial phages (137, 138). Recently, the groE proteins are shown to be associated with newly synthesized peptides and to assist in assembly of multimeric enzyme complexes (139-141). The groE proteins are required for growth at all temperatures (142).

dnaK protein is another abundant heat-shock protein (143). It possesses ATPase and phosphorylase activities (144). dnaK functions with dnaJ, another heat-shock protein in replication of lambda phage (145). Mutations in dnaK or dnaJ also inhibit cellular DNA replication and RNA synthesis (146, 147, 148).

grpE, also involved in lambda replication, is a member of heat-shock proteins (149). Like dnaK and dnaJ, mutations in groE also cause reduced synthesis of both RNA and DNA (149). Detailed functions of grpE protein are unknown.

Protease La, an ATP dependent protease encoded by lon, is the sixth member of the heat-shock protein family (150-151). It provides a major route by which E. coli degrades abnormal proteins (153, 154). The last two members of the heat-shock proteins known so far are the sigma 70 subunit of RNA polymerase and the lysU gene product, a minor form of lysyl-tRNA synthetase (128).

B. The Heat-shock Regulatory Protein, Sigma32

Expression of heat-shock proteins is controlled by the product of rpoH gene, a 32 KDa sigma factor of RNA polymerase (130-133). Sigma32 RNA polymerase recognizes specific patterns of -10 and -35 regions characteristic of heat-shock promoters (155).

The level of sigma32 increases transiently up to 15 fold after a temperature shift (156, 157). The transient accumulation of sigma32 is a result of both increased synthesis and increased stability. A temperature shift from 30° to 42°C has been shown to lengthen the half life of sigma32 from about 1 minute to 8 minutes within the first 4 minutes following the shift. Thereafter, the half life returns to about 1 minute, which causes a relative decrease in the level of sigma32 (157, 158). The amount of sigma32, as well as levels of the heat-shock proteins, then stabilizes at a level characteristic of the growth temperature.

The regulation of sigma32 has been examined at transcriptional level (159-161). Four promoters has been mapped in the regulatory region, designated rpoH1P, 2P, 3P, and 4P, with 4P being the most proximal. 2P is only observed with one specific strain; other promoters are observed both in vivo and in vitro. Transcripts from 1P are most abundant at all temperatures. Its level increases moderately (2 fold from 30°C to 42°C) with temperature. Transcripts of 3P and 4P are present at low levels at 30°C, and increase 5-15 fold transiently after heat shock. This increase correlates well with rate of synthesis of sigma32. The presence of high levels of 1P RNA even at 30°C where levels of sigma32 are extremely low suggests posttranscriptional

regulation on expression of the 1P transcript.

C. Regulation of the Heat-shock Response

Current evidence indicates that dnaK protein plays an important role in the control of the heat-shock response. Mutants of dnaK show increased levels of heat-shock proteins and prolonged synthesis of these proteins at high levels after heat shock (162, 163). These results indicate a negative regulatory function of dnaK protein.

The level of ppGpp, the messenger of stringent response when amino acid is limiting, has been observed to increase transiently after heat shock in a similar pattern as that of sigma32 and of heat-shock proteins (166, 167). High levels of ppGpp are associated with mutations in dnaK and dnaJ (148). dnaK mutants also fail to phosphorylate at least five proteins. Two of them are identified as glutaminyl-tRNA synthetase and threonyl-tRNA synthetase (164). dnaJ protein also participates in the phosphorylation of the latter two. The accumulation of ppGpp seems to be a result rather than a cause of the absence of phosphorylation of the tRNA synthetases in dnaK and dnaJ mutants (164). Related to these observations is the finding that stringent response induces expression of heat shock proteins (165). These results indicate a direct correlation among dnaK (dnaJ), the level of ppGpp, and synthesis of heat shock proteins.

A model is formulated from the above evidence. 1. A temperature shift might result in elevated levels of ppGpp through the following connection. Glutamate and threonine are precursors of many other amino acids (168). A

sudden increase in temperature might result in an increase in demand for these two amino acid in several biochemical pathways. This, in turn, may cause a temporary shortage of glutamate and threonine. High level of ppGpp could then produced as a result. 2. Synthesis of heat shock proteins could be stimulated by ppGpp (165). 3. Production of dnaK and dnaJ protein might modulate the response. High levels of dnaK and dnaJ protein, resulting from the elevated synthesis of heat shock proteins, might modulate the activities of glutaminyl-tRNA and threonyl-tRNA synthetase by phosphorylation, which presumably could result in decreased synthesis of ppGpp. The levels of ppGpp, sigma32, and the rate of synthesis of heat shock proteins will decrease until reaching a new balance.

III. Objectives of the Project

dnaA protein recognition sequences are present not only in oriC, the dnaA and 16 KDa promoter regions but also in promoter regions of several other genes, including rpoH, polA, uvrB, and nrd. This project was undertaken to determine if dnaA protein regulates expression of these and other genes. Further understanding of dnaA protein as a regulatory protein will help to correlate DNA replication with other cellular processes during cell growth.

REFERENCES

1. Avery, O. T., MacLeod, C. M., and McCarty, M. (1944) J. Exptl. Med. 79, 137.

2. Lewin, B. (1983) Gene. John Wiley & Sons

3. Meselson, M., and Stahl, F. W. (1958) Proc. Natl. Acad. Sci. USA 44, 671-682.

4. Jacob, F., and Wollman, E. L. (1961) Sexuality and the genetics of bacteria. Academic Press, New York.

5. Cairns, J. (1963) J. Mol. Biol. 6, 208-213.

6. Marsh, R. C., and Worcel, A. (1977) Proc. Natl. Acad. Sci. USA 74, 2720-2724.

7. Prescott, D. M., and Kuempel, P. L. (1972) Proc. Natl. Acad. Sci. USA 69, 2842-2845.

8. Louarn, J., and Funderburgh, M., and Bird, R. E. (1974) J. Bacteriol. 120. 1-5.

9. Jacob, F., Brenner, S., and Cuzin, F. (1963) Cold Spr. Harb. Symp. Quant. Biol. 28, 329-348.

10. Maaloe, O., and Kjeldgaard, N. O. (1966) Control of macromolecular synthesis. W. A. Benjamin, inc.

11. Helmstetter, C. E., and Cooper, S. (1968) J. Mol. Biol. 31, 507-518.

12. Cooper, S., and Helmstetter, C. E. (1968) J. Mol. Biol. 31, 519-540.

13. Donachie, W. D. (1968) Nature (London) 219, 1077-1079.

14. Hirota, Y., Mordoh, J. and Jacob, F. (1970) J. Mol. Biol. 53, 369-387.

15. Zyskind, J. W., and Smith, D. W. (1977) J. Bacteriol. 129, 1476-1486.

16. Wolf, B. (1972) Genetics 72, 569-593.

17. Beyersmann, D., Messer, W., and Schlicht, M. (1974) J. Bacteriol. 118, 783-789.

18. Wada, C., and Yura, T. (1974) Genetics 77, 199-220.

19. Hanna, M. H., and Carl, P. L. (1975) J. Bacteriol. 121, 219-226.

20. Helmstetter, C. E. (1974) J. Mol. Biol. 84, 1-19.

21. Lark, K. G. (1972) J. Mol. Biol. 64, 47-60.

22. Lark, K. G., Repko, T., and Hoffman, E. J. (1963) Biochim. Biophys. Acta 76, 9-24.

23. Zyskind, J. W., Deen, L. T., and Smith, D. W. (1977) J. Bacteriol. 129, 1466-1475.

24. Kung, F.-C., and Glaser, D. A. (1978) J. Bacteriol. 133, 755-762.

25. Nishimura, Y., Caro, L., Berg, C. M., and Hirota, Y. (1971) J. Mol. Biol. 55, 441-456.

26. Lindahl, G., Hirota,Y., and Jacob, F. (1971) Proc. Natl. Acad. Sci. USA 68, 2407-2411.

27. Tresguerres, E. F., Nandadasa, H. G., and Pritchard, R. H. (1975) J. Bacteriol. 121, 554-561.

28. Kogoma, T. (1978) J. Mol. Biol. 121, 55-69.

29. Kogoma, T. (1986) J. Bacteriol. 166, 361-363.

30. Kogoma, T., and von Meyenburg, K. (1983) EMBO J. 2, 463-468.

31. Torrey, T. A., Atlung, A., and Kogoma, T. (1984) Mol. Gen. Genet. 196, 350-355.

32. Lindahl, G., and Lindahl, T. (1984) Mol. Gen. Genet. 196, 283-289.

33. Ogawa, T., Pickett, G. G., Kogoma, T., and Kornberg, A. (1984) Proc. Natl. Acad. Sci. USA 81, 1040-1044.

34. Horiuchi, T., Maki, H., and Sekiguchi, M. (1984) Mol. Gen. Genet. 195, 17-22.

35. Tippe-Schindler, R., Zahn, G., and Messer, W. (1979) Mol. Gen. Genet. 168, 185-195.

36. Eberle, H., and Forrest, N. (1982) Mol. Gen. Genet. 186, 66-70.

37. Evens, I. M., and Eberle, H. (1975) J. Bacteriol. 121, 883-891.

38. Eberle, H., Forrest, N., Hrynyszyn, J., and Knapp, J. V. (1982) 186, 57-65.

39. LaDuca, R. J., and Helmstetter, C. E. (1983) J. Bacteriol. 154, 1371-1380.

40. Helmstetter, C. E., Krajewski, C. A., Leonard, A. C., and Weinberger, M. (1986) J. Bacteriol. 165, 631-637.

41. Kellenberger-Gujer, G., Podhajska, A. J., and Caro, L. (1978) Mol. Gen. Genet. 162, 9-16.

42. Bernander, R., Merryweather, A., Nordstrom, K. (1989) J. Bacteriol. 171, 674-683.

43. Churchward, G., Holmans, P., and Bremer, H. (1983) Mol. Gen. Genet. 192, 506-508.

44. Bremer, H., and Churchward, G. (1985) J. Bacteriol. 164, 922-924.

45. Xu, Y.-C., Bremer, H. (1988) Mol. Gen. Genet. 211, 138-142.

46. Atlung, T., Lobner-Olesen, A., and Hansen, F. G. (1987) Mol. Gen. Genet. 206, 51-59.

47. Pierucci, O., Helmstetter, C. E., Rickert, M., Weinberger, M., and Leonard, A. C. (1987) J. Bacteriol. 169, 1871-1877.

48. Oka, A., Sugimoto, K., Takanami, M., and Hirota, Y. (1980) Mol. Gen. Genet. 178, 9-20.

49. Zyskind, J. W., Cleary, J. M., Brusilow, W. S. A., Harding, N. E., and Smith, D. W. (1983) Proc. Natl. Acad. Sci. USA 80, 1164-1168.

50. Sternberg, N. (1985) J. Bacteriol. 164, 490-493.

51. Smith, D. W., Garland, A. M., Herman, G., Enns, R. E., Baker, T. A., and Zyskind, J. W. (1985) EMBO J. 4, 1319-1326.

52. Messer, W., Bellekes, U., and Lother, H. (1985) EMBO J. 4, 1327-1332.

53. Russell, D. W., and Zinder, N. D. (1987) Cell 50, 1071-1079.

54. Ogden, G. B., Pratt, M. J., and Schaecher, M. (1988) Cell 54, 127-135.

55. Hansen, F. G., and Rasmussen, K. V. (1977) Mol. Gen. Genet. 155, 219-225.

56. Fralick, J. A. (1978) J. Mol. Biol. 122, 217-286.

57. Zahn, G., and Messer, W. (1979) Mol. Gen. Genet. 168, 197-209.

58. Bachmann, B. J. (1983) Microbiology Reviews 47, 180-230.

59. Messer, W., Bergmans, H. E. N., Meijer, M., and Womack, J. E. (1978) Mol. Gen. Genet. 162, 269-275.

60. Yasuda, S., and Hirota, Y. (1977) Proc. Natl. Acad. Sci. USA 74, 5458-5462.

61. Meijer, M., Beck, E., Hansen, F. G., Bergmans, H. E. N., Messer, W., von Meyenburg, K., and Schaller, H. (1979) Proc. Natl. Acad. Sci. USA 76, 580-584.

62. Sugimoto, K., Oka, A., Sugisaki, H., Takanami, M., Nishimura, A., Yasuda, Y., and Hirota, Y. (1979) Proc. Natl. Acad. Sci. USA 76, 575-579.

63. von Meyenburg, K., Hansen, F. G., Riise, E., Bergmans, H. E. N., Meijer, M., and Messer, W. (1979) Cold Spring Harbor Spmp. Quant. Biol. 43, 121-128.

64. Leonard, A., Hucul, J. A., and Helmstetter, C. E. (1982) 149, 499-507.

65. Meijer, M., and Messer, W. (1980) J. Bacteriol. 143, 1049-1053.

66. Yoshimoto, M., Nagai, K., and Tamura, G. (1986) Mol. Gen. Genet. 204, 214-220.

67. Tabata, S., Oka, A., Sugimoto, K., Takanami, M., Yasuda, S., and Hirota, Y. (1983) Nucleic Acid Res. 11, 2617-2626.

68. Kornberg, A. (1980 and 1982) DNA replication, and Supplement to DNA replication. W. H. Freeman and Company, San Francisco.

69. Lehman, R. I., Bessman, M. J., Simms, E. S., and Kornberg, A. (1958) J. Biol. Chem. 233, 163.

70. Gieder, K., and Kornberg, A. (1974) J. Biol. Chem. 249, 3999-4005.

71. Kaguni, J. M., and Kornberg, A. (1982) J. Biol. Chem. 257, 5437-5443.

72. Zechel, K., Bouche, J.-P., and Kornberg, A. (1975) J. Biol. Chem. 250, 4684-4689.

73. Bouche, J.-P., Zechel, K., and Kornberg, A. (1975) J. Biol. Chem. 250, 5995-6001.

74. Stayton, M. M., and Kornberg, A. (1983) 258, 13205-13212.

75. Rowen, L., and Kornberg, A. (1978) J. Biol. Chem. 253, 758-764.

76. Rowen, L., and Kornberg, A. (1978) J. Biol. Chem. 253, 770-774.

77. Bouche, J.-P., Rowen, L., and Kornberg, A. (1978) J. Biol. Chem. 253, 765-769.

78. McMacken, R., and Kornberg, A. (1978) J. Biol. Chem. 253, 3313-3319.

79. Weiner, J. H., McMacken, R., and Kornberg, A. (1976) Proc. Natl. Acad. Sci. USA 73, 752-756.

80. Arai, K., Low, R., Kobori, J., Shlomai, J., and Kornberg, A. (1981) J. Biol. Chem. 256, 5273-5280.

81. Shlomai, J., and Kornberg, A. (1980) Proc. Natl. Acad. Sci. USA 77, 799-803.

82. Arai, K., and Kornberg, A. (1981) Proc. Natl. Acad. Sci. USA 78, 69-73.

83. McMacken, R., Ueda, K., and Kornberg, A. (1977) Proc. Natl. Acad. Sci. USA 74, 4190-4194.

84. Low, R. L., Arai, K., and Kornberg, A. (1981) Proc. Natl. Acad. Sci. USA 78, 1436-1440.

85. Arai, K., Low, R. L., and Kornberg, A. (1981) Proc. Natl. Acad. Sci. USA 78, 707-711.

86. Arai, K., and Kornberg, A. (1979) Proc. Natl. Acad. Sci. USA 76, 4308-4312.

87. Wahle, E., Lasken, R. S., and Kornberg, A. (1989) J. Biol. Chem. 264, 2463-2468.

88. Wahle, E., Lasken, R. S., and Kornberg, A. (1989) J. Biol. Chem. 264, 2469-2475.

89. Hansen, E. B., Hansen, F. G., and von Meyenburg, K. (1982) Nucleic Acid Res. 10, 7373-7385.

90. Hansen, F. G., Hansen, E. B., and Atlung, A. (1982) EMBO J. 1, 1043-1048.

91. Ohmori, H., Kimura, M., Nagata, T., and Sakakibara, Y. (1984) Gene 28, 159-170.

92. Fuller, R. S., Kaguni, J. M., and Kornberg, A. (1981) Proc. Natl. Acad. Sci. USA 78, 7370-7374.

93. Kaguni, J. M., Fuller, R. S., and Kornberg, A. (1982) Nature (London) 296, 623-626.

94. Fuller, R. S., and Kornberg, A. (1983) Proc. Natl. Acad. Sci. USA 80, 5817-5821.

95. Fuller, R. S., Funnell, B. E., and Kornberg, A. (1984) Cell 38, 899-900.

96. Matsui, M., Oka, A., and Takanami, M. (1985) J. Mol. Biol. 184, 529-533.

97. Kaguni, J. M., and Kornberg, A. (1984) Cell 38, 183-190.

98. Ogawa, T., Baker, T. A., van der Ende, A., and Kornberg, A. (1985) Proc. Natl. Acad. Sci. USA 82, 3562-3566.

99. van der Ende, A., Baker, T. A., Ogawa, T., and Kornberg, A. (1985) Proc. Natl. Acad. Sci. USA 82, 3954-3958.

100. Bramhill, D., and Kornberg, A. (1988) Cell 52, 743-755.

101. Sekimizu, K., Bramhill, D., and Kornberg, A. (1987) Cell, 50, 259-265.

102. Baker, T. A., Sekimizu, K., Funnell, B. E., and Kornberg, A. (1986) Cell, 45, 53-64.

103. Baker, T. A., Funnell, B. E., and Kornberg, A. (1987) J. Biol. Chem. 262, 6877-6885.

104. Wechsler, J. S. (1975) J. Bacteriol. 121, 594-599.

105. Funnell, B. E., Baker, T. A., and Kornberg, A. (1987) J. Biol. Chem. 262, 10327-10334.

106. Baker, T. A., and Kornberg, A. (1988) Cell 55, 113-123.

107. Hasunuma, K., and Sekiguchi, M. (1977) Mol. Gen. Genet. 154, 225-230.

108. Felton, J., and Wright, A. (1979) Mol. Gen. Genet. 175, 231-233.

109. Frey, J., Chandler, M., and Caro, L. (1979) Mol. Gen. Genet. 174, 117-126.

110. Nagata, T., Murakami, Y., and Imai, M. (1988) Mol. Gen. Genet. 213, 163-165.

111. Hansen, E. B., and Yarmolinsky, M. B. (1986) Proc. Natl. Acad. Sci. USA 83, 4423-4427.

112. Murakami, Y., Ohmori, H., Yura, T., and Nagata, T. (1987) J. Bacteriol. 169, 1724-1730.

113. Tresguerres, E. F., Nieto, C., Casquero, I., and Canovas, J. L. (1986) J. Bacteriol. 165, 424-427.

114. Kline, B. C., Kogoma, T., Tam, J. E., and Shields, M. (1986) J. Bacteriol. 168, 440-443.

115. Kogoma, T., and Kline, B. C. (1987) Mol. Gen. Genet. 210, 262-269.

116. Seufert, W., and Messer, W. (1987) Cell 48, 73-78.

117. Seufert, W., Dobrinski, B., Lurz, R., and Messer, W. (1988) J. Biol. Chem. 263, 2719-2723.

118. Sompayrac, L., and Maaloe, O. (1973) Nature (London) New Biology 241, 133- 135.

119. Braun, R. E., O'Day, K., and Wright, A. (1985) Cell 40, 159-169.

120. Atlung, T., Clausen, E. S., and Hansen, F. G. (1985) Mol. Gen. Genet. 200, 442-450.

121. Kucherer, C., Lother, H., Kolling, R., Schauzu, M-A., and Messer, W. (1986) Mol. Gen. Genet. 205, 115-121.

122. Lother, H., Kolling, R., Kucherer, C., and Schauzu, M. (1985) EMBO J. 4, 555-560.

123. Schauzu, M., Kucherer, C., Kolling, R., Messer, W., and Lother H. (1987) Nucleic Acid Res. 15, 2479-2497.

124. de Wind, N., Parren, P., Stuitje, A. R., and Meijer, M. (1987) Nucleic Acid Res. 15, 4901-4915.

125. Stuitje, A. R., and Meijer, M. (1983) Nucleic Acid Res. 11, 5775- 5791.

126. Stuitje, A. R., de Wind, N., van der Spek, J. C., Pors, T. H., and Meijer, M. (1986) Nucleic Acid Res. 14, 2333-2345.

127. Jones P. G., VanBogelen, R. A., and Neidhardt, F. C. (1987) J. Bacteriol. 169, 2091-2095.

128. Neidhardt, F. C., VanBogelen, R. A., and Vaughn, V. (1984) Ann. Rew. Genet. 18, 295-239.

129. Lemaux, P. G., Herendeen, S. L., Bloch, P. L., and Neidhardt, F. C. (1978) Cell 13, 427-434.

130. Yamamori, T., and Yura, T. (1982) Proc. Natl. Acad. Sci. USA 79, 860-864.

131. Yura, T., Tobe, T., Ito, K., and Osawa, T. (1984) Proc. Natl. Acad. Sci. USA 81, 6803-6807.

132. Landick, R., Vaughn, V., Lau, E. T., VanBogelen, R. A., Erickson, J. W., and Neidhardt, F. C. (1984) Cell 38, 175-182.

133. Grossman, A. D., Erickson, J. W., and Cross, C. A. (1984) Cell 38, 383-390.

134. Hendrix, R. W. (1979) J. Mol. Biol. 129, 375-392.

135. Hohn, T., Hohn, B., Engel, A., Wurtz, M., and Smith, P. R. (1979) J. Mol. Biol. 129, 359-373.

136. Chandrasekhar, G. N., Tilly, K., Woolford, C., Hendrix, R., Georgopoulos, C. (1986) J. Biol. Chem. 261, 12414-12419.

137. Tilly, K., and Georgopoulos, C. (1982) J. Bacteriol. 149, 1082- 1088.

138. Tilly, K., Murialdo, H., and Georgopoulos, C. (1981) Proc. Natl. Acad. Sci. USA 78, 1629-1633.

139. Bochkareva, E. S., Lissin, N. M., and Girshovich, A. S. (1988) Nature (London) 336, 254-257.

140. Goloubinoff, P., Gatenby, A. A., and Lorimer, G. H. (1989) Nature (London) 337, 44-47.

141. Hemmingsen, S. M., and Woodford, C., van der Vies, S. M., Tilly, K., Dennis, T. D., Georgopoulos, C. P., Hendrix, R. W., and Ellis, R. J. (1988) Nature (London) 333, 330-334.

142. Fayet, O., Ziegelhoffer, T., and Georgopoulos, C. (1989) J. Bacteriol. 171, 1379-1385.

143. Zylicz, M., and Georgopoulos, C. (1984) J. Biol. Chem. 259, 8820-

8825.

144. Zylicz, M., LeBowitz, J. H., McMacken, R., and Georgopoulos, C. (1983) Proc. Natl. Acad. Sci. USA 80, 6431-6435.

145. Liberek, K., Georgopoulos, C., and Zylicz, M. (1988) Proc. Natl. Acad. Sci. USA 85, 6632-6636.

146. Itikawa, H., and Ryu, J.-I. (1979) J. Bacteriol. 138, 339-344.

147. Wada, M., Kadokami, Y., and Itikawa, H. (1982) Jpn. J. Genet. 57, 407-413.

148. Nanaka, M., Wada, M., and Itikawa, H. (1985) Jpn. J. Gent. 60, 27-38.

149. Ang, D., Chandrasekhar, G. N., Zylicz, M. and Georgopoulos, C. (1986) J. Bacteriol. 167, 25-29.

150. Chung, C. H., and Goldberg, A. L. (1981) Proc. Natl. Acad. Sci. USA 78, 4931-4935.

151. Charette, M. F., Henderson, G. W., and Markovitz, A. (1981) Proc. Natl. Acad. Sci. USA 78, 4728-4732.

152. Phillips, T. A., VanBogelen, R. A., and Neidhardt, F. C. (1984) J. Bacteriol. 159, 283-287.

153. Goff, S. A., and Goldberg, A. L. (1985) Cell 41, 587-595.

154. Waxman, L., and Goldberg, A. L. (1986) Science 232, 500-503.

155. Cowing, D. W., Bardwell, J. C. A., Craig, E. A., Woolford, C., Hendrix, R. W., and Cross, C. A. (1985) Proc. Natl. Acad. Sci. USA 82, 2679-2683.

156. Lesley, S. A., Thompson, N. E., and Burgess, R. R. (1987) J. Biol. Chem. 262, 5404-5407.

157. Straus, D. B., Walter, W. A., and Cross, C. A. (1987) Nature (London) 329, 348-351.

158. Tilly, K., Spence, J., and Georgopoulos, C. (1989) J. Bacteriol. 171, 1585-1589.

159. Erickson, J. W., Vaughn, V., Walter, W. A., Neidhardt, F. C., and Cross, C. A. (1987) Genes & Develop. 1, 419-432.

160. Tobe, T., Kusukawa, N., and Yura, T. (1987) J. Bacteriol. 169, 4128-

4134.

161. Fujita, N., and Ishihama, A. (1987) Mol. Gen. Genet. 210, 10-15.

162. Tilly, K., McKittrick, N., Zylicz, M., and Georgopoulos, C. (1983) Cell 34, 641-646.

163. Paek, K-H., and Walker, G. C. (1987) J. Bacteriol. 169, 283-290.

164. Wada, M., Sekine, K., and Itikawa, H. (1986) J. Bacteriol. 168, 213-220.

165. Grossman, A. D., Taylor, W. E., Burton, Z. F., Burgess, R. R., and Gross, C. A. (1985) J. Mol. Biol. 186, 357-365.

166. Gallant, J., Palmer, L., and Pao, C. C. (1977) Cell 11, 181-185.

167. Chaloner-Larsson, G., and Yamazaki, H. (1977) Biochem. Biophys. Res. Comm. 77, 503-508.

168. Bender, D. A. (1975) Amino acid metabolism. John Wiley & Son.

169. Kogoma, T. (1976) J. Mol. Biol. 103, 191-197.

170. von Meyenburg, K., and Hansen, F. G. (1987) in Escherichia coli and Salmonella typhimurium, cellular and molecular boilogy. Vol. 2, eds. Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., Umbarger, H. E., editor in chief, Neidhardt, F. C. American Society of Microbiology, Washington, D.C.

Chapter II

TRANSCRIPTIONAL REPRESSION OF THE dnaA GENE OF Escherichia coli BY dnaA PROTEIN

ABSTRACT

The promoter regions of the dnaA gene and of a gene which encodes a 16KDa protein contain sites which are recognized and bound by dnaA protein. Using assays of run-off transcription of restriction fragments, purified dnaA protein specifically repressed transcription from both dnaA promoters and from the promoter for the 16 KDa gene to almost undetectable levels. This repressive effect is observed at levels of dnaA protein required for specific binding of dnaA protein to restriction fragments containing the promoters for these genes. These results indicate that transcription of these genes is regulated by binding of dnaA protein to the promoter regions of these genes.

INTRODUCTION

The dnaA gene of E. coli is an essential gene involved in the initiation of DNA replication from the chromosomal origin, oriC. Genetic, physiological, and biochemical experiments indicate that the dnaA product acts at an early stage in initiation of DNA replication (21, 41, 28, 15). Its mechanism of action in initiation of replication remains to be completely clarified. Various results have indicated that this initiator protein may play a central role in the regulation of chromosomal replication. Although temperature sensitive mutants of dnaA fail to initiate new rounds of DNA synthesis when shifted to the nonpermissive temperature, some mutants of dnaA accumulate excess initiation capacity at the nonpermissive temperature (18, 11, 39, 29). When these strains are returned to the permissive temperature, new initiations occur in the absence of protein synthesis. The capacity to initiate DNA replication under such conditions has led to the suggestion that the dnaA product regulates the initiation of DNA replication and also represses its own expression (18, 26, 12). Evidence supporting this is that overproduction of dnaA protein, when under control of the lambdaP_L or lacUV5 promoters, resulted in an increased frequency of initiation of DNA replication (2, 6).

The dnaA gene is the first gene in an operon also containing the dnaN gene which encodes the β subunit of DNA polymerase III holoenzyme (34, 8). The dnaA gene has been cloned and its DNA sequence determined (19, 33, 17, 32). S1 nuclease mapping experiments of transcripts produced in vivo indicate

that two promoters, dnaA1P and dnaA2P located approximately 230 bp and 150 bp upstream from the translational start site, are responsible for transcription of the dnaA gene (20, 4).

Using assays of in vitro replication of plasmids containing the chromosomal origin of E. coli in conjunction with overproduction of the dnaA gene product, highly purified preparations of dnaA protein have been obtained (14). Characterization of such preparations has indicated that dnaA protein is a sequence-specific DNA binding protein (10, 13). DNA fragments bound by dnaA protein include those containing the oriC sequence, the promoter region of a gene adjacent to oriC which encodes a 16 KDa protein, and the dnaA promoter region. From comparative sequence analysis and DNase I footprinting experiments, it is postulated that dnaA protein recognizes a specific 9 base pair sequence in binding to these DNA fragments (13).

Recent studies have examined the in vivo expression of the dnaA gene by using transcriptional and translational gene fusions of the dnaA promoter region to the structural region of the lacZ gene (4, 2) or by S1 nuclease mapping (27). Over-production of the dnaA gene product led to decreased expression from the dnaA promoters. In contrast, introduction of the dnaA46 allele resulted in elevated expression from the dnaA promoters at the nonpermissive temperature. In the former studies, deletion of one of the two dnaA promoters (dnaA1P) and the dnaA protein recognition sequence resulted in the loss of this autoregulatory effect. These results suggest that dnaA protein autoregulates its own expression by recognizing and binding to the dnaA promoter region. The inability of the dnaA46 gene product to repress

expression of β-galactosidase activity when under control of the dnaA regulatory region is presumably due to its inability to bind to this region.

Transcriptional repression of a gene adjacent to oriC by dnaA protein has also been observed by use of gene fusions of the 16 KDa promoter to the galK gene (30, 38). Expression of galactokinase in vivo or in coupled transcription/translation assays was dependent on the promoter region of the 16 KDa gene.

A biochemical understanding of the binding of dnaA protein to the chromosomal origin, to its promoter region, and to promoter regions of other genes in relation to its effects on transcription may contribute to the understanding of the regulation of initiation. The results presented in this report indicate that purified dnaA protein functions to specifically repress transcription of the dnaA gene and of the gene encoding a 16 KDa protein. This inhibition is observed at levels which correspond to those required for binding of dnaA protein to DNA fragments containing the promoter region of these genes.

MATERIALS AND METHODS

Plasmids and DNAs

The plasmid pdnaA/dnaN is a recombinant of pMOB45 which contains the dnaA and dnaN genes within the inserted 1.0 and 3.3 kilobase pair EcoR1 fragments (8). Plasmid pBF1509 contains the E. coli chromosomal DNA fragment of pBF1209 (13) inserted into the vector pAD329 (23). This DNA fragment was modified near the ClaI site by attachment of a BamH1 linker. These plasmids, M13oriC26 RF (25), and pBR322 were purified from cleared lysates by centrifugation in ethidium bromide-CsCl gradients. The 203 bp restriction fragment containing the lacUV5 promoter was a gift of D. Lorimer of this department.

Restriction enzyme digests for filter binding assays and for purification of restriction fragments were performed according to the manufacturers' recommendations. Restriction fragments for run-off transcription assays were fractionated on polyacrylamide gels, visualised by ethidium bromide staining, purified by electroelution with an ISCO Model 1750 electrophoretic concentrator, and ethanol precipitated. DNA concentrations were determined spectrophotometrically, or by comparison of the purified DNA fragments to different known amounts of electrophoretically separated restriction enzyme digests visualized by ethidium bromide staining.

Enzymes

Restriction enzymes EcoRI, BamHI, and TaqI were purchased from New England Biolabs, HaeIII and HinfI were from IBI, and HpaII was from Boehringer/Mannheim. DNA polymerase I (large fragment) was from New England Biolabs. RNA polymerase holoenzyme was purified from W3110 as described except that Biogel A5m (Biorad) chromatography was replaced by chromatography on a TSK 3000SW (Altex) high performance gel permeation column (9, 16). dnaA protein was purified as described (24). Protein concentrations were determined by the dye-binding method with bovine serum albumin as a standard (3).

DNA binding

Fragment retention assays were performed using Millipore HAWP nitrocellulose filters (13). Filters, boiled (5 min), and stored in H_2O at 4°C, were equilibrated in binding buffer containing 40 mM HEPES-KOH pH 7.6, 5 mM magnesium acetate, 2 mM dithiothreitol, and 50 mM KCl prior to use. Binding assays (25 µl) were performed in the above buffer containing 0.025 pmol of purified restriction fragment digested with the indicated restriction enzyme and end-labeled with DNA polymerase I (large fragment) (40). Various amounts of dnaA protein were added in 1 µl of dnaA buffer containing 25 mM HEPES-KOH pH 7.6, 15% glycerol, 0.1 mM EDTA, 2 mM dithiothreitol, and 0.1 M $(NH_4)_2SO_4$. Reactions, incubated for 10 min at 30°C, were immediately filtered under gentle suction and the filters were washed with 250 µl of binding buffer. DNA retained on the filters was eluted in buffer

containing 25 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.5 M sodium acetate and 0.5% sodium dodecyl sulfate at 65°C for 20 min. DNA eluted from the filters and in the flow through were concentrated by ethanol precipitation with 10 µg of E. coli tRNA (Sigma) as carrier, dissolved in buffer containing 5.0% glycerol, 0.1% bromophenol blue, 10 mM Tris-HCl pH 8, and 1 mM EDTA, and electrophoresed in 1.5% agarose gels in 80 mM Tris-borate pH 8.3, and 1 mM EDTA. After electrophoresis, the gels were stained with ethidium bromide, photographed, dried onto Whatman DE81 paper and autoradiographed at -70°C with Kodak XAR-5 film and Cronex Quanta III intensifying screens.

Transcription assays

Run-off transcription assays (10 µl) contained 0.025 pmol of the indicated restriction fragment, 0.65 pmol of RNA polymerase, and varying amounts of dnaA protein in 1 µl of dnaA buffer in 40 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 M NaCl, 1 mM dithiothreitol, 10% glycerol, 0.1 mg/ml bovine serum albumin, 300 µM each of ATP, GTP, and CTP, and 20 µM [α^{32}p] UTP (5). Incubations of 10 min. at 37°C were terminated by addition of an equal volume of stop buffer (0.1% SDS, 20 mM EDTA, 0.3 M sodium acetate, and 100 µg/ml E. coli tRNA). Reaction products were ethanol precipitated, resuspended in 20 µl of 95% formamide, and 40 mM Tris-HCl pH 7.8, incubated at 100°C for 2 min., chilled on ice, and electrophoresed in 5 or 8% polyacrylamide gels in 7 M urea, 90 mM Tris-borate pH 8.3, and 1 mM EDTA. pBR322 DNA digested with EcoRI and HpaII and end-labeled

with DNA polymerase I (large fragment) was used as a molecular weight marker. After electrophoresis, gels were soaked for 10 min. in H_2O before drying and autoradiography as described above. Autoradiograms were quantitated by densitometry with a Hoefer gel scanner interfaced with an IBM personal computer.

Radioactive labelling of DNA

Restriction fragments (1-5 µg) for DNA-binding experiments were end-labeled with 2 µCi of [α^{32}P] dCTP (New England Nuclear, 800 Ci/mmol) and one unit of of DNA polymerase I (large fragment) in 10 µl of 10 mM Tris-HCl pH 7.3, 10 mM $MgCl_2$, and 50 mM NaCl (HpaII digested DNA) or 100 mM NaCl (TaqI digested DNA) for 20 min at room temperature. The end-labeled restriction fragments (0.5-1 x 10^5 cpm/µg of DNA by acid insoluble radioactivity) were used directly in filter binding experiments.

RESULTS

dnaA protein specifically inhibits transcription from the dnaA promoters

In fragment retention assays and DNase I protection experiments, purified dnaA protein has been shown to bind in a cooperative fashion to a region of 100 bp containing the dnaA promoters and to the promoter region for a gene encoding a 16 KDa protein (Figures 1,2) (13). Levels of dnaA protein sufficient for specific binding and for protection correspond to a ratio of approximately 50 monomers of this 52 KDa protein per bound DNA fragment.

An EcoRI restriction fragment of 945 bp was isolated from a recombinant of pMOB45 (8) which contains the promoters required for divergent transcription of the rpmH and dnaA genes (20, 32, 4). Addition of RNA polymerase to run-off transcription assays containing this restriction fragment as template resulted in the synthesis of several products (Figure 3a). Two transcripts of approximately 500 and 380 nucleotides were observed which correspond in size to those products expected by transcription from rpmH1P and rpmH2P (505 and 378 nucleotides, respectively) (20). Two other transcripts of approximately 300 and 215 nucleotides were observed which are consistent in length with transcription from dnaA1P and dnaA2P (20, 4). A transcript of 130 nucleotides was also observed which may result from transcriptional termination from rpmH1P (20). The largest product is most likely due to end to end transcription of the restriction fragment since its size

Figure 1. Map of the dnaA and rpmH promoter regions and coding regions contained in the 945 bp EcoRI fragment (20).

Restriction sites: E, EcoRI; Hf, HinfI; Hp, HpaII; H, HaeIII; C, ClaI. The approximate positions of the promoters for the dnaA gene, dnaA1P and dnaA2P (⊂--->), and for the rpmH gene, rpmH1P and rpmH2P (<---⊃) are indicated. The direction of transcription is indicated by the arrows. The stippled box indicates the position of the dnaA protein recognition sequence; cross hatched boxes indicates open reading frames for known gene products. The dashed box indicates an open reading frame for a putative protein. Restriction fragments used in run-off transcription assays are indicated.

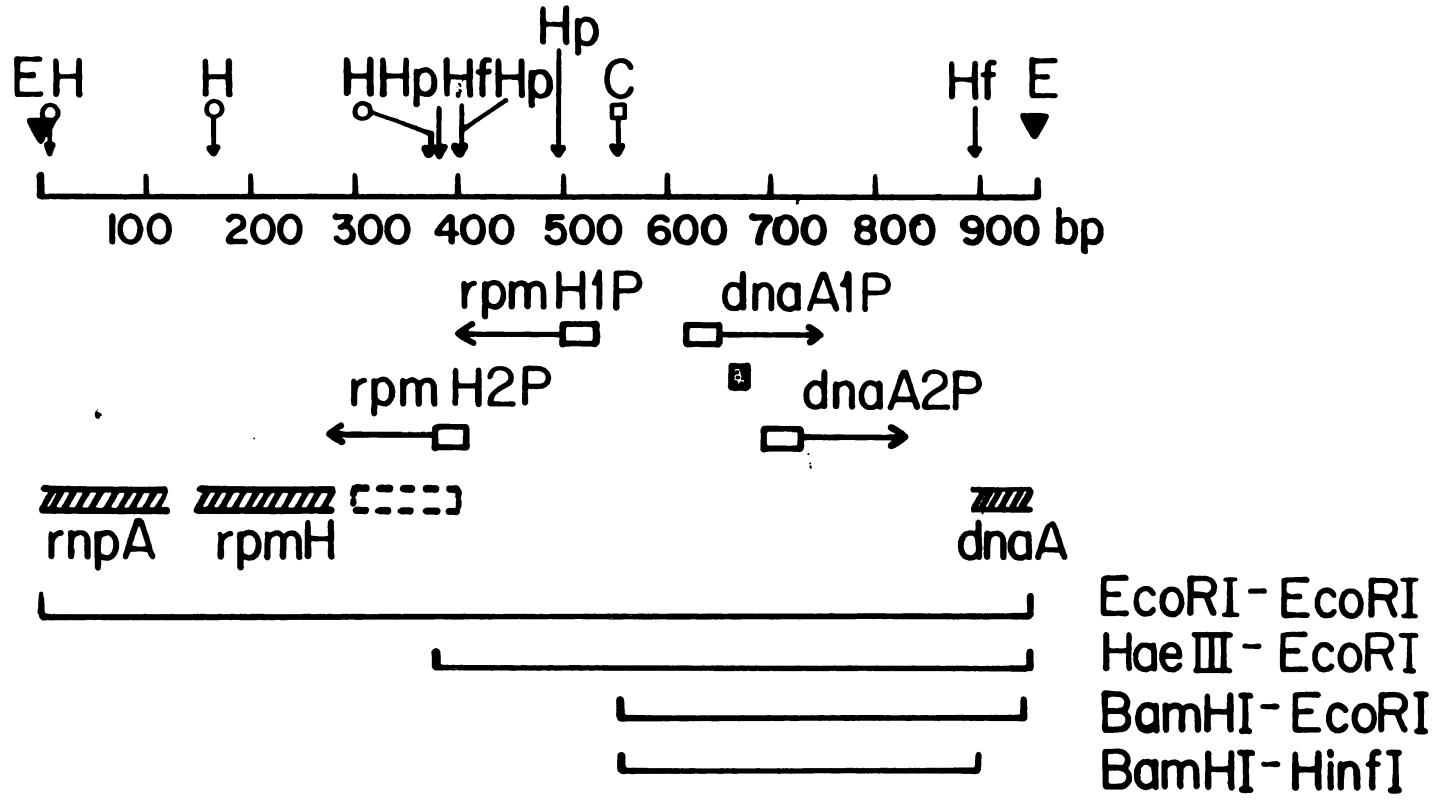

Hp
EH
H
HHpHfHp
C
Hf
E
100 200 300 400 500 600 700 800 900 bp
rpmH1P
dnaA1P
rpmH2P
dnaA2P
rnpA
rpmH
dnaA
EcoRI-EcoRI
HaeIII-EcoRI
BamHI-EcoRI
BamHI-HinfI

Figure 2. Map of the gene encoding the 16 KDa protein and adjacent asnC gene (22, 42).

Restriction sites: T, TaqI; Hp, HpaII. The approximate positions of the promoters for the gene encoding the 16 KDa protein (<---), and for the adjacent asnC gene (<---), and the direction of transcription are indicated. The restriction fragment used in run-off transcription assays is also indicated. Other designations are as in Figure 1.

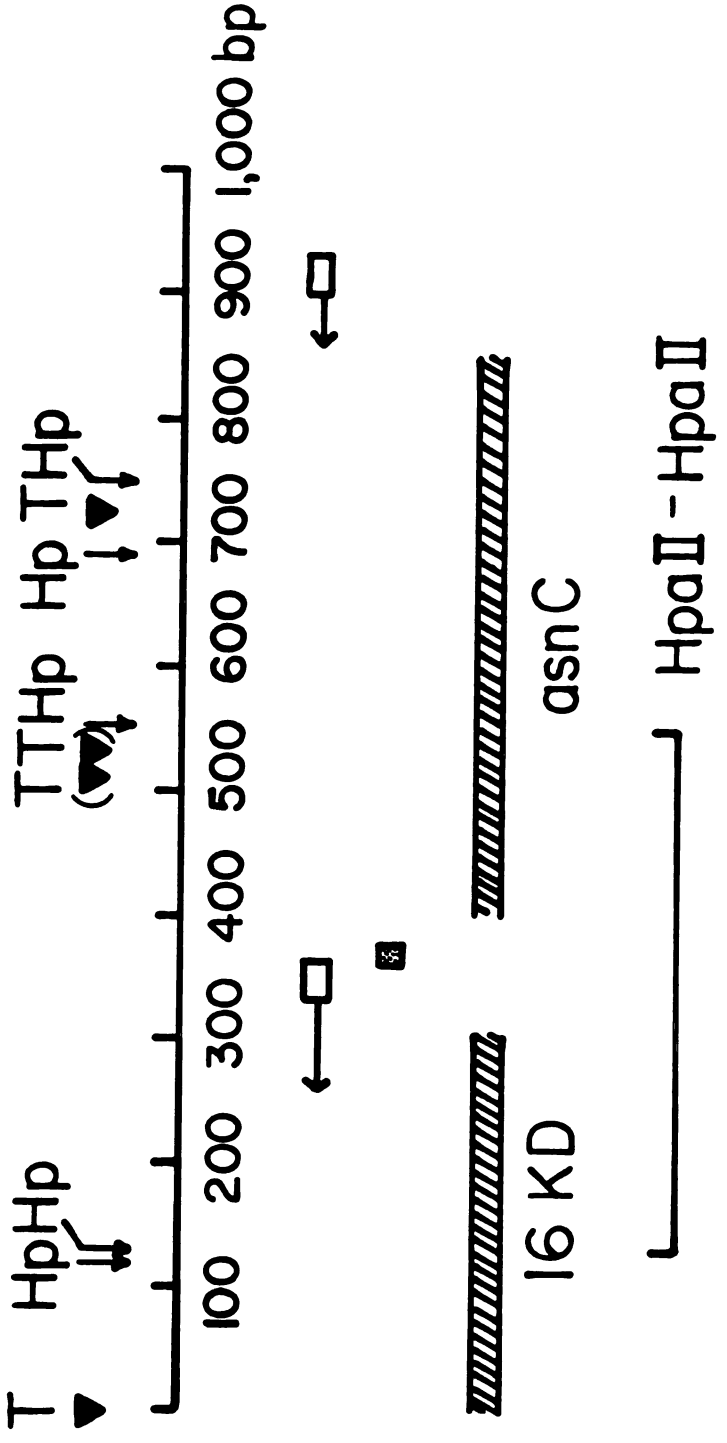
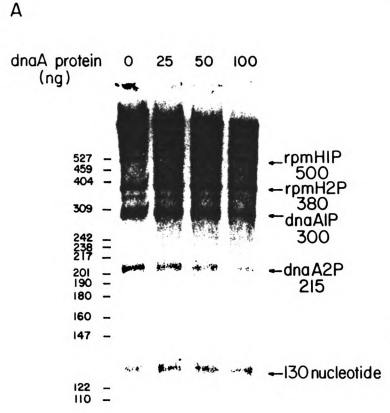
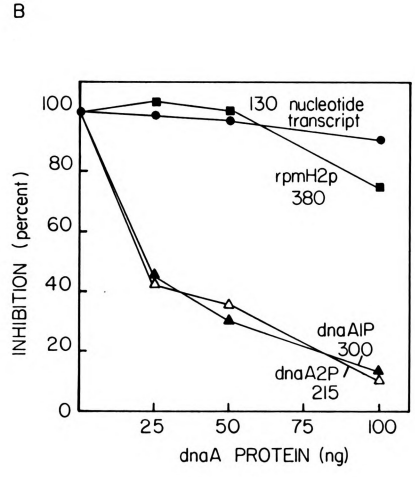

T
HpHp
TTHp
(
Hp THp
100 200 300 400 500 600 700 800 900 1,000 bp
16 KD
asnC
HpaII-HpaII

Figure 3. Run-off transcription using the 945 bp EcoRI restriction fragment.

(A) Assays were performed as described in Materials and Methods with the indicated amounts of dnaA protein. The approximate sizes of the transcripts were determined relative to EcoRI, HpaII digested pBR322 DNA as a size standard (left column).

(B) The amounts of each transcript determined as described in Materials and Methods are expressed as a ratio compared to the control with no dnaA protein added.


A
dnaA protein (ng)
0
25
50
100
527
459
404
309
242
238
217
201
190
180
160
147
122
110
rpmHIP 500
rpmH2P 380
dnaAIP 300
dnaA2P 215
130 nucleotide


B
INHIBITION (percent)
100
80
60
40
20
0
25
50
75
100
dnaA PROTEIN (ng)
130 nucleotide transcript
rpmH2p 380
dnaAIP 300
dnaA2P 215

is approximately equal to the length of the fragment. Similar products attributed to transcription from dnaA1P and dnaA2P have been observed by others in run-off transcription assays with preparations of the same EcoRI restriction fragment (32, 5).

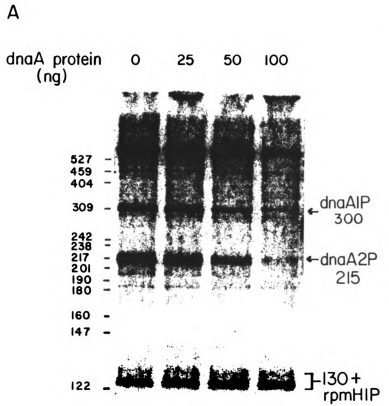
Addition of various amounts of purified dnaA protein to run-off transcription assays containing this EcoRI fragment resulted in a preferential inhibition of synthesis of both 300 and 215 nucleotide transcripts (Figure 3A,B). This result is consistent with inhibition from dnaA1P and dnaA2P by dnaA protein. At the highest level of dnaA protein added, synthesis of the 300 and 215 nucleotide transcripts was inhibited about 10 fold (Figure 3B). The amount of dnaA protein sufficient for maximal inhibition of transcription is comparable to that amount required for preferential binding of dnaA protein to restriction fragments containing the dnaA promoter region (13, see below). In contrast, synthesis of transcripts of 380 and 130 nucleotides was inhibited by less than 2 fold at this level. It was not possible to quantitate accurately the amount of the 500 nucleotide transcript due to high background in this region of the autoradiogram.

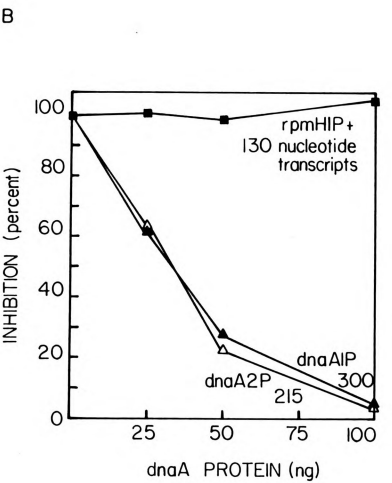
Cleavage of this 945 bp EcoRI fragment with HaeIII restriction enzyme removes the rpmH2P promoter and coding sequences distal to and downstream from the rpmH promoters (Figure 1). Run-off transcription assays with this 569 bp restriction fragment as a template resulted in transcripts of approximately 300 and 215 nucleotides (Figure 4A) which are similar in size to those observed with the larger 945 bp restriction fragment as a template (Figure 3A). In addition, two transcripts close to 130 nucleotides in length

Figure 4. Run-off transcription using the 569 bp HaeIII-EcoRI restriction fragment.

(A) Reactions were performed as described in Materials and Methods with the indicated amounts of dnaA protein. The sizes of the transcripts were determined relative to EcoRI, HpaII digested pBR322 DNA as a size standard (left column).

(B) The amounts of each transcript determined as described in Materials and Methods are expressed as a ratio compared to the amount of transcript produced in the absence of dnaA protein.


A
dnaA protein (ng)
0
25
50
100
527
459
404
309
242
238
217
201
190
180
160
147
122
dnaAIP 300
dnaA2P 215
130+ rpmHIP


B
INHIBITION (percent)
100
80
60
40
20
0
25
50
75
100
dnaA PROTEIN (ng)
rpmHIP+ 130 nucleotide transcripts
dnaAIP 300
dnaA2P 215

were observed while products of 500 and 380 nucleotides were absent. The appearance of one of these transcripts with this shortened restriction fragment is consistent with the termination product observed in Figure 3A formed by transcription from rpmH1P. The slightly longer transcript is consistent with run-off transcription from rpmH1P. Based on size, the largest product is presumed to result from end to end transcription of the restriction fragment. The addition of increased amounts of dnaA protein to reactions containing RNA polymerase and this restriction fragment resulted in up to a twenty-fold repression of synthesis of the 300 and 215 nucleotide transcripts. Marginal inhibition by dnaA protein was observed with the 130 nucleotide transcript (Figure 4B). Levels of dnaA protein sufficient for maximal and preferential inhibition of synthesis of the 300 and 215 nucleotide transcripts were similar to that of Figure 3.

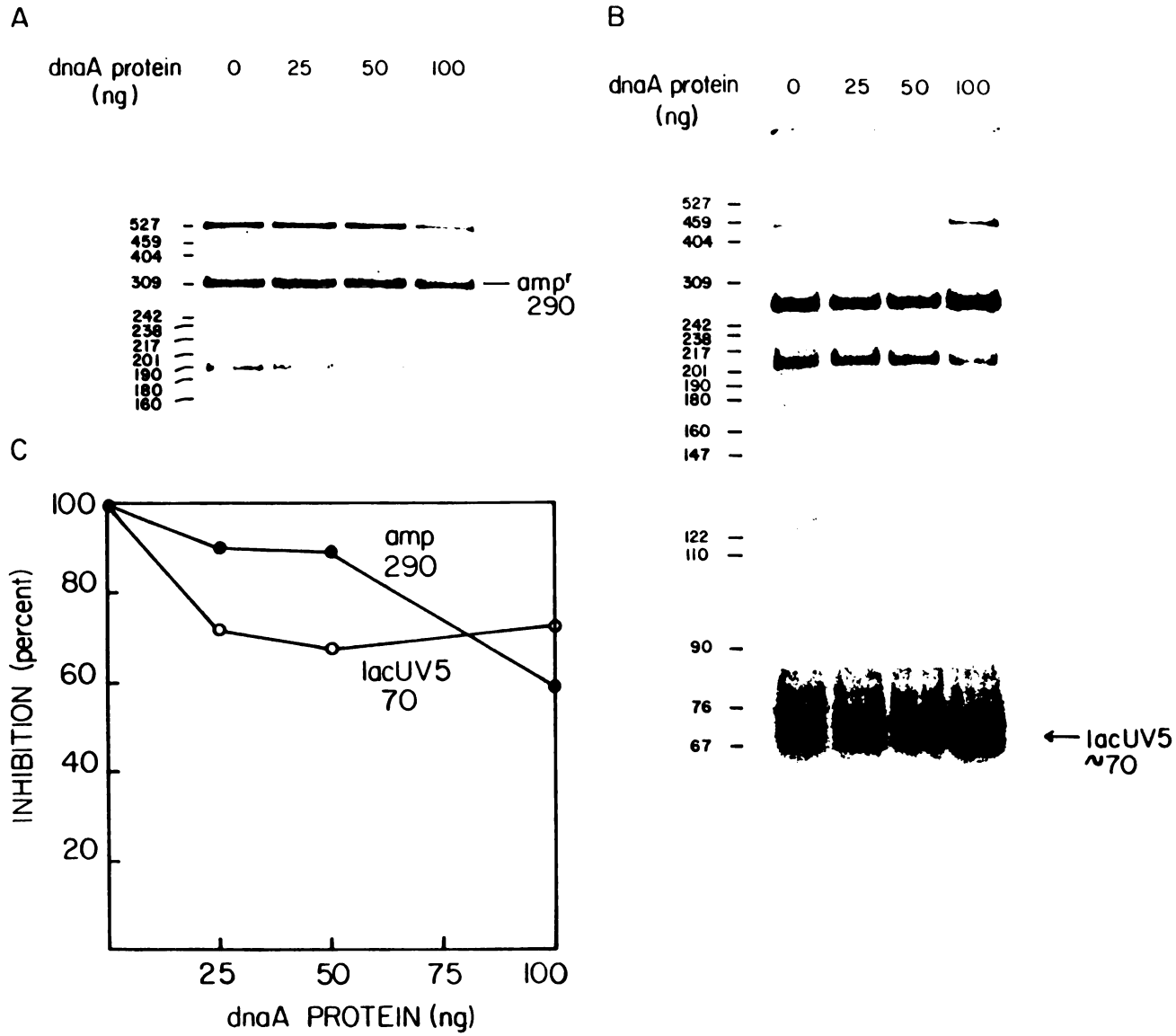
Digestion of pBR322 with EcoRI and HpaII restriction enzymes produces a 459 bp restriction fragment containing the promoter and N-terminal coding region of the β-lactamase gene. This DNA fragment is poorly bound by dnaA protein and contains no striking homologies to the dnaA protein recognition sequence (13). Other studies which have determined the location of the promoter and transcriptional start site for this gene predict a 288 nucleotide run-off product upon transcription of this restriction fragment (7). The observed product of about 290 nucleotides is in agreement with the expected size (Figure 5A). Addition of dnaA protein to run-off transcription assays containing this restriction fragment resulted in a modest level of inhibition of transcription from the β-lactamase promoter (Figure 5A, C). The modest level

Figure 5. Run-off transcription from the β-lactamase promoter and from the lacUV5 promoter.

(A) Assays were performed with the 459 bp EcoRI-HpaII restriction fragment containing the β-lactamase promote.

(B) Run-off transcription assayed with the 203 bp restriction fragment containing the lacUV5 promoter. Sizes of transcripts were determined relative to EcoRI, HpaII digested pBR322 DNA as a size standard (left column).

(C) The amounts of each transcript determined as described in Materials and Methods are expressed as a ratio compared to the amount of transcript produced in the absence of dnaA protein.


A
dnaA protein (ng)
0
25
50
100
527
459
404
309
242
238
217
201
190
180
160
amp^r 290
B
dnaA protein (ng)
0
25
50
100
527
459
404
309
242
238
217
201
190
180
160
147
122
110
90
76
67
lacUV5 ~70
C
INHIBITION (percent)
100
80
60
40
20
amp 290
lacUV5 70
25
50
75
100
dnaA PROTEIN (ng)

of inhibition observed is similar to the effect observed for transcription from rpmH1P and rpmH2P (Figure 3,4). As above, the largest transcript can be attributed to end to end transcription of the restriction fragment. A transcript of about 190 nucleotides was also observed. The presence of this transcript is unexpected.

Run-off transcription experiments were also performed with a 203 bp restriction fragment containing the lacUV5 promoter. By sequence analysis, this DNA fragment lacks sequences homologous to the consensus dnaA protein recognition sequence and is poorly bound by dnaA protein (see below). The observed transcripts of 60-70 nucleotides are similar to those previously observed by transcription from the lacUV5 promoter with RNA polymerase in the absence of cyclic AMP binding protein (Figure 5B) (31). The addition of increasing amounts of dnaA protein to these assays minimally affected on synthesis of these transcripts (Figure 5B, C). The product of 205-210 nucleotides is prsumably due to end-to-end transcription of the restriction fragment. The product of about 280 nucleotides was unexplained but has been observed by others in transcription of the same fragment containing the lacUV5 promoter (43).

Construction of pBF1509 results in a 397 bp BamHI-EcoRI fragment which separates the dnaA promoter region from the rpmH promoter region (13). Run-off transcription with this restriction fragment resulted in synthesis of 215 and 300 nucleotide transcripts (data not shown). Addition of dnaA protein inhibited synthesis of both transcripts to almost undetectable levels (data not shown). The 397 bp BamHI-EcoRI fragment containing the dnaA

promoter region was digested with HinfI which removes additional coding sequences of the dnaA gene. If the 215 and 300 nucleotide products observed above were due to transcription from dnaA1P and dnaA2P, transcripts of 231 and 146 nucleotides are expected with this 328 bp BamHI-HinfI fragment. As indicated in Figure 6A, transcripts of approximately 230 and 140 nucleotides were observed. The addition of dnaA protein in increasing amounts resulted in a corresponding decrease in synthesis of both of these transcripts to undetectable levels (Figure 6B). The largest product corresponds in size to end to end transcription of the restriction fragment. A transcript of about 70 nucleotides was also observed. Although the appearance of this transcript is unexplained, its synthesis is not inhibited by the addition of dnaA protein. Parallel reactions were performed of run-off transcription from the lacUV5 promoter and the β-lactamase promoter. These transcripts were marginally inhibited upon addition of similar levels of dnaA protein (data not shown).

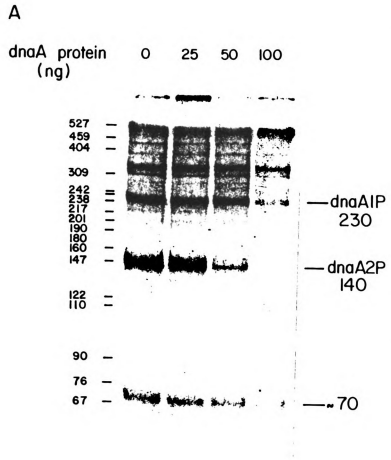
dnaA protein specifically inhibits transcription from the 16 KDa promoter

Experiments to determine the influence of dnaA protein on transcription of the 16 KDa gene adjacent to oriC were performed. The promoter region for this gene encoding a 16 KDa protein is contained in a 414 bp HpaII restriction fragment (22). Run-off transcription assays with this restriction fragment resulted in formation of a 200 nucleotide transcript which was inhibited up to 20-fold upon addition of increasing amounts of dnaA protein (Figure 7A,B). As in Figure 5, addition of similar amounts of dnaA protein to parallel run-off transcription assays with a restriction fragment containing

Figure 6. Run-off transcription using the 328 bp BamHI-HinfI restriction fragment.

(A) Assays were performed as described in Materials and Methods with the indicated amounts of dnaA protein. The sizes of transcripts were determined relative to EcoRI, HpaII digested pBR322 DNA as a size standard (left column).

(B) The amounts of each transcript determined as described in Materials and Methods are expressed as a ratio compared to the amount of transcript produced in the absence of dnaA protein.


A
dnaA protein (ng)
0
25
50
100
527
459
404
309
242
238
217
201
190
180
160
147
122
110
90
76
67
dnaAIP 230
dnaA2P 140
70

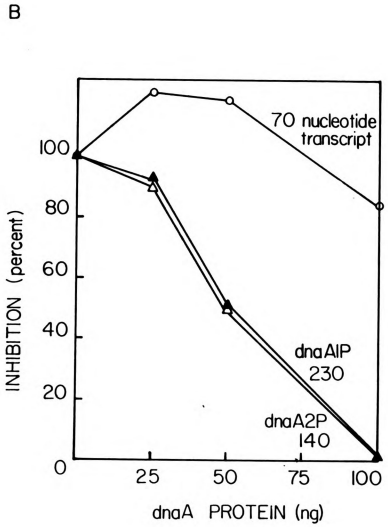
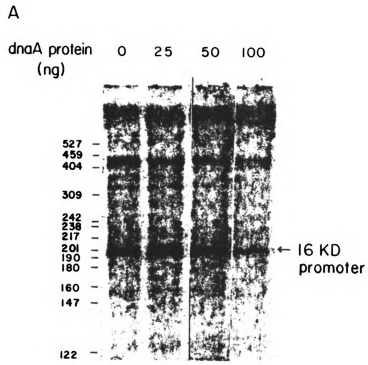
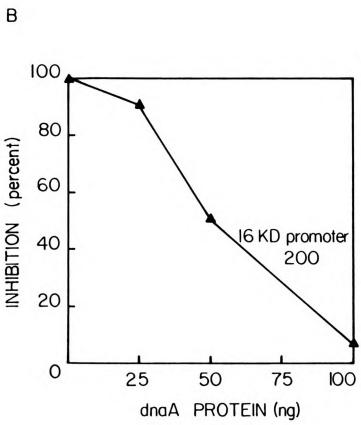

B
70 nucleotide transcript
INHIBITION (percent)
100
80
60
40
20
0
dnaAIP 230
dnaA2P 140
25
50
75
100
dnaA PROTEIN (ng)

Figure 7. In vitro transcription using the 414 bp HpaII restriction fragment containing the promoter region for the 16 KDa protein.

(A) Assays were performed as described in Materials and Methods with the indicated amounts of dnaA protein. The approximate sizes of transcripts are indicated.

(B) The amounts of each transcript determined as described in Materials and Methods are expressed as a ratio compared to the control with no dnaA protein added.


A
dnaA protein (ng)
0
25
50
100
527
459
404
309
242
238
217
201
190
180
160
147
122
16 KD promoter


B
INHIBITION (percent)
100
80
60
40
20
0
25
50
75
100
dnaA PROTEIN (ng)
16 KD promoter
200

the lacUV5 promoter resulted in less than 2-fold inhibition of transcription (data not shown).

Specific binding of dnaA protein to restriction fragments

Fragment retention assays were performed with dnaA protein and DNA fragments used in run-off transcription assays to determine whether inhibition of transcription by dnaA protein correlated with its ability to bind to restriction fragments containing dnaA protein recognition sequences.

The 945 bp EcoRI fragment was digested with HpaII restriction endonuclease and 3' end-labeled with the large fragment of DNA polymerase I and α^{32}P-dCTP. After incubation with various amounts of dnaA protein, fragments which bound to or flowed through nitrocellulose filters were visualized after gel electrophoresis and autoradiography (Figure 8A). The largest fragment of 445 bp contains the dnaA protein recognition sequence between dnaA1P and dnaA2P and the promoter, rpmH1P. This fragment was more tightly bound by dnaA protein than the 378 bp fragment containing the rpmH and rnpA genes (Figure 8B). The 95 bp and 21 bp fragment between rpmH1P and rpmH2P was poorly bound under these reaction conditions.

Fragment retention experiments were also performed with the 414 bp restriction fragment containing the promoter region for the 16 KDa gene combined with an equimolar amount of the 203 bp restriction fragment containing the lacUV5 promoter. The addition of dnaA protein resulted in preferential retention of the 16 KDa promoter-containing fragment compared to binding to the lacUV5 promoter fragment (Figure 9A,B). The amount of

dnaA protein required for preferential binding to restriction fragments containing the promoters for the dnaA or 16 KDa genes corresponded to the amount of dnaA protein required for specific inhibition of transcription from these promoters.

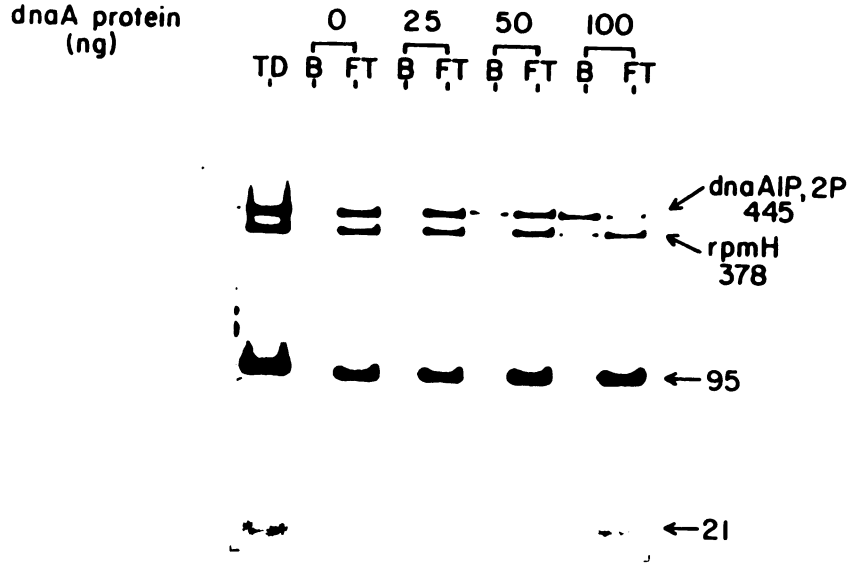
Figure 8. Preferential binding of dnaA protein to a restriction containing the dnaA promoters.

Fragment retention assays were performed as described in Materials and Methods with 0.025 pmol of the 945 bp EcoRI fragment digested with HpaII and the indicated amounts of dnaA protein.

(A) Positions and sizes of the subfragments produced by HpaII digestion are indicated. TD, total digest; B, bound fragments eluted from filters; FT, fragments which flowed through the filter.

(B) Amounts of fragments bound were determined as described in Materials and Methods.

A


dnaA protein (ng)
0
25
50
100
TD
B
FT
dnaAIP, 2P 445
rpmH 378
95
21

B

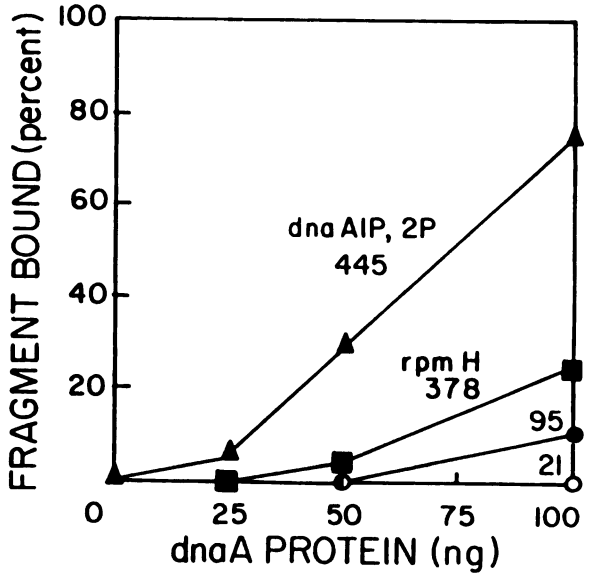

FRAGMENT BOUND (percent)
100
80
60
40
20
0
25
50
75
100
dnaA PROTEIN (ng)
dnaAIP, 2P 445
rpmH 378
95
21

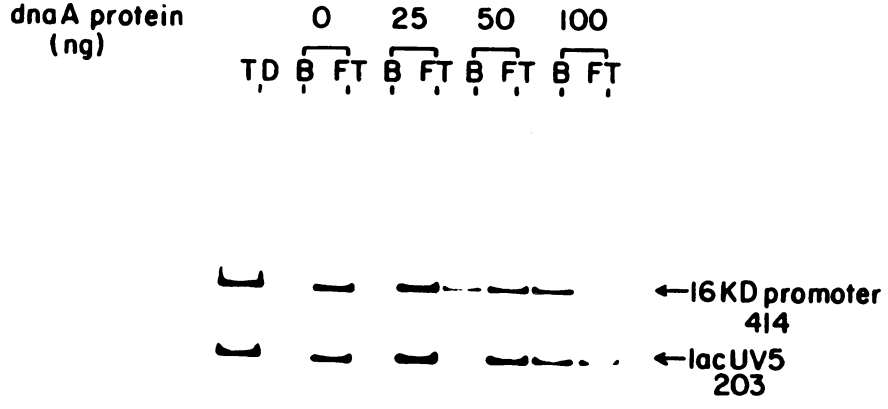
Figure 9. Preferential binding of dnaA protein to a restriction fragment containing the 16 KDa promoter.

Fragment retention assays were performed as described in Materials and Methods with 0.025 pmol each of the 414 bp fragment containing the 16 KDa promoter and of the 203 bp fragment containing the lacUV5 promoter.

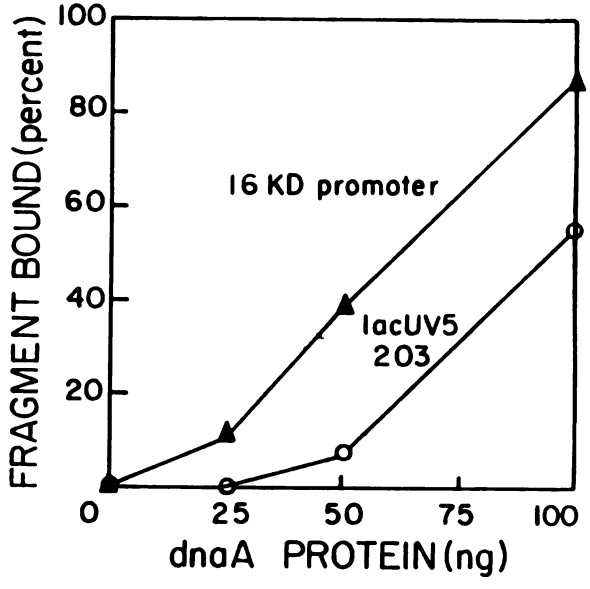
(A) Positions and sizes of the restriction fragments are indicated. TD, B, and FT are as in Figure 8.

(B) Amounts of fragments bound were determined as described in Materials and Methods.

A


dnaA protein (ng)
0
25
50
100
TD B FT B FT B FT B FT
←16KD promoter 414
←lacUV5 203

B


FRAGMENT BOUND (percent)
100
80
60
40
20
0
25
50
75
100
dnaA PROTEIN (ng)
16 KD promoter
lacUV5 203

DISCUSSION

In the studies reported here, transcriptional repression of the dnaA gene by dnaA protein was observed at levels similar to those required for specific binding of dnaA protein to DNA fragments containing the dnaA promoter region. Inhibition of transcription by dnaA was observed to affect both dnaA promoters. Under most reaction conditions, 100 ng of dnaA protein resulted in about 10 fold inhibition of both of the dnaA promoters. Transcriptional inhibition of the 16 KDa gene by dnaA protein was also observed at levels similar to those required for specific binding to DNA fragments containing the promoter region for this gene. dnaA protein did not markedly inhibit transcription from the promoter for either the β-lactamase gene, or the lacUV5 promoter in separate run-off transcription assays, or from the rpmH promoters contained on the same restriction fragment as the dnaA promoters. These results indicate that inhibition is specific. Recognition of the dnaA protein recognition sequence and binding are directly related to its repressive effect.

The transcripts observed in these experiments confirm the positions of the promoters for the dnaA, rpmH, lacUV5, and β-lactamase genes (19, 22, 31, 7). In one experiment, transcription of the BamHI-HinfI fragment resulted in an unexpected product of 70 nucleotides. This transcript remains unexplained as larger restriction fragments do not direct the synthesis of a corresponding transcript. A transcription experiment of a DNA fragment

containing the β-lactamase promoter resulted in synthesis of a 190 nucleotide transcript. This product was observed in addition to transcripts attributed to initiation from the β-lactamase promoter and end-to-end transcription. Based on S1 nuclease mapping experiments and electron microscopic mapping of RNA polymerase-promoter complexes, this restriction fragment only contains one of the two promoters involved in transcription of the β-lactamase gene of pBR322 (7, 36). This transcript may have arisen by premature termination from the β-lactamase promoter in this DNA fragment. A termination product of 190 nucleotides would not have been observed in these studies.

Recent studies relying on transcriptional and translational fusions of the dnaA promoter region to the lacZ gene have indicated that overproduction of the dnaA gene product resulted in decreased expression of β-galactosidase activity while introduction of the dnaA46 allele resulted in elevated expression at the nonpermissive temperature (4, 1). Deletion of sequences upstream from dnaA2P and including dnaA1P resulted in the lack of this repressive effect. This deletion also removes the dnaA protein recognition sequence between dnaA1P and dnaA2P involved in binding of dnaA protein to this region of DNA.

Other studies in which the promoter for the 16 KDa gene was linked to the galK structural gene have indicated that galactokinase activity was decreased with elevated levels of dnaA protein (30, 38). No influence of dnaA protein on transcription from the 16 KDa promoter was observed with recombinants lacking 6 bp of the dnaA protein recognition sequence (38). The results from these in vivo studies suggest that binding of dnaA protein to the

promoter regions regulates expression of these genes.

The studies presented here are in agreement with these in vivo studies. Using purified components, maximal inhibition of transcription is observed at a ratio of 50 to 100 monomers of dnaA protein per recognition site. This level is consistent with its cooperative binding to the dnaA and 16 KDa promoter regions, and with the sizes of dnaA protein-DNA complexes observed by electron microscopy (13). The dnaA protein recognition sequence is located between the two promoters involved in transcription of the dnaA gene (13). In comparison, this recognition sequence is located slightly upstream from the -35 region of the 16 KDa promoter (38). These results suggest that transcriptional inhibition may involve occlusion of RNA polymerase from promoters bound by dnaA protein. If dnaA protein interacts directly with RNA polymerase to influence its activity at promoters containing dnaA protein recognition sequences, this interaction does not dramatically influence transcription from the promoters for β-lactamase, rpmH, or lacZ.

Experiments of fragment retention on nitrocellulose filters showed that dnaA protein was weakly bound to the DNA fragment containing the lacUV5 promoter and to the fragment containing the rpmH coding region. The result that transcription of these DNAs was slightly inhibited by dnaA protein may be due to the ability of nonspecifically bound dnaA protein to inhibit transcription.

Expression of the dnaA gene involves transcriptional initiation from two promoters both in vivo and in vitro. Promoters utilized in vitro appear to be identical to those recognized in the cell (20, 4). Whereas transcription in vivo

is reported to occur predominantly from the dnaA2P promoter, the in vitro studies reported here indicate that both dnaA1P and dnaA2P promoters are utilized with about equal efficiencies at an RNA polymerase:DNA template ratio of 26:1. Varying the ratio of RNA polymerase holoenzyme to the DNA restriction fragment appeared to influence the efficiency of promoter usage (data not shown). At a 1:1 ratio of RNA polymerase to DNA template, dnaA2P appeared to be more efficiently utilized. Other factors may modulate the usage of the dnaA promoters. The observation that dnaA protein binds with higher affinity to supercoiled than linearized oriC plasmid DNA (14) suggests that the superhelical state of the template may influence the regulatory effect of dnaA protein on expression of its gene.

The levels of dnaA protein required for autoregulation in vitro are approximately equal to the levels of dnaA protein required for initiation of replication on oriC plasmid DNAs (15). It has been shown that the rate of DNA replication is strictly coordinated to the growth rate of the bacterium such that the ratio of DNA content to cell mass remains constant (35). An autorepressor model for control of DNA replication was proposed (37). This model proposes that an initiator protein is contained in an operon which also encodes a gene product that autoregulates expression of the operon. The activities of dnaA protein are consistent with its role as an initiator of replication and as an autoregulator of its expression. From genetic and biochemical studies, dnaA protein appears to play a central role in initiation of replication (21, 41, 28, 13). Experiments in which expression of dnaA protein is elevated by overproduction resulted in an increased frequency of

initiation (2, 6). That these new initiations are aborted in the former studies indicates that the coordination of DNA replication and cell growth is interrupted or that other factors may become rate limiting.

Whether the dnaA gene product also functions to coordinate DNA replication to cell growth is unknown. The observation that expression of the 16 KDa gene is negatively regulated by the dnaA gene product and the presence of sequences homologous to the concensus dnaA protein recognition sequence in promoter regions for other genes suggests that dnaA protein may act to influence expression of these genes. The influence of dnaA protein on expression of these genes can be measured directly with the availability of cloned DNA fragments containing these genes of interest.

REFERENCES

1. Atlung, T., Clausen, E., and Hansen, F. G. (1985) Mol. Gen. Genet. 200, 442-450.

2. Atlung, T., Rasmussen, K. V., Clausen, E., and Hansen, F. G. (1985) in Molecular biology of bacterial growth. Jones and Bartlett Publ. Inc., Portola Valley, CA:p 282-297.

3. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254.

4. Braun, R. E., O'Day, K., and Wright, A. (1985) Cell 40, 159-169.

5. Braun, R. E., and Wright, A. (1986) Mol. Gen. Genet. 202, 246-250.

6. Bremer, H., and Churchward, G. (1985) J. Bacteriol. 164, 922-924.

7 Brosius, J., Cate, R. L., and Perlmutter, P. A. (1982) J. Biol. Chem. 257, 9205-9210.

8 Burgers, P. M. J., Kornberg, A., and Sakakibara, Y. (1981) Proc. Natl. Acad. Sci. USA 78, 5391-5395.

9 Burgess, R. R., and Jendrisak, J. J. (1975) Biochemistry 14, 4634-4638.

10. Chakraborty, T., Yoshinaga, K., Lother, H., and Messer, W. (1982) EMBO J. 1, 1545-1549.

11. Fralick, J. A. (1978) J. Mol. Biol. 122, 271-286.

12. Frey, J., Chandler, M., and Caro, L. (1981) Mol. Gen. Genet. 182, 364-366.

13. Fuller, R. S., Funnell, B. E., and Kornberg, A. (1984) Cell 38, 889-900.

14. Fuller, R. S., and Kornberg, A. (1983) Proc. Natl. Acad. Sci. USA 80, 5817-5821.

15. Funnell, B. E., Baker, T. A., and Kornberg, A. (1986) J. Biol. Chem. 261, 5616-5624.

16. Gonzalez, N., Wiggs, J., and Chamberlin, M. J. (1977) Arch. Biochem. Biophys. 182, 404-408.

17. Hansen, E. B., Hansen, F. G., and von Meyenburg, K. (1982) Nucleic Acids Res. 10, 7373-7385.

18. Hansen, F. G., and Rasmussen, K. V. (1977) Mol. Gen. Genet 155:219-255.

19. Hansen, F. G., and von Meyenburg, K. (1979) Mol. Gen. Genet. 175, 135-144.

20. Hansen, F. G., Hansen, E. B., and Atlung, T. (1982) EMBO J. 1, 1043-1048.

21. Hirota, Y., Mordoh, J., and Jacob, F. (1970) J. Mol. Biol. 53, 369-387.

22. Hirota, Y., Yamada, M., Nishimura, A., Oka, A., Sugimoto, K., Asada, K., and Takanami, M. (1981) Prog. Nucleic Acid Res. Mol. Biol. 26, 33-47.

23. Hoyt, M. A., Knight, D. M., Das, A., Miller, H. I., and Echols, H. (1982) Cell 31, 565-573.

24. Hwang, D. S., and Kaguni, J. M. (1988) J. Biol. Chem. 263, 10625-10632.

25. Kaguni, J. M., LaVerne, L. S., and Ray, D. S. (1979) Proc. Natl. Acad. Sci. USA 76, 6250-6254.

26. Kellenberger-Gujer, G., Podhajska, A. J., and Caro, L. (1978) Mol. Gen. Genet. 162, 9-16.

27. Kucherer, C., Lother, H., Kolling, R., Schauzu, M., and Messer, W. (1986) Mol. Gen. Genet. 205, 115-121.

28. Kung, F. C., and Glaser, D. A. (1978) J. Bacteriol. 133, 755-762.

29. Lycett, G., Orr, E., and Pritchard, R. (1980) Mol. Gen. Genet. 178, 329-336.

30. Lother, H., Kolling, R., Kucherer, C., and Schauzu, M. (1985) EMBO J. 4, 555-560.

31. Maizels, N. (1973) Proc. Natl. Acad. Sci. USA 70, 3585-3589.

32. Ohmori, H., Kimura, M., Nagata, T., and Sakakibara, Y. (1984) Gene 28, 159-170.

33. Miki, T., Kimura, M., Hiraga, S., Nagata, T., and Yura, T. (1979) J. Bacteriol. 140, 235-247.

34. Sakakibara, Y., and Mizukami, T. (1980) Mol. Gen. Genet. 178, 541-553.

35. Skarstad, K., Boye, E., and Steen, H. B. (1986) EMBO J. 5, 1711-1717.

36. Stuber, D., and Bujard, H. (1981) Proc. Natl. Acad. Sci. USA 78, 167-171.

37. Sompayrac, L., and Maaloe, O. (1973) Nature New Biol. 241, 133-135.

38. Stuitje, A., de Wind, N., van der Spek, J. C., Pors, T. H., and Meijer, M. (1986) Nucleic Acids Res. 14, 2333-2344.

39. Tippe-Schindler, R., Zahn, G., and Messer, W. (1979) Mol. Gen. Genet. 168, 185-195.

40. Wu, R. (1970) J. Mol. Biol. 51, 501-521.

41. Zyskind, J. W., Deen, L., and Smith, D. (1977) J. Bacteriol. 129, 1466-1475.

42. Kolling, R. and Lother, H. (1985) J. Bacteriol. 164, 310-315.

43. Lorimer, D. D. and Revzin, A. (1986) Nucl. Acids Res. 14, 2921-2938.

Chapter III

TRANSCRIPTIONAL REPRESSION OF THE *rpoH* GENE OF *Escherichia coli* BY dnaA PROTEIN

ABSTRACT

The rpoH (htpR) gene of Escherichia coli encodes a sigma factor which confers upon RNA polymerase the ability to recognize the promoters for genes responsive to the phenomenon termed the heat shock response. dnaA protein, a sequence-specific DNA binding protein, is required for initiation of chromosomal replication by binding to sites within the chromosomal origin. dnaA protein also autoregulates its expression by binding to a site in the dnaA promoter region. Two copies of the dnaA protein recognition sequence are present within the rpoH promoter region. Using filter binding assays, dnaA protein was observed to bind specifically to DNA fragments containing the rpoH promoter region with greater affinity than its binding to the dnaA promoter region. By contrast, reduced binding to a DNA fragment containing the lacUV5 promoter was observed. DNase I footprint analysis indicated that dnaA protein protected specific sites within the rpoH promoter region. The binding of dnaA protein to the rpoH promoter region resulted in transcriptional repression from two of the three promoters of the rpoH gene in vitro. Elevated levels of dnaA protein repressed transcription from these two rpoH promoters in vivo. These results indicate that dnaA protein regulates rpoH transcription to influence the expression of genes under rpoH control.

INTRODUCTION

dnaA protein is required for initiation of DNA replication from the E. coli chromosomal origin, oriC, in vivo (26, 57, 32) and in vitro (17, 6). In this process, dnaA protein recognizes and binds cooperatively to sites within the oriC region (11, 18). Upon binding, this protein appears to induce a localized unwinding of oriC in an ATP-dependent fashion to create a structure required for subsequent steps of replication (3, 46, 6). These and other results suggest that dnaA protein functions at an early stage in the initiation process.

dnaA protein not only binds to oriC but to other DNA fragments including those containing the dnaA promoter, the 16 KDa promoter, and to origin sequences of some plasmids (18). DNA fragments specifically bound by dnaA protein share a nine base-pair consensus sequence, TTAT(A/C)CA(A/C)A, which is postulated to be recognized by dnaA protein. Other experiments indicate that dnaA protein represses transcription of the dnaA and the 16 KDa genes (7, 2, 31, 36, 48, 54). This repressive effect was observed at levels of dnaA protein required for specific binding to these promoter-containing DNA fragments (54). The ability of dnaA protein to autoregulate its expression and its role in initiation of E. coli chromosomal replication may contribute to regulating the frequency of initiation. The binding of dnaA protein to plasmid origins has also been correlated with the involvement of dnaA protein in plasmid DNA replication (25, 16, 43, 30, 24, 40, 47). These observations suggest a biochemical function in site-specific binding by dnaA

protein.

E. coli exposed to high temperature is induced to express seventeen specific proteins in a phenomenon termed the heat shock response (42). Other treatments which elicit this response include ethanol, bacteriophage infection, or UV irradiation. A sigma factor, σ^{32}, encoded by the rpoH (htpR) gene confers upon RNA polymerase the ability to recognize and transcribe from the promoters of these heat shock genes (33, 23, 14, 4).

Recent reports indicate that the level of σ^{32} is relatively low at 30°C and increases by a temperature shift to 42°C (34, 49). The increase in σ^{32} appears to occur by increased transcription and by stabilization of rpoH mRNA (50, 15). rpoH transcripts start at promoters designated here as rpoH1P, 2P, 3P, and 4P (15, 51). Promoters 1P and 4P are recognized by σ^{70}-RNA polymerase and account for 90% of the total rpoH mRNA at 30°C (15). rpoH3P may be recognized by a novel form of RNA polymerase. A fourth promoter, 2P, appears to be strain dependent. A temperature increase results in an increase in rpoH transcript levels with 2P and 3P increasing the most. Despite the response of rpoH expression to increased temperature, its expression does not appear to involve σ^{32} (4, 15).

Computer analysis revealed two presumptive dnaA protein recognition sequences (dnaA boxes) in the promoter region of the rpoH gene. This observation led us to consider whether dnaA protein might regulate expression of the rpoH gene.

In this study, we report that dnaA protein binds specifically to the rpoH promoter region as determined by nitrocellulose filter binding assays and

DNase I protection experiments. dnaA protein repressed transcription from two promoters of the rpoH gene in vivo and in vitro.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli W3110 and AB1157 thr-1, ara-14, leuB6, del(gpt-proA)62, lacY1, tsx-33, supE-44, galK2, hisG4, rpsL31, xyl-5, mtl-1, argE3, thi-1 were from the E. coli Genetic Stock Center.

Plasmid pING1 (29) was from Dr. Dan S. Ray, UCLA. pDS596 contains the dnaA gene inserted in the vector pING1 and under control of the araB promoter (28). The plasmid pdnaA/dnaN contains the dnaA and adjacent dnaN genes within the 0.94 and the 3.4 kb EcoR1 fragments inserted in the vector pMOB45 (9). pFN82, from Dr. Frederick C. Neidhardt, University of Michigan, consists of a 2.5 kb PstI fragment containing the N-terminal coding region of the rpoH gene inserted at the single PstI site of pBR322 (41). pBR322 and pUC19 were from this laboratory. Plasmid DNAs were purified from cleared lysates by centrifugation in ethidium bromide-CsCl gradients. Individual restriction fragments separated by electrophoresis were purified by gel electroelution with an ISCO 1750 electrophoretic concentrator followed by ethanol precipitation. DNA concentration was determined spectrophotometrically, or by comparison of the purified DNA fragments to different known amounts of electrophoretically separated restriction enzyme digests visualized by ethidium bromide staining. The 203 bp EcoR1 fragment containing the lacUV5 promoter was a gift from D. Lorimer of this department (35).

Enzymes

Restriction enzymes HindIII, EcoR1, ClaI, EcoRV, and PvuII, DNA polymerase I (large fragment), and T4 polynucleotide kinase were purchased from New England Biolabs; PstI was from BRL; HpaII and S1 nuclease from Pharmacia; DNase I from Worthington. RNA polymerase holoenzyme was purified from W3110 as described except that Biogel A5m (Biorad) chromatography was replaced by chromatography on a TSK 3000SW (Altex) high performance gel permeation column (10, 22). dnaA protein was purified from an overproducing strain (28). Protein concentrations were determined by the dye-binding method with bovine serum albumin as a standard (5).

Radioactive labelling of DNA

Restriction enzyme digests or purified DNA fragments were either 5' end-labeled with T4 polynucleotide kinase and [γ^{32}P] ATP according to the manufacturer's instructions or 3' end-labeled with DNA polymerase I (large fragment) and [α^{32}P]dCTP as described (54).

DNA binding assays

Nitrocellulose filter binding assays were performed as described (54). DNase I protection assays (19) (10 µl) contained the indicated amounts of dnaA protein and end-labeled DNA fragments in buffer containing 40 mM Tris-HCl pH 7.6, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol 100 µg/ml bovine serum albumin, and 10% glycerol. The

PvuII-ClaI fragment labeled at the 5' end as described above was cleaved with HpaII and used without further purification in these assays. Cleavage with HpaII produces a 27 bp PvuII-HpaII fragment and a 156 bp HpaII-ClaI fragment. For the other strand, the PvuII-ClaI fragment was 3' end-labeled at the ClaI site. Reactions were incubated at 37°C for 10 min. DNase I (4 ng) was added and incubations were continued for 2 min. After addition of an equal volume of stop buffer containing 0.1% sodium dodecyl sulfate, 0.3 M sodium acetate, 20 mM EDTA, and 100 µg/ml tRNA, the samples were ethanol precipitated, resuspended in 5 µl of gel loading buffer containing 80% formamide, 10 mM NaOH, 1 mM EDTA and 0.025% bromophenol blue, boiled for 2 min, then placed on ice. Electrophoresis was in an 8% sequencing gel.

Maxam-Gilbert sequencing reactions of the end-labeled DNA served as markers for DNase I protection experiments (38).

Run-off transcription assays

In vitro transcription assays were performed as described with the following modifications (54). A prior incubation was performed at 37°C for 10 min in buffer containing 0.025 pmol of the indicated DNA fragment, 0.65 pmol of RNA polymerase, and the indicated amounts of dnaA protein but in the absence of ribonucleotides. Heparin (100 µg/ml), 300 µM each of CTP, GTP, ATP, and 50 µM [α^{32}P] UTP were added to inhibit open complex formation and to initiate transcription. Incubation was continued at 37°C for 10 min. Reactions were stopped, and samples were ethanol precipitated, resuspended, and electrophoresed in 8% polyacrylamide gels containing 7M

urea.

Gels were dried onto Whatman DE81 paper, and autoradiographed with Kodak XAR-5 film either at room temperature or at -70°C with Cronex Quanta III intensifying screens. The autoradiograms were quantitated by densitometry with a Hoefer gel scanner interfaced with an IBM personal computer.

RNA preparation

RNA was prepared (8) with the following modifications. E. coli AB1157 containing either pING1 or pDS596 was grown in LB media (120 ml) (39) at 30°C in a shaking water bath to a turbidity at 595 nm of 0.1. Arabinose was then added to 0.75% (w/v) to induce synthesis of dnaA protein. Incubation was continued at 30°C with shaking and portions were removed prior to or at the indicated times after arabinose addition. Where indicated, cultures were shifted to 42°C after induced expression of dnaA protein for 2 hr at 30°C and portions were removed at the indicated times. Portions of the culture (3 ml) from each time point were immediately centrifuged in a Fisher microcentrifuge for 20 seconds at room temperature. Resuspended cells were lysed as described (8). The lysate was phenol extracted three times, ether extracted, and total cellular RNA was obtained by ethanol precipitation. RNA concentration was determined spectrophotometrically (1 absorbance unit at 260nm equals 40 μg/ml).

S1 nuclease assays

Quantitative S1 nuclease assays (20 µl) were performed in buffer containing 40 mM PIPES pH 6.4, 1 mM EDTA, 0.4 M NaCl, 25 µg of yeast tRNA (Sigma), and 80% formamide by hybridization at 45°C for 12 to 16 h with 25 µg of the isolated RNA to 0.025 pmol of 5' end-labeled EcoRV restriction fragment containing the rpoH promoter region. At the end of hybridization, 300 µl of cold S1 nuclease buffer containing 50 mM sodium acetate pH 4.6, 0.25 M NaCl, 4.5 mM ZnSO4, 20 µg/ml heat-denatured salmon sperm DNA and 50 units of S1 nuclease was added. Reactions were incubated at 37°C for 15 min. Ammonium acetate and EDTA were added to 0.25 M and 5 mM, respectively. The samples were precipitated by addition of 2.5 volumes of ethanol, and resuspended in 20 µl of gel loading buffer described above. Electrophoresis was in an 8% polyacrylamide gel containing 7 M urea. Autoradiography, and its quantitation were as described above.

RESULTS

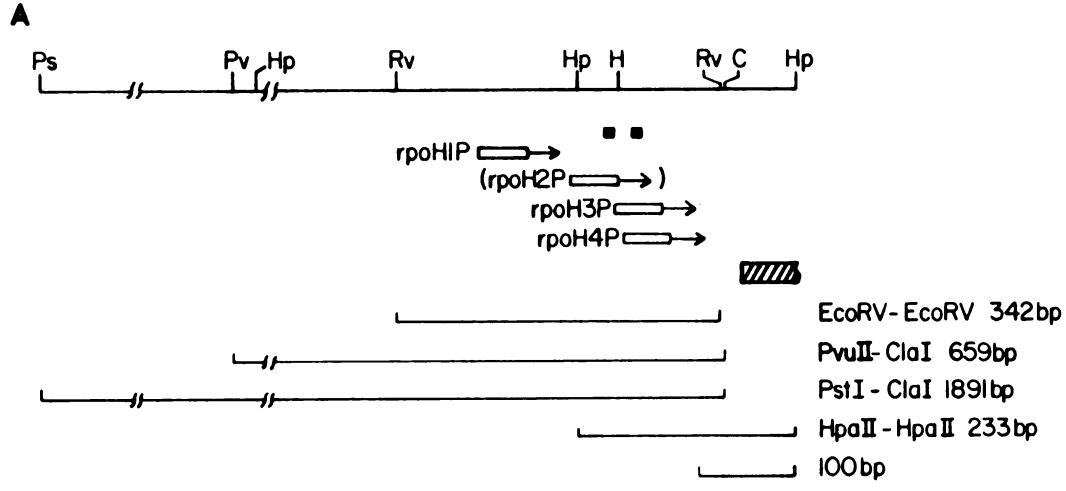
dnaA protein binds specifically to restriction fragments containing the rpoH promoter region

Two presumptive dnaA protein recognition sequences (dnaA boxes) were observed in the promoter region of the rpoH gene (Figure 1A,B). To examine the binding affinity of dnaA protein to the rpoH promoter region, nitrocellulose filter binding assays were performed. Plasmid pFN82 contains the promoter region and the N-terminal rpoH coding sequence in a 2.5 kb PstI fragment inserted at the PstI site of pBR322 (41). HpaII digestion of pFN82 results in fragments including those containing the rpoH promoter region (233 bp), and the pBR322 origin (527 bp). The latter DNA fragment contains a dnaA box near the pBR322 origin. dnaA protein binds to this fragment with an affinity similar to its binding to the dnaA promoter region (18; data not shown). Filter binding assays were performed with increasing amounts of dnaA protein (Figure 2). A DNA fragment near the size expected for the rpoH promoter-containing fragment (233 bp) was bound with higher affinity by dnaA protein than its binding to the 527 bp pBR322 origin fragment (Figure 2).

Based on DNA sequence information, HindIII digestion of the fragment containing the rpoH promoter (233 bp) is expected to produce two fragments of 186 and 43 bp. A second HindIII site is located in the 622 bp HpaII fragment of the vector. It is not bound by dnaA protein under these reaction

Figure 1. (A) Physical map of the rpoH promoter region. Pertinent restriction enzyme sites: C, ClaI; H, Hind III; Hp, HpaII; Rv, EcoRV; Ps, PstI; Pv, PvuII. The approximate positions of dnaA boxes (▩), rpoH promoters (⇒), and the rpoH coding region (▨), are indicated. Restriction fragments used in the experiments are also indicated.

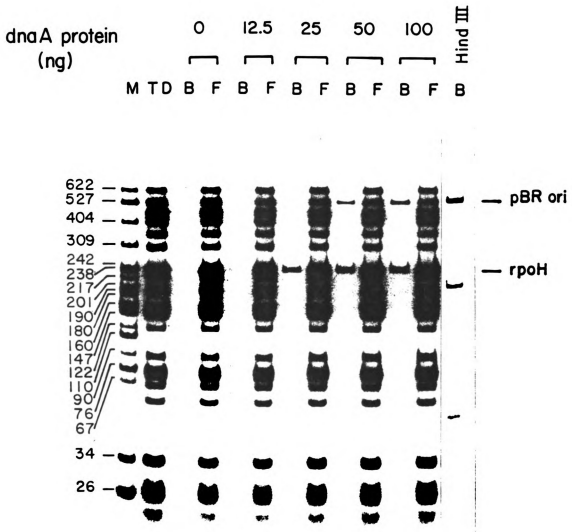
(B) DNA sequence of the rpoH promoter region (33, 56, 20) (coding strand). The C-terminal coding region of ftsX, positions of transcriptional start sites for rpoH mRNA (15), dnaA protein recognition sequences (▭), the N-terminal coding region of rpoH, and the restriction enzyme sites are as indicated.


A
Ps
Pv
Hp
Rv
Hp
H
Rv
C
Hp
rpoH1P
(rpoH2P
)
rpoH3P
rpoH4P
EcoRV-EcoRV 342bp
PvuII-ClaI 659bp
PstI-ClaI 1891bp
HpaII-HpaII 233bp
100bp


B
rpoH1P
GTACAACATTTACGCCACTTTACGCCTGAATAATAAAAGCGTGTTATACTCTTTCCCTGCAATGGGTTCCGTAGC
ftsX
(rpoH2P)
AGGGAAAGAGACCCCGTTGTCTCTTCCCGGTATTTCATCTCTATGTCACATTTTGTGCGTAATTTATTCACAAGC
HpaII
dnaA box
HindIII
rpoH3P
rpoH4Pa
TTGCATTGAACTTGTGGATAAAATCACGGTCTGATAAAACAGTGAATGATAACCTCGTTGCTCTTAAGCTCTGGC
dnaA box
rpoH4Pb
ClaI
ACAGTTGTTGCTACCACTGAAGCGCCAGAAGATATCGATTGAGGAGGATTTGAATGACTGACAAAATGCAAAGTT
EcoRV
rpoH

Figure 2. Preferential binding of dnaA protein to a restriction fragment containing the rpoH promoter region.

Filter binding assays were performed as described in Experimental Procedures with the indicated amounts of dnaA protein and 100 ng of 3' end-labeled pFN82 digested with HpaII. M, 3' end-labeled pBR322 DNA digested with HpaII used as a size standard; TD, total digest of pFN82; B, bound fragments eluted from the filter; F, fragments which flowed through the filter. HindIII, DNA fragments retained on the filters by 100 ng of dnaA protein were isolated, and digested with HindIII prior to electrophoresis.


dnaA protein (ng)
0
12.5
25
50
100
Hind III
M TD B F B F B F B F B F B
622
527
404
309
242
238
217
201
190
180
160
147
122
110
90
76
67
34
26
pBR ori
rpoH

conditions. DNA fragments bound with 100 ng of dnaA protein and retained on a nitrocellulose filter were isolated and digested with HindIII restriction enzyme (Figure 2). The 527 bp fragment containing the pBR322 origin and lacking a HindIII site was not digested. Absence of the fragment near 233 bp and production of subfragments of about 190-195 bp and 40 bp by HindIII confirm that the HpaII fragment bound with greatest affinity by dnaA protein contains the rpoH promoter region.

The relative binding affinity of dnaA protein to fragments containing the promoter regions of dnaA (945 bp), rpoH (659 bp), and lacUV5 (203 bp) was measured in filter binding assays (Figure 3). With equimolar amounts (in fragment) of each, higher affinity binding by dnaA protein to the rpoH promoter-containing fragment (659 bp) was observed compared to the dnaA promoter-containing fragment (945 bp). The lacUV5 fragment (203 bp) was poorly bound. The increased binding affinity to the rpoH promoter-containing fragment may be due to the presence of two dnaA protein recognition sequences in the rpoH promoter region compared to one such sequence in the region of the dnaA promoters. Alternatively, nucleotides flanking the recognition sequences may influence binding affinity.

dnaA protein binds to the dnaA boxes in the rpoH promoter

In order to locate the sites of binding by dnaA protein in the rpoH promoter region more precisely, DNase I protection assays (19) were performed. Restriction fragments containing the rpoH promoter region were 5' or 3' end-labeled at the ClaI site and incubated with varying amounts of

Figure 3. Preferential binding of dnaA protein to restriction fragments containing the dnaA promoter region, or the rpoH promoter region.

(A) Filter binding assays were performed as described in Experimental Procedures with 0.025 pmol each of 5' end-labeled DNA fragments containing the promoter regions of dnaA, rpoH, and lacUV5. The amounts of dnaA protein added are indicated. TD, total DNA used in the binding assay; B, bound fragments eluted from the filter; F, fragments which flowed through the filter.

(B) The fraction (in percent) of a DNA fragment retained and eluted from filters is expressed as a ratio to the total amount of that fragment in both the "bound" and "flow-through" lanes measured as described in Experimental Procedures.

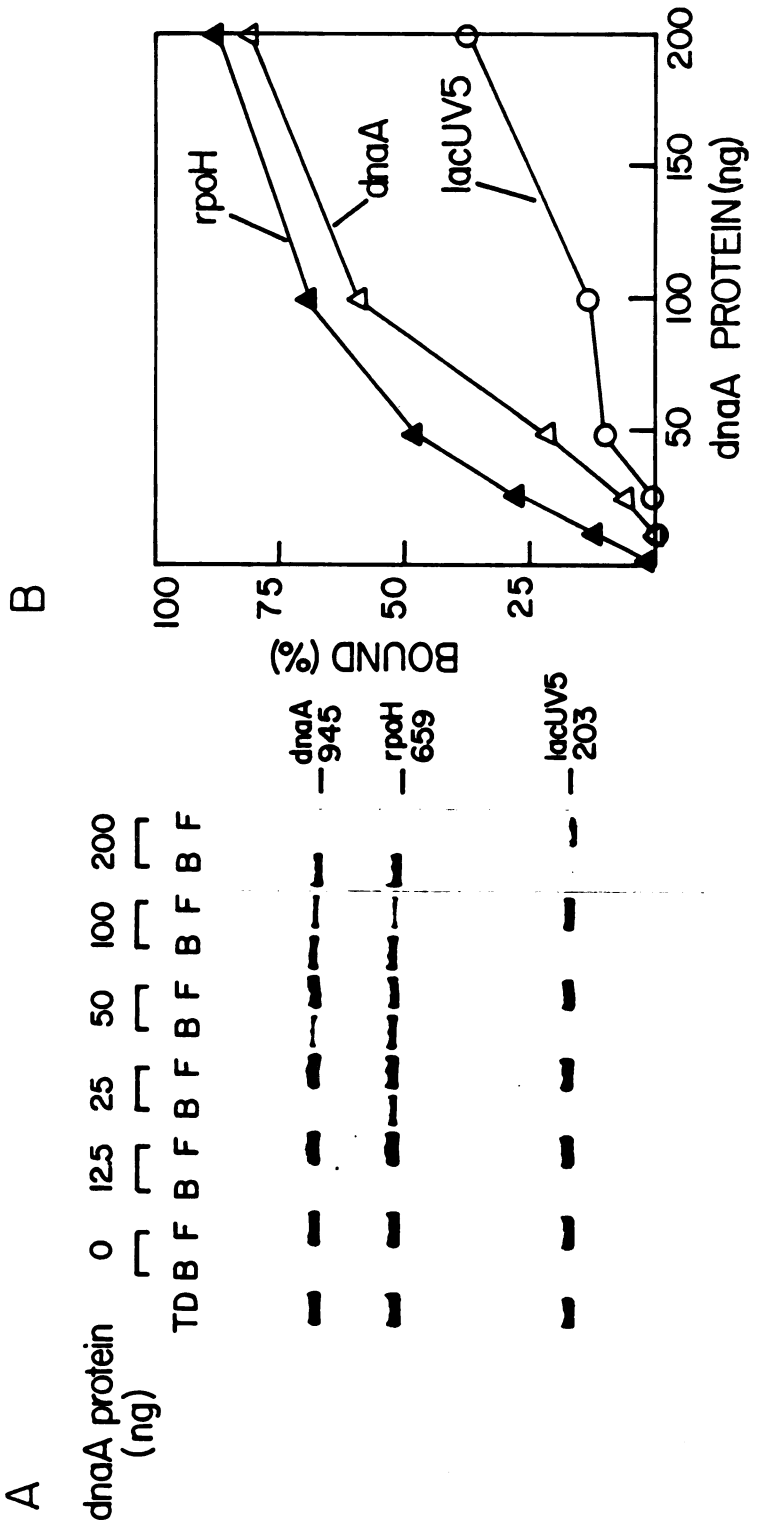
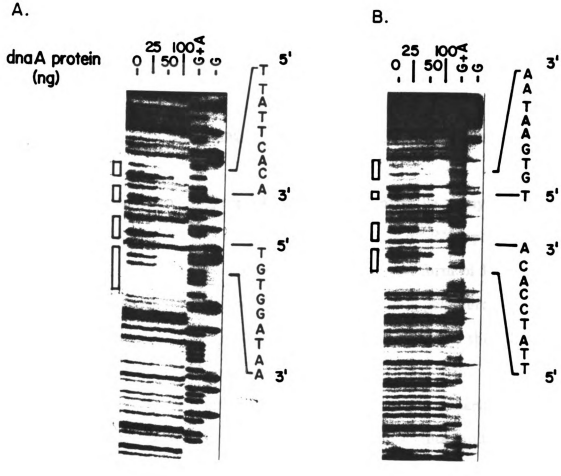

A
dnaA protein (ng)
TD B F 0 B F 12.5 B F 25 B F 50 B F 100 B F 200 B F
dnaA 945
rpoH 659
lacUV5 203
B
BOUND (%)
100
75
50
25
rpoH
dnaA
lacUV5
50
100
150
200
dnaA PROTEIN (ng)

Figure 4. dnaA protein binds to both dnaA boxes in the rpoH promoter region.

DNase I protection assays were performed with the indicated amounts of dnaA protein and (A) 3' end-labeled PvuII-ClaI fragment, or (B) 5' end-labeled HpaII-ClaI fragment as described in Experimental Procedures. The protected regions, ([]), dnaA protein recognition sequences, and lanes containing the products of Maxam-Gilbert sequencing reactions of the end-labeled DNA fragments used in the DNaseI protection assays are also indicated.


A.
dnaA protein (ng)
0
25
50
100
G+A
G
5'
TTATTCACA
3'
5'
TGTGGATAA
3'
B.
0
25
50
100
G+A
G
3'
ATAAGTGT
5'
3'
ACACCTATT
5'

dnaA protein. A limited digestion with DNase I was performed and the denatured products were separated on a sequencing gel (Figure 4A,B). Four regions of protection were observed on either strand in the vicinity of the two nine base pair dnaA boxes. This pattern of protection was observed at levels of dnaA protein sufficient for specific retention of rpoH promoter-containing fragments on nitrocellulose filters.

dnaA protein inhibits transcription from two of the three rpoH promoters in vitro

Transcription of the rpoH gene apparently involves as many as four promoters (15, 51). rpoH1P and 4P are recognized by σ^{70}-RNA polymerase. A third promoter, 3P, is apparently recognized by a novel form of RNA polymerase which may be distinct from σ^{32}-, or σ^{70}-RNA polymerase. Detection of the fourth promoter, 2P, by S1 mapping was dependent on the E. coli strain from which the RNA was isolated (15).

Different restriction fragments were used in run-off transcription assays to define the start sites and strandedness of the transcripts, and to determine the influence of dnaA protein on rpoH transcription. Run-off transcription assays from the rpoH promoters were performed with various preparations of σ^{70}-RNA polymerase purified as described (15, 22). Most preparations resulted in transcription from 1P and 4P (data not shown). This is consistent with results of others that σ^{70}- RNA polymerase mediates transcription from these promoters. One preparation resulted additionally in transcription from 3P presumably due to the presence of a novel factor sigma or a positive

regulatory protein. Due to the proximity of the dnaA protein recognition sequences to 3P, this preparation of RNA polymerase was used in the following experiments.

The DNA templates used included the PvuII-ClaI, PstI-ClaI, and EcoRV fragments containing the rpoH promoter region (Figure 1). Transcription of the 659 bp PvuII-ClaI fragment resulted in three transcripts of about 220, 75, and 65 nucleotides relative to single-stranded DNA marker fragments electrophoresed in a separate lane (Figure 5). The PstI-ClaI fragment which extends 1.2 kb upstream from the PvuII site was used as a template. Three transcripts were observed with this template and were of similar sizes as those with the PvuII-ClaI fragment. Compared to the PvuII-ClaI fragment, the 342 bp EcoRV fragment is truncated by four nucleotides on the template strand near the N-terminal rpoH coding region (Figure 1B) and is about 320 bp shorter at the other end. Three transcripts observed with the EcoRV fragment were each slightly shorter than those observed with the PvuII-ClaI fragment. ClaI restriction within the rpoH promoter region of the template DNA strand produces a 5' end 16 nucleotides from the first codon. Taking this distance into account, these experiments indicate transcriptional start sites at approximately 235, 90, and 80 nucleotides upstream from the coding sequence. Whereas these transcripts have not been mapped relative to the products of sequencing reactions of the template fragment, these results are consistent with results of others (15, 51) in identification of promoters 1P, 3P, and 4Pa (referred to henceforth as 4P) respectively (Figure 1). Transcripts from 2P or 4Pb were not observed in this or other experiments (Figure 6,

Figure 5. Run-off transcription assays with DNA fragments containing the rpoH promoters.

Assays were performed as described in Experimental Procedures with the indicated DNA fragments as templates. The positions of transcripts from the rpoH promoters are indicated. M, 3' end-labeled pBR322 DNA digested with HpaII was included as a size standard.

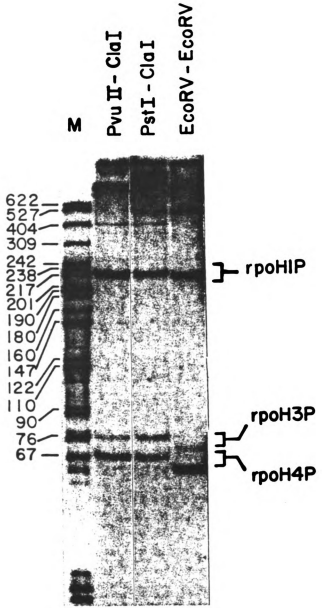
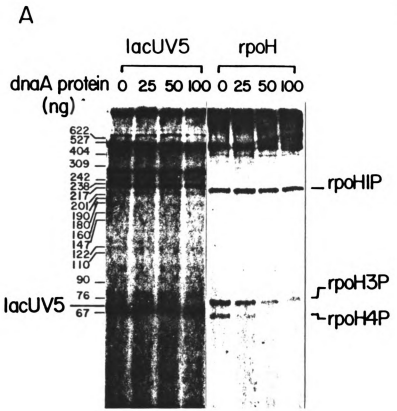
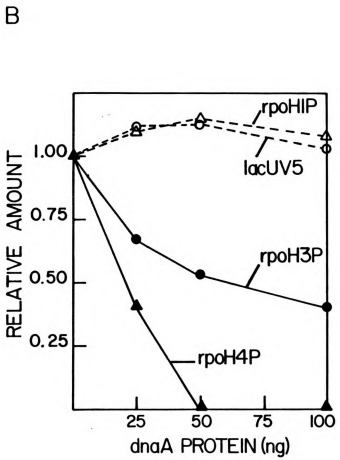

M
PvuII-ClaI
PstI-ClaI
EcoRV-EcoRV
622
527
404
309
242
238
217
201
190
180
160
147
122
110
90
76
67
rpoH1P
rpoH3P
rpoH4P

Figure 6. dnaA protein inhibits transcription from rpoH3P and rpoH4P.

(A) Run-off transcription assays were performed as described in Experimental Procedures with the 203 bp EcoR1 fragment containing the lacUV5 promoter or the 1891 bp PstI-ClaI fragment containing the rpoH promoter region. The amounts of dnaA protein added are indicated. The size standard was as in Figure 5.

(B) The relative amounts of each transcript determined as described in Experimental Procedures are expressed as a ratio compared to the amount produced with no dnaA protein added.


A
lacUV5
rpoH
dnaA protein (ng)
0 25 50 100 0 25 50 100
622
527
404
309
242
238
217
201
190
180
160
147
122
110
90
76
67
lacUV5
rpoHIP
rpoH3P
rpoH4P


B
RELATIVE AMOUNT
1.00
0.75
0.50
0.25
rpoHIP
lacUV5
rpoH3P
rpoH4P
25
50
75
100
dnaA PROTEIN (ng)

data not shown). Such experiments should have resolved 4Pa transcripts (82 or 83 nucleotides) from 4Pb transcripts (78 nucleotides). Radioactive products near the top of the autoradiogram (and in Figure 6A) are presumably due to end-to-end transcription of the restriction fragment.

Run-off transcription assays were performed with the PstI-ClaI fragment to determine the influence of dnaA protein on rpoH transcription (Figure 6A,B). Transcription from rpoH4P was markedly inhibited with comparatively less inhibition from 3P with increasing amounts of purified dnaA protein added. At these levels of dnaA protein, specific binding of dnaA protein to the rpoH promoter region was observed (Figure 2,3). Similar results of transcriptional inhibition of rpoH3P and 4P by dnaA protein were obtained with the HpaII or EcoRV DNA fragments containing the rpoH promoter region (data not shown). No inhibition by dnaA protein was observed on transcription from rpoH1P.

Run-off transcription assays were also performed with a 203 bp restriction fragment containing the lacUV5 promoter. By sequence analysis, this DNA fragment lacks sequences similar to the consensus dnaA protein recognition sequence, and is poorly bound by dnaA protein (Figure 4). The observed transcript of about 67 nucleotides is consistent with transcription from the lacUV5 promoter in the absence of cyclic AMP binding protein (Figure 6A) (37). Addition of increasing amounts of dnaA protein to these assays marginally affected the synthesis of this transcript (Figure 6B). The product of about 240 nucleotides is presumably due to end-to-end transcription of the restriction fragment. Its slightly greater apparent size

appears to be due to its anomolous electrophoretic migration in this experiment compared to other experiments of run-off transcription with this fragment (54; data not shown). Other high molecular weight products are unexplained but have been observed by others in transcription of this fragment (35).

dnaA protein inhibits rpoH transcription in vivo

The plasmid pDS596 contains the dnaA gene under inducible expression from the araB promoter (28). In the uninduced state, transcription from the araB promoter is repressed by the araC gene product encoded by the vector pING1. Based on the above in vitro results, increased levels of dnaA protein by induced expression of the cloned dnaA gene were expected to inhibit rpoH transcription.

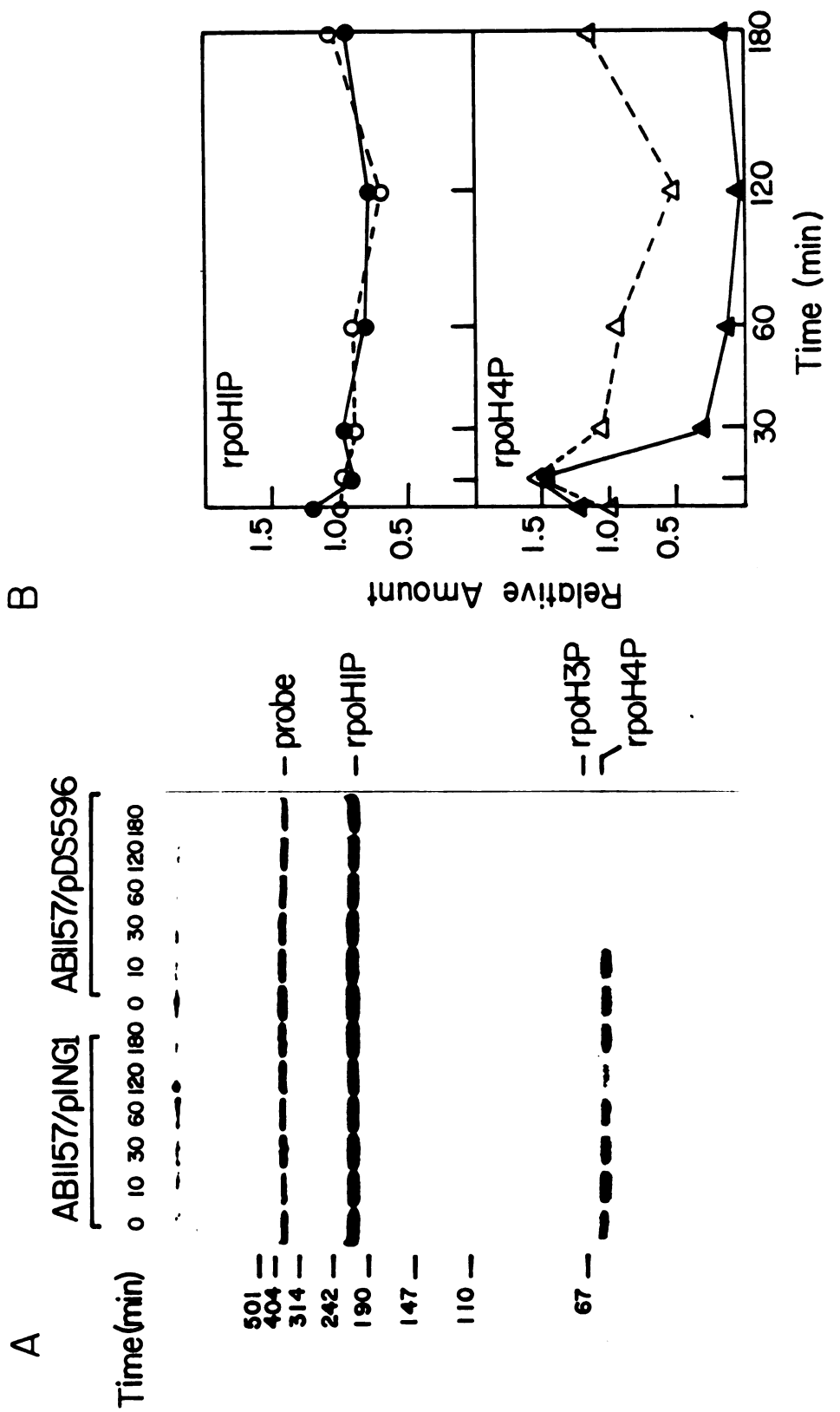
E. coli AB1157 containing either pDS596 or the vector pING1 was grown in LB media at 30°C. Expression from the araB promoter was induced by addition of arabinose. Total cellular RNA was isolated from portions of each culture removed prior to and at various times after addition of arabinose. RNA was hybridized to an excess of 5' end-labeled EcoRV restriction fragment containing the rpoH promoter region, treated with S1 nuclease, electrophoresed, and autoradiographed (Figure 7A). The relative amounts of each transcript from the rpoH promoters were determined (Figure 7B).

S1 mapping experiments with RNA from E. coli AB1157 containing the vector pING1 indicated a relatively constant level of transcript from rpoH1P prior to and at various times after addition of arabinose. Its addition

Figure 7. Inhibition of rpoH4P transcription by overproduction of dnaA protein in vivo.

(A) E. coli AB1157 harboring either the vector pING1, or pDS596 containing the dnaA gene were grown in LB media at 30°C to a turbidity at 595 nm of 0.1. Arabinose was then added to 0.75% (w/v) to induce synthesis of dnaA protein. RNA was isolated from portions of each culture removed prior to or at the indicated times after arabinose addition and used in S1 mapping experiments as described in Experimental Procedure. The molecular weight markers were 3' end-labeled pUC19 DNA digested with EcoR1 and HpaII.

(B) rpoH1P and 4P transcripts were quantitated as described in Experimental Procedures. The level of each respective transcript is expressed as a ratio compared to that contained in RNA from AB1157 harboring the vector pING1 and removed just prior to the time of arabinose addition. Open symbols (▲,○), rpoH from AB1157 containing pING1; closed symbols (▲,●), rpoH mRNA from AB1157 containing pDS596.


A
ABI157/pING1
ABI157/pDS596
Time(min)
0 10 30 60 120 180 0 10 30 60 120 180
501
404
314
242
190
147
110
67
probe
rpoHIP
rpoH3P
rpoH4P
B
Relative Amount
rpoHIP
rpoH4P
1.5
1.0
0.5
30
60
120
180
Time (min)

appeared to result in a transient increase in 4P transcript at 10 min before returning to the original level. The reason for this transient increase is not understood. Transcripts initiating from 3P were barely detectable compared to 1P and 4P. Whereas transcripts from 2P were not detected, the relative levels of other rpoH transcripts prior to arabinose addition are in agreement with observations of Erickson et al. (15), and Tobe et al. (51). The influence of arabinose addition on 3P transcription was not determined due to its low abundance prior to arabinose addition. The S1-resistant material migrating near the bottom of the gel and near 100-130 nucleotides appeared to have arisen by hybrid formation between the radioactive probe and yeast tRNA and/or the denatured salmon sperm DNA included in the reactions (data not shown).

S1 mapping experiments were performed in parallel with RNA isolated from AB1157 harboring pDS596 prior to and at various times after induction (Figure 7A). As above, a transient increase in the level of 4P transcript was observed 10 min after addition of arabinose (Figure 7B). Almost no rpoH4P transcript was detected by thirty minutes of induced expression of dnaA protein. This interval corresponds to the time after induction when the replication activity of dnaA protein was detectable in extracts from similar cultures (Hwang and Kaguni, unpublished results). Levels of 4P transcript were further reduced at later time points. By contrast, the relative level of 1P transcript was marginally altered by elevated levels of dnaA protein. As above, the effect of elevated levels of dnaA protein on transcription from rpoH3P was not determined due to its low abundance prior to arabinose

addition.

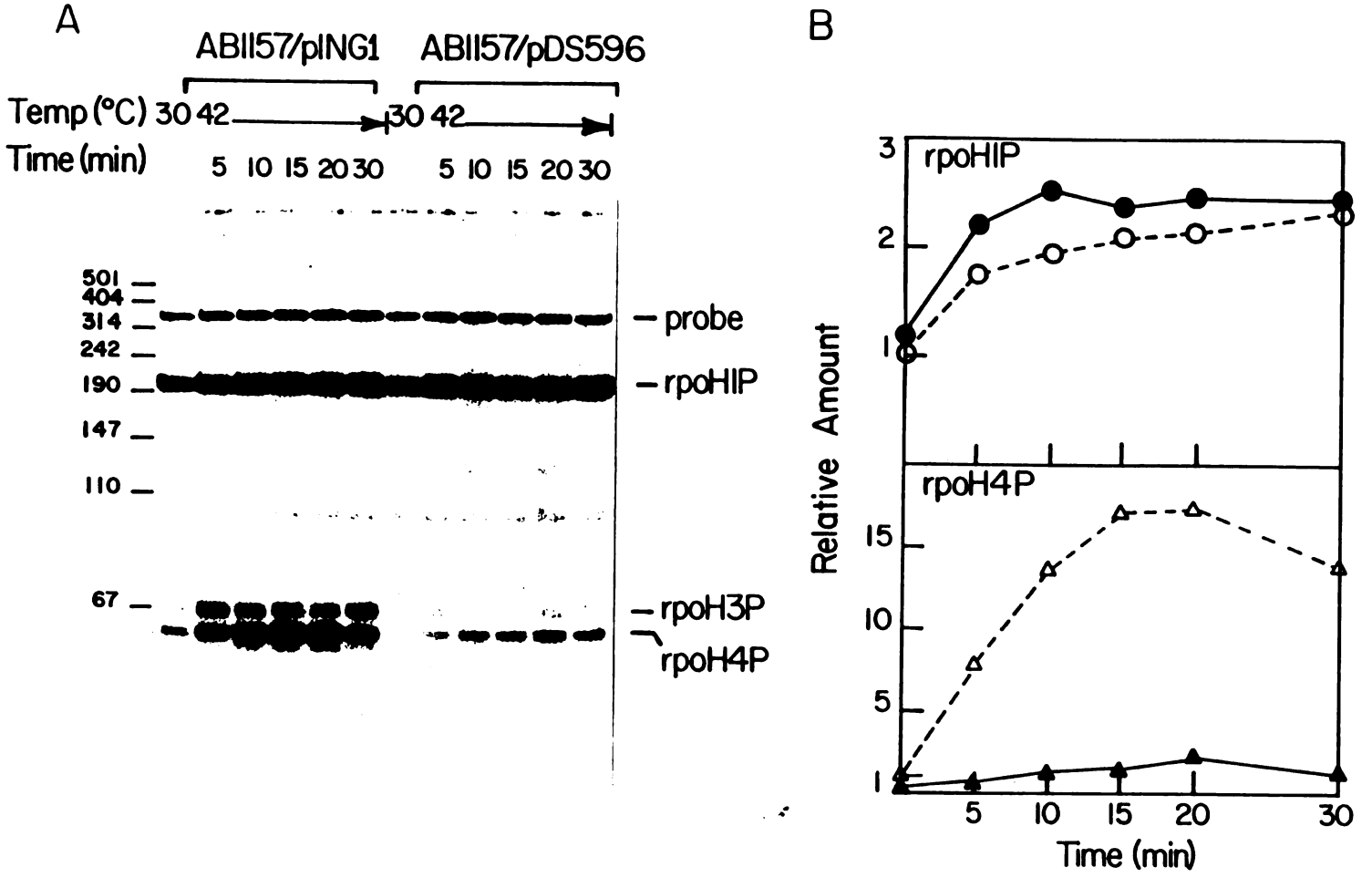
Reports indicate that the levels of rpoH transcripts increase with an increase in temperature (50, 15). Transcripts from 2P and 3P were observed to increase the most (15). This increased transcription contributes, in part, to elevated expression of heat shock genes upon a temperature upshift. To determine whether elevated levels of dnaA protein would inhibit this increase in transcript levels, AB1157 containing either pDS596 or pING1 were grown at 30°C in LB media, arabinose was added, and induced expression of dnaA protein proceeded for 2 hr at 30°C. The culture was then shifted to 42°C. RNA was isolated from portions of each culture prior to and at various times after the temperature shift.

S1 mapping experiments with RNA from AB1157 containing the vector pING1 showed a two- to three-fold increase with the temperature shift in the level of rpoH1P transcripts (Figure 8A,B). Consistent with previous observations (15), 3P transcripts were most dramatically elevated by the temperature shift. Due to the low levels of 3P transcript detected prior to the temperature shift, it was not possible to quantitate accurately the relative increase upon temperature upshift. In addition, an increase greater than 15-fold in the level of 4P transcript was observed 15 to 20 min after the temperature shift. Transcriptional start sites mapped by S1 analysis on sequencing gels for rpoH1P, 3P, and 4Pa were in agreement with Erickson et al. (15; data not shown). Transcripts from 4Pb were not detected. As in Figure 7A, the S1-resistant material near the bottom of the gel and near 100-130 nucleotides was attributed to annealing between the probe and yeast

Figure 8. Inhibition of rpoH3P and rpoH4P transcription by dnaA protein after a temperature shift.

(A) E. coli AB1157 harboring either the vector pING1, or pDS596 containing the dnaA gene were grown in LB media at 30°C to a turbidity at 595 nm of 0.1. Arabinose was added to 0.75% (w/v), the cultures were incubated at 30°C for two hours then shifted to 42°C. RNA was isolated from portions of each culture prior to or at the times indicated after the temperature shift. S1 nuclease mapping experiments were performed as described in Experimental Procedures. The size standard was as in Figure 7.

(B) rpoH1P and 4P transcripts were quantitated as described in Experimental Procedures and normalized to the level of each respective transcript contained in RNA from AB1157 harboring pING1 at 30°C. Open symbols (△,○), rpoH mRNA from AB1157 containing pING1; closed symbols (▲,●), rpoH mRNA from AB1157 containing pDS596.


A
ABI157/pING1
ABI157/pDS596
Temp (°C) 30 42 30 42
Time (min) 5 10 15 20 30 5 10 15 20 30
501
404
314
242
190
147
110
67
probe
rpoHIP
rpoH3P
rpoH4P
B
rpoHIP
rpoH4P
Relative Amount
3
2
1
15
10
5
1
5 10 15 20 30
Time (min)

tRNA and/or the denatured salmon sperm DNA included in the reactions (data not shown). Transcripts from 2P were not observed.

S1 mapping experiments with RNA isolated from AB1157 containing pDS596 indicated that rpoH1P transcripts increased to between two- to three-fold (Figure 8A,B). This result was similar to levels observed with RNA from AB1157 containing the vector. By contrast, the large increase in 3P and 4P transcripts was not observed after the temperature shift. Based on other experiments (28), this strain presumably contains elevated levels of dnaA protein which inhibits transcription from 3P upon the temperature shift. For reasons described above, the levels of 3P transcripts were not quantitated. While the amount of 4P transcripts increased to three- fold with time after the temperature shift, levels of this mRNA were far less than those observed with AB1157 containing the vector pING1 at similar times. These results indicate that dnaA protein preferentially inhibits transcription from rpoH3P and 4P in vivo.

DISCUSSION

Upon a shift to high temperature, E. coli expresses a set of about seventeen proteins in a phenomenon termed the heat shock response. This response appears to have been conserved in all organisms suggesting its essential role in cell physiology.

Due to the presence of two presumptive dnaA protein recognition sequences in the rpoH promoter region, we were interested in determining whether dnaA protein would specifically bind to these sites to influence rpoH expression. The results shown here indicate that dnaA protein specifically bound to the rpoH promoter region to repress transcription from promoters 3P and 4P. This inhibition may involve occlusion of RNA polymerase by dnaA protein. That transcription from 1P was not influenced suggests that dnaA protein bound at a down stream site does not interfere markedly with the progress of a transcribing RNA polymerase. This result is consistent with the observation that lac repressor bound at a distal site only transiently blocks a transcriptional elongation complex (44, 27).

Although dnaA protein can be isolated as a monomer, its form as a transcriptional repressor remains to be determined. In studies of dnaA protein binding to the dnaA promoter region (18, 7), a complex DNase I footprint was observed which extended 40 to 50 bp on either side of the dnaA protein recognition sequence. This result appears to have been due to addition of excess amounts of dnaA protein. At levels of dnaA protein (2 pmol of protein

with 0.025 pmol of fragment) sufficient to specifically inhibit rpoH (this study) and dnaA transcription (54; unpublished results), protection from DNase I was limited to a 30 to 40 bp region encompassing the dnaA protein recognition sequence. In comparison, lac repressor, a tetramer of identical subunits, protects a 25 bp region when bound to its operator (19).

The role of the heat shock response is not well understood. Proposed models relate the heat shock response to thermotolerance, protein degradation, or cell division. In E. coli, thermotolerance upon temperature upshift is dependent on a brief incubation at an elevated but nonlethal temperature (55). In this previous study, exposure of cells to this intermediate temperature was presumed necessary for expression of heat shock proteins. However, induction of the rpoH gene product under control of the inducible tac promoter at low temperature did not confer thermotolerance at high temperature (53). Thermotolerance did not appear to depend on rpoH-dependent expression of heat shock proteins.

The production of abnormal proteins in E. coli by incorporation of puromycin, the arginine analog, canavanine, or by induced synthesis of a foreign protein results in elevated levels of heat shock proteins (21). Other conditions which induce the heat shock response may also generate abnormal polypeptides (1). Since Lon, an ATP-dependent protease (13, 12), is a heat shock protein, the heat shock response may function in protein degradation.

The role of the heat shock response in cell division is based on studies of rpoH mutants. Whereas rpoH nonsense (and some missense) mutants are viable up to 30°C in nonsuppressing strains due to the production of trace

amounts of σ^{32}, an rpoH::kan mutant is viable only at or below 20°C (51). σ^{32} appears to be essential at higher growth temperatures. When such mutants are shifted to 42°C, cell division stops with an increase in mass to form long filaments (52).

Other treatments which result in expression of heat shock proteins include ethanol, UV irradiation, or bacteriophage infection. That dnaA protein negatively regulates rpoH expression suggests that these treatments may inactivate dnaA protein as a repressor or, in the case of bacteriophage infection, that a site(s) on the replicating viral DNA or a viral gene product acts as a competitor for binding of dnaA protein.

In E. coli as in many organisms, DNA replication and cell growth are tightly coordinated events. dnaA protein functions at an early step in initiation of DNA replication from the chromosomal origin, oriC (57, 32, 6). That expression of rpoH is influenced by this initiator protein for DNA replication and that rpoH mutants render the cell incapable of proper cell division (52) suggest a regulatory mechanism whereby dnaA protein may coordinate initiation of DNA replication to the expression of genes under control of rpoH which are required for cell division.

With regard to this, an allele of dnaK, a heat shock gene, has been isolated which is conditionally defective in initiation of DNA replication (45). We have recently determined that dnaK protein stimulates the replication activity of a mutant form of dnaA protein (Hwang and Kaguni, unpublished results). dnaK protein also appears to influence the activity of wild type dnaA protein in replication (Carr and Kaguni, unpublished results). The ability of

dnaA protein to regulate rpoH expression may influence the level of dnaK protein which in turn influences the activity of dnaA protein in initiation.

REFERENCES

1. Anathan, J.A., Goldberg, A.L., and Voellmy, R. (1986). Science 232, 522-524.

2. Atlung, T., Clausen, E.S., and Hansen, F.G. (1985). Mol. Gen. Genet. 200, 442-450.

3. Baker, T.A., Sekimizu, K., Funnell, B.E., and Kornberg, A. (1986). Cell 45, 53-64.

4. Bloom, M., Skelly, S., VanBogelen, R.A., Neidhardt, F.C., Brot, N., and Weissbach, H. (1986). J. Bacteriol. 166, 380-384.

5. Bradford, M.M. (1976). Anal. Biochem. 72, 248-254.

6. Bramhill, D., and Kornberg, A. (1988). Cell 52, 743-755.

7. Braun, R.E., O'Day, K., and Wright, A. (1985). Cell 40, 159-169.

8. Brosius, J., Cate, R.L., and Perlmutter, P.A. (1982). J. Biol. Chem. 257, 9205-9210.

9. Burgers, P.M.J., Kornberg, A., and Sakakibara, Y. (1981). Proc. Natl. Acad. Sci. USA 78, 5391-5395.

10. Burgess, R.R., and Jendrisak, J.J. (1975). Biochem. 14, 4634-4638.

11. Chakraborty, T., Yoshinaga, K., Lother, H., and Messer, W. (1982). EMBO J. 1, 1545-1549.

12. Charette, M.F., Henderson, G.W., and Markovitz, A. (1981). Proc. Natl. Acad. Sci. USA 78, 4728-4732.

13. Chung, C.H., and Goldberg, A.L. (1981). Proc. Natl. Acad. Sci. USA 78, 4931-4935.

14. Cowing, D.W., Bardwell, J.C.A., Craig, E.A., Woolford, C., Hendrix, R., and Gross, C.A. (1985). Proc. Natl. Acad. Sci. USA 80, 2679-2683.

15. Erickson, J.W., Vaughn, V., Walter, W.A., Neidhardt, F.C., and Gross, C.A. (1987). Genes and Development 1, 419-432.

16. Frey, J., Chandler, M., and Caro, L. (1979). Mol. Gen. Genet. 174, 1117-1126.

17. Fuller, R.S., and Kornberg, A. (1983). Proc. Natl. Acad. Sci. USA 80, 5817-5821.

18. Fuller, R.S., Funnell, B.E., and Kornberg, A. (1984). Cell 38, 889-900.

19. Galas, A., and Schmitz, D.J. (1978). Nucl. Acids Res. 5, 3157-3170.

20. Gill, D.R., Hatfull, G.F., and Salmond, G.P.C. (1986). Mol. Gen. Genet. 205, 134-145.

21. Goff, S.A., and Goldberg, A.L. (1985). Cell 41, 587-595.

22. Gonzalez, N., Wiggs, J., and Chamberlin, M.J. (1977). Arch. Biochem. Biophys. 182, 404-408.

23. Grossman, A.D., Erickson, J.W., and Gross, C.A. (1984). Cell 38, 383-390.

24. Hansen, E.B., and Yarmolinsky, M.B. (1986). Proc. Natl. Acad. Sci. USA 83, 4423-4427.

25. Hasunuma, K., and Sekiguchi, M. (1977). Mol. Gen. Genet. 154, 225-230.

26. Hirota, Y., Mordoh, J., and Jacob, F. (1970). J. Mol. Biol. 53, 369-387.

27. Horowitz, H., and Platt, T. (1982). Nucl. Acids Res. 10, 5447-5465.

28. Hwang, D.S., and Kaguni, J.M. (1988). J. Biol. Chem. 263, 10625-10632.

29. Johnston, S., Lee, J.-H., and Ray, D.S. (1985). Gene 34, 137-145.

30. Kline, B.C., Kogoma, T., Tam, J.E., and Shields, M.S. (1986). J. Bacteriol. 168, 440-443.

31. Kucherer, C., Lother, H., K olling, R., Schauzu, M.-A., and Messer, W. (1986). Mol. Gen. Genet. 205, 115-121.

32. Kung, F.C., and Glaser, D.A. (1978). J. Bacteriol. 133, 755-762.

33. Landick, R., Vaughn, V., Lau, E.T., VanBogelen, R.A., Erickson, J.W., and Neidhardt, F.C. (1984). Cell 38, 175-182.

34. Lesley, S.A., Thompson, N.E., and Burgess, R.R. (1987). J. Biol. Chem. 262, 5404-5407.

35. Lorimer, D.D., and Revzin, A. (1986). Nucl. Acids Res. 14, 2921-2938.

36. Lother, H., Kolling, R., Kucherer, C., and Schauzu, M. (1985). EMBO J. 4, 555-560.

37. Maizels, N. (1973). Proc. Natl. Acad. Sci. USA 70, 3585-3589.

38. Maxam, A.M., and Gilbert, W. (1980). Meth. Enzymol. 65, 499-560.

39. Miller, J.H. (1972). Experiments in Molecular Genetics. p. 433. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

40. Murakami, Y., Ohmori, H., Yura, T., and Nagata, T. (1987). J. Bacteriol. 169,1724-1730.

41. Neidhardt, F.C., VanBogelen, R.A., and Lau, E.T. (1983). J. Bacteriol. 153, 597-603.

42. Neidhardt, F.C., and VanBogelen, R.A. (1987). In Cellular and Molecular Biology (Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M., and Umbarger, H.E., eds), Vol. 2, pp 1334-1345, American Society for Microbiology, Washington, D.C.

43. Ortega, S., Lanka, E., and Diaz, R. (1986). Nucl. Acids Res. 14, 4865-4879.

44. Reznikoff, W.S., Miller, J.H., Scaife, J.G., and Beckwith, J.R. (1969). J. Mol. Biol. 43, 201-213.

45. Sakakibara, Y. (1988). J. Bacteriol. 170, 972-979.

46. Sekimizu, K., Bramhill, D., and Kornberg, A. (1987). Cell 50, 259-265.

47. Seufert, W., and Messer, W. (1987). Cell 48, 73-78.

48. Stuitje, A.R., de Wind, N., van der Spek, J.C., Pors, T.H., and Meijer, M. (1986). Nucl. Acids Res. 14, 2333-2344.

49. Straus, D.B., Walter, W.A., and Gross, C.A. (1987). Nature 329, 348-351.

50. Tilly, K., Erickson, J., Sharma, S., and Georgopoulos, C. (1986). J. Bacteriol. 168, 1155-1158.

51. Tobe, T., Kusukawa, N., and Yura, T. (1987). J. Bacteriol. 169, 4128-4134.

52. Tsuchido, T., VanBogelen, R.A., and Neidhardt, F.C. (1986). Proc. Natl. Acad. Sci. USA 83, 6959-6963.

53. VanBogelen, R.A., Acton, M.A., and Neidhardt, F.C. (1987). Genes &

Development 1, 525-531.

54. Wang, Q., and Kaguni, J.M. (1987). Mol. Gen. Genet. 209, 518-525.

55. Yamamori, T., and Yura, T. (1982). Proc. Natl. Acad. Sci. USA 79, 860-864.

56. Yura, T., Tobe, T., Ito, K., and Osawa, T. (1984). Proc. Natl. Acad. Sci. USA 81, 6803-6807.

57. Zyskind, J.W., Deen, L.T., and Smith, D.W. (1977). J. Bacteriol. 129, 1446-1475.

Chapter IV

A NOVEL SIGMA FACTOR INVOLVED IN EXPRESSION OF THE rpoH GENE OF Escherichia coli

ABSTRACT

The E. coli rpoH gene encoding σ^{32} involved in the heat shock response is transcribed by as many as four promoters. We have isolated a novel sigma factor of about 24 KDa from a preparation of RNA polymerase by electroelution from SDS-polyacrylamide gels. The renatured protein conferred upon core RNA polymerase the ability to transcribe preferentially from an rpoH promoter which is regulated by dnaA protein. Whereas the stringent response which induces expression of heat shock proteins resulted in a decrease in transcript levels from this promoter, other rpoH transcripts were not elevated. This result suggests that induction of heat shock proteins by the stringent response is not mediated by increased transcription of the rpoH gene.

INTRODUCTION

The heat shock response in E. coli induced by such treatments as a sudden temperature upshift, bacteriophage infection, or UV irradiation results in preferential synthesis of at least 17 heat shock proteins (reviewed in 28). The rpoH gene product, σ^{32}, plays a central role in this response by conferring upon RNA polymerase the ability to initiate transcription from promoters of heat shock genes (8, 15, 22). Upon temperature upshift, the increased expression of heat shock proteins is attributable to an increase in σ^{32} due to increased expression and stability (10, 12, 32, 33). A reprogramming of RNA polymerase ensues.

Recent experiments have examined transcription of the rpoH gene (10, 12, 33). Four promoters designated rpoH1P, 2P, 3P, and 4P have been mapped at about 220, 130, 90, and 80 bp, respectively, upstream from the coding sequence (10, 12). Promoters 1P and 4P are recognized by σ^{70}-RNA polymerase ($E\sigma^{70}$) (3, 10, 12). A third promoter, rpoH2P appears to be strain-specific. In vitro transcription from the fourth promoter, rpoH3P, does not involve $E\sigma^{70}$ or $E\sigma^{32}$ (3, 10). These results suggest that a novel sigma factor or a positive regulatory protein may be required for transcription from rpoH3P. In related experiments examining regulation of the rpoH gene, we identified a preparation of RNA polymerase able to initiate transcription from rpoH1P, 3P, and 4P (35). From this, we have isolated a protein of 24 KDa which confers rpoH3P specific transcription to core RNA polymerase. We also

report on transcriptional regulation of the rpoH gene during the stringent response.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli W3110 and AB1157 thr-1, ara-14, leuB6, del(gpt-proA)62, lacY1, tsx-33, supE-44, galK2, hisG4, rpsL31, xyl-5, mtl-1, argE3, thi-1 were from the E. coli Genetic Stock Center. Plasmid pFN82 was from Dr. Frederick C. Neidhardt (University of Michigan). To construct this plasmid, a 2.5 kb PstI fragment containing the rpoH promoter region and adjacent N-terminal coding region was inserted at the single PstI site of pBR322 (27). Plasmid pUC19 was from this laboratory. After restriction and gel electrophoresis, DNA fragments were purified by electroelution with an ISCO 1750 concentrator followed by ethanol precipitation. The DNA concentration of a purified fragment was determined by comparison to different amounts of electrophoretically separated restriction enzyme digests stained with ethidium bromide.

Enzymes

Restriction enzymes EcoRI, EcoRV, ClaI, and T4 polynucleotide kinase were purchased from New England Biolabs; PstI was from BRL; HpaII and S1 nuclease were from Pharmacia. Samples of $E\sigma^{70}$ or core RNA polymerase designated holo B and core B, respectively, lacking this novel sigma factor were purified from W3110 as described (6, 14). This procedure, modified by chromatography on a TSK 3000SW (Altex) high performance gel permeation

column instead of Biogel A5m (Bio-Rad), may have resulted in the presence of this novel sigma factor in the resultant core and holoenzyme fractions cited as core A and holo A, respectively. σ^{70} was obtained by separation of σ^{70} from $E\sigma^{70}$ on a Bio-Rex 70 (Bio-Rad) column (6). Protein determination was by the method of Bradford with bovine serum albumin as a standard (4).

Elution of proteins from SDS-polyacrylamide gels

The preparation of core RNA polymerase containing the novel sigma factor (1 mg of core A) was electrophoresed on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide vertical slab gel (15 cm x 15 cm x 1.5 mm) essentially as described (17). The gel was cut into 10 fractions to separate proteins by size. Proteins were electroeluted using an ISCO 1750 concentrator. The eluted samples were divided into two equal parts, and precipitated by four volumes of acetone (HPLC grade). One part of each fraction was resuspended in 10 µl of 67 mM Tris-HCl pH 7.0, 0.001% bromophenol blue, 10% glycerol (v/v), 5% mercaptoethanol (v/v), and 2% SDS, boiled for two minutes, separated on a 6%-20% SDS-polyacrylamide gradient gel, stained with Coomassie brilliant blue, then silver stained (36) to visualize proteins. The other part was renatured in 500 µl as described (17), and assayed for rpoH3P stimulatory activity by addition to run-off transcription assays containing core RNA polymerase (core B). The recovery of protein judged by Coomassie blue staining, and activity of this novel sigma factor relative to the sample loaded on the SDS-polyacrylamide gel was about 10-20% and 8%, respectively.

Run-off transcription assays

Run-off transcription assays were performed with 0.025 pmol of the PstI-ClaI fragment and 0.65 pmol of RNA polymerase, unless indicated, in 10 µl of transcription buffer (34) at 37°C for 10 minutes in the absence of ribonucleotides. Heparin (100 µg/ml), 300 µM each of ATP, GTP, CTP, and 50 µM of [α^{32}P] UTP (2.5 µCi) (New England Nuclear) were added to a final volume of 11 µl to inhibit further open complex formation and to initiate transcription. Incubation was continued at 37°C for 10 minutes. Reactions were then stopped, and samples were ethanol precipitated, resuspended, and electrophoresed in an 8% polyacrylamide gel containing 7 M urea. Open complex formation was in 5 µl when [^{35}S] UTPαS (New England Nuclear) was used as the radioactive nucleotide (1,37). Heparin, ATP, GTP, and CTP at the above concentrations, and 0.5 uM (2.5 uCi) of [^{35}S] UTPαS were added to a final volume of 5.5 µl. After incubation at 37°C for 5 minutes, UTP was added to 300 uM and the reactions (6 µl) were further incubated for another 10 minutes to complete one cycle of transcription. An equal volume of buffer containing 80% formamide, 10 mM NaOH, 1 mM EDTA, and 0.025% bromophenol blue was added to stop the reactions. Samples were incubated at 100°C for 2 min and electrophoresed as described above. Dried gels were autoradiographed with Kodak XAR-5 film.

Induction of the stringent response

AB1157 was grown to a turbidity at 595 nm of 0.3 in M9 minimal media (26) supplemented with 0.4% glucose and 18 L-amino acids (without valine

and isoleucine) each at 100 µg/ml (21). The culture was then split into two equal parts. RNA was isolated from portions of each culture (10 ml) removed before and at various times after the addition of valine (500 µg/ml), or valine and isoleucine (each at 500 µg/ml) (5). Valine addition results in isoleucine limitation. The efficacy of the stringent response by valine addition was confirmed by the reduction of rpmH mRNA of about 20 fold which encodes ribosomal protein L34 (data not shown). As a control, simultaneous addition of isoleucine and valine did not markedly change rpmH transcript levels measured by S1 nuclease protection assays.

Detection of in vivo synthesized transcripts

Quantitative S1 nuclease protection assays were performed as described (35) by hybridization at 45°C for 12-16 hr in 20 µl of buffer containing 40 mM PIPES pH 6.4, 1 mM EDTA, 0.4 M NaCl, and 80% formamide with 50 µg of the isolated RNA to 0.025 pmol of 5' end-labeled EcoRV restriction fragment containing the rpoH promoter region. The restriction fragment was confirmed to be present in excess (data not shown). After hybridization, 300 µl of cold S1 buffer containing 50 mM sodium acetate pH 4.6, 0.25 M NaCl, 4.5 mM ZnSO4, 20 µg/ml of denatured salmon sperm DNA and 50 units of S1 nuclease were added. Reactions were incubated at 37°C for 15 minutes. Ammonium acetate and EDTA were added to 0.25 M and 5 mM, respectively. The samples were then ethanol precipitated, resuspended, electrophoresed, and autoradiographed as described above.

RESULTS

Identification of a factor required for rpoH3P transcription

The rpoH promoters are contained in a 1.89 kb PstI-ClaI restriction fragment (10, 12) (Figure 1). While coding sequences for ftsX and part of ftsE are present, the promoter for this operon is not (13). Run-off transcription assays with this fragment were performed with various $E\sigma^{70}$ preparations purified as described (6, 14). Such preparations have been shown to contain σ^{32}, and dnaK protein among others (23, 31). Most preparations examined resulted in RNAs of 210 and 67 nucleotides and correspond to transcription from 1P and 4P. These promoters are recognized by $E\sigma^{70}$ (10). The results from such a preparation of $E\sigma^{70}$ (holo B) are in Figure 2. RNA polymerase purified by a modification of the above procedure (Materials and Methods) was chromatographed to separate the core enzyme from $E\sigma^{70}$ (14). The holoenzyme fraction (holo A) was able to transcribe not only from 1P and 4P but also from 3P to form a 72 nucleotide RNA (Figure 2). Transcription from 3P with this and the core fraction (core A) were presumably due to the presence of a contaminating factor (see below). Due to residual amounts of σ^{70} in the core fraction, low levels of 1P transcript also were observed. The strandedness of these transcripts was confirmed by use of different DNA fragments (35, data not shown). Transcripts near the top of the gel presumably resulted from nonspecific transcription. The 190 nucleotide transcript is unexplained and has not been detected in similar

Figure 1. Physical map of the rpoH promoter region (13,22,38).

Approximate positions of relevant restriction sites, rpoH promoters (⇨), coding sequences, polarity (➡) of the rpoH, ftsX, and ftsE genes, and restriction fragments are indicated.

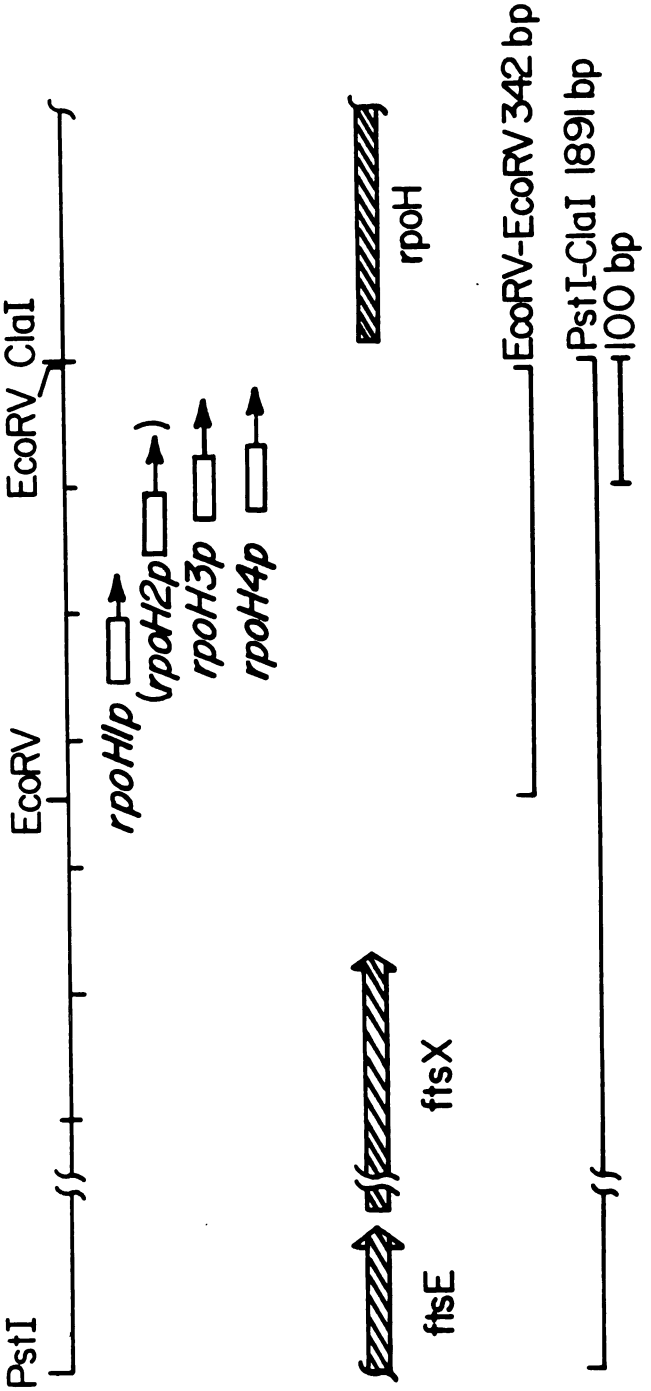
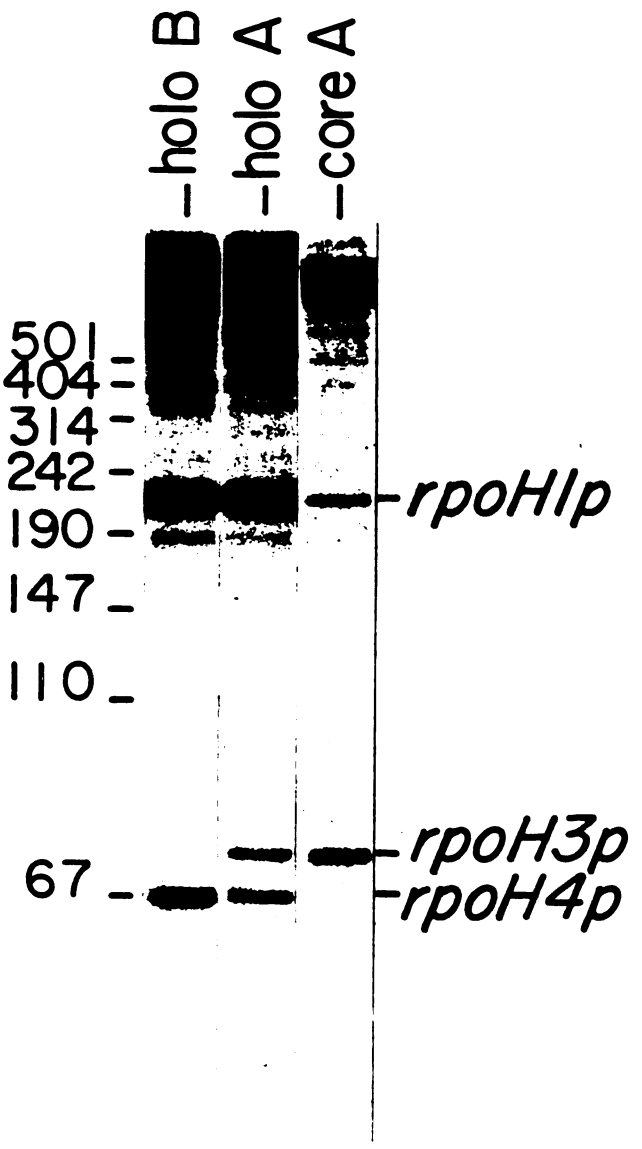

PstI
EcoRV
EcoRV ClaI
rpoHIp
(rpoH2p
rpoH3p
rpoH4p
rpoH
ftsE
ftsX
EcoRV-EcoRV 342 bp
PstI-ClaI 1891 bp
100 bp

Figure 2. Run-off transcription assays with preparations of RNA polymerase.

Assays were performed as described in Materials and Methods with the indicated preparations of RNA polymerase. Transcripts from the rpoH promoters and sizes of denatured restriction fragments of pUC19 digested with HpaII and EcoRI are indicated.


-holo B
-holo A
-core A
501
404
314
242
190
147
110
67
rpoH1p
rpoH3p
rpoH4p

experiments(35).

The rpoH3P transcription factor is a heat-stable sigma factor

Preliminary experiments indicated that this transcription factor was relatively heat-stable (data not shown, see below). This property was utilized to address its mechanism of action. If this factor functions as a sigma factor, its addition to a preparation of core enzyme is expected to confer specific transcription from rpoH3P. A sample of core RNA polymerase containing this transcription factor (core A), when heated at 65°C for 5 min., was unable to transcribe from rpoH1P, 3P, or 4P (Figure 3). An unrelated preparation of core RNA polymerase (core B) was obtained which cannot initiate transcription from 3P. This preparation contained low levels of σ^{70} as indicated by the relative level of transcription from rpoH1P. Upon addition of the heat treated sample to this core fraction, transcription from 3P was observed suggesting that this stimulatory factor acts as a novel sigma factor (see below also). This presumed sigma factor retained about 70% of its transcriptional activity after heat treatment.

The rpoH3P transcription factor is a protein of 24 KDa

To determine the size of this sigma factor, the core enzyme preparation containing this activity (core A) was electrophoresed in an SDS-polyacrylamide gel. Proteins were electroeluted from gel slices and renatured as described (17). Renatured fractions were assayed for 3P transcriptional activity by addition to core enzyme (core B) lacking this activity (Figure 4A). Most of the

Figure 3. The sigma factor which confers rpoH3P recognition is heat stable.

A preparation of core RNA polymerase (core A) containing the rpoH3P stimulatory factor was not heated, or heated at 65°C for 5 min. in 10 µl of transcription buffer in the absence of DNA. The untreated or treated samples were assayed in run-off transcription (11 µl reaction volume) in the presence or absence of a preparation of core enzyme (core B) lacking this stimulatory activity. The reaction with both core B and heat treated core A were with 0.65 pmol of each.

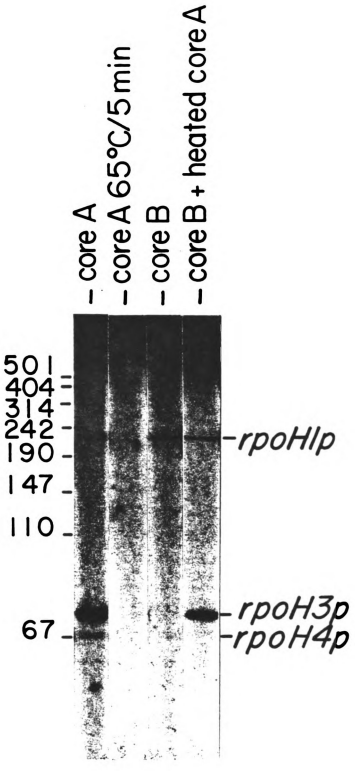
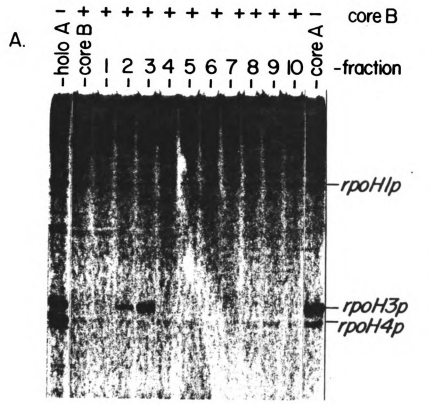

– core A
– core A 65°C/5 min
– core B
– core B + heated core A
501
404
314
242
190
147
110
67
rpoH1p
rpoH3p
rpoH4p

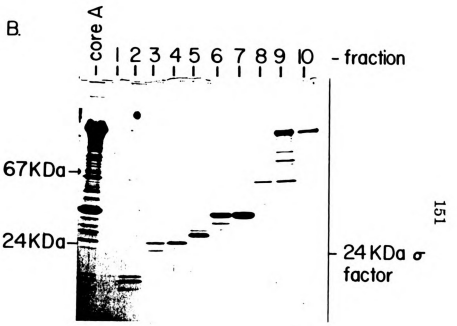
Figure 4. Isolation of a novel sigma factor by electroelution from an SDS-polyacrylamide gel.

The core enzyme preparation (core A) containing the stimulatory factor was electrophoresed in a 10% SDS-polyacrylamide slab gel. The gel was fractionated into 10 slices. Proteins were recovered by electroelution as described in Materials and Methods.

(A) Proteins from each fraction indicated were renatured and assayed for rpoH3P sigma factor activity in run-off transcription assays with [^{35}S]UTP by addition of 1 µl to a preparation of core enzyme (core B) lacking this sigma factor. Control assays were with RNA polymerase preparations containing this activity (holo A, core A). Transcripts from rpoH1P, 3P, and 4P are indicated.

(B) Proteins recovered from the indicated fractions were electrophoresed on an SDS-polyacrylamide gel as described in Materials and Methods and silver-stained. Positions of the 24 KDa sigma factor, and molecular weight markers bovine serum albumin (67 KDa), and trypsinogen (24 KDa) are indicated. For comparison, 20 µg of the core enzyme preparation (core A) from which this sigma factor was isolated was also electrophoresed on this gel.


A.
holo A
core B
1 2 3 4 5 6 7 8 9 10
core A
core B
fraction
rpoH1p
rpoH3p
rpoH4p


B.
core A
1 2 3 4 5 6 7 8 9 10
fraction
67KDa
24KDa
24 KDa σ factor

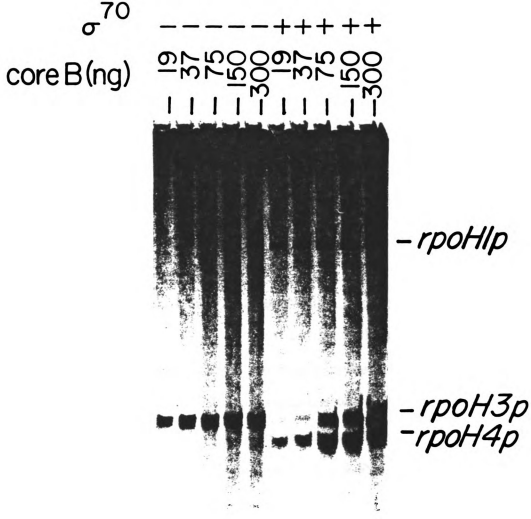
3P transcriptional activity was in fraction 3. A minor amount was in fraction 2. A silver-stained polyacrylamide gel of proteins recovered from these fractions revealed two bands near 24 KDa (Figure 4B). As fraction 4 was enriched in the larger polypeptide contained in fraction 3 yet lacked the activity of this novel sigma factor, it is unlikely that this polypeptide represents this novel sigma factor. The presence of the smaller polypeptide in fractions 2 and 3 correlates well with activity levels in these fractions. This protein migrated slightly faster than trypsinogen of 24 KDa. Related experiments in which the gel was cut into narrower slices to separate these two polypeptides of similar sizes confirmed that the smaller polypeptide is this novel sigma factor (data not shown). Assuming that polypeptides are equally silver-stained, this sigma factor is a trace contaminant relative to other contaminating polypeptides in this core enzyme preparation.

The 24 KDa protein acts as a sigma factor

Sigma factors of RNA polymerase are physically associated with the core enzyme to confer promoter recognition. Whereas other contaminating polypeptides are present in the core preparation (Figure 4B), this novel sigma factor in preparations of core and holoenzyme (Figure 2) suggests its physical association. Experiments were performed to examine whether the renatured 24 KDa protein would compete with σ^{70} in interacting with the core enzyme. Increasing levels of core enzyme (core B) lacking this novel activity were added to run-off transcription assays containing a constant level of the renatured polypeptide (Figure 5). Transcripts from 3P increased then

Figure 5. Competition between the 24 KDa protein and σ^{70} for core enzyme.

A constant amount (estimated to be less than 1 ng from the Coomassie blue stained gel) of the renatured 24 KDa protein and increasing amounts of core B, as indicated, were mixed in the absence or the presence of 50 ng (0.7 pmol) of σ^{70} in run-off transcription assays. Assays were performed as described in Materials and Methods with [^{35}S-thiol]UTP. Transcripts from rpoH1P, 3P, and 4P are indicated.


σ70
– – – – – + + + + + +
core B (ng)
19
37
75
150
300
19
37
75
150
300
– rpoHIp
– rpoH3p
– rpoH4p

remained relatively constant with increasing amounts of core enzyme indicating that the 24 KDa protein was limiting at higher levels. The increase in transcription from rpoH1P was presumably due to increasing levels of σ^{70} by addition of this core enzyme preparation.

In the presence of constant levels of σ^{70} and the renatured 24 KDa polypeptide, increasing amounts of core enzyme resulted in increased transcription from rpoH1P and 4P. At low levels of core RNA polymerase, reduced transcription from rpoH3P was observed in the presence of excess σ^{70} relative to assays lacking it. This result indicates that σ^{70} competes with the 24 KDa protein in interacting with the core enzyme and that this 24 KDa protein acts as a sigma factor.

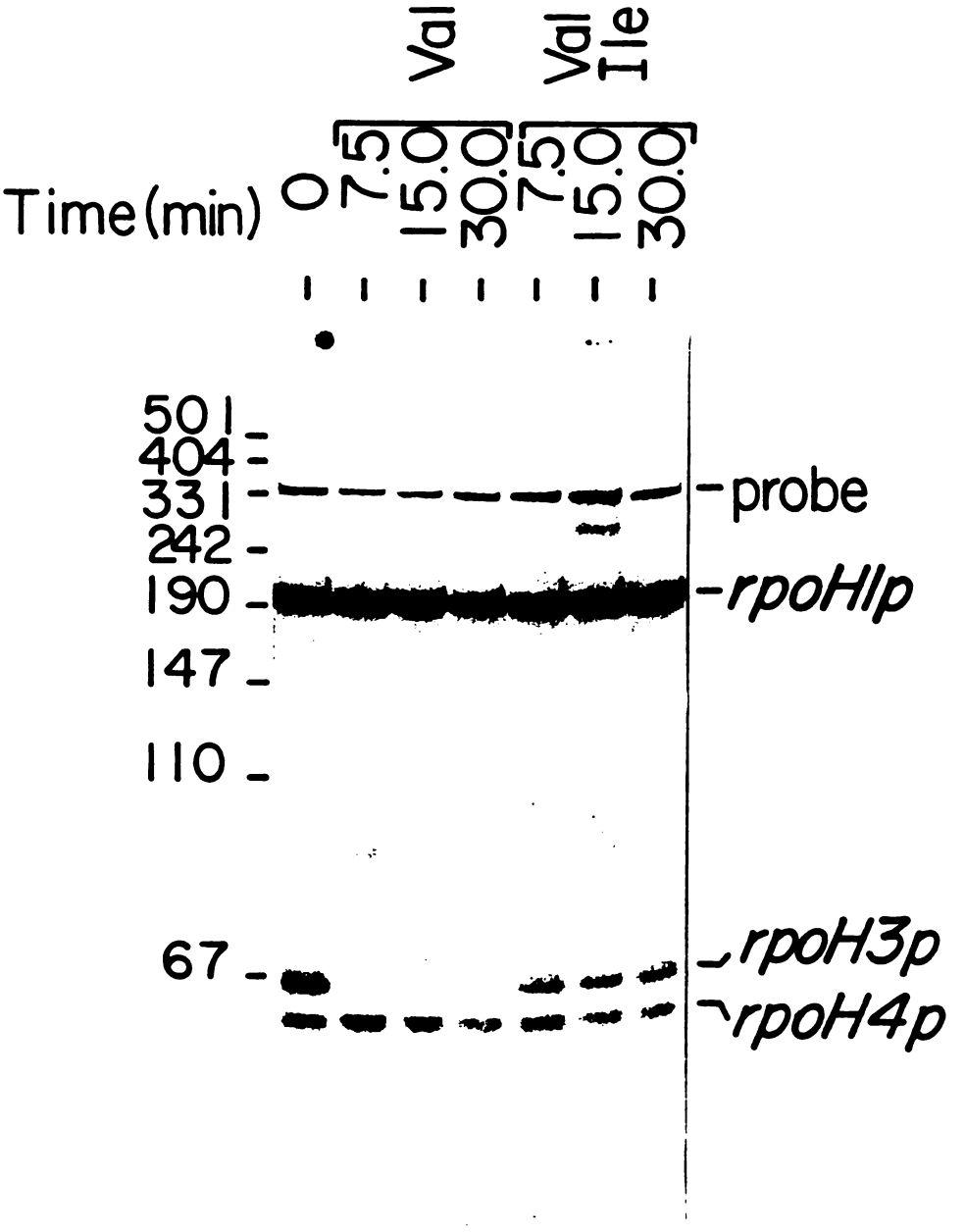
Sigma 24 is not identical to stringent starvation protein

The stringent response in E. coli can be induced by amino acid starvation (reviewed in 7). A protein, stringent starvation protein, is predominantly synthesized (29). This protein, stably associated with σ^{70}-RNA polymerase (20) and formerly thought to be 22.5 KDa, is calculated to be 24.3 KDa from the DNA sequence of the gene (30). It migrates at the F24.5 position on two-dimensional polyacrylamide gel electrophoresis (30). Amino acid starvation also induces synthesis of heat shock proteins (16). We considered that the stringent starvation protein might be identical to this novel sigma factor. If so, its elevated levels may result in increased transcription from rpoH3P under the condition of stringent response. Experiments were performed to determine whether σ^{24} was identical to stringent starvation protein.

A culture was grown in a synthetic medium and divided. The stringent response was induced by addition of valine to one part of the culture to inhibit isoleucine biosynthesis. As a control, both valine and isoleucine were added to the other half. Transcriptional regulation of the rpoH gene was measured by S1 nuclease protection assays with RNA isolated from portions of each culture removed at the indicated times (Figure 6). rpoH transcript levels were only slightly influenced by valine and isoleucine addition in the control culture. By comparison, induction of the stringent response resulted in a striking reduction in rpoH3P transcripts whereas 1P and 4P transcript levels were marginally affected. These results suggest that stringent starvation protein and σ^{24} are not identical.

Figure 6. Repression of rpoH3P under amino acid starvation.

Cells were grown at 37°C in glucose minimal media supplemented with 18 amino acids (without valine and isoleucine) as described in Materials and Methods. Valine was then added to induce isoleucine limitation. Valine and isoleucine were added to a parallel culture as a control. RNA was isolated from cells prior to and at 7.5, 15, 30 minutes after the addition of valine. S1 assays were performed as described in Materials and Methods with the 5' end-labeled EcoRV fragment as a probe. Positions of transcripts from rpoH promoters are indicated. Size markers are denatured HpaII restriction fragments of pUC19.


Val
Val
Ile
Time(min)
0
7.5
15.0
30.0
7.5
15.0
30.0
501
404
331
242
190
147
110
67
probe
rpoHIp
rpoH3p
rpoH4p

DISCUSSION

Sigma factors confer upon core RNA polymerase an ability to recognize a specific group of promoters. The roles of alternate sigma factors in expression of SPO1 genes during its propagation or in uninfected B. subtilis are well studied examples (9, 24, 25). Four sigma factors encoded by the E. coli genome and distinguished by size have been identified. σ^{70} encoded by the rpoD gene is the major sigma factor. σ^{32} encoded by rpoH (htpR) confers recognition of heat shock promoters (8, 15, 22). σ^{54} encoded by rpoN (ntrA, glnF) is required for expression of genes involved in nitrogen utilization (18, 19). The fourth, σ^{F} of 28 KDa, controls transcription of flagellar and chemotaxis genes (2). We have identified a fifth sigma factor of 24 KDa which confers recognition of one of the rpoH promoters, 3P. Antibodies raised against a synthetic peptide corresponding to the most highly conserved region of bacterial sigma factors were used in Western-blot analysis of whole cell lysates of E. coli (11). These experiments resulted in cross-reactivity with σ^{70}, σ^{32}, and proteins of 75, 27, and 23 KDa. We presume that the latter two proteins correspond to σ^{F} and σ^{24}. Identification of other genes transcribed by this form of σ^{24}-RNA polymerase is important in understanding the role of this sigma factor in the growth of E. coli. A consensus promoter sequence derived from a sequence analysis of promoters recognized by $E\sigma^{24}$ may reveal how different holoenzymes recognize their respective promoters.

The expression of the rpoH gene appears very complex. rpoH1P and 4P are transcribed by $E\sigma^{70}$ (10) while rpoH3P is transcribed by $E\sigma^{24}$ as shown here. rpoH2P appears to be strain specific. Second, dnaA protein involved in initiation of DNA replication regulates expression from 3P and 4P (35). Third, upon temperature upshift, rpoH transcript levels increase with 2P and 3P increasing the most (10). Fourth, a downshift in temperature also results in increased levels of rpoH1P and 3P mRNA (unpublished results). The variety of treatments such as an increase in temperature, ethanol, bacteriophage infection, and others (reviewed in 1) which induce expression of heat shock proteins may occur by different mechanisms resulting in increased transcription from one or more of the rpoH promoters.

Expression of heat shock proteins is also induced by the stringent response (16). This condition also results in synthesis of stringent starvation protein comprising at least half of the proteins synthesized (29). We considered whether σ^{24} and stringent starvation protein were identical. The observation that rpoH3P transcription was not elevated but diminished to undetectable levels under stringent response conditions indicates that σ^{24} and stringent starvation protein are not the same. That rpoH transcripts were not elevated by this treatment also suggests that stringent induction of heat shock proteins does not require σ^{32} but an alternate sigma factor. Alternatively, stringent induction may not act at the transcriptional level of rpoH expression but post-translationally by stabilization of σ^{32}.

Note: While these studies were being completed, J.W. Erickson and C.A. Gross reported to have identified a novel sigma factor of 24 KDa which promotes transcription from rpoH3P at the Molecular Genetics of Bacteria and Phages Meeting, Cold Spring Harbor, N.Y., August, 1988.

REFERENCES

1. Armstrong, V.W., Yee, D., and Eckstein, F. (1979) Biochem. 18, 4120-4123.

2. Arnosti, D.N., and Chamberlin, M.J. (1989) Proc. Natl. Acad. Sci. USA 86, 830-834.

3. Bloom, M., Skelly, S., VanBogelen, R.A., Neidhardt, F.C., Brot, N., and Weissbach, H. (1986) J. Bacteriol. 166, 380-384.

4. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.

5. Brosius, J., Cate, R.L., and Perlmutter, P.A. (1982) J. Biol. Chem. 257, 9205-9210.

6. Burgess, R.R., and Jendrisak, J.J. (1975) Biochem. 14, 4634-4638.

7. Cashel, M., and Rudd, K.E. (1987) Escherichia coli and Salmonella typhimurium, Cellular and Molecular Biology (Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B.L., Schaechter, M., and Umbarger, H.E., eds). Vol. 2, pp 1410-1438, American Society for Microbiology, Washington, D.C.

8. Cowing, D.W., Bardwell, J.C.A., Craig, E.A., Woolford, C., Hendrix, R.W., and Gross, C.A. (1985) Proc. Natl. Acad. Sci. USA 82, 2679-2683.

9. Doi, R.H., and Wang, L.-F. (1986) Microbiol. Rev. 50, 227-243.

10. Erickson, J.W., Vaughn, V., Walter, W.A., Neidhardt, F.C., and Gross, C.A. (1987) Genes and Development 1, 419-432.

11. Fujita, N., Ishihama, A., Nagasawa, Y., and Ueda, S. (1987) Mol. Gen. Genet. 210, 5-9.

12. Fujita, N., and Ishihama, A. (1987) Mol. Gen. Genet. 210, 10-15.

13. Gill, D.R., Hatfull, G.F., and Salmond, G.P.C. (1986) Mol. Gen. Genet. 205, 134-145.

14. Gonzalez, N., Wiggs, J., and Chamberlain, M.J. (1977) Arch. Biochem. Biophys. 182, 404-408.

15. Grossman, A.D., Erickson, J.W., and Gross, C.A. (1984) Cell 38, 383-390.

16. Grossman, A.D., Taylor, W.E., Burton, Z.F., Burgess, R.R., and Gross, C.A. (1985) J. Mol. Biol. 186, 357-365.

17. Hager, D.A., and Burgess, R.R. (1980) Anal. Biochem. 109, 76-86.

18. Hirschman, J., Wong, P.K., Sei, K., Keener, J., and Kustu, S. (1985) Proc. Natl. Acad. Sci. USA 82, 7525-7529.

19. Hunt, T.P., and Magasanik, B. (1985) Natl. Acad. Sci. USA 82, 8453-8457.

20. Ishihama, A., and Saitoh, T. (1979) J. Mol. Biol. 129, 517-530.

21. Lamond, A.I., and Travers, A.A. (1985) Cell 40, 319-326.

22. Landick R., Vaughn, V., Lau, E.T., VanBogelen, R.A., Erickson, J.W., and Neidhardt, F.C. (1984) Cell 38, 175-182.

23. Lesley, S.A., Thompson, N.E., and Burgess, R.R. (1987) J. Biol. Chem. 262, 5404-5407.

24. Losick, R., and Pero, J. (1981) Cell 25, 582-584.

25. Losick, R., Youngman, P., and Piggot, P.J. (1986) Annu. Rev. Genet. 20, 625-629.

26. Miller, J.H. (1972) Experiments in Molecular Genetics. p 431. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

27. Neidhardt, F.C., VanBogelen, R.A., and Lau, E.T. (1983) J. Bacteriol. 153, 597-603.

28. Neidhardt, F.C., and VanBogelen, R.A. (1987) Escherichia coli and Salmonella typhimurim, cellular and Molecular Biology (Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B.L., Schaechter, M., and Umbarger, H.E., eds.), Vol. 2, pp 1334-1345, American Society for Microbiology, Washington, D.C.

29. Reeh, S., Pederson, S., Friesen, J.D. (1976) Mol. Gen. Genet. 149, 279-289.

30. Serizawa, H., and Fukuda, R. (1987) Nucleic Acids Res. 15, 1153-1163.

31. Skelly, S., Fu, C.-F., Dalie, B., Redfield, B., Coleman, T., Brot, N., and Weissbach, H. (1988) Proc. Natl. Acad. Sci. USA 85, 5497-5501.

32. Straus, D.B., Walter, W.A., and Gross, C.A. (1987) Nature 329, 348-351.

33. Tilly, K., Erickson, J., Sharma, S., and Georgopoulos, C. (1986) J. Bacteriol. 168, 1155-1158.

34. Wang, Q., and Kaguni, J.M. (1987) Mol. Gen. Genet. 209, 518-525.

35. Wang, Q., and Kaguni, J.M. (1989) J. Biol. Chem. 264,7338-7344.

36. Wray, W., Boulikas, T., Wray, V.P., and Hancock, R. (1981) Anal. Biochem. 118, 197-202.

37. Yee, D., Armstrong, V.W., and Eckstein, F. (1979) Biochem. 18, 4116-4120.

38. Yura, T., Tobe, T., Ito, K., and Osawa, T. (1984) Proc. Natl. Acad. Sci. USA 81, 6803-6807.

Chapter V

PREFERENTIAL BINDING OF dnaA PROTEIN TO DNA FRAGMENTS CONTAINING PROMOTER REGIONS OF polA, uvrB, AND nrd, AND TO FRAGMENTS CONTAINING SOME UNIDENTIFIED SITES

ABSTRACT

Binding activity of dnaA protein to DNA fragments containing the promoter region of polA, uvrB, or nrd was tested by nitrocellulose filter binding assays. In comparison to fragments containing the dnaA promoters or the pBR322 origin, the polA, uvrB, and nrd fragments were retained with greater affinity. Filter binding assays were also performed with a restriction enzyme digest of chromosomal DNA. Some unidentified fragments were preferentially bound. A cloned EcoRI-HindIII fragment of about 700 bp was bound by dnaA protein more strongly than the dnaA promoter fragment.

INTRODUCTION

dnaA protein is an essential protein for initiation of DNA replication from the chromosomal origin of E. coli (1-3). It binds to the oriC region and functions in an early stage of initiation (4). dnaA protein also binds to other DNA fragments containing a consensus sequence of TTAT(A/C)CA(A/C)A (5). Among these are the dnaA promoter fragment and a fragment containing the promoter region of a gene encoding a 16 KDa protein. The binding at those promoter regions results in transcriptional repression of these genes by dnaA protein (6-9).

In addition to E. coli genes described in previous chapters, the dnaA protein binding sequence is present in regulatory regions of several other genes including polA, uvrB, and nrd (10-14). polA is the gene coding for the DNA polymerase I (11). The product of the uvrB gene is a subunit of the uvrABC endonuclease (18). Both uvrB protein and pol I are involved in DNA repair. Ribonucleotide reductase which catalyzes the conversion of ribonucleotide to deoxyribonucleotide is encoded by the nrdAB operon (13).

The presence of dnaA binding sites in these regulatory regions prompted us to test the influence of dnaA protein on the expression of these genes. Experiments described in this chapter indicate that dnaA protein binds to promoter regions of these genes. However the effect of binding was not conclusively determined.

Since only a limited portion (about one fourth) of the E. coli genome is currently sequenced, effort was also made to isolate DNA fragments bound by dnaA protein from a chromosomal DNA digest in attempt to identify unsequenced genes controlled by dnaA protein. Although this approach did not result in any positively identified genes, it did show that certain DNA fragments are preferentially bound by dnaA protein.

MATERIALS AND METHODS

Strains, DNAs, and enzymes

E. coli W3110 (wild type) was from the E. coli genetic stock center. Chromosomal DNA for binding assays was prepared from W3110 as described (16). Plasmid pCJ60 containing the 592 bp HpaII fragment of the polA promoter region was obtained from Drs C. Joyce and N. Grindley (Yale University) (17). pDR1494, from Dr A. Sancar (Yale University) (18), contains the 6.5 kb PstI fragment of the uvrB gene and its promoter region. Plasmid pPS2, carrying the 12 kb PstI fragment of the nrd gene and the promoter region, was from Dr. B. Sjoberg (Swedish University) (19). All three plasmids are pBR322 derivatives. The plasmid pUVRB is a recombinant of pKO1 (22) constructed by cloning the uvrB promoter region contained in a 600 bp TaqI fragment at the SmaI site. A shorter fragment (1.6 kb EcoRI-KpnI) containing the nrd promoter region was subcloned into pUC19, resulting in pNRD. Plasmid DNAs and DNA fragments were prepared as described in previous chapters.

Restriction endonucleases and dnaA protein are from the same sources as described in chapter IV.

Nitrocellulose filter binding assays

Binding assays were performed as described in previous chapters. Assays were performed with 100 ng plasmid DNA digest or with 0.025 pmol of

purified DNA fragments. 10 μg of EcoRI digested chromosomal DNA was used where as indicated. DNA fragments were end-labeled either at 3' end or at 5' end. The labeling was performed as described in previous chapters. The amounts of dnaA protein used were as indicated.

RESULTS

dnaA protein recognition sequences in promoter regions of polA, uvrB, and nrd

Three copies of the dnaA protein binding sequence (dnaA box) (one 9/9 and two 8/9 matches) are present at 232-253 and 374-383 bp upstream of the coding sequence of the polA gene (Figure 1A) (11). Since transcriptional start sites have not been mapped, positions of these dnaA boxes relative to promoters cannot be determined. In the case of uvrB, transcriptional start sites have been mapped at three positions (Figure 1B) (12). The two dnaA boxes (one 9/9 and one 8/9 match) are located at 22-45 bp upstream of the 3P RNA start site. One copy of dnaA recognition sequence (9/9) is found at 43-52 bp upstream of the only RNA start site of the nrd gene (Figure 1C) (13).

Binding of dnaA protein to the three promoter regions

The binding activity of dnaA protein to promoter regions of polA, uvrB, and nrd was tested by nitrocellulose filter binding assays. Plasmid pCJ60, which contains the 592 bp HpaII fragment of the polA regulatory region in a pBR322 derivative, was digested with HpaII and used in binding assays (Figure 2). Two fragments were retained as increasing amounts of dnaA protein were added, one about 527 bp containing the pBR322 origin and the other about 1.2 kb (markers not shown). One dnaA box is present near the replication origin of pBR322. DNA fragments containing the origin were

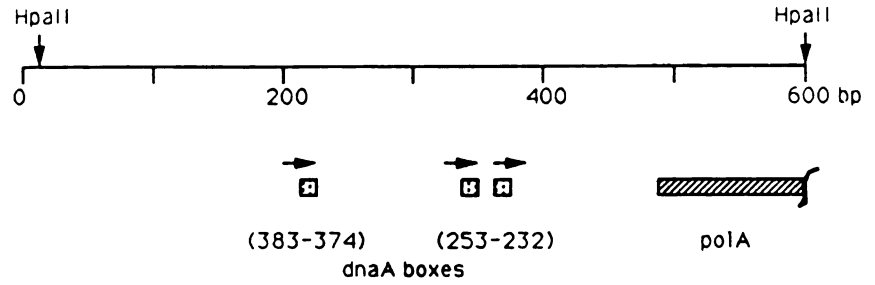
Figure 1. Map of the promoter regions of polA, uvrB, and nrd.

(A) polA. The coding region is indicated by a cross hatched box. The dnaA recognition sequences (dnaA boxes) are the stippled boxes. The arrows indicate the relative orientation of the dnaA boxes. The numbers are positions of the dnaA boxes relative to the initiation codon.

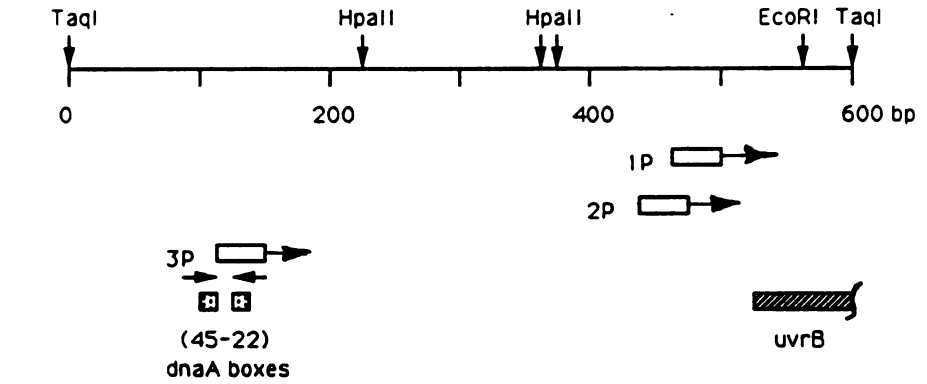
(B) uvrB. Relevant restriction sites are as indicated. Promoters are indicated by arrow boxes. Positions of the dnaA boxes are compared to the 3P mRNA start site. Other designations are as in (A).

(C) nrd. Designations are as in (B).

A.


HpaII
HpaII
0
200
400
600 bp
(383-374)
(253-232)
dnaA boxes
polA

B.


TaqI
HpaII
HpaII
EcoRI
TaqI
0
200
400
600 bp
1P
2P
3P
(45-22)
dnaA boxes
uvrB

C.

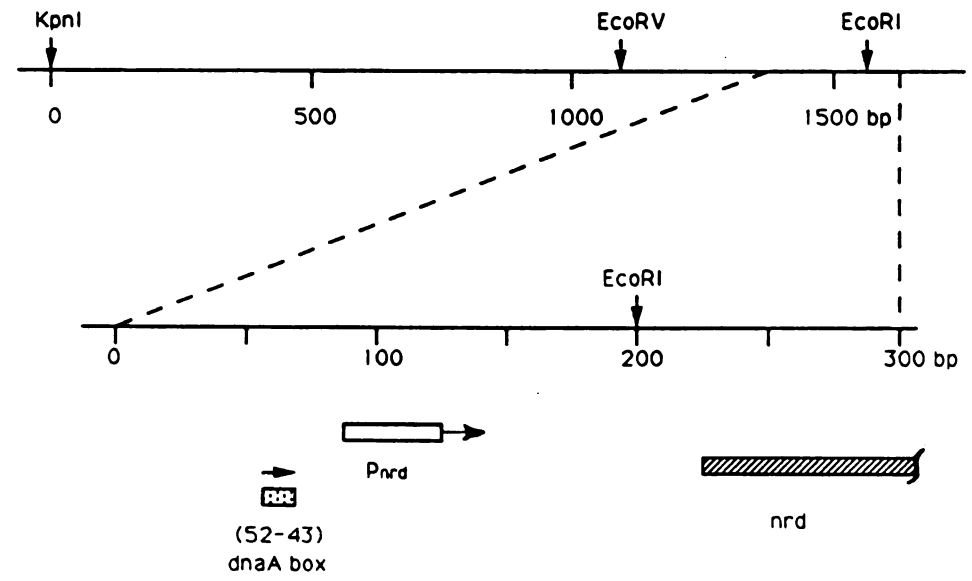

KpnI
EcoRV
EcoRI
0
500
1000
1500 bp
EcoRI
0
100
200
300 bp
Pnrd
(52-43)
dnaA box
nrd

Figure 2. Preferential binding of dnaA protein to a restriction fragment containing the polA regulatory region.

Filter binding assays were performed as described in Materials and Methods with the indicated amounts of dnaA protein and 100 ng of 3' end-labeled pCJ60 digested with HpaII. TD, total DNA digest; B, bound fragments eluted from the filter; F, fragments which flowed through the filter. The positions of DNA fragments containing the pBR322 replication origin or the regulatory region of the polA gene are as indicated.


0
25
50
100
dnaA protein (ng)
TD
B
F
B
F
B
F
B
F
-polA
-pBR322 ori

observed previously to be bound by dnaA protein with a similar affinity as that of the dnaA promoter fragment. The other retained fragment of about 1.2 kb contains the cloned polA sequence and part of the pBR322 sequence--a 622 bp HpaII fragment of pBR322. Since the 622 bp vector fragment was not bound by dnaA protein (chapter 2, Figure 2), the binding affinity of the 1.2 kb fragment must have originated from the polA sequence. The relative amounts of the two fragments in the bound lanes indicate that the binding affinity to the polA fragment is similar to or greater than that to the pBR322 origin fragment.

The uvrB promoter region is contained in pDR1494. pDR1494 was digested with TaqI and the digest was used in filter binding assays (Figure 3). Two fragments were retained: one 1.3 kb containing the pBR322 replication origin; the other 600 bp presumably containing the uvrB promoter region. The bound DNA was further digested with EcoRI to confirm that the retained fragment contains the uvrB promoter region. Only two EcoRI sites are present in pDR1494, one in a 368 bp vector fragment, the other in the uvrB promoter region (18). The 600 bp fragment was cleaved to a shorter fragment, indicating that it contains the uvrB promoters. To further compare the binding affinity of the uvrB fragment to the pBR322 origin fragment, assays were performed with HpaII digest of pUVRB, which contains only the 600 bp TaqI fragment in pKO1 (a pBR322 derivative) (Figure 4). A 400 bp fragment was retained in addition to the 527 bp pBR322 origin fragment (Figure 4A). A fragment from the vector pKO1 coelectrophoresed with the 400 bp fragment. This vector fragment was not bound by dnaA protein as shown

Figure 3. Binding of dnaA protein to restriction fragment containing the uvrB promoter region.

Filter binding assays were performed as described in Materials and Methods with 100 ng of dnaA protein and 100 ng of 3' end-labeled pDR1494 digested with TaqI. B, bound fragments eluted from the filter; F, fragments which flowed through the filter. EcoRI, DNA fragments eluted from the filter was further digested with EcoRI. Positions of DNA fragments containing the uvrB promoter region or the pBR322 replication origin are as indicated.

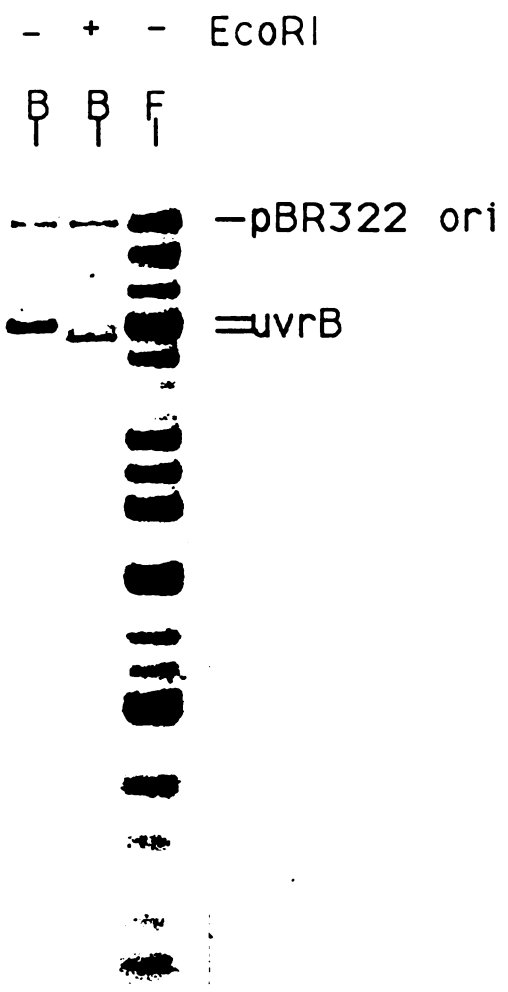
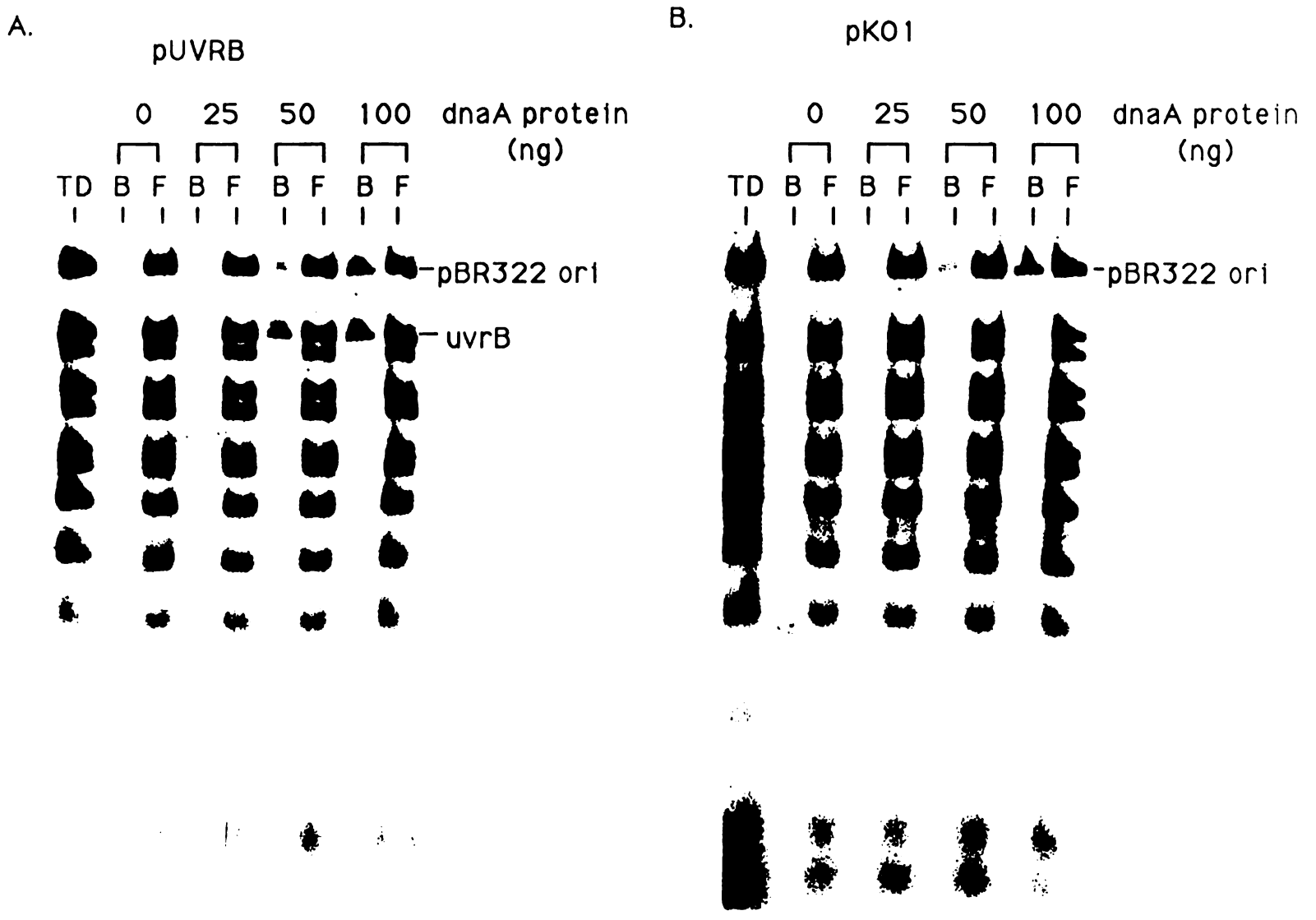

- + - EcoRI
B B F
—pBR322 ori
=uvrB

Figure 4. Preferential binding of dnaA protein to the uvrB promoter region.

(A) Filter binding assays were performed as described in Materials and Methods with 100 ng 3' end-labeled pUVRB digested with HpaII. The amounts of dnaA protein used are as indicated. Positions of the pBR322 origin fragment and the uvrB promoter fragment are also indicated. TD, DNA digest; B, bound fragments eluted from the filter; F, fragments which flowed through the filter.

(B) Binding assays were performed with 3' end-labeled pKO1 digested with HpaII. Other designations are as in (A).


A.
pUVRB
0
25
50
100
dnaA protein
(ng)
TD
B
F
B
F
B
F
B
F
pBR322 ori
uvrB
B.
pKO1
0
25
50
100
dnaA protein
(ng)
TD
B
F
B
F
B
F
B
F
pBR322 ori

in parallel assays performed with pKO1 (Figure 4B). The amounts of the two fragments in the bound lanes indicate that the 400 bp uvrB fragment was bound with greater affinity than the origin fragment.

Binding assays were performed with a purified DNA fragment containing the nrd promoter region (Figure 5). A 511 bp EcoRV-EcoRI nrd fragment was mixed with equal molar amounts of DNA fragments containing the dnaA promoter (945 bp), the rpoH promoter (342 bp), and the lacUV5 promoter (203 bp). As described in the preceding chapters, the rpoH promoter region was bound by dnaA protein with higher affinity than that of dnaA promoter region. The lacUV5 fragment was poorly bound by dnaA protein and used here as a negative control. The binding affinity of the nrd fragment appears to be greater than that of the dnaA promoter fragment, but less than that of the rpoH fragment.

Transcription run-off assays were performed in attempt to assess the effect of binding. However the results were circumstantial and inconclusive.

Chromosomal DNA fragments selectively bound by dnaA protein

To date, about one fourth of the E. coli genome is sequenced. In order to obtain DNA fragments bound by dnaA protein in the unsequenced portion of the genome, filter binding assays were performed with chromosomal DNA digested with EcoRI (Figure 6). The assays were with 10 μg of radioactively labeled DNA and a limiting amount of dnaA protein (260 ng) to obtain fragments bound with high affinity. About 1% of the total input was retained on the filter. DNA fragments retained were separated on an agarose gel

Figure 5. Binding affinity of dnaA protein to DNA fragment containing the nrd promoter region.

Binding assays were with 0.025 pmol each of DNA fragments containing the promoter region of dnaA, rpoH, nrd, or lacUV5. DNA fragments were 5' end-labeled. The amounts of dnaA protein used are as indicated. TD, the mixture of DNA fragments used in the assay; B, fragments eluted from the filter; F, fragments that flowed through the filter.

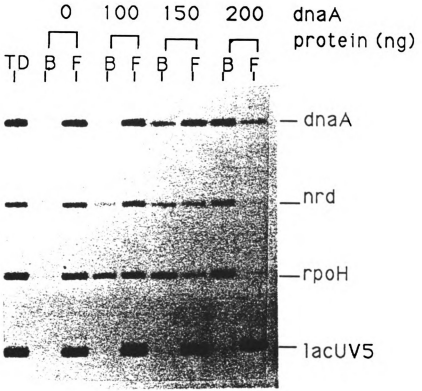
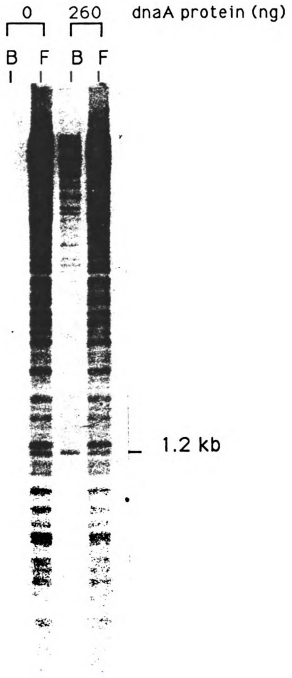

0
100
150
200
dnaA
protein (ng)
TD
B
F
B
F
B
F
B
F
dnaA
nrd
rpoH
lacUV5

Figure 6. Preferential binding of dnaA protein to chromosomal DNA fragments.

Binding assays were performed with 10 μg of chromosomal DNA digested with EcoRI and 260 ng of dnaA protein. B, fragments eluted from the filter; F, one tenth of the fragments that flowed through the filter.

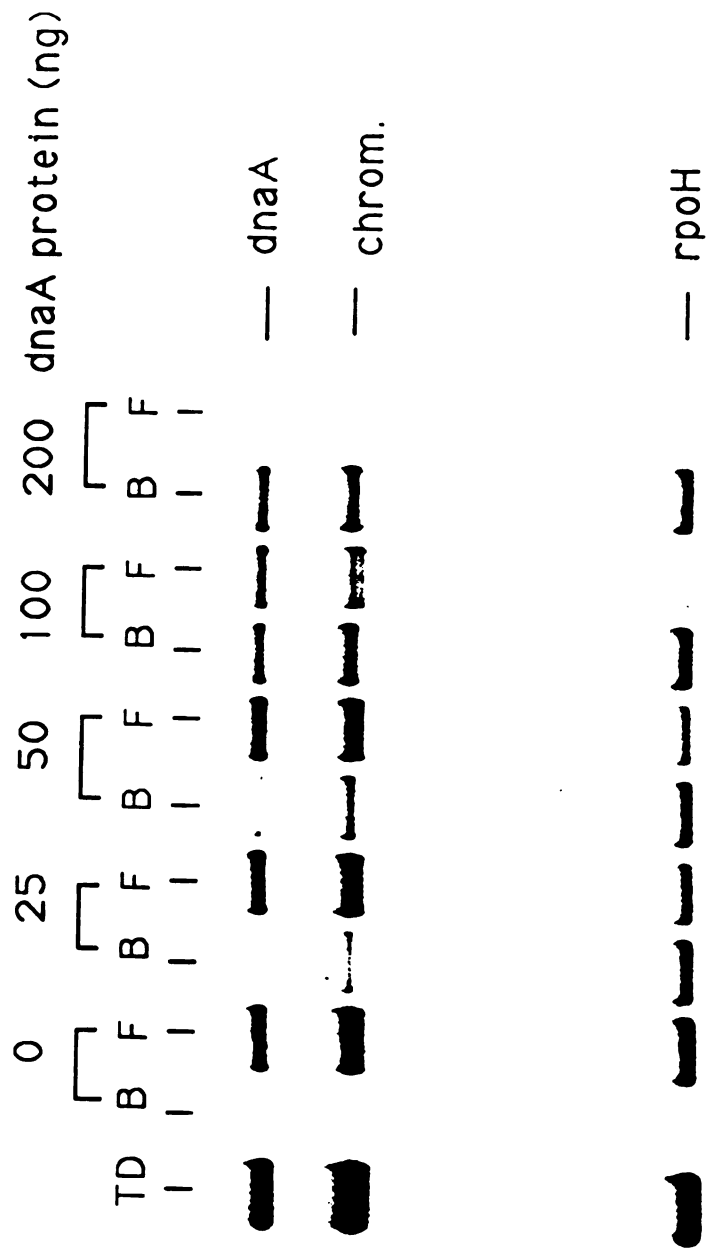

0
260
dnaA protein (ng)
B
F
B
F
1.2 kb

together with one tenth of the DNA in the flow through. dnaA protein preferentially retained several fragments, most obviously a fragment of about 1.2 kb.

Since none of the known binding sites are expected in a 1.2 kb EcoRI fragment (20, 21, 10-13), the fragment retained from the chromosomal DNA must represent a new binding site. To facilitate further characterization, this fragment was purified from the bound DNA after electrophoresis, and a 700 bp EcoRI-HindIII subfragment carrying the binding site (data not shown) was cloned into M13mp18. Binding assays were performed with purified fragments. The 700 bp EcoRI-HindIII fragment was mixed in equal molar amounts with the dnaA promoter fragment (945 bp) and the rpoH promoter fragment (342 bp). The binding affinity of the cloned 700 bp fragment is between that of the rpoH and dnaA fragments. The minor band below the cloned fragment may be a deletion product. This fragment was not bound well by dnaA protein presumably because the binding site has been removed. Initial efforts in cloning the 700 bp fragment into plasmid vectors (pBR322 and pUC19) were unsuccessful, presumably because the presence of the 700 bp chromosomal fragment in high copy number is deleterious to cell growth. Similar lethal effect has been observed with the uvrB promoter region (23). This may be due to titration of dnaA protein from the essential sites in the E. coli chromosome. Further effort in characterization of this fragment was unsuccessful. Sequencing of the fragment may be required for any further experimentation.

Figure 7. Binding affinity of a 700 bp cloned fragment.

Binding assays were performed with 0.025 pmol each of 5' end-labeled DNA fragments containing the promoter region of dnaA or rpoH, and a fragment containing the 700 bp cloned chromosomal fragment. The amounts of dnaA protein used are as indicated. TD, the mixture of DNA fragments used in the assay; B, fragments eluted from the filter; F, fragments flowed through the filter.


dnaA protein (ng)
0
25
50
100
200
TD
B
F
dnaA
chrom.
rpoH

DISCUSSION

Experiments presented here indicate that dnaA protein binds to promoter regions of genes polA, uvrB, and nrd. Several unidentified chromosomal fragments are also preferentially bound by dnaA protein. The binding affinities are comparable to the binding at the dnaA promoter region which results in transcriptional repression of the dnaA gene. This may suggest the relative importance of these binding events.

Preliminary results of in vitro transcription run-off assays indicate that transcription from promoters of the polA gene and of the uvrB gene may be inhibited by dnaA protein. However, effort in detection of transcripts from these promoters in vivo was not successful due to technical difficulties. The possible regulation of polA and uvrB by dnaA protein suggests that the DNA replication process could be correlated with DNA repair. Transcription from the nrd promoter seems not to be influenced by dnaA protein in vitro. Since inhibition of transcription by dnaA protein may occur by promoter occlusion, the lack of repression on the nrd promoter could be due to the distance between the dnaA binding site and the promoter. Alternatively, other factors required for any repressive (or stimulatory) effect may be absent in the run-off assays.

A current computer analysis of about 1000 kb of the sequenced portion of the E. coli genome yielded 27 sites of TTAT(A/C)CA(A/C)A (Table 1). Among them are 8 copies of TTATCCACA (Table 2), 8 of TTATACAAA (Table 3), 9 of

Table 1. Number of sites of the dnaA binding sequences in the sequenced *E. coli* genome.

Sequences[a]	Number of Sites	Coding Region	Regulatory[b] Region	Others
TTATCCACA	8	1	7	
TTATACAAA	8	4	4	
TTATCCAAA	9	4	5	
TTATACACA	2		1	1[c]

a Only the four sequences are listed.

b The binding sequences are within 506 bp upstream of initiation codon or within 137 bp upstream of mRNA start site.

c This binding site is present in a bent DNA fragment (22).

Table 2.

a The location of the dnaA binding sites are compared to mRNA start sites if promoters have been mapped, or compared to the position of the initiation codon where promoter positions are unknown. Reference sites are listed in parenthesis. The numbers listed are the distance between the closest nucleotide in the binding sequence and the reference position. "-" means the binding site is upstream of the reference site and "+" indicates down stream sites. In the case of multiple promoters, the distant one is designated 1P.

b The reference position is the left boundary of the minimum oriC sequence (Chapter 1).

c Designations of the three promoters are changed from the published order so that the distant one is named 1P.

Table 2. Locations of TTATCCACA Sites.

Gene	Protein	Function	Location[a]	Ref.
dnaA	dnaA protein	DNA Replication; Gene Regulation	+23 (1P); -51 (2P)	24
guaB	IMP Dehydrogenase	Purine Metabolism	+205 (ATG)	25
nrd	Ribonucleotide Reductase	dNTP synthesis	-43 (P_{nrd})	13
oriC		DNA Replication	+58 (left boundary)[b]	26
oriC		DNA Replication	+238 (left boundary)	26
polA	DNA Polymerase I	DNA Synthesis	-132 (ATG)	11
rpoH	sigma32	Heat Shock Response	+104 (1P); +15 (2P); -19 (3P); -24 (4P)	10
uvrB	uvrB protein	DNA repair	-22 (1P); -332 (2P); -363 (3P)[c]	18

Table 3. Locations of TTATACAAA Sites[a]

Gene	Protein	Function	Location	Ref.
argF	Ornithine Carbamoyltransferase	Amino Acid Synthesis	+9 (ATG)	27
dld	D-lactate Dehydrogenase	Sugar and Amino Acid Transport	+1598 (ATG)	28
ermBC	23 rRNA methylase	rRNA modification	-67 (ATG)	29
fur	Iron Regulatory Gene	Iorn Transport	-142 (ATG)	30
ileR	ile Repressor	Amino Acid Synthesis	-81 ($1P_{ile}$); -129 ($2P_{ile}$); -2 ($1P_{orf83}$); -20 ($2P_{orf83}$)[b]	31
nirB	Nitrate Reductase	Nitrogen Assimilation	-86 (P_{nir})	32
pin	DNA Invertase	DNA Inversion	+521 (ATG)	33
rrnB	rRNA Operon	rRNA	+566 (23 rRNA)	34

a Designations are as described in table 2.

b The orf83 is located adjacent to ileR in the opposite direction.

Table 4. Locations of TTATCCAAA Sites[a]

Gene	Protein	Function	Location	Ref.
dedB	ded Protein	Unknown	-119 (ATG)	35
dgk	Diglyceride Kinase	Phospholipid Synthesis	+1 (Termination)	36
folC Operon	Unknown	Unknown	-119 (ATG)	37
glnA	Glutamine Synthetase	Nitrogen Assimilation	-39 (1P); -154 (2P)	38
malT	malT Protein	Maltose Metabolism	+821 (ATG)	39
oriC		DNA Replication	+164 (left boundary)	26
pyrB	Aspartate Transcarbamylase	Pyrimidine Synthesis	-127 (1P); -340 (2P)	40
tdh	Threonine Dehydrogenase	Amino Acid Metabolism	+9 (ATG)	41
xylE	xylE Protein	Xylose Transport	-130 (ATG)	42

a Designations are as described in table 2.

Table 5. Locations of TTATACACA sites[a]

Gene	Function	Location	Ref.
bent5	unknown	bending site	22
oriC	DNA replication	+200 (left boundary)	26

a Designations are as described in table 2.

TTATCCAAA (Table 4), and only 2 of TTATACACA (Table 5). The number of sites of the former three sequences is in good agreement with the expected frequency--31 sites each in the 4000 kb genome. The frequency of the fourth sequence is lower than the expected. The reason for this is unknown. Analysis of locations of the sequences TTATACAAA and TTATCCAAA indicates that they are distributed about equally in coding regions and in regulatory regions upstream of open reading frames. On the other hand, all copies of TTATCCACA but one are in regulatory regions, including two in the oriC region. The difference in location may suggest that the sequences TTAT(A/C)CAAA are less significant functionally than the sequence TTATCCACA so that the latter is preferentially selected in promoter regions through evolution whereas the distribution of the former is rather random. In another word, the sequences TTAT(A/C)CAAA are less well bound than TTATCCACA. In supporting this conclusion, a TTATACAAA site within M13 genome and a TTATCCAAA site in the promoter region of the gln gene (encoding glutamine synthetase in nitrogen assimilation) are poorly bound by dnaA protein (5, data not shown).

Transition of A to T at the third position of the nine base-pair dnaA protein binding sequence does not seem to significantly influence the binding affinity in the case the 16 KDa gene. One TTTTCCACA site in the promoter region of the 16 KDa gene has a similar binding affinity compared to the dnaA promoter region which contains a TTATCCACA site. The binding to either promoter regions results in transcriptional repression to a similar degree. However, a TTTTCCACA site in the coding region of the dnaA gene

appears to have much lower affinity than the site in the dnaA promoter region. Sequences outside the nine base-pair may also affect the binding activity. The exact sequence requirement for dnaA binding awaits further experimentation.

Among the 8 TTATCCACA sites, two are in the oriC minimum sequence; one copy is at each promoter region of dnaA, rpoH, polA, uvrB, and nrd; and one is in the coding region of the guaBA gene coding for IMP dehydrogenase in the metabolism of purine (Table 2). All these sites were shown to be bound by dnaA protein except the site in guaBA which was not tested. The binding resulted in functional involvement of dnaA protein in initiation of replication and expression of dnaA and rpoH gene. Further experiments are needed to clarify the role of dnaA protein in expression of polA, uvrB, and nrd.

Binding assays with chromosomal DNA fragments indicate that there are some unidentified binding sites on the chromosome. A total of 31 dnaA recognition sequence TTATCCACA are expected in the 4000 kb genome, not including the other three variants. This seems to be a large number for a regulatory protein. The location of the binding sites may be critical for functional involvement, which may reduce the number of effective binding sites. On the other hand, dnaA protein may regulate a large number of genes. The level of dnaA protein is expected to be constant independent of growth rate due to autoregulation of the dnaA gene. As a result, the concentration of proteins under its control will also be kept constant. Many E. coli proteins may have a relatively fixed concentration independent of growth rate. These genes may be potential candidates to be regulated by dnaA protein. The exact

function of dnaA protein in regulating gene expression awaits further exploration of genes under control of dnaA protein.

REFERENCES

1. Hirota, Y., Mordoh, J., and Jacob, F. (1970) J. Mol. Biol. 53, 369-387.

2. Fuller, R. S., Kaguni, J. M., and Kornberg, A. (1981) Proc. Natl. Acad. Sci. USA 78, 7370-7374.

3. Kaguni, J. M., and Kornberg, A. (1984) Cell 38, 183-190.

4. Bradmill, D., and Kornberg, A. (1988) Cell 52, 743-755.

5. Fuller, R. S., Funnell, B. E., and Kornberg, A. (1984) Cell, 38, 899-900.

6. Braun, R. E., O'Day, K., and Wright, A. (1985) Cell 40, 159-169.

7. Atlung, T., Clausen, E. S., and Hansen, F. G. (1985) Mol. Gen. Genet. 200, 442-450.

8. Lother, H., Kolling, R., Kucherer, C., and Schauzu, M. (1985) EMBO J. 4, 555-560.

9. Wang, Q., and Kaguni, J. M. (1987) Mol. Gen. Genet. 209, 518-525.

10. Landick, R., Vaughn, V., Lau, E. T., VanBogelen, R. A., Erickson, J. W., and Neidhardt, F. C. (1984) Cell 38, 175-182.

11. Joyce, C. M., Kelley, W. S., and Grindley, N. D. F. (1982) J. Biol. Chem. 257, 1958-1964.

12. Sancar, G. B., Sancar, A., Little, J. W., and Rupp, W. D. (1982) Cell 28, 523-530.

13. Carlson, J., Fuchs, J. A., and Messing, J. (1984) Proc. Natl. Acad. Sci. USA 81, 4294-4297.

14. Grossman, A. D., Erickson, J. W., and Cross, C. A. (1984) Cell 38, 383-390.

15. Wang, Q., and Kaguni, J. M. (1989) J. Biol. Chem. 264, 7338-7344.

16. Saito, H., and Miura, K. (1963) Biochim. Biophy. Acta 72, 619-629.

17. Joyce, C. M. Personal communication.

18. Sancar, A., Clarke, N. D., Griswold, J., Kennedy, W. J., and Rupp, W. D. (1981) J. Mol. Biol. 148, 63-67.

19. Platz, A., and Sjoberg, B. (1980) J. Bacteriol. 143, 561-568.

20. Yasuda, S., and Hirota, Y. (1977) Proc. Natl. Acad. Sci. USA 74, 5458-5462.

21. Gill, D. R., Hatfull, G. F., and Salmond, G. P. C. (1986) Mol. Gen. Genet. 205, 134-145.

22. Mizuno, T. (1987) Nucl. Acid Res. 15, 6827-6841.

23. van den Berg, E. A., Geerse, R. H., Memelink, J., Bovenberg, R. A. L., Magnee, F. A., and van de Putte, P. (1985) Nucl. Acid Res. 13, 1829-1840.

24. Hansen, E. B., Hansen, F. G., and von Meyenburg, K. (1982) Nucl. Acid Res. 10, 7373-7385.

25. Tiedeman, A. A., and Smith, J. M. (1985) Nucl. Acid Res. 13, 1303-1316.

26. Zyskind, J. W., Cleary, J. M., Brusilow, W. S. A., Harding, N. E., and Smith, D. W. (1983) Proc. Natl. Acad. Sci. USA 80, 1164-1168.

27. van Vliet, F., Cunin, R., Jacob, A., Piette, J., Gigot, D., Lauwerey, M., and Pierard, A., and Glansdorff, N. (1984) Nucl. Acid Res. 12, 6277-6289.

28. Campbell, H. D., Rogers, B. L., and Young, I. G. (1984) E. J. Biochem. 144, 367-372.

29. Brisson-Noel, A., Arthur, M., and Courvalin, P. (1988) J. Bacteriol. 170, 1739-1745.

30. Schaffer, S., Hantke, K., and Braun, V. (1985) Mol. Gen. Genet. 200, 110-113.

31. Weiss, D. L., Johson, D. I., Weith, H. L., and Somervill, R. L. (1986) J. Biol. Chem. 261, 9966-9971.

32. Jayaraman, P. S., Peakman, T. C., Busby, S. J. W., Quincey, R. V., and Cole, J. A. (1987) J. Mol. Biol. 196, 781-788.

33. Plasterk, R. H. A., Brinkman, A., and van de Putte, P. (1983) Proc. Natl. Acad. Sci. USA 80, 5355-5358.

34. King, T. C., Sirdeshmukh, R., and Schlessinger, D. (1984) Proc. Natl. Acad. Sci. USA 81, 185-188.

35. Nonet, M. L., Marvel, C. C., and Tolan, D. R. (1987) J. Biol. Chem.

262, 12209-12217.

36. Lightner, V. A., Bell, R. M., and Modrich, P. (1983) J. Biol. Chem. 258, 10856-10861.

37. Bognar, A. L., Osborne, C., and Shane, B. (1987) J. Biol. Chem. 262, 12337-12342.

38. Reitzer, L. J., and Magasanik, B. (1985) Proc. Natl. Acad. Sci. USA 82, 1979-1983.

39. Cole, S. T., and Raibaud, O. (1986) Gene 42, 201-208.

40. Turnbough, C. L., Hicks, K. L., and Donahue, J. P. (1983) Proc. Natl. Acad. Sci. USA 80, 368-372.

41. Aronson, B. D., Ravnikar, P. D., and Somerville, R. L. (1988) Nucl. Acid Res. 16, 3586.

42. Davis, E. O., and Henderson, P. J. E. (1987) J. Biol. Chem. 262, 13927-13932.

Chapter VI

SUMMARY AND PERSPECTIVES

SUMMARY AND PERSPECTIVES

dnaA protein binds to oriC and functions in initiation of DNA replication. It also binds to promoter regions of its own gene, the 16 KDa protein gene, rpoH, polA, uvrB, and nrd. The binding results in transcriptional repression of the former three genes, and the effect on the later three are not determined. Autoregulation of the dnaA gene provides a relatively constant concentration of dnaA protein, so that its accumulation is proportional to the increase in cell volume, that is the growth rate. Assuming initiation occurs when a certain amount of dnaA protein has accumulated, initiation of replication can then be kept in pace with cell growth. Transcription from the 16 KDa promoter seems to influence initiation from oriC. Regulation of transcription from the 16 KDa promoter by dnaA protein may directly contribute in regulation of the initiation event.

Regulation of the rpoH expression appears to indirectly influence its activity in DNA replication. The rpoH gene encodes a sigma factor, sigma32, that recognizes promoters of heat-shock genes. The level of sigma32 controls levels of heat-shock proteins. Heat-shock proteins, in turn, seem to modulate the replication activity of dnaA protein. Both in vitro and in vivo evidence indicates that dnaK protein is involved in this process. dnaK protein was first observed to stimulate the activity of purified dnaA46 protein in vitro (Hwang and Kaguni, unpublished results). The wild type dnaA protein also seems to be modified by dnaK protein (Carr and Kaguni, unpublished results). Consistent with these observations, a dnaK mutant has been obtained which

is defective in initiation of replication presumably because the unmodified dnaA protein is not as active in supporting initiation (1). groEL and groES proteins also appear to interact with dnaA protein in vivo (2, 3). Mutations in the rpoH gene result in inhibition of cell division, possibly caused by the defect in initiation (4).

A regulatory circuit between dnaA protein and the heat-shock system is summarized as follows. dnaA protein regulates expression of heat-shock genes through rpoH. In turn, heat-shock proteins modulate the activity of dnaA protein. A balance between dnaA protein and the heat-shock proteins might be maintained at a specific level in cells grown at a fixed temperature. Regulation by dnaA protein provides a constant concentration of heat-shock proteins under normal growth conditions. On the other hand, conditions detrimental to cell growth might reduce the activity of dnaA protein, blocking DNA replication, and simultaneously increasing synthesis of heat-shock proteins, the function of which has been known as stress coping. One such example is the SOS response. Transcription from the dnaA promoters increases significantly after induction of the SOS response, indicating that the level of active dnaA protein as a repressor is reduced (5). A different pathway of DNA replication independent of dnaA protein is induced (6). Synthesis of heat-shock proteins is also induced (7). These observations fit well into a picture where dnaA protein is inactivated during the SOS response. If dnaA protein represses expression of polA and uvrB, inactivation of dnaA protein could induce the synthesis of pol I and uvrB protein, both of which are involved in DNA repair. Further proof of inactivation of dnaA protein will

support this model. Identification of new genes controlled by dnaA protein will also provide insights into the role of dnaA protein in the process of cell growth.

REFERENCES

1. Sakakibara, Y. (1988) J. Bacteriol. 170, 972-979.

2. Jenkins, A. J., March, J. B., Oliver, I. R., and Masters, M. (1986) Mol. Gen. Genet. 202, 446-454.

3. Fayet, O., Lourarn, J.-M., and Georgopoulos, C. (1986) Mol. Gen. Genet. 202, 435-445.

4. Tsuchido, T., VanBogelen, R. A., and Neidhardt, F. C. (1986) Proc. Natl. Acad. Sci. USA 83, 6959-6963.

5. Quinones, A., Kaasch, J., Kaasch, M., and Messer, W. (1989) EMBO J. 8, 587-593.

6. Walker, G. C. (1984) Microbiol. Rev. 48, 63-93.

7. Krueger, J. H., and Walker, G. (1984) Proc. Natl. Acad. Sci. USA 81, 1499-1450.