THE ROLE OF THE *Escherichia coli* HEAT SHOCK PROTEIN, grpE, IN *Escherichia coli* GROWTH AND λ DNA REPLICATION

by

Deborah Ang

A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Cellular, Viral, and Molecular Biology

The University of Utah

December 1988

THE UNIVERSITY OF UTAH GRADUATE SCHOOL

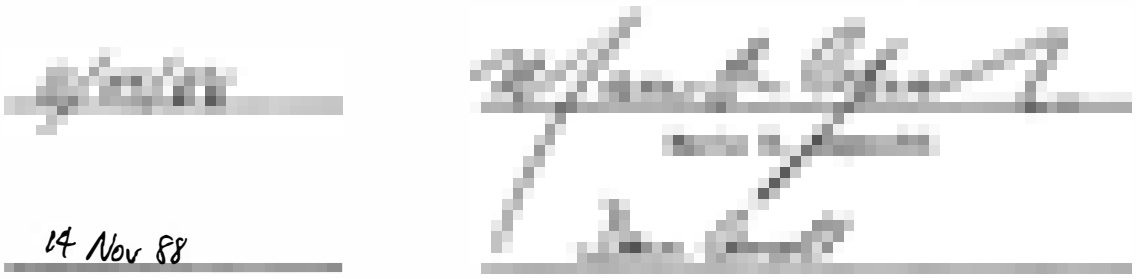
SUPERVISORY COMMITTEE APPROVAL

of a dissertation submitted by

Deborah Ang

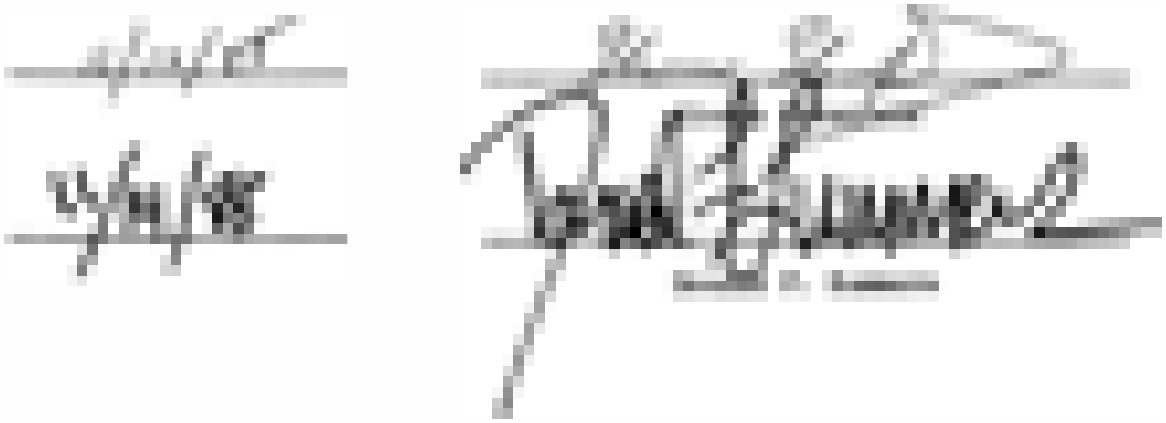
This dissertation has been read by each member of the following supervisory committee and by majority vote has been found to be satisfactory.

Chair: Constantine P. Georgopoulos



14 Nov 88

Dana Carroll



THE UNIVERSITY OF UTAH GRADUATE SCHOOL

FINAL READING APPROVAL

To the Graduate Council of the University of Utah:

I have read the dissertation of [redacted]
in its final form and have found that (1) its format, citations, and bibliographic style are consistent and acceptable; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the Supervisory Committee and is ready for submission to the Graduate School.

[redacted]
Date

[redacted]
Constantine P. Georgopoulos
Chair, Supervisory Committee

[redacted]

Approved for the Graduate Council

[redacted]
B. Gale Dick
Dean of The Graduate School

ABSTRACT

The *Escherichia coli grpE* gene was first recognized as one of several host functions (termed *groP* or *grp*) required by bacteriophage λ for replication of its DNA. Through a combination of genetic and biochemical analysis, its effects on *E. coli* growth and initiation of λ DNA replication have been examined. The *E. coli grpE*280 mutant is temperature-sensitive for growth above 43°C and both RNA and DNA syntheses are affected in the mutant at the nonpermissive temperature. The *grpE* gene product has been identified as the heat shock protein, B25.3, whose expression is regulated at the transcriptional level by the *htpR* gene product, σ^{32}. It interacts with the dnaK protein, which is the bacterial analog of the eukaryotic heat shock protein Hsp 70; this complex is specifically disrupted in the presence of ATP. Attempts to delete the *grpE* gene in a wild type background were unsuccessful, demonstrating that the *grpE* gene product is essential for *E. coli* growth at all temperatures, and not only at high temperatures. Surprisingly, the deletion was viable at 30°C in a strain with a *dnaK*$^-$ mutation. This particular *dnaK*$^-$ strain was shown to have extragenic suppressor(s) which allow loss of both *dnaK* and *grpE* functions, suggesting that the two proteins function in the same pathway.

To study the interaction between the grpE and dnaK proteins in greater detail, wild type grpE and mutant grpE280 proteins were purified. Wild type grpE and dnaK form a complex which is stable in glycerol. In contrast, under similar conditions, grpE280 and dnaK do not form an isolatable complex. Unexpectedly, in the presence of glutaraldehyde, grpE280 is crosslinked to dnaK more efficiently than wild type

grpE. Further experiments, however, suggest that the *grpE*280 mutation results in a more transient interaction with dnaK. The interaction also appears to be more hydrophobic in nature.

Others have shown that inhibition of λ DNA replication in *groP*$^-$ mutants occurs at the level of initiation. DnaK and grpE are required in initiation at the step of unwinding of the DNA at *oriλ*. Addition of grpE to the purified *in vitro* λ DNA replication assay and the λdv DNA unwinding assay allows a ten-fold reduction in the amount of dnaK added. This reduction might be due to interaction of grpE with either dnaK and/or λP.

To Mommy, Daddy, and Jenny

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

"...I know for sure that friendship and people and things done together are the most important aspects of life."

B. Lipinska, '88

I have been very fortunate these last few years to have made many dear friends without whom graduate school would have been endured rather than enjoyed. Of my friends who have since left the lab, I especially appreciate Susan Sell for her sense of humor at all hours of the day and night, and Basia Lipinska (and her daughter Magda) for the many wonderful camping trips we took together. I would like to thank my friends outside of the lab, especially Juliet Ouyoung, Angie and Akbar Matinkhah, Teiko Nakamura, and June Han-Kohl, for being my family away from home.

The two people to whom I am most indebted are Maciej Zylicz and Costa Georgopoulos. Besides having common sense in "doing science," they have been excellent examples of generosity, encouragement and good humor.

I would like to thank Anna Maddock for allowing me to present her data in Ch. 5.

I would also like to thank the American Society for Microbiology, the American Society for Biochemistry and Molecular Biology, Inc., and IRL Press Limited for allowing me to reprint Ch. 2, App. A, and App. B, respectively. Since November of 1986, I have been supported by an NIH Genetics Training grant.

CHAPTER 1

INTRODUCTION TO THE *Escherichia coli groP* GENES

Knowledge of the gram-negative bacterium, *Escherichia coli*, has accumulated to the point where entire volumes have been devoted to it. Even so, our understanding of how this minute creature carries out processes fundamental to all living things is far from complete. The many and various metabolic pathways used by *E. coli* are interconnected by global regulatory networks, resulting in a highly intricate situation. This can be likened to playing the game of chess in three dimensions. One begins to appreciate the complexity of the situation as one realizes that the position of a piece affects not only pieces on the same board, but also pieces on other boards.

In *E. coli*, an example of this complexity is demonstrated by a small group of proteins originally identified as host factors required by bacteriophage λ for replication of its DNA (32, 35, 69, 79). These proteins - dnaB, dnaK, dnaJ and grpE - were subsequently shown to be essential for *E. coli* growth, affecting host RNA and DNA syntheses (35, 43, 69, 70, 88) and, in the cases of dnaK, dnaJ and grpE, in the response to stress [also known as the heat shock response (3, 37; for reviews, see 20, 53, 60, 61)]. The fundamental role of these proteins in *E. coli* growth is emphasized by the fact that the corresponding genes cannot be deleted or inactivated in the absence of extragenic suppressors (4, 16; S. Sell, Ph.D. dissertation, University of Utah, 1987). In addition, there may be other pathways in which these proteins participate, either together or separately, since some of these extragenic suppressors appear to map in hitherto unexplored regions of the *E. coli* chromosome (see ch. 5). Although the mechanism(s) by which these proteins influence *E. coli* growth is still unclear, physical interaction among the proteins appears to be a significant underlying factor.

In this chapter, the identification and properties of this set of proteins will be discussed in the context of initiation of λ DNA replication . Understanding the role of these proteins in this somewhat simpler system may facilitate our understanding of their mechanism of action in *E. coli*. In addition, a brief description of the heat shock

response in *E. coli* and the effect of null mutations in these genes will be summarized in the latter half of this chapter.

Initiation of λ DNA Replication

Dependence of λ on *E. coli* for replication of its DNA.

The temperate bacteriophage λ depends upon its host, *E. coli*, for many of the functions necessary to propagate itself. Because of its relative simplicity (its genome of 48,502 bp is approximately 100 times smaller than *E. coli*'s), it is a convenient system for studying the mechanism of action of *E. coli* functions. DNA replication is a process fundamental to all organisms. While elongation of newly synthesized chains is fairly similar in most systems, a wide range of mechanisms to initiate replication has been encountered (45, 47, 48). One reason to specifically study initiation is that it is the frequency of initiation and not the velocity of elongation which regulates the extent of DNA synthesis (45). The following sections will cover a) the genetic selections used to identify host factors involved in initiation of λ DNA replication, b) the effect which mutations in these genes have on the host, c) host protein interactions with each other and with the λO and λP initiator proteins, and finally, d) a model correlating the various documented observations with the probable steps leading to initiation of replication. Other aspects of DNA replication in *E. coli* and its bacteriophages are extensively discussed in several recent comprehensive reviews (45, 47, 48, 59).

Identification of *E. coli* genes. Two independent selections were carried out to identify *E. coli* genes necessary for bacteriophage λ DNA replication. Georgopoulos and Herskowitz (35) mutagenized wild type *E. coli* cells with nitrosoguanidine and then selected for large colonies resistant to both λ*b2c*I and 434*c*I phage. Three classes were observed: a) mucoid colonies, b) colonies resistant to adsorption by both λ and 434, and c) *gro*$^-$ colonies which inhibit phage development

in a step beyond adsorption, as shown by the fact that *gro*⁻ *gal*⁻ strains are transduced to *gal*⁺ by λ*gal*⁺ phage. Subsequently it was shown that a specific subset of this last class of mutants, termed *groP*⁻, blocks the growth of most lambdoid phages by inhibiting replication of their DNA. Growth of nonlambdoid phages, such as the T phages, is unaffected in these hosts (35).

The second selection, carried out by Saito and Uchida (69), involved mutagenesis, again with nitrosoguanidine, of an *E. coli* lysogen harboring a λ*N*⁻*c*Its prophage. The cells were incubated at 42°C for 24 hours, with survivors expected only if replication of the prophage is inhibited. Two classes of survivors were observed: a) the majority which carried mutations mapping in the prophage genome, and b) a minority which carried mutations mapping in the host genome. Members of this latter class were distinguished by their inability to propagate superinfecting heteroimmune lambdoid phages. They were named *grp*⁻ (*groP*-like).

Eventually, the locations of these mutations (herein collectively referred to as *groP*⁻ to indicate their inability to allow λ DNA replication) on the *E. coli* chromosome were mapped and their designations changed to reflect the not so surprising fact that they also affect *E. coli* DNA synthesis at nonpermissive temperatures (35, 69, 70) [The *grpE*280 mutation affects host RNA and DNA synthesis, also (2, 3); however, its name has not been changed]. The majority of the *groP*A⁻ and *groP*B⁻ mutations of Georgopoulos and Herskowitz (35) and the *grpA* mutations of Saito and Uchida (69) were shown to be coincident with the previously identified *dnaB* gene (47). The original *groP*AB756 mutation (35), also known as *groP*C756 (32), is now called *dnaK*756. The *dnaK* gene forms an operon with the *dnaJ* gene (94). The *grpC*⁻ mutations, which comprise the majority of mutations isolated by Saito and Uchida (69), map in this operon. The *groP*C259 mutation of Sunshine et al. (79) also maps

here and is now known as *dnaJ*259 (70). Fig. 1.1 shows the positions of these genes as well as the *grpE* and *grpD* genes on the *E. coli* genetic map.

The genes *groEL* and *groES* [also known as *mopA* and *mopB* (6)], which form an operon at 94.2 min (27, 33) and are required by λ for proper head assembly (34, 76), were also identified in the two selection methods described. Presently, there is no evidence that their gene products directly participate in initiation of λ DNA replication, although mutations in either gene do affect host RNA and DNA syntheses at the nonpermissive temperature (87).

λ mutants which suppress the GroP⁻ phenotype. Lambda encodes two proteins, λO and λP, which are absolutely essential for replication of its DNA (64). These two proteins have been shown to interact with each other in the initiation of λ DNA replication (83). Spontaneous mutants of λ able to propagate on *E. coli groP*⁻ mutants can be isolated at a frequency of 10^{-8} - 10^{-6} (35, 69, 79). These suppressor mutations, called π (35) or *reg* (69), map in the λ*P* gene, suggesting that interaction between the λ*P* gene product and the various host factors occurs *in vivo* and that the *groP* genes are involved in initiation of λ DNA replication. Density shift experiments as well as other data (32, 35, 69) also suggest that an early event in λ DNA replication is affected in the *groP*⁻ mutants. As shown in Table 1.1, λ*c*I⁻πA1 phage, originally selected on *E. coli dnaB*15, propagate best on *E. coli dnaB*15 mutants. However, λ*c*I⁻πB1 phage, originally selected on *E. coli dnaB*558, propagate well on *E. coli dnaB*15, and *dnaK*756 mutants, in addition to *E. coli dnaB*558. And, although the plaque size is small, the efficiency of plating of λ*c*I⁻πB1 is fairly high on *E. coli dnaJ*259 and *grpE*280, suggesting that the λ*P* gene has mutated to allow partial functional interaction with each host protein. Most λ*reg* mutants isolated on a given mutant strain are also able to propagate on other *groP*⁻ strains (69).

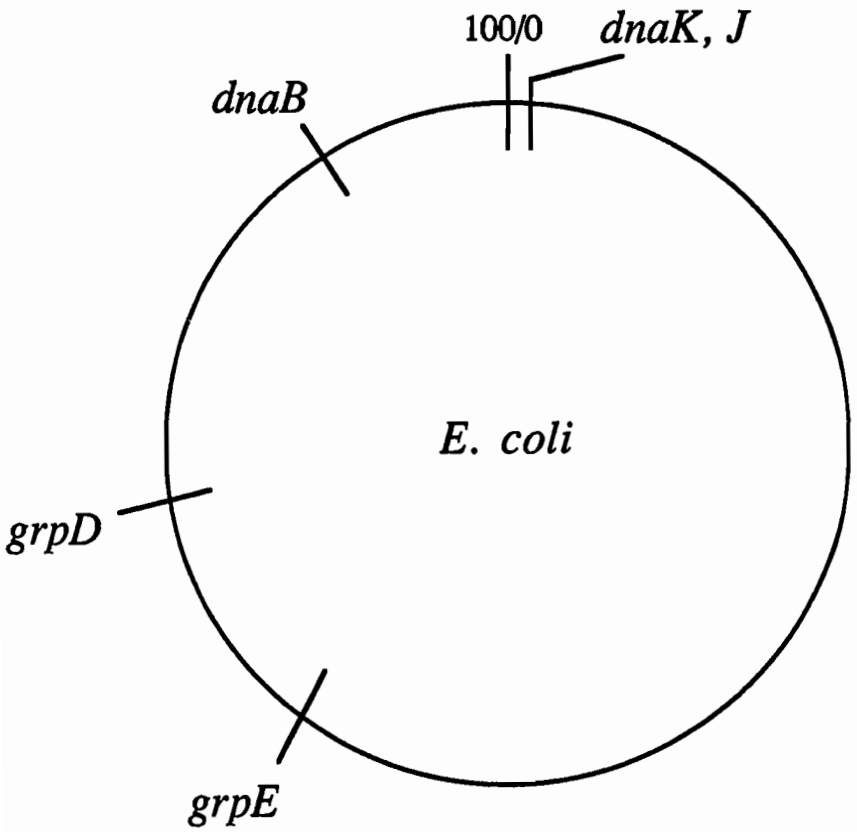


Figure 1.1. Locations of *groP* genes on the *E. coli* genetic map.

Table 1.1

Efficiency of plating of various λπ mutants on various restrictive bacterial hosts

Bacterial strain	λ*c*I⁻	λ*c*I⁻πA1	λ*c*I⁻πB1	λ*c*I⁻πC1	λ*c*I⁻πC2	λ*c*I⁻πE6
B178 wild type	1.0	1.0	1.0	1.0	1.0	1.0
*dnaB*15	$<10^{-4}$	0.7	0.9	0.8	0.02*	0.5*
*dnaB*558	$<10^{-4}$	$<10^{-5}$	0.5	$<10^{-4}$	$<10^{-4}$	$<10^{-5}$
*dnaJ*259	$<10^{-4}$	0.1*	0.4*	0.7*	$<10^{-4}$	$<10^{-5}$
*dnaK*756	$<10^{-4}$	0.05*	1.0	$<10^{-5}$	0.4	0.9
*grpE*280	$<10^{-4}$	0.15*	0.6*	0.05*	0.8*	1.0

Efficiency of plating denotes the number of plaques produced on a given bacterial host at 37°C relative to the number on B178 wild type bacteria. The phage mutants λ*c*I⁻πA1, λ*c*I⁻πB1, λ*c*I⁻πE6 were isolated as plaque fomers on *dnaB*15, *dnaB*558 and *grpE*280 bacteria respectively; λ*c*I⁻πC1 and λ*c*I⁻πC2 were isolated on *dnaJ*259 bacteria. The symbol (*) indicates very small plaque size (32, 35, 79, 94; D.A. and C. Georgopoulos, unpublished data).

Effect of *groP*⁻ mutations on *E. coli*. Although mutations in this group of genes were selected at temperatures as high as 42°C, further studies showed that temperatures above 42°C are lethal to most of the mutant hosts (3, 35, 69, 79). Early experiments showed that host DNA synthesis is defective at the nonpermissive temperature (35, 69, 70). Subsequently, it was shown that RNA synthesis, but not protein synthesis, is also affected by mutations in these genes (3, 43, 88). The *dnaB* gene product is a helicase (49) and is required for unwinding of the DNA duplex in both *ori*λ-specific and *E. coli ori*C-specific replication (8, 98). Thus far, it is unclear what role, directly or indirectly, the other *groP* gene products play in *E. coli ori*C-specific replication. However, Sakakibara (72) has shown that an *E. coli dnaK* mutant is defective in reinitiation of *ori*C-specific replication at the nonpermissive temperature.

As discussed below, the *dnaK*, *dnaJ* and *grpE* genes are members of the heat shock regulon. The *dnaK*⁻, *dnaJ*⁻ and *grpE*⁻ mutations not only interfere with host macromolecular syntheses but also result in an abnormal heat shock response (81; S. Sell, Ph.D. dissertation, University of Utah, 1987).

Factors required for initiation of λ DNA replication.

All the factors discussed in this section have been purified and studied in defined *in vitro* systems (25, 47, 58, 98). The factors discussed here and in Table 1.2, with the exception of RNA polymerase, were defined by virtue of the fact that they are necessary in the purified *in vitro ori*λ-specific DNA replication system described by Zylicz et al. (98). It is possible, however, that these are not necessarily the only elements required for λ DNA initiation *in vivo*.

Cis-acting *ori*λ sequence. A region of the λ genome, approximately 82% from the left end, has been defined by partial denaturation mapping of replicating λ

Table 1.2

Proteins required for initiation of λ DNA replication *in vitro*

	Heat shock protein	Subunit Mr	Native form	Map position	Properties, activities
λ-Encoded proteins					
λO	--	33,800	dimer	38,686-39,583[a]	binds specifically to *ori*λ
λP	--	26,500	dimer	39,582-40,281	inhibits dnaB and dnaK ATPase activity
***E. coli*-encoded proteins**					
dnaB	?	52,300	hexamer	92 min[b]	5'-3' helicase; DNA-dependent NTPase inhibited by λP; binds ss DNA
dnaG	no	65,600	monomer (?)	67 min	primase; utilizes both ribose and deoxyribose triphosphates to synthesize primers at dnaB - ss DNA complexes
dnaJ	yes	41,100	dimer	0.3 min	membrane protein, ss DNA-binding protein
dnaK	yes	69,100	monomer (?)	0.3 min	5'-NTPase inhibited by λP, stimulated by λO; autophosphorylase
grpE	yes	22,500	monomer (?)	57 min	reduces by 10-fold the amount of dnaK necessary for *in vitro* replication and unwinding; autophosphorylase (?)
gyrA, B	no	97,000 89,800	$\alpha_2 \beta_2$	48 min 83 min	gyrase, type II topoisomerase; DNA-dependent ATPase
ssb	no	18,800	tetramer	92 min	binds and stabilizes ss DNA; inhibits dnaB binding to ss DNA

[a] Coordinates on λ DNA sequence (73).

[b] Location on the E. coli genetic map (6).

molecules as the site of initiation of λ DNA replication. It is a sequence of approximately 130 bp and is found within the coding sequences of the λ*O* gene. The first 86 bp comprise four 19-bp direct repeats, followed by an AT-rich region of approximately 40 bp (review in 29, 45). It has been demonstrated that the 19-bp DNA repeats exhibit bending properties (95), a feature which may be important with respect to binding of λO protein and initiation of λ DNA replication (74, 96). The AT-rich region is conserved among lambdoid phages and is most likely involved in melting of the duplex following binding of λO protein (15). Its importance is suggested by the isolation of in-frame deletions in this region which still encode functional λO protein but are incapable of initiating replication (22).

λ-encoded O and P proteins. Lambda encodes only two proteins, λO and λP, for replication of its DNA (reviewed in 28, 29,45; see Table 1.2). The λO protein is required for specific recognition of the *ori*λ sequence. In its native form, λO is a dimer and a minimum of four such dimers are required to bind the four 19-bp repeats at the origin (85, 100). In addition, the λO protein equivalent of other lambdoid phages such as ϕ80 and ϕ82 cannot substitute for λO at *ori*λ (29). This was elegantly demonstrated by Furth and Yates (30) who constructed hybrid λ*O* genes in which approximately each half of the gene was derived from a different lambdoid phage. They showed that λO is composed of two domains, the amino-terminal region which recognizes and binds specifically to the *ori* sequence, and the carboxy-terminal region which interacts with the P protein.

In vivo, the λO protein has a half-life of less than 2 minutes (55). This feature could be important in terms of regulating the frequency of initiation from *ori*λ or arresting DNA replication following integration of λ into the genome.

The λP protein is required for attracting host proteins to *ori*λ. It acts as a go-between by interacting with both λO and host proteins such as dnaB, as suggested by

the fact that mutations which suppress the GroP⁻ phenotype map in the λ*P* gene (see above). Some of these interactions have been observed *in vitro* and will be discussed below. Like λO, the native form of λP also appears to be a dimer (100). However, unlike λO, the λP protein is fairly stable (55).

The dnaB protein. The *dnaB* gene product is required by *E. coli* for replication of its DNA. It has also been shown that many phages including φX174, P2, and λ depend on the *dnaB* gene product for replication of their DNA (45, 47, 48, 59). [P1 encodes its own dnaB analog, called the ban protein (21,63).] In its native form, dnaB forms a hexamer and binds single-stranded DNA (47, 92). It is a single-stranded DNA-dependent nucleoside triphosphatase, in addition to being a 5' to 3' helicase which processively unwinds duplex DNA (49). It has been shown that the dnaB - single-stranded DNA complex is recognized specifically by the dnaG primase in order to initiate synthesis of a primer (47, 48). Recently, it has been shown by insertion mutagenesis that the last six amino acids of the carboxy-terminus of dnaB are not required for *E. coli* replication at temperatures below 37°C; however, λ replication is affected at all temperatures (S.-F. Chang, D. Ng and C. Georgopoulos, unpublished results). This result suggests that the λP protein may be interacting with the carboxy-terminal domain of the dnaB protein.

The dnaK and dnaJ proteins. The *dnaK* and *dnaJ* genes form an operon located at 0.3 min on the *E. coli* genetic map, with the order being promoter-*dnaK*-*dnaJ* (94). The dnaK protein is found in higher copy number in the cell than dnaJ (99, 102). One possible explanation for the differential is the formation of a potential hairpin loop in the RNA sequence between *dnaK* and *dnaJ*, which may cause premature termination of RNA polymerase following transcription of *dnaK* (12). The dnaK protein has been purified on the basis of its ability to bind specifically to ATP (S. Sell, Ph.D. dissertation, University of Utah, 1987). Its activity can be monitored

by its ability to complement both the *in vitro* λO-, λP-dependent M13 single-stranded DNA replication system (50) and the *in vitro* λdv DNA replication system, using a crude soluble extract (FII) deficient in wild type dnaK protein (99). The dnaK protein has both autophosphorylase and 5'-nucleotidase activities (13, 101). The 5'-nucleotidase activity is heat-resistant and weak in comparison to other known nucleotidases (13). *In vitro*, this activity is inhibited and stimulated by purified λP and λO proteins, respectively (101).

The dnaJ protein was purified from the membrane fraction of bacteria transformed with a plasmid which overproduces the protein (102). Its activity was monitored by its ability to complement the *in vitro* λdv DNA replication assay using a FII extract deficient in wild type dnaJ protein (102). The dnaJ protein, unlike dnaK and grpE, is a basic protein (12). In addition, it binds to both single-and double-stranded DNA in a sequence-independent manner (102).

The grpE protein. The product of the *grpE* gene was first identified by analysis of proteins encoded by a λ*grpE*$^+$ transducing phage and its deletion derivatives (71). It was purified on the basis of its well-documented ability to form a complex with the dnaK protein and the disruption of this complex by ATP (2, 97). The grpE protein is required in the *in vitro* λdv DNA replication assay using a FII extract derived from mutant *E. coli grpE*280 cells or *E. coli grpE*$^\Delta$ cells in which the majority of the *grpE* coding sequence has been deleted (4, 97; J. Skorko and M. Zylicz, unpublished data). However, in an *in vitro* system using only purified components, λdv DNA can be replicated in the absence of grpE (58, 98). Interestingly, the addition of grpE to this purified system allows at least a ten-fold reduction in the concentration of dnaK required in this system (98).

It has been shown both genetically and biochemically that the dnaK and grpE proteins interact (2, 44, 97). The importance of this interaction is demonstrated by the

fact that the mutant phenotypes of the *E. coli dnaK*756 and *E. coli grpE*280 strains are correlated to a significant decrease in complex formation between the mutant dnaK756 and grpE280 proteins and the wild type grpE and dnaK proteins, respectively. In terms of λ DNA replication, the isolation of λπ mutants which can propagate on *E. coli grpE*$^-$ cells (69) and the *in vitro* isolation of a grpE-λP complex following glutaraldehyde crosslinking (D. A., M. Zylicz and C. Georgopoulos, unpublished data) suggest that interaction between grpE and λP is also important for initiation.

DNA gyrase. *E. coli* DNA gyrase is the prototype of Type II topoisomerases (reviewed in refs. 31, 47, 90,91). It is a DNA-dependent ATPase and in the presence of ATP, converts relaxed duplex DNA to a negatively supercoiled form. In the absence of ATP, relaxation of supercoiled DNA occurs. Gyrase is a tetramer composed of two subunits which are the gene products of *gyrA* and *gyrB*. The *gyrA* and *gyrB* genes map at 48 min and 82 min on the *E. coli* genetic map, respectively (31, 47). The gyrA subunit is sensitive to nalidixic and oxolinic acid, while the gyrB subunit is sensitive to coumermycin A_1 and novobiocin (47, 98), with all four drugs inhibiting DNA replication in *E. coli*. Mutations which confer drug resistance map in the corresponding gene (31, 47). *In vitro*, gyrase has been shown to be required for both *ori*C-specific and *ori*λ-specific DNA replication (47, 98), most likely to assist melting of the duplex at the origin, thus allowing entrance of the dnaB helicase, and to subsequently maintain the negative superhelicity of the DNA as positive supercoils are introduced during the unwinding of the duplex at the replication fork. In addition, it may be required to decatenate the newly synthesized daughter circles. *In vivo*, the opposing actions of gyrase and topoisomerase I, which relaxes negative supercoils, are required to homeostatically regulate the superhelical state of the chromosome (31, 90). The importance of the superhelical state of the DNA with respect to replication is

emphasized by the facts that *in vivo* replication is blocked in *gyr*$^-$ or *top*$^-$ mutants and that a relaxed plasmid does not support replication *in vitro* (1).

Single-stranded DNA binding protein. *E. coli* single-stranded DNA binding protein (ssb) is a stable tetramer of 18.5 kd subunits (reviewed in refs. 17, 47). It binds to single-stranded DNA preferentially and cooperatively in a sequence-independent manner. DNA bound by ssb appears shorter in length, suggesting that the DNA is coiled around the tetramer (17). The amino-terminal region of the protein contains the DNA binding domain while the carboxy-terminal region, in analogy to T4 gp32, makes contact with other proteins involved in DNA metabolism (17). One of the roles of ssb in DNA replication is to assist dnaB in destabilization of the duplex at the replication fork (47). Its ability to bind single-stranded DNA allows DNA polymerase III to migrate along the template unimpeded by secondary structure (47).

The dnaG primase. The dnaG primase has the specific role in *E. coli* and λ DNA replication of synthesizing primers for initiating leading strand synthesis and also primers for synthesis of Okazaki fragments on the lagging strand (reviewed in 47, 59). As the dnaB helicase migrates 5' to 3' along the lagging strand template, unwinding the duplex at the replication fork, dnaG, in the presence of ATP, may join this dnaB - single-stranded DNA complex. DnaG then periodically lays down primers which are extended into DNA by DNA polymerase III holoenzyme.

The dnaG primase, unlike RNA polymerase, is not sensitive to rifampicin (47). In addition, it is capable of polymerizing both ribo- and deoxyribonucleoside triphosphates (47).

RNA polymerase. Transcription near the *oriλ* sequence is required for initiation of λ DNA replication *in vivo* (29). Historically, two possibilities have been considered to explain the need for this so-called "transcriptional activation" phenomenon, namely, a) to separate the strands in the duplex so that the prepriming

proteins can recognize and bind to the site, or b) to provide an RNA primer for leading strand DNA synthesis. Thus far, there is no evidence that either possibility is correct. McMacken et al. have shown recently that *in vitro* "transcriptional activation" can be bypassed provided the *E. coli* HU and other nonspecific DNA binding proteins, such as integration host factor (IHF), are absent from the reaction (57). The HU protein is the *E. coli* equivalent of histones in eukaryotes (47). Transcription in the region of *ori*λ may displace HU and other binding proteins, allowing λO and other prepriming proteins proper access to the *ori*λ region.

Assembly of the preprimosomal complex.

Known interactions among proteins. Protein-protein interactions among the phage- and host-encoded gene products required for prepriming (that is, events which occur prior to RNA primer synthesis) are shown in Table 1.3. In most instances, the inferred protein-protein interaction is substantiated by both genetic and biochemical evidence, e. g., λP and dnaB. However, in other instances the evidence for a putative interaction is weak, e. g., λP and dnaJ, and perhaps should be taken *cum grano salis*, until further studies are completed.

Steps in initiation of λ DNA replication. In a recent review by Bramhill and Kornberg (15), a model for initiation of replication at various origins similar in size and structure to *E. coli oriC* is presented. It proposes that the primary functions of an origin-recognizing protein, e. g., dnaA, are a) to bind to repeat sequences at the origin to form an "initial complex," b) subsequently to melt a nearby AT-rich region to form an "open complex," and c) to direct the formation of a prepriming complex within the melted region which will ultimately result in progression of the replication forks.

Table 1.3

Interactions among λ- and *E. coli*-encoded proteins required for initiation of λ DNA replication *in vitro*

	oriλ	λO	λP	dnaB	dnaK	dnaJ	grpE
oriλ		1					
λO		2	3		4		5
λP			6	7	8	9	10
dnaB				11		12	
dnaK						13	14
dnaJ						15	
grpE							16

1. The amino-terminus of λO binds preferentially to DNA fragments containing the 19-bp repeats found in *oriλ* (85).

2. λO sediments as a dimer under native conditions (100).

3. Mutations in λ*P* can suppress a λ*O*ts mutation (83). In hybrid λO proteins, the carboxy-terminal region determines which lambdoid P protein can be used for replication (30). A λO-λP complex can be isolated by gel filtration (93).

4. λO protein stimulates dnaK 5'-nucleotidase activity *in vitro* (10). λO binds to a dnaK-affinity column and is eluted at 0.6 M KCl (52). A λO - dnaK complex can be isolated by glutaraldehyde crosslinking (M. Zylicz, unpublished data) and by gel filtration (52).

5. A λO - grpE complex can be isolated by glutaraldehyde crosslinking (M. Zylicz and D. A., unpublished data).

6. λP sediments as a dimer under native conditions (100).

7. Active λP protein can be copurified with dnaB as a complex (46, 93). λP inhibits the DNA-dependent ATPase activity of dnaB *in vitro* (93). λπ mutants which suppress the GroP$^-$ phenotype in *E. coli dnaB*$^-$ mutants can be isolated (35).

8. λP inhibits dnaK 5'-nucleotidase activity *in vitro* (101). λπ mutants can be isolated which propagate on the *E. coli dnaK*756 mutant (35). λP binds to a dnaK-affinity column and is eluted in 10 mM ATP (52). λP-dnaK complexes can be isolated by glutaraldehyde crosslinking and by gel filtration (52; M. Zylicz, unpublished data).

9. λπ mutants able to propagate on *E. coli dnaJ*259 can be isolated (79). λP binds to a dnaJ-affinity column, and is eluted in 50 mM KCl (K. Liberek and M. Zylicz, unpublished data).

10. λπ mutants able to propagate on *E. coli grpE*280 can be isolated (69). A λP - grpE complex can be isolated by glutaraldehyde crosslinking (D. A. and M. Zylicz, unpublished data).

11. DnaB forms a hexamer under native conditions (5, 47, 67).

12. DnaB binds to a dnaJ-affinity column and is eluted by 0.2 M KCl (K. Liberek and C. Georgopoulos, unpubished data).

13. DnaJ binds to a dnaK-affinity column and is eluted by 0.5 M KCl (S. Sell, Ph.D. dissertation, University of Utah, 1987). Some extragenic suppressors of *dnaJ*259 map in the region of the *dnaK* gene (S. Sell, Ph.D. dissertation, University of Utah, 1987).

14. DnaK and grpE are co-immunoprecipitated by either anti-dnaK or anti-grpE antibodies (2, 44). GrpE binds to a dnaK-affinity column, is not eluted in 2 M KCl, but is eluted in 10 mM ATP. GrpE cosediments with dnaK in glycerol gradients except in the presence of ATP (97). A dnaK-grpE complex can be isolated by glutaraldehyde crosslinking (Ch. 4; D. A. and M. Zylicz, unpublished data). A dnaK-grpE280 complex can also be isolated but this complex is less stable (Ch. 4). Some Tr^+ revertants of *E. coli grpE*280 have mutations which map in the *dnaK* gene (44).

15. Under native conditions, dnaJ acts as a dimer (102).

16. In glycerol gradients, grpE behaves as a monomer (97). However, dimers can be isolated by glutaraldehyde crosslinking (D. A. and M. Zylicz, unpublished data). Under native conditions, ^{35}S-grpE behaves as an aggregate of undetermined size by gel filtration (D. A., unpublished data).

There is ample evidence that the events leading to *in vitro* initiation at *oriλ* occur as described above. First, a stable *oriλ* - λO complex can be isolated which can be visualized by electron microscopy and shown to be active in λ DNA replication (25). From measurements of the diameter of the complex and the entry and exit points of the DNA in the complex, it was inferred that the DNA is folded or wound around λO (25), forming the "initial complex" or "O-some" structure. Recently, it has been shown that binding of λO to *oriλ*, in an ATP-independent, superhelicity-dependent fashion, causes destabilization of the duplex within the AT-rich region adjacent to *oriλ* (74), resulting in formation of the "open complex." The well-documented λO - λP and λP - dnaB interactions (see Table 1.3) and the fact that addition of λP and dnaB proteins to *oriλ*-containing DNA and λO results in the formation of a larger complex at *oriλ* (whereas λP and dnaB, in the absence of λO, do not form any visible complex) suggest that λO provides the base upon which the prepriming complex is built (25).

The λP protein has been described as having a Dr. Jekyll - Mr. Hyde complex, strongly depending on the position of the moon relative to Skourochori, Greece (C. Georgopoulos, personal communication). That is, although λP is crucial for localizing dnaB at *oriλ*, in the absence of other factors it inhibits dnaB activity (52, 92). The subsequent addition of dnaK, dnaJ and ssb results in local unwinding of the duplex in an ATP-dependent manner (24, 98). It has been shown that the action of dnaK and dnaJ proteins results in the release of λP, thus liberating dnaB from the *oriλ* - λO - λP complex (52) to move through adjoining DNA. The ssb protein prevents reannealing of the duplex as dnaB carries out ATP-dependent unwinding. The requirement for grpE in this reaction has only recently been elucidated. Its presence results in at least a ten-fold reduction in the amount of dnaK protein required to liberate the dnaB protein (98).

Once dnaB-dependent unwinding has occurred, replication can commence. DnaG primase synthesizes primers along the template which are elongated into DNA by DNA polymerase III holoenzyme. The series of events which occur at this step *in vivo* are still unclear since only unidirectional replication is seen in the *in vitro* replication system composed of purified components (24). In addition, it is not known whether other accessory proteins besides dnaB and dnaG make up the mobile replication complex. That is, as dnaB migrates processively along the lagging strand template with dnaG generating primers, do dnaK, dnaJ and/or grpE (in the presence or absence of λP) remain bound to dnaB to help stabilize it and allow it to be processive?

The Heat Shock Response in *E. coli*

The first observation of a heat shock effect is generally ascribed to the localized puffing of polytene chromosomes in Drosophila salivary glands upon experiencing a sudden increase in ambient temperature (68). Since then, this phenomenon has been documented in many systems (reviewed in refs. 20, 53, 60, 61) and appears to be a highly conserved cellular response to stress. The most obvious effect of the heat shock response is the rapid yet transient increase in the rate of transcription of a defined set of genes in response to changes in environmental conditions. These genes are said to form a "regulon", defined as a group of genes dispersed throughout the genome but regulated by a common mechanism (61). Although heat shock is an efficient method of inducing the response, other agents such as ethanol, DNA damaging agents and cadmium also induce transcription of at least a subset of these genes (60, 61, 86). It is thought that one of the consequences of the induction of the heat shock or stress response is to enable an organism to better withstand the harsh environmental conditions it may encounter (66). In this section, the heat shock response in *E. coli* will be emphasized. In this system, several of the host functions

identified as necessary for λ growth have been shown to be heat shock proteins, including groEL, groES, dnaK, dnaJ and grpE. Their roles in λ growth may provide clues not only to their roles in *E. coli* but also in higher organisms since these genes appear to be highly conserved in evolution (20, 60, 61).

Description of the heat shock response in *E. coli*.

When *E. coli* growing at 30°C is suddenly shifted to a higher temperature, e. g., 43°C, and the proteins pulse-labeled with [^{35}S]-methionine, electrophoresis (on a two-dimensional gel which separates proteins by both charge and size) of the crude lysate shows a group of approximately 20 proteins which are preferentially labeled (61). The higher rate of expression of these proteins is transient; that is, the rate of synthesis increases rapidly the first few minutes after the shift but then begins decreasing at approximately 8 minutes after the shift, returning to steady state levels by 15 minutes (reviewed in refs. 60, 61). If the cells are kept continuously at the high temperature, the rate of expression does not return to the initial basal level but, instead, decreases to an approximately two-fold higher steady state level (61).

The response in *E. coli* is positively regulated by the *rpoH* (*htpR*) gene product, σ^{32} (38, 39). This sigma factor, when complexed with the RNA polymerase core, allows the recognition of a promoter sequence common to most heat shock-regulated genes sequenced thus far (19, 38). The *rpoH* gene product itself is under a complex, heat-dependent mechanism of regulation. The *rpoH*-specific mRNA levels are higher after a shift to 42°C, resulting from a combination of both an increase in transcription and mRNA stabilization (26, 78, 80). Recently, it has been shown that a new σ factor, called σ^{24} or σ^{E}, in combination with the *E. coli* RNA polymerase core transcribes one of the *rpoH* promoters. The level of σ^{24}-promoted *rpoH* gene transcription is highest at 50°C, resulting in preferential synthesis of *rpoH* mRNA (26;

J. Erickson and C. Gross, personal communication). The increased σ^{32} levels at 50°C, in turn, result in higher levels of expression of the heat shock genes. Lambda, upon infection of *E. coli*, is also able to induce the heat shock response (7, 89). The mechanism employed by λ involves a stabilization of σ^{32} by the λcIII gene product (7).

Known σ^{32}-regulated genes in *E. coli*.

GroEL, groES. The *groEL, groES* genes of *E. coli* encode polypeptides identified as the heat shock proteins B56.5, and C15.4, respectively (62, 82). They form an operon, located at 94.2 min on the *E. coli* genetic map (6), whose transcription is regulated from two promoters, one of which is heat shock-regulated (19, 27). GroEL is an abundant protein which has been highly conserved throughout evolution, with analogs found in plant and animal cells (40). Its native form in *E. coli* is a decatetramer of two 7-subunit rings stacked on top of each other (41, 42). It has been proposed that its role in cellular function is as a "chaperonin." That is, it chaperones or assists other proteins to form larger protein complexes required for their function, but is not part of the final complex (40). Some examples supporting this role are a) the assembly of proheads prior to packaging of λ DNA: although it is not included in the final product, groEL is thought to provide a scaffold for the proper assembly of the λB dodecameric head-tail connector structure of λ (36); b) suppression of the *dnaA*46 mutation of *E. coli* by overproduction of groEL (27): binding of the dnaA protein to "dnaA boxes" at *ori*C is required for proper initiation of *E. coli* DNA replication (15); interaction of groEL with the mutant dnaA protein may sufficiently stabilize an inherently unstable structure to allow assembly of the prepriming complex; c) assembly of the ribulose bisphosphate carboxylase-oxygenase (Rubisco) subunits by the groEL analog in plants, called the Rubisco binding protein

(reviewed in 40). The role of groEL and groES in heat shock is not known. One possibility entertained is that they help disaggregate complexes of denatured proteins which result from stress.

***DnaK, dnaJ,* and *grpE*.** These three gene products were originally identified as necessary for λ DNA replication (see above). The dnaK and grpE proteins were subsequently identified as the B66.0 and B25.3 heat shock proteins of *E. coli* (3, 37, 61). DnaK is the prokaryotic analog of the Hsp70 protein of higher organisms with 48% of its amino acid sequence identical to that of the Drosophila Hsp70 (9). DnaJ and grpE have not been studied as extensively as dnaK. However, a grpE analog has been observed in Chlamydomonas using anti-grpE antibody (D. A., unpublished observation).

DnaK is a negative regulator of the heat shock response in *E. coli*. In the *E. coli* *dnaK*756 mutant, the basal level of heat shock proteins at 30°C is higher than in the wild type parent. Although a shift to higher temperature induces the heat shock response normally, the subsequent decrease in heat shock gene expression is not observed (81). The *dnaJ* and *grpE* gene products may also have a role in regulation of the heat shock response since mutations in these genes also result in overproduction of heat shock proteins at 30°C (S. Sell, Ph.D. dissertation, University of Utah, 1987).

Besides regulation of the heat shock response, dnaK has been implicated in the refolding of denatured proteins during recovery from stress. C. Johnson (Ph.D. dissertation, University of Utah, 1988) has shown that dnaK binds preferentially to an improperly folded, denatured form of bovine pancreatic trypsin inhibitor (BPTI) as compared to the native form . Whether dnaK truly facilitates protein renaturation in *E. coli* is not known yet. However, evidence for another potential role of dnaK, translocation of proteins across membrane (18, 23), supports the idea that dnaK is able to manipulate or influence the conformation of other proteins. This ability may be

related to its function in initiation in λ DNA replication, that of freeing the dnaB helicase from its complex with λP (see above). There is evidence for a dnaK - λP interaction (Table 1.3); one possible scenario is that dnaK binds to λP, changing λP's conformation and resulting in the release of dnaB. Again, neither dnaJ nor grpE have been studied as extensively as dnaK. However, their observed interaction with dnaK (Table 1.3) indicates perhaps a role in regulating dnaK activity.

HtpG, lon, lysU, and rpoD. The C62.5, H94.0, D60.5 and B83.0 heat shock proteins of *E. coli* have been shown to be encoded by the *htpG*, *lon*, *lysU* and *rpoD* genes, respectively (for review, see 61). The C62.5 protein has been purified and characterized but no activities have been ascribed to it nor is its function known (75). It has been shown that the C62.5 protein is 42% identical at the amino acid level to the Drosophila Hsp90 protein (10). Its gene is not essential for *E. coli* growth and can be deleted from the genome with no apparent major consequence under the conditions tested (11). The *lon* gene product is an ATP-dependent protease and, in addition to belonging to the heat shock regulon, is also induced by the SOS response (61). *LysU* encodes one of the two lysyl-tRNA synthetases in *E. coli* whose function is to charge $tRNA^{lys}$ with the amino acid lysine. However, under normal growth conditions, the *lysU* gene is not expressed. One of the important functions of tRNA synthetases, especially *lysU*, is to synthesize the dinucleotide ApppA which has been postulated to be a signal of heat stress (14, 51). The *rpoD* gene product is the σ^{70} subunit of RNA polymerase which allows the polymerase to recognize normal promoters of genes expressed under non-heat shock conditions (61). It may have been placed under heat shock regulation as part of the recovery mechanism of the cell. That is, it may be functioning as a negative regulator of the response. As its level transiently rises during heat shock, it may competitively inhibit σ^{32} from associating with RNA polymerase, resulting in the subsequent decrease in the transcription of heat

shock genes. The role of dnaK and possibly dnaJ and grpE, in regulation of the heat shock response may also be at this level. In a manner similar to their role in liberating the dnaB helicase from λP, these three heat shock proteins may be displacing σ^{32} from the RNA polymerase core, thus allowing σ^{70} entry.

A new heat shock-induced regulon? Genes essential for *E. coli* growth only at high temperature were identified by insertion mutagenesis at 30°C and subsequent screening for the inability to grow at 43°C. Transcription of one of the genes identified, *htrA* (high temperature required), was shown to be heat-inducible but σ^{32}-independent (56). Interestingly, the promoter of *htrA* is identical to the promoter for the σ^E gene, a newly discovered sigma factor of *E. coli* (26; J. Erickson and C. Gross, personal communication). These two genes, *htrA* and σ^E, may define a new heat-inducible regulon separate from that controlled by σ^{32}. It has been shown that the *htrA* gene is specifically transcribed both *in vivo* and *in vitro* by the $E\sigma^E$ holoenzyme, but not by the $E\sigma^{32}$ and $E\sigma^{70}$ holoenzymes (J. Bardwell, C. Gross, B. Lipinska and C. Georgopoulos, unpublished results). The *htrA* gene has been sequenced (56) and its product, a processed protein (54), was fortuitously identified as the *degP* gene product, a protease (77).

Essentiality of *groP* Genes in *E. coli*

Because *dnaK*, *dnaJ*, *groEL*, *groES* and *grpE* belong to the heat shock regulon, and because strains with mutations in these genes appear normal for bacterial (but not phage) growth at 30°C, it was postulated that perhaps these genes would not be essential for *E. coli* at 30°C. Attempts to construct strains with null mutations in these genes has led to the conclusion that, in a wild type background, these genes are essential for *E. coli* at all temperatures. However, it is possible to isolate extragenic suppressors which allow the deletion of *dnaK*, *dnaJ* and *grpE* (4, 16, 65; S. Sell,

Ph.D. dissertation, University of Utah, 1987). Potential mechanisms of such suppression include: a) a protein which normally interacts with dnaK, dnaJ and/or grpE may be altered or overproduced such that the interaction is no longer required, b) a protein which is not normally involved in dnaK, dnaJ and grpE functions may be altered or overproduced such that it can now substitute for the missing protein, c) activation of a cryptic gene of *E. coli* whose product can substitute for the missing function, or d) the mechanism may be indirect, i. e., it could be that *E. coli* bacteria can grow in the absence of dnaK or dnaJ or grpE if the rate of growth is slow. Mutations which result in slow growth may allow *E. coli* to survive the lack of either dnaK, dnaJ or grpE. The significance of identifying the genes in which these suppressors map is that they may reveal new regulons in which the three proteins participate, and provide a better understanding of the mechanism of dnaK, dnaJ and grpE action.

Literature Cited

1. **Alfano, C. and R. McMacken.** 1988. The role of template superhelicity in the initiation of bacteriophage λ DNA replication. Nucleic Acids Res. **16**:9611-9630.

2. **Ang, D., G. N. Chandrasekhar, C. Johnson, M. Zylicz and C. Georgopoulos.** 1987. A genetic and biochemical analysis of the *Escherichia coli grpE* gene and its product. *In* R. McMacken and T. Kelly (eds.), UCLA Symp. Mol. Cell. Biol. New Series, **47**:521-532, Alan R. Liss, Inc., New York.

3. **Ang, D., G. N. Chandrasekhar, M. Zylicz and C. Georgopoulos.** 1986. *Escherichia coli grpE* gene codes for heat shock protein B25.3, essential for both λ DNA replication at all temperatures and host growth at high temperature. J. Bacteriol. **167**:25-29.

4. **Ang, D. and C. Georgopoulos.** The heat shock-regulated *grpE* gene of *Escherichia coli* is required for bacterial growth at all temperatures, but is dispensable in certain mutant backgrounds. Submitted to J. Bacteriol.

5. **Arai, K., S. Yasuda and A. Kornberg.** 1981. Mechanism of DnaB protein action. I. Crystallization and properties of DnaB protein, an essential replication protein in *Escherichia coli*. J. Biol. Chem. **256**:5247-5252.

6. **Bachmann, B. J.** 1987. Linkage map of *Escherichia coli* K-12, edition 7. *In* F. C. Neidhardt et al. (eds.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology, pp. 807-876, American Society for Microbiology, Washington D. C.

7. **Bahl, H., H. Echols, D. B. Straus, D. Court, R. Crowl and C. P. Georgopoulos.** 1987. Induction of the heat shock response of *E. coli* through stabilization of σ^{32} by the phage λ*c*III protein. Genes Dev. **1**:57-64.

8. **Baker, T. A., K. Sekimizu, B. W. Funnell and A. Kornberg.** 1986. Extensive unwinding of the plasmid template during staged enzymatic initiation of DNA replication from the origin of the *Escherichia coli* chromosome. Cell **45**:53-64.

9. **Bardwell, J. C. A. and E. A. Craig.** 1984. Major heat shock gene of Drosophila and the *Escherichia coli* heat-inducible *dnaK* gene are homologous. Proc. Natl. Acad. Sci. USA **81**:848-852.

10. **Bardwell, J. C. A. and E. A. Craig.** 1987. Eukaryotic Mr 83,000 heat shock protein has a homologue in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84**:5177-5181.

11. **Bardwell, J. C. A. and E. A. Craig.** 1988. Ancient heat shock gene is dispensable. J. Bacteriol. **170**:2977-2983.

12. **Bardwell, J. C. A., K. Tilly, E. Craig, J. King, M. Zylicz and C. Georgopoulos.** 1986. The nucleotide sequence of the *Escherichia coli* K12 *dnaJ*$^{+}$ gene: a gene that encodes a heat shock protein. J. Biol. Chem. **261**:1782-1785.

13. **Bochner, B. R., M. Zylicz and C. Georgopoulos.** 1986. *Escherichia coli* dnaK protein possesses a 5'-nucleotidase activity that is inhibited by AppppA. J. Bacteriol. **168**:931-935.

14. **Bochner, B. R., P. C. Lee, S. W. Wilson, C. W. Cutler and B. N. Ames.** 1984. AppppA and related adenylylated nucleotides are synthesized as a consequence of oxidation stress. Cell **37**:225-232.

15. **Bramhill, D. and A. Kornberg.** 1988. A model for initiation at origins of DNA replication. Cell **54**:915-918.

16. **Bukau, B., C. E. Donnelly and G. C. Walker.** 1988. The role of the *Escherichia coli* heat shock proteins. *In* M. L. Pardue, J. Feramisco and S. Lindquist (eds.), Stress-induced Proteins, UCLA Symposia on Molecular and Cellular Biology, Alan R. Liss Inc., N. Y., *in press*.

17. **Chase, J. W. and K. R. Williams.** 1986. Single-stranded DNA binding proteins required for DNA replication. Ann. Rev. Biochem. **55**:103-136.

18. **Chirico, W. J., M. G. Waters and G. Blobel.** 1988. 70 K heat shock related proteins stimulate protein translocation into microsomes. Nature **332**:805-810.

19. **Cowing, D. W., J. C. A. Bardwell, E. A. Craig, C. Woolford, R. W. Hendrix and C. A. Gross.** 1985. Consensus sequence for *Escherichia coli* heat shock gene promoters. Proc. Natl. Acad. Sci. USA **82**:2679-2683.

20. **Craig, E. A.** 1985. The heat shock response. Crit. Rev. Biochem. **18**:239-280.

21. **D'Ari, R. A., A. Jaffe-Brochet, D. Touati-Schwartz and M. Yarmolinsky.** 1975. A dnaB analogue specified by bacteriophage P1. J. Mol. Biol. **94**:341-366.

22. **Denniston-Thompson, K., D. D. Moore, K. Kruger, M. Furth and F. R. Blattner.** 1977. Physical structure of the replication origin of bacteriophage lambda. Science **198**:1051.

23. **Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig and R. Schekman.** 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature **332**:800-805.

24. **Dodson, M., H. Echols, S. Wickner, C. Alfano, K. Mensa-Wilmot, B. Gomes, J. H. LeBowitz, J. D. Roberts and R. McMacken.** 1986. Specialized nucleoprotein structures at the origin of replication of bacteriophage λ: Localized unwinding of duplex DNA by six-protein reaction. Proc. Natl. Acad. Sci. U. S. A. **83**:7638.

25. **Dodson, M., J. Roberts, R. McMacken and H. Echols.** 1985. Specialized nucleoprotein structures at the origin of replication of bacteriophage λ: complexes with λO, λP and *Escherichia coli* DnaB proteins. Proc. Natl. Acad. Sci. U. S. A. **82**:4678-4682.

26. **Erickson, J. W., V. Vaughn, W. A. Walter, F. C. Neidhardt and C. A. Gross.** 1987. Regulation of the promoters and transcripts of *rpoH*, the *Escherichia coli* heat shock regulatory gene. Genes Dev. **1**:419-432.

27. **Fayet, O., J.-M. Louarn and C. Georgopoulos.** 1986. Suppression of the Escherichia coli *dnaA*46 mutation by amplification of the *groES* and *groEL* genes. Mol. Gen. Genet. **202**:435-445.

28. **Friedman, D. E., E. R. Olson, C. Georgopoulos, K. Tilly, I. Herskowitz and F. Banuett.** 1984. Interactions of bacteriophage and host

macromolecules in the growth of bacteriophage λ. Microbiol. Rev. **48**:299-325.

29. **Furth, M. E. and S. H. Wickner.** 1983. Lambda DNA replication. *In* R. W. Hendrix, J. W. Roberts, F. W. Stahl and R. A. Weisberg (eds.), Lambda II, pp. 145-173, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

30. **Furth, M and J. Yates.** 1978. Specificity determinants for bacteriophage lambda DNA replication. II. Structure of O protein of λ-ϕ80 and ϕ-82 hybrid phages and of a λ mutant defective in the origin of replication. J. Mol. Biol. **126**:227-240.

31. **Gellert, M.** 1981. DNA topoisomerases. Annu. Rev. Biochem. **50**:879-910.

32. **Georgopoulos, C. P.** 1977. A new bacterial gene (*groPC*) which affects λ DNA replication. Mol. Gene. Genet. **151**:35-39.

33. **Georgopoulos, C. P. and H. Eisen.** 1974. Bacterial mutants which block phage assembly. J. Supramol. Struct. **2**:349-359.

34. **Georgopoulos, C. P., R. W. Hendrix, S. R. Casjens and A. D. Kaiser.** 1973. Host participation in bacteriophage lambda head assembly. J. Mol. Biol. **76**:45-60.

35. **Georgopoulos, C. P. and I. Herskowitz.** 1971. *Escherichia coli* mutants blocked in lambda DNA synthesis. *In* A. D. Hershey (ed.), The bacteriophage lambda, pp. 553-564, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

36. **Georgopoulos, C., K. Tilly and S. Casjens.** 1983. Lambdoid phage head assembly. *In* R. W. Hendrix, J. W. Roberts, F. W. Stahl and R. A Weisberg (eds.), Lambda II, pp. 279-304, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

37. **Georgopoulos, C. P., K. Tilly, D. Drahos and R. Hendrix.** 1982. The B66.0 protein of *Escherichia coli* is the product of the *dnaK* gene. J. Bacteriol. **149**:1175-1177.

38. **Grossman, A. D., J. W. Erickson and C. A. Gross.** 1984. The *htpR* gene product of *E. coli* is a sigma factor for heat-shock promoters. Cell **38**:383-390.

39. **Grossman, A. D., D. B. Straus, W. A. Walter and C. A. Gross.** 1987. σ^{32} synthesis can regulate the synthesis of heat shock proteins in *Escherichia coli.* Genes Dev. **1**:179-184.

40. **Hemmingsen, S. M., C. Woolford, S. M. van der Vies, K. Tilly, D. T. Dennis, C. P. Georgopoulos, R. W. Hendrix and R.J. Ellis.** 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. Nature **333**:330-334.

41. **Hendrix, R. W.** 1979. Purification and properties of GroE, a host protein involved in bacteriophage assembly. J. Mol. Biol. **129**:375-392.

42. **Hohn, T., B. Hohn, A. Engel, M. Wurtz and P. R. Smith.** 1979. Isolation and characterization of the host protein GroE involved in bacteriophage lambda assembly. J. Mol. Biol. **129**:359-373.

43. **Itikawa, H. and J.-I. Ryu.** 1979. Isolation and characterization of a temperature-sensitive *dnaK* mutant of *Escherichia coli* B. J. Bacteriol. **138**:339-344.

44. **Johnson, C., G. N. Chandrasekhar and C. Georgopoulos.** The dnaK and grpE heat shock proteins in *Escherichia coli* interact both *in vivo* and *in vitro*. Submitted to J. Bacteriol.

45. **Keppel, F., O. Fayet and C. Georgopoulos.** 1988. Strategies of bacteriophage DNA replication. *In* R. Calendar (ed.), The bacteriophages, 2:145-262, Plenum Press, N. Y.

46. **Klein, A., E. Lanka and E. Schuster.** 1980. Isolation of a complex between the P protein of phage λ and the *dnaB* protein of *Escherichia coli*. Eur. J. Biochem. **105**:1-6.

47. **Kornberg, A.** 1980. DNA replication. W. H. Freeman and Co., San Francisco.

48. **Kornberg, A.** 1982. 1982 supplement to DNA replication. W. H. Freeman and Co., San Francisco.

49. **LeBowitz, J. H. and R. McMacken.** 1986. The *Escherichia coli* DnaB replication protein is a DNA helicase. J. Biol. Chem. **261**:4738-4748.

50. **LeBowitz, J. H., M. Zylicz, C. Georgopoulos and R. McMacken.** 1985. Initiation of DNA replication on single-stranded DNA templates catalyzed by purified replication proteins of bacteriophage λ and *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **82**:3988-3992.

51. **Lee, P. C., B. R. Bochner and B. N. Ames.** 1983. AppppA, heat-shock stress, and cell oxidation. Proc. Natl. Acad. Sci. U. S. A. **80**:7496-7500.

52. **Liberek, K., C. Georgopoulos and M. Zylicz.** 1988. The role of the *Escherichia coli* DnaK and DnaJ heat shock proteins in the initiation of bacteriophage λ DNA replication. Proc. Natl. Acad. Sci. U. S. A. **85**:6632-6636.

53. **Lindquist, S.** 1986. The heat shock response. Ann. Rev. Biochem. **55**:1151-1191.

54. **Lipinska, B., O. Fayet, L. Baird and C. Georgopoulos.** 1988. Identification, characterization and mapping of the *Escherichia coli htrA* gene, whose product is essential for bacterial viability only at elevated temperatures. J. Bacteriol., *in press*.

55. **Lipinska, B., A. Podhajska and K. Taylor.** 1980. Synthesis and decay of λ DNA replication proteins in minicells. Biochem. Biophys. Res. Commun. **92**:120.

56. **Lipinska, B., S. Sharma and C. Georgopoulos.** 1988. Sequence analysis and transcriptional regulation of the *htrA* gene of *Escherichia coli*: a σ^{32}-independent mechanism of heat-inducible transcription. Nucleic Acids Res., *in press*.

57. **McMacken, R., C. Alfano, B. Gomes, J. H. LeBowitz, K. Mensa-Wilmot, J. D. Roberts and M. Wold.** 1987. Biochemical mechanisms in the initiation of bacteriophage λ DNA replication. *In* T. J. Kelly and R. McMacken (eds.), DNA replication and recombination, pp. 227-245, Alan R. Liss, Inc., N. Y.

58. **McMacken, R., K. Mensa-Wilmot, C. Alfano, R. Seaby, K. Carroll, B. Gomes and K. Stephens.** 1988. Reconstitution of purified protein systems for the initiation and regulation of bacteriophage λ DNA replication. *In* T. Kelly and B. Stillman (eds.), DNA replication in eukaryotes, **6**:25-34, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (Cancer Series).

59. **McMacken, R., L. Silver and C. Georgopoulos.** 1987. DNA replication. *In* F.C. Neidhardt et al. (eds.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology, pp. 564-612, American Society for Microbiology, Washington, D.C.

60. **Neidhardt, F. C. and R. A. VanBogelen.** 1987. Heat shock response. *In* F. C. Neidhardt et al. (eds.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology, pp. 1334-1345, American Society for Microbiology, Washington, D. C. .

61. **Neidhardt, F. C., R. A. VanBogelen and V. Vaughn.** 1984. The genetics and regulation of heat shock proteins. Ann. Rev. Genet. **18**:295-329.

62. **Neidhardt, F. C., T. A. Philips, R. A. VanBogelen, M. W. Smith, Y. Georgalis and A. R. Subramanian.** 1981. Identity of the B56.5 protein, the A protein, and the *groE* gene product of *Escherichia coli*. J. Bacteriol. **145**:513-520.

63. **Ogawa, T.** 1975. Analysis of the *dnaB* function of *Escherichia coli* K12 and the *dnaB*-like function of P1 prophage. J. Mol. Biol. **94**:327.

64. **Ogawa, T. and J.-I. Tomizawa.** 1968. Replication of bacteriophage DNA. I. Replication of DNA of lambdoid phage defective in early functions. J. Mol. Biol. **38**:217.

65. **Paek, K. and G. C. Walker.** 1987. *Escherichia coli dnaK* null mutants are inviable at high temperature. J. Bacteriol. **169**:283-290.

66. **Pelham, H. R. B.** 1986. Speculations on the functions of the major heat shock and glucose-regulated proteins. Cell **46**:959-961.

67. **Reha-Krantz, L. J. and J. Hurwitz.** 1978. The *dnaB* gene product of *Escherichia coli*. I. Purification, homogeneity, and physical properties. J. Biol. Chem. **253**:4043-4050.

68. **Ritossa, F.** 1962. A new puffing pattern induced by temperature shock and DNP in Drosophila. Experentia **18**:571-573.

69. **Saito, H. and H. Uchida.** 1977. Initiation of the DNA replication of bacteriophage lambda in *Escherichia coli* K12. J. Mol. Biol. **113**:1-25.

70. **Saito, H. and H. Uchida.** 1978. Organization and expression of the *dnaJ* and *dnaK* genes of *Escherichia coli* K12. Mol. Gen. Genet. **164**:1-8.

71. **Saito, H., Y. Nakamura and H. Uchida.** 1978. A transducing λ phage carrying *grpE*, a bacterial gene necessary for λ DNA replication, and two

ribosomal protein genes, *rpsP* (S16) and *rplS* (L19). Mol. Gen. Genet. **165**:247-256.

72. **Sakakibara, Y.** 1988. The *dnaK* gene of *Escherichia coli* functions in initiation of chromosome replication. J. Bacteriol. **170**:972-979.

73. **Sanger, F., A. R. Coulson, G.F. Hong, D.F. Hill and G.B. Petersen.** 1982. Nucleotide sequence of bacteriophage λ DNA. J. Mol. Biol. **162**:729.

74. **Schnos, M., K. Zahn, R. B. Inman and F. R. Blattner.** 1988. Initiation protein induced helix destabilization at the λ origin: A prepriming step in DNA replication. Cell **52**:385-395.

75. **Spence, J. and C. Georgopoulos.** 1988. Purification and properties of the *Escherichia coli* heat shock protein HtpG. J. Biol. Chem., *in press*.

76. **Sternberg, N.** 1973. Properties of a mutant of *Escherichia coli* defective in bacteriophage λ head formation (*groE*). I. Initial characterization. J. Mol. Biol. **67**:1-24.

77. **Strauch, K. L. and J. Beckwith.** 1988. An *Escherichia coli* mutation preventing degradation of abnormal periplasmic proteins. Proc. Natl. Acad. Sci. U. S. A. **85**:1576-1580.

78. **Straus, D. B., W. A. Walter and C. A. Gross.** 1987. The heat shock response of *E. coli* is regulated by changes in the concentration of σ^{32}. Nature **329**:348-351.

79. **Sunshine, M., M. Feiss, J. Stuart and J. Yochem.** 1977. A new host gene (*groPC*) necessary for lambda DNA replication. Mol. Gen. Genet. **151**:27-34.

80. **Tilly, K., J. Erickson, S. Sharma and C. Georgopoulos.** 1986. Heat shock regulatory gene *rpoH* mRNA level increases after heat shock in *Escherichia coli*. J. Bacteriol. **168**:1155-1158.

81. **Tilly, K., N. McKittrick, M. Zylicz and C. Georgopoulos.** 1983. The dnaK protein modulates the heat-shock response of *Escherichia coli*. Cell **34**:641-646.

82. **Tilly, K., R. A. VanBogelen, C. Georgopoulos and F. C. Neidhardt.** 1983. Identification of the heat-inducible protein C15.4 as the *groES* gene product in *Escherichia coli*. J. Bacteriol. **154**:1505-1507.

83. **Tomizawa, J.** 1971. Functional cooperation of genes *O* and *P*. *In* A. D. Hershey (ed.), The bacteriophage lambda, pp. 549-552, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

84. **Tsurimoto, T. and K. Matsubara.** 1982. Replication of λdv plasmid *in vitro* by purified λO and P proteins. Proc. Natl. Acad. Sci. U. S. A. **79**:7639-7643.

85. **Tsurimoto, T. and K. Matsubara.** 1981. Purified bacteriophage λO protein binds to four repeating sequences at the λ replication origin. Nucleic Acids Res. **9**:1789-1799.

86. **VanBogelen, R. A., P. M. Kelley and F. C. Neidhardt.** 1987. Differential induction of heat shock, S. O. S., and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli*. J. Bacteriol. **169**:26-32.

87. **Wada, M. and H. Itikawa.** 1984. Participation of *Escherichia coli* K-12 *groE* gene products in the synthesis of cellular DNA and RNA. J. Bacteriol. **157**:694-696.

88. **Wada, M., Y. Kadokami and H. Itikawa.** 1982. Thermosensitive synthesis of DNA and RNA in *dnaJ* mutants of *Escherichia coli* K-12. Jpn. J. Genet. **57**:407-413.

89. **Waghorne, C. and C. R. Fuerst.** 1985. Involvement of the *htpR* gene product of *Escherichia coli* in phage lambda development. Virology **141**:51-64.

90. **Wang, J. C.** 1985. DNA topoisomerases. Annu. Rev. Biochem. **54**:665-697.

91. **Wang, J. C.** 1987. Recent studies of DNA topoisomerases. Biochim. Biophys. Acta **909**:1-9.

92. **Wickner, S.** 1978. DNA replication proteins of *Escherichia coli* and phage λ. Cold Spring Harbor Symp. Quant. Biol. **43**:303-310.

93. **Wickner, S. H. and K. Zahn.** 1986. Characterization of the DNA binding domain of bacteriophage λO Protein. J. Biol. Chem. **261**:7537-7543.

94. **Yochem, J., H. Uchida, M. Sunshine, H. Saito, C. Georgopoulos and M. Feiss.** 1978. Genetic analysis of two genes, *dnaJ* and *dnaK*, necessary for *Escherichia coli* and bacteriophage lambda DNA replication. Mol. Gen. Genet. **164**:9-14.

95. **Zahn, K. and F. R. Blattner.** 1985a. Sequence-induced DNA curvature at the bacteriophage λ origin of replication. Nature **317**:451.

96. **Zahn, K. and F. R. Blattner.** 1985b. Binding and bending of the λ replication origin by the phage O protein. EMBO J. **4**:3605.

97. **Zylicz, M., D. Ang and C. Georgopoulos.** 1987. The grpE protein of *Escherichia coli*: Purification and properties. J. Biol. Chem. **262**:17437-17442.

98. **Zylicz, M., D. Ang, K. Liberek and C. Georgopoulos.** Initiation of λ DNA replication with purified host- and bacteriophage-encoded proteins: The role of the dnaK, dnaJ and grpE heat shock proteins. Submitted to EMBO J.

99. **Zylicz, M. and C. Georgopoulos.** 1984. Purification and properties of the *Escherichia coli* dnaK replication protein. J. Biol. Chem **259**:8820-8825.

100. **Zylicz, M., I. Gorska, K. Taylor and C. Georgopoulos.** 1984. Bacteriophage λ replication proteins: Formation of a mixed oligomer and binding to the origin of λ DNA. Mol. Gen. Genet. **196**:401-406.

101. **Zylicz, M., J. H. LeBowitz, R. McMacken and C. Georgopoulos.** 1983. The dnaK protein of *Escherichia coli* possesses an ATPase and autophosphorylating activity and is essential in an *in vitro* DNA replication system. Proc. Natl. Acad. Sci. U. S. A. **80**:6431-6435.

102. **Zylicz, M., T. Yamamoto, N. McKittrick, S. Sell and C. Georgopoulos.** 1985. Purification and properties of the DnaJ replication protein of *Escherichia coli*. J. Biol. Chem. **260**:7591-7598.

CHAPTER 2

Escherichia coli grpE GENE CODES FOR HEAT SHOCK PROTEIN B25.3, ESSENTIAL FOR BOTH λ DNA REPLICATION AT ALL TEMPERATURES AND HOST GROWTH AT HIGH TEMPERATURE

J. Bacteriol. **167**:25-29.

Abstract

We have identified the *grpE* gene product as the B25.3 heat shock protein of *Escherichia coli* on the following evidence: (i) a protein similar in size and isoelectric point to B25.3 was induced after infection of UV-irradiated bacteria by λ*grpE*$^+$ transducing phage, (ii) mutant phage λ*grpE40*, isolated by its inability to propagate on *grpE*280 bacteria, failed to induce the synthesis of the B25.3 protein, and (iii) λ*grpE*$^+$ revertants, derived from phage *grpE*40 as able to propagate on *grpE*280 bacteria, simultaneously recovered the ability to induce synthesis of the B25.3 protein. In addition, we show that *E. coli* bacteria carrying the *grpE*280 mutation are temperature-sensitive for bacterial growth at 43.5°C. Through transductional analysis and temperature reversion experiments, it was demonstrated that the *grpE*280 mutation is responsible for both the inability of λ to replicate at any temperature tested and the lack of colony formation at high temperature. At the nonpermissive temperature the rates of synthesis of DNA and RNA were reduced in *grpE*280 bacteria.

Introduction

The identification of *Escherichia coli* proteins necessary for bacteriophage λ DNA replication has been facilitated by the isolation of bacterial mutants (originally termed *groP* or *grp*) that fail to support phage growth (6,7). The combined efforts of many different laboratories have demonstrated that the host *dnaB*, *dnaK*, *dnaJ*, *grpD*, and *grpE* gene products are absolutely necessary for λ DNA replication (6-8, 10, 14, 21, 23). The block to λ DNA replication can be overcome by phage mutations (originally termed π [10] or *reg* [21]) that map in the *P* gene, suggesting that the five host proteins and the phage-coded P protein functionally interact. In support of this, it has been demonstrated that the purified P protein of λ physically interacts with both the dnaB (29) and dnaK bacterial proteins (31). Recently it has been shown that the dnaK

and dnaJ proteins belong to the heat shock class of proteins (2, 12). The rate of synthesis of the heat shock proteins is positively regulated by the *htpR* (*rpoH*) gene product and is coordinately and transiently increased after a temperature shift (13, 19). In this paper we demonstrate through genetic and biochemical analysis that the grpE protein is identical to the B25.3 heat shock protein of *E. coli* originally identified by Neidhardt and colleagues (19). The *grpE* gene, which encodes a 24,000-M_r protein (20), was discovered because a mutation in it, *grpE*280, blocked bacteriophage λ DNA replication at all temperatures (21). Thus, at least three of the host proteins, dnaJ, dnaK, and grpE, known to participate in λ DNA replication are heat shock proteins. In addition we demonstrate that the grpE protein is essential for host RNA and DNA syntheses at high temperature.

Materials and Methods

Bacterial and phage strains

E. coli C600 *thr leu supE*, from our collection, served as the wild type strain in this work. *E. coli* K-12 159 *uvrA*, used in the UV irradiation experiments, was also from our collection. Strain K2801 *grpE*280 was kindly provided by Hisao Uchida. Strain NK6024 *pheA*::Tn*10* Hfr Hayes, used in cotransduction experiments with the nearby *grpE* locus, was provided by Nancy Kleckner. Strain MF687, carrying the *mutD5* allele isolated by Degnen and Cox (5), was used in phage mutagenesis experiments.

Phage λ*c*I857 was from our collection. Transducing phages λ*c*I857 *grpE*$^+$ and λ*c*I857 *grpE*$^+$ Δ7 (20) were a gift of Hisao Uchida. An *E. coli* DNA library, prepared by partial digestion with *Sau*3A restriction nuclease, in λ vector L47.1 (16) was obtained from Sam Cartinhour. Phage P1L4, used in the transduction studies, was obtained from Lucien Caro.

Media

L broth contained 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter. The pH was adjusted to 7.4 by the addition of NaOH. T broth was L broth without yeast extract. T agar and L agar plates were prepared from the corresponding broths with the addition of 10 g of agar (Difco Laboratories) per liter. The composition of high-sulfur M9 medium for labeling with [^{35}S]methionine has been described previously (11).

P1 transduction experiments

Phage P1L4 was grown and used in transduction experiments as described by Miller (17).

One- and two-dimensional gel electrophoresis

The procedures used for isoelectric focusing in the first dimension [1.6% (v/v) ampholines pH 5 to 7, 0.4% (v/v) ampholines pH 3.5 to 10) and sodium dodecyl sulfate (SDS)-polyacrylamide [12.5% (w/v)] gel electrophoresis in the second dimension have been described previously (12).

Labeling experiments

The procedure used for labeling UV-irradiated *E. coli* K-12 159 *uvrA* bacteria (45,000 ergs/mm^2 at 254 nm) after infection with various derivative phage strains has been described (11). To preferentially label heat shock proteins, strain C600 *grpE*$^+$ bacteria were grown in M9 high-sulfur medium at 30°C to approximately 3 x 10^8 cells per ml. A portion (usually 1 ml) was transferred to a 15-ml Corex tube prewarmed at 43°C and labeled with 10 μCi of [^{35}S]methionine (Amersham) between 5 and 15 min

after the temperature shift. The bacteria were centrifuged for 1 min in an Eppendorf microfuge, suspended in two-dimensional lysis buffer, lysed by three freeze-and-thaw cycles, and stored at -20°C until used.

DNA, RNA, and protein synthesis experiments

Cells were grown at 30°C in high-sulfur M9 medium. Portions (0.5 ml) were distributed into prewarmed (44°C) culture tubes at time zero. Five microliters of label (final concentrations were 4 [29 Ci/mmol], 1.4 [39 Ci/mmol], and 3.3 [1,480 Ci/mmol] μCi of [^{3}H]thymidine [ICN], [^{3}H]uridine [ICN], and [^{35}S]methionine [Amersham], per ml, respectively) were added to each tube at the appropriate times. Following incubation for 2 min, 0.5 ml of 10% cold trichloroacetic acid was added. Samples were chilled on ice prior to further processing. The cells were washed on glass fiber filters (Whatman GF/C) with cold 5% trichloroacetic acid, followed by washes with cold distilled water and then with cold ethanol. The dried filters were counted in a Beckman LS 333 liquid scintillation counter.

Results

Isolation of *grpE* transducing phage

To characterize the *grpE* gene product, we sought to obtain *grpE* point mutations. This was done by growing the λ*c*I857 *grpE*$^+$ Δ7 transducing phage (20) on the *E. coli mutD* strain. The mutagenized phage were plated on a mixture of C600 *grpE*$^+$ and C600 *grpE280* isogenic bacteria (1:5). Small and turbid-looking plaques were tested for growth on both C600 *grpE*$^+$ and C600 *grpE*280 bacterial lawns. Phage which grew on C600 *grpE*$^+$ but not on C600 *grpE*280 bacteria were presumed to be λ*grpE* mutants. Approximately 20 such phage mutants were isolated, at an estimated frequency of 3 x 10^{-4}.

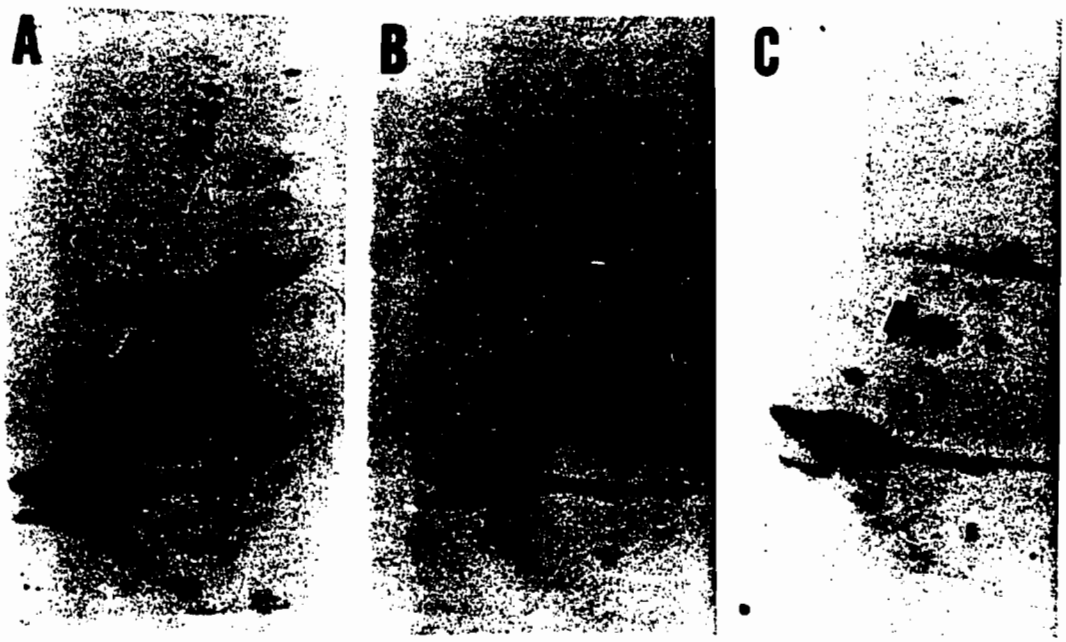
Isolation of λ*grpE*$^+$ revertants

Mutant *grpE* phage were plated on C600 *grpE*280 bacteria, and revertant plaques, appearing at a frequency of 10^{-5} to 10^{-3}, were purified for one cycle and tested further. The majority of the λ*grpE* mutant phage isolated as described above behaved this way. However, we found that some λ*grpE* phage did not revert (frequency less than 10^{-7}), suggesting that they had large deletions of the *grpE* gene carried on the transducing phage.

grpE gene product is a 24,000-M_r protein

The *grpE* locus was originally identified because a single mutation in it, *grpE*280, blocked phage λ DNA replication at all temperatures (20; our unpublished results). The isolation of a λ*grpE*$^+$ transducing phage and subsequent deletion analysis resulted in the tentative identification of grpE as a 24,000-M_r, mildly acidic protein (20). We confirmed this conclusion by showing that the presence of this protein in two-dimensional gels correlated with the phenotype of the λ*grpE*$^+$ transducing phage (Fig. 2.1A, filled arrow). Specifically, the majority of the λ*grpE* derivative phage that we isolated failed to induce or induced substantially less of the 24,000-M_r protein. Infection of the UV-irradiated bacteria by λ*grpE*40 mutant phage did not induce synthesis of the 24,000-M_r protein (Fig. 2.1B). The *grpE*40 mutation is not suppressible by supD, supE, or supF (17), suggesting that it is not an amber mutation. The production of a fragment suggests that *grpE*40 may be a nonsense or frameshift mutation. λ*grpE*40R, a GrpE$^+$ revertant of phage λ*grpE*40, induced the synthesis of normal amounts of the 24,000-M_r protein (Fig. 2.1C). The ability to induce the synthesis of normal amounts of the 24,000-M_r protein was associated with all GrpE$^+$ revertants of phage λ*grpE*40. All of these results together demonstrated that

Figure 2.1. Two-dimensional gel eletrophoresis of UV-irradiated strain 159 *uvrA* bacteria infected with λ *grpE*. (A) λ*grpE*$^+$ Δ7; (B) λ*grpE*40; (C) λ*grpE*40R (revertant). Proteins were labeled with [^{35}S] methionine (30 μCi/ml) for 15 min at 37°C. Only the region of each gel ranging from pH 4.5 to 5.5 (horizontal axis) is shown. Filled arrows indicate the position of the wild type 24,000-M_r polypeptide. The open arrow indicates the position of a new spot whose synthesis was induced by λ*grpE*40 mutant phage (see text for details). In all instances the acidic side of the gel is on the right and the basic side is on the left.


A
B
C

the *grpE* gene product is the 24,000-M_r acidic protein, as originally suggested by the results of Saito et al. (20).

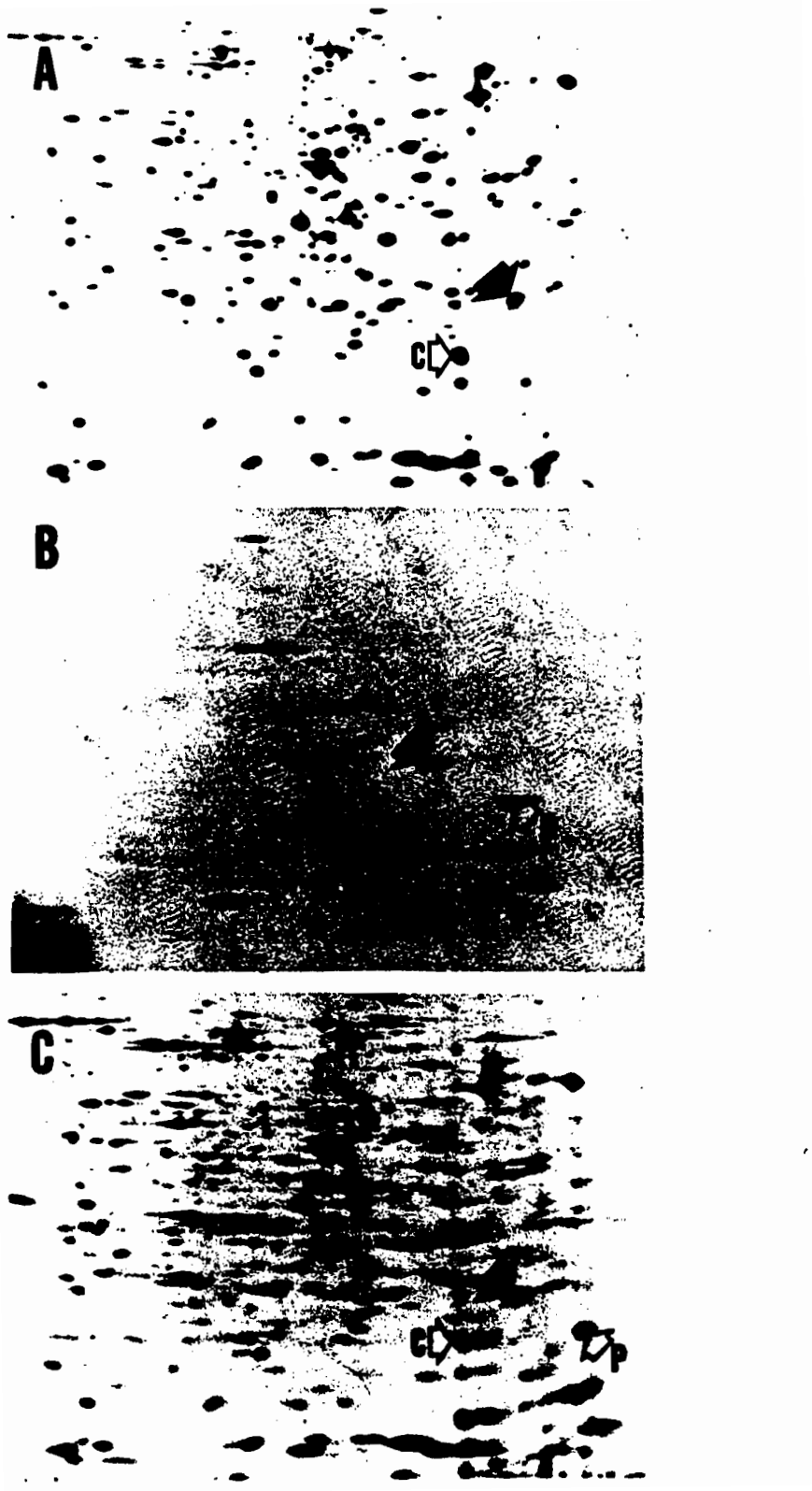
Isolation of additional λ*grpE*$^+$ transducing phage

An *E. coli* DNA library was prepared by partially digesting *E. coli* DNA with *Sau*3A restriction nuclease and ligating the fragments into the unique *Bam*HI restriction site of the phage λ vector L47.1 (16). By plating this phage library on *grpE*280 bacteria, it was found that plaque-formers arose at a frequency of 10^{-4} to 10^{-3}, which is approximately 10,000-fold higher than the expected frequency of occurrence of π mutations in the phage replication gene *P* (20; our unpublished results). We verified that these phages were indeed λ*grpE*$^+$ transducing phage by demonstrating that (i) they induced synthesis of the 24,000-M_r polypeptide after infection of UV-irradiated hosts (data not shown) and (ii) after lysogenization of *grpE*280 bacteria, they simultaneously conferred the GrpE$^+$ phenotype for both heteroimmune phage growth and bacterial colony formation at 43°C (data not shown).

GrpE protein is *E. coli* B25.3 heat shock protein

Neidhardt and his colleagues have identified over 17 *E. coli* proteins as belonging to the heat shock family of proteins (19). A prominent member of this group is protein B25.3. Because the size and position in two-dimensional gels of the B25.3 protein were very close to those of the grpE protein and because the dnaK and dnaJ proteins of *E. coli*, which are also necessary for λ DNA replication, have been shown to be heat shock proteins (2, 12), we investigated the possible identity of the grpE and B25.3 proteins. Fig. 2.2A shows the two-dimensional electrophoresis pattern of heat-shocked *E. coli* cells. The filled arrow points to the position of the B25.3 protein. Extracts of UV-irradiated bacteria infected with phage λ*grpE*$^+$Δ7 (Fig. 2.2B) and

Figure 2.2. Two-dimensional gel electrophoresis of heat-shocked C600 *grpE*$^+$ bacteria. (A) Uninfected culture labeled between 5 and 15 min after a shift to 43°C; (B) UV-irradiated cells infected with λ*grpE*$^+$ Δ7, as described in the legend to Fig. 2.1; (C) co-electrophoresis of a mixture of the extracts shown in panels A and B. Arrows indicate the positions of the grpE and B25.3 proteins. C, host-coded protein; P, phage-coded protein.


A
C
B
C
C
P

extracts of heat-shocked *E. coli* (Fig. 2.2A) containing approximately equal amounts of radioactivity in the grpE and B25.3 protein, respectively, were mixed, and their proteins were separated by two-dimensional gel electrophoresis; the grpE and B25.3 protein spots coincided (Fig. 2.2C).

The identity of the two proteins was verified in the following way. The ^{35}S-labeled grpE and B25.3 protein bands were excised from SDS-polyacrylamide gels and partially digested with *Staphylococcus aureus* V8 protease by the method of Cleveland et al. (3). The partial digestion patterns of the grpE (induced by the λ*grpE*$^+$ transducing phage) and B25.3 (induced after heat shock) proteins were identical (data not shown). We conclude that the grpE protein is identical to the B25.3 heat shock protein of *E. coli*.

grpE gene product required for *E. coli* growth at high temperature

The *grpE*280 bacterial mutant was isolated as a temperature-resistant survivor at 42°C (20). However, upon testing, we found that this strain, K2801 *grpE*280, was unable to form colonies at temperatures above 43.5°C. To prove that the temperature-sensitive phenotype is due to the *grpE*280 mutation and not to another mutation caused by the original mutagenesis procedure, we carried out the P1 transduction experiments shown in Table 2.1. We took advantage of the existence of a transposon Tn*10* insertion in the *pheA* gene to select for transfer of the *grpE*$^+$and *grpE*280 markers between strains. Previously, Saito et al. (20) had shown that the P1 cotransduction frequency between *pheA* and *grpE* is 61%. We confirmed this result by showing that the cotransduction frequency of the Tet^r and GrpE$^+$ markers was 58 to 70% (Table 2.1). The Tet^r transductants of the crosses shown in Table 2.1 were further analyzed for the ability to support phage λ growth and the inability to form colonies at 43.5°C. In no case (0 of 200) were we able to separate these two

Table 2.1

P1 transduction studies with the *grpE*280 mutation

Donor	Recipient	Selected marker	Unselected markers[b]	No.
NK6024 *pheA*::Tn*10*	K2801 *grpE*280	Tet^r	λ^- Ts^-	30
			λ^+ Ts^+	70
			λ^- Ts^+	0
			λ^+ Ts^-	0
K2801 *grpE*280 *pheA*::Tn*10*	C600	Tet^r	λ^- Ts^-	58
			λ^+ Ts^+	32
			λ^- Ts^+	0
			λ^+ Ts^-	0

[a] Tetracycline-resistant transductants were selected by incubation for 36 h at 37°C on L agar plates supplemented with 20 μg of tetracycline per ml and 5 x 10^{-3} M sodium citrate (to prevent P1 reinfection). Inability to propagate phage was tested either by streaking the colonies against phage λ*c*I857 or by spot tests. In all cases it was verified that the λ*c*I857 *grpE*$^+$ transducing phage grew well. The inability to form colonies at high temperature (Ts^-) was tested by streaking individual colonies on L agar plates containing tetracycline and incubating at 44°C for 36 h.

[b] λ^-, block to phage λ infection; λ^+, susceptibility to phage λ infection.

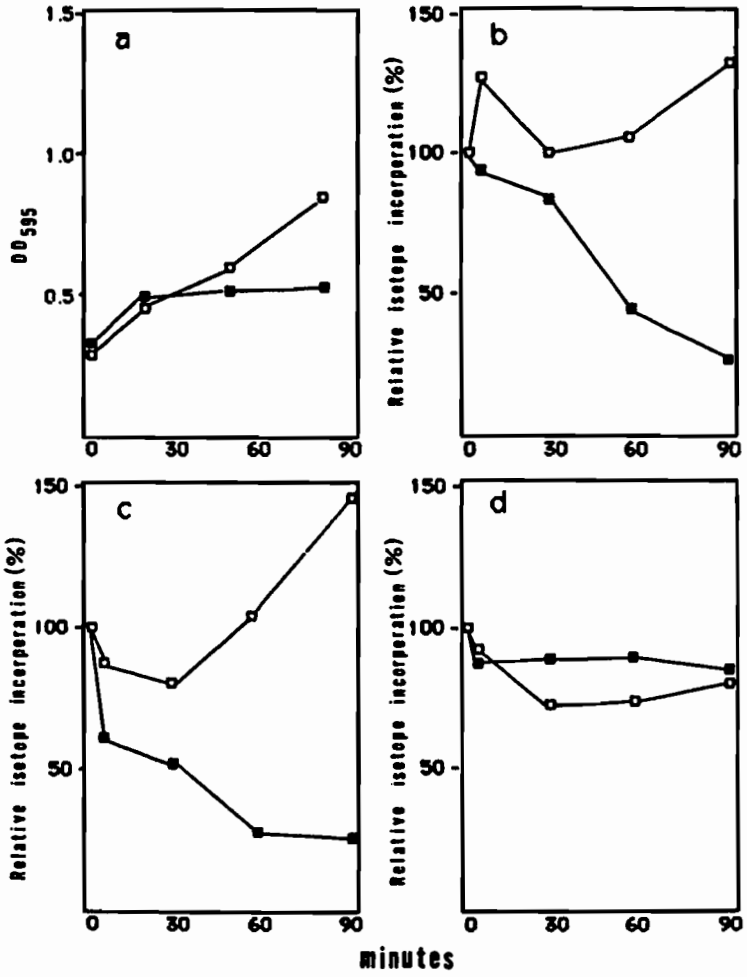
phenotypes. We conclude that the *grpE*280 mutation is responsible for the block to both phage growth at all temperatures and bacterial colony formation at high temperature. A possibility that cannot be excluded by these data is that another mutation, tightly linked to *grpE*280, is responsible for the inability to form colonies at high temperature. This possibility was examined through an analysis of Ts^+ bacterial revertants. Bacteria carrying the *grpE*280 mutation reverted to Ts^+ at a frequency of 10^{-7} to 10^{-5} at 43.5°C and at a frequency of 10^{-8} to 10^{-7} at 44.5°C. A substantial fraction (5 of 17) of the Ts^+ revertants at 44.5°C simultaneously recovered the ability to propagate phage λ. This result suggests that the *grpE*280 mutation is indeed responsible for both phenotypes.

*grpE*280 interferes with both DNA and RNA synthesis

The rates of synthesis for DNA, RNA, and protein were determined in C600 *grpE*$^+$ and C600 *grpE*280 isogenic bacteria at both the permissive (30°C) and nonpermissive (44°C) temperatures. At 44°C the rate of synthesis for DNA and RNA in the *grpE*280 mutant declined substantially over a period of 90 min, whereas that of protein did not (Fig. 2.3). At 30°C, the permissive temperature, the rates of DNA, RNA, and protein synthesis were virtually identical in *grpE*$^+$ and *grpE*280 bacteria (data not shown). We conclude that the grpE protein directly or indirectly affects both DNA and RNA synthesis in *E. coli*, at least at high temperature. Similar observations have been made for mutations in both the *dnaK* (14, 21) and *dnaJ* (27) genes.

The evidence that the *E. coli* heat shock protein B25.3 is identical to the grpE protein can be summarized as follows: (i) λ *grpE*$^+$ transducing phages, selected as plaque-formers on *grpE*280 bacteria, always induced the synthesis of a 24,000-M_r protein; (ii) most of the λ*grpE* mutant phage, isolated as unable to propagate on *grpE*280 bacteria, did not induce synthesis of the 24,000-M_r protein; (iii) bona fide

Figure 2.3. Rates of DNA, RNA, and protein synthesis in C600 *grpE*280. (a) Rate of cell growth after a shift in temperature from 30°C to 44°C at time zero [optical density (OD) at 595nm]; (b) rate of [^{3}H]thymidine incorporation; (c) rate of [^{3}H]uridine incorporation; (d) rate of [^{35}S]methionine incorporation. Percent incorporation is determined relative to incorporation at time zero, designated at 100%. The values at time zero were 2,341 and 4,960 cpm for [^{3}H]thymidine, 65,907 and 76,047 cpm for [^{3}H]uridine, and 321,204 and 297,733 cpm for [^{35}S]methionine for C600 *grpE*$^{+}$ (□) and C600 *grpE*280 (■), respectively.


a
b
c
d
OD_{595}
Relative isotope incorporation (%)
minutes
0
30
60
90
0.5
1.0
1.5
50
100
150

B25.3 protein, synthesized by heat-shocked *E. coli* cells, and the grpE protein induced by λ*grpE*$^+$ transducing phage comigrated in two-dimensional acrylamide gels; and (iv) the grpE and B25.3 proteins exhibited identical patterns of partial proteolytic products.

Discussion

The *dnaK*, *dnaJ*, and *grpE* genes exhibit a number of similarities. (i) All three were originally discovered because mutations in them interfered with phage λ replication at all temperatures (8, 21, 23). (ii) All three gene products interact with the λ *P* gene replication protein inasmuch as phage mutations mapping in the *P* gene (8, 10, 21, 23) can bypass the bacterial mutation blocks. (iii) All three were shown to code for proteins belonging to the heat shock group of proteins whose expression requires the *htpR* (σ^{32}) gene product (13, 19): *dnaK* codes for a 70,000-M_r protein called B66.0 (12, 19), *dnaJ* codes for a 37,000-M_r heat shock protein called H26.5 (2, 19), and *grpE* codes for a 24,000-M_r protein called B25.3 (19, 20; this work). (iv) All three gene products are essential for *E. coli* growth, at least at high temperature (8, 22, 23; this work). (v) Mutations in all three directly or indirectly interfere with the overall rates of synthesis of both bacterial DNA and RNA at the nonpermissive temperature (14, 21, 22, 27; this work).

Both the dnaK (30) and dnaJ (32) proteins have been purified to homogeneity. The dnaK protein has been shown to exist mostly as a monomer in solution and to possess both nonspecific 5'-nucleotidyl phosphatase (B. Bochner, personal communication; our unpublished data) and autophosphorylating activities (30, 31). In addition, it has been shown to be 48% identical at the amino acid sequence level with the *Drosophila melanogaster* Hsp70 (1). The purified dnaJ protein has been shown to exist mostly as a dimer in solution and to bind to both single- and double-stranded DNA without obvious specificity (32). Both proteins have been shown to be

absolutely essential for λ DNA replication at all temperatures in an *in vitro* system (15, 30-32). The level of action of the dnaK and dnaJ proteins in λ DNA replication has been shown to be at a step following the need for the λ O and P replication proteins, the bacterial dnaB protein, and "transcriptional activation," but preceding that of the dnaG primase action (M. Zylicz, unpublished observation). It would be interesting to know whether the need for grpE protein in λ DNA replication is at the same level.

In addition to its dependence on the dnaK, dnaJ, and grpE heat shock proteins for its DNA replication, we have previously demonstrated through the isolation of bacterial mutants that phage λ requires the groES and groEL morphogenetic proteins of *E. coli* for proper assembly of its head (6, 9). We have also previously shown that both the groES and groEL proteins are essential for *E. coli* colony-forming ability at 42°C (9, 26). Surprisingly, at the nonpermissive temperature both RNA and DNA syntheses are affected (26), a result reminiscent of the effect of the *grpE280* mutation. This similarity extends to the fact that the expression of both the groES and groEL proteins is also under heat shock regulation (18, 24, 25). Thus it appears that phage λ has tapped the *E. coli* heat shock regulon as a source of proteins to help carry out its DNA replication and morphogenesis processes. Recently, Waghorne and Fuerst (28) have shown that λ does not propagate in an *htpR* mutant host. One interpretation of this result is the need for wild type intracellular levels of heat shock proteins for proper λ growth in *E. coli*, at least at high temperatures. Interestingly, infection of *E. coli* by phage λ results in a transient increase in the rate of synthesis of the heat shock proteins (reviewed in ref. 24). Thus, phage λ has not only adapted some of the heat shock proteins for its growth but has found a mechanism for turning on their synthesis following infection of a sensitive host.

Acknowledgments

This work was supported by Public Health Service grants GM-23917 and AI-21029 from the National Institutes of Health.

We would like to thank Jerri Cohenour for preparation of the manuscript and Takis Vouganis for technical assistance.

Literature Cited

1. **Bardwell, J. C. A. and E. A. Craig.** 1984. Major heat shock gene of *Drosophila* and the *Escherichia coli* heat inducible *dnaK* gene are homologous. Proc. Natl. Acad. Sci. U. S. A. **81**:848-852.

2. **Bardwell, J. C. A., K. Tilly, E. Craig, J. King, M. Zylicz and C. Georgopoulos.** 1986. The nucleotide sequence of the *Escherichia coli* K12 *dnaJ* gene: a gene that encodes a heat shock protein. J. Biol. Chem. **261**:1782-1785.

3. **Cleveland, D. W., S. G. Fischer, M. W. Kirschner and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102-1106.

4. **Cowing. D. W., J. C. A. Bardwell, E. A. Craig, C. Woolford, R. W. Hendrix and C. A. Gross.** 1985. Consensus sequence for *Escherichia coli* heat shock gene promoters. Proc. Natl. Acad Sci. U. S. A. **82**:2679-2683.

5. **Degnen, G. E. and E. C. Cox.** 1974. Conditional mutator gene in *Escherichia coli*: isolation, mapping, and effector studies. J. Bacteriol. **117**:477-487.

6. **Friedman, D. I., E. R. Olson, K. Tilly, C. Georgopoulos, I. Herskowitz and F. Banuett.** 1984. Interactions of bacteriophage λ and host macromolecules in the growth of bacteriophage λ. Microbiol. Rev. **48**:299-325.

7. **Furth, M. E. and S. H. Wickner.** 1983. Lambda DNA replication. *In* R. W. Hendrix, J. W. Roberts, F. W. Stahl and R. A. Weisberg (eds.), Lambda II, pp. 145-173, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

8. **Georgopoulos, C. P.** 1977. A new bacterial gene (*groPC*) which affects λ DNA replication. Mol. Gen. Genet. **151**:35-39.

9. **Georgopoulos, C. P. and H. Eisen.** 1974. Bacterial mutants which block phage assembly. J. Supramol. Struct. **2**:349-359.

10. **Georgopoulos, C. P. and I. Herskowitz.** 1971. *Escherichia coli* mutants blocked in lambda DNA synthesis. *In* A.D. Hershey (ed.), The bacteriophage lambda, pp. 553-564, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

11. **Georgopoulos, C. P. and B. Hohn.** 1978. Identification of a host protein necessary for bacteriophage morphogenesis (the *groE* gene product). Proc. Natl. Acad. Sci. U. S. A. **75**:131-135.

12. **Georgopoulos, C. P., K. Tilly, D. Drahos and R. Hendrix.** 1982. The B66.0 protein of *Escherichia coli* is the *dnaK*$^+$ gene product. J. Bacteriol. **149**:1175-1177.

13. **Grossman, A. D., J. W. Erickson and C. A. Gross.** 1984. The *htpR* gene product of *E. coli* is a sigma factor for heat shock promoters. Cell **38**:383-390.

14. **Itikawa, H. and J.-I. Ryu.** 1979. Isolation and characterization of a temperature-sensitive *dnaK* mutant of *Escherichia coli* B. J. Bacteriol. **138**:339-344.

15. **LeBowitz, J., M. Zylicz, C. Georgopoulos and R. McMacken.** 1985. Initiation of DNA replication on single-stranded DNA templates catalyzed by purified replication proteins of bacteriophage λ and *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **82**:3988-3992.

16. **Loenen, W. A. M. and W. J. Brammar.** 1980. A bacteriophage lambda vector for cloning large DNA fragments made with several restriction enzymes. Gene **10**:249-259.

17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

18. **Neidhardt, F. C., T. A. Phillips, R. A. VanBogelen, M. W. Smith, Y. Georgalis and A. R. Subramanian.** 1981. Identity of the B56.5 protein, the A-protein, and the *groE* gene product of *Escherichia coli*. J. Bacteriol. **145**:513-520.

19. **Neidhardt, F. C., R. A. VanBogelen and V. Vaughn.** 1984. The genetics and regulation of heat-shock proteins. Annu. Rev. Genet. **18**:295-329.

20. **Saito, H., Y. Nakamura and H. Uchida.** 1978. A transducing λ phage carrying *grpE*, a bacterial gene necessary for λ DNA replication, and two ribosomal protein genes, *rpsP* (S16) and *rplS* (L19). Mol. Gen. Genet. **165**:247-256.

21. **Saito, H. and H. Uchida.** 1977. Initiation of the DNA replication of bacteriophage lambda in *Escherichia coli* K-12. J. Mol.Biol. **113**:1-25.

22. **Saito, H. and H. Uchida.** 1978. Organization and expression of the *dnaJ* and *dnaK* genes of *Escherichia coli* K-12. Mol. Gen. Genet. **164**:1-8.

23. **Sunshine, M., M. Feiss, J. Stuart and J. Yochem.** 1977. A new host gene (*groPC*) necessary for lambda DNA replication. Mol. Gen. Genet. **151**:27-34.

24. **Tilly, K., G. N. Chandrasekhar, M. Zylicz, and C. Georgopoulos.** 1985. Relationships between the bacterial heat shock response and bacteriophage lambda growth. *In* L. Leive (ed.), Microbiology-1985, pp. 322-326, American Society for Microbiology, Washington, D.C.

25. **Tilly, K., R. A. VanBogelen, C. Georgopoulos and F. C. Neidhardt.** 1983. Identification of the heat-inducible protein C15.4 as the *groES* (*mopB*) gene product of *Escherichia coli*. J. Bacteriol. **154**:1505-1507.

26. **Wada, M. and H. Itikawa.** 1984. Participation of *Escherichia coli* K-12 *groE* gene products in the synthesis of cellular DNA and RNA. J. Bacteriol. **157**:694-696.

27. **Wada, M., Y. Kadokami and H. Itikawa.** 1982. Thermosensitive synthesis of DNA and RNA in *dnaJ* mutants of *Escherichia coli* K-12. Jpn. J. Genet. **57**:407-413.

28. **Waghorne, C. and C. R. Fuerst.** 1985. Involvement of the *htpR* gene product of *Escherichia coli* in phage λ development. Virology **141**:51-64.

29. **Wickner, S.** 1978. DNA replication proteins of *Escherichia coli* and phage λ. Cold Spring Harbor Symp. Quant. Biol. **43**:303-310.

30. **Zylicz, M. and C. Georgopoulos.** 1984. Purification and properties of the *Escherichia coli* dnaK replication protein. J. Biol. Chem. **259**:8820-8825.

31. **Zylicz, M., J. H. LeBowitz, R. M. McMacken and C. Georgopoulos.** 1983. The dnaK protein of *Escherichia coli* possesses an ATPase and autophosphorylating activity and is essential in an *in vitro* DNA replication system. Proc. Natl. Acad. Sci. U. S. A. **80**:6431-6435.

32. **Zylicz, M., T. Yamamoto, N. McKittrick, S. Sell and C. Georgopoulos.** 1985. Purification and properties of the dnaJ replication protein of *Escherichia coli*. J. Biol. Chem. **260**:7591-7598.

CHAPTER 3

THE HEAT SHOCK-REGULATED *grpE* GENE OF *Escherichia coli* IS REQUIRED FOR BACTERIAL GROWTH AT ALL TEMPERATURES, BUT IS DISPENSABLE IN CERTAIN MUTANT BACKGROUNDS *

* D. Ang and C. Georgopoulos, submitted to J. Bacteriol.

Abstract

Previous work has established that the *grpE*$^+$ gene product is a heat shock protein that is essential for bacteriophage λ growth at all temperatures and for *E.coli* growth at temperatures above 43°C. Here it is shown that the *grpE*$^+$ gene product is essential for bacterial viability at all temperatures. The strategy required constructing a *grpE* deletion derivative carrying a selectable chloramphenicol drug resistance marker provided by an Ω insertion, and showing that this deletion construct can be crossed into the bacterial chromosome if, and only if, a functional *grpE*$^+$ gene is present elsewhere in the same cell. As a control, it is shown that the same Ω insertion can be placed immediately downstream of the *grpE*$^+$-coding sequence without any observable effects on host growth. This result demonstrates that the inability to construct a *grpE*-deleted *E. coli* strain is not simply due to a lethal polar effect on neighboring gene expression.

Unexpectedly, it was found that the *grpE* deletion derivative can be crossed into the bacterial chromosome in a strain which is defective in dnaK function. Further analysis has shown that it is not the lack of dnaK function *per se* that now allows *E. coli* to tolerate a deletion in its *grpE*$^+$ gene. Rather, it is the presence of unknown extragenic suppressors of a *dnaK*$^-$ mutation which somehow compensate for the deficiency in both dnaK and grpE function.

Introduction

The *grpE*$^+$ gene product of *E. coli* was originally identified as necessary for λ DNA replication (25, 26). It was subsequently shown to be essential for *E. coli* growth at temperatures above 43°C, to be involved in host RNA and DNA metabolism, and to be a heat shock protein (1, 2). There is ample genetic and biochemical data which support a direct, physical interaction between the *grpE*$^+$ and

dnaK+ gene products of *E. coli* (1, 2, 33; C. Johnson, G. N. Chandrasekhar, and C. G., submitted for publication). Mutations in the *dnaK*+ gene result in many of the same phenotypes seen in the *grpE*280 mutant (2, 13). The dnaK protein, which is also required for initiation of λ DNA replication (26), is the bacterial equivalent of the eukaryotic Hsp70, a highly conserved heat shock protein family found in all organisms (6, 18, 22). Recently, genetic and biochemical data have been presented demonstrating that *in vivo* at 42°C a functional dnaK protein is required for *E. coli* *oriC*-dependent DNA initiation (27).

A question recently addressed in the literature was whether the *dnaK*+ gene of *E. coli*, which is required for growth at temperatures above 42°C (10, 11), is also required at lower temperatures, e.g., 30°C (23). Since it has been shown that the *dnaK*+ gene product is a negative regulator of the heat shock response (28), it was postulated that it may be required only at high temperatures. Evidence was presented at that time which supported the conclusion that the *dnaK*+ gene could be deleted from the chromosome at temperatures below 37° (23). Because mutations in *dnaK*+ and *grpE*+ affect the same host functions and because the grpE protein interacts with dnaK (1, 33; C. Johnson, G. N. Chandrasekhar, and C. G., submitted for publication), this result suggested to us that it also may be possible to delete the *grpE*+ gene from the chromosome. However, since the original publication (23), it has been shown that a deletion in the *dnaK*+ gene is only marginally tolerated within a specific temperature range, and even then, only in the presence of extragenic suppressors (4). Here we present data that demonstrate that the *grpE*+ gene product is also required for *E. coli* growth at temperatures ranging from 18°C to 43°C. In addition, the data suggest that selection for extragenic suppressors upon deletion of *grpE*+, resulting in cell viability, occurs rarely, if at all. However, to our surprise, we found that in strains which appear to already have a suppressor mutation compensating for a defective *dnaK* gene

product, the *grpE* deletion is readily crossed into the bacterial chromosome at the expected frequencies. This phenomenon is allele-specific in that not every strain with a mutation in the *dnaK* gene can tolerate deletion of *grpE*$^+$.

Several methods for replacing *E. coli* genes with deleted versions have been reported in the literature (14, 15, 16, 20). The method reported here introduces an inactivated copy of the target gene by integration of a λ transducing phage at the target gene site. Spontaneous excision of the prophage in which the deleted version of the target gene remains on the chromosome can be selected for in the presence of extrachromosomal copies of the gene. This strain is subsequently used as the donor in a transductional analysis, using a closely linked drug resistance marker, in this case, kanamycin-resistance, to select for the region containing the target gene without any *a priori* selection for the deletion itself. The transductants are then screened for the presence of the deletion which is marked with a gene encoding resistance to a second drug, in this case, chloramphenicol. If the gene is not essential, normal linkage between the two markers occurs in the presence or absence of extrachromosomal copies of the gene in the recipient. However, if the gene is essential, normal linkage occurs only in the presence of additional, extrachromosomal copies. In their absence, retention of the deletion on the chromosome occurs through rare events, which have been shown to somehow leave behind one or more copies of the wild type gene in the cell.

Materials and Methods

Bacteria, bacteriophage and plasmids

The various bacterial, bacteriophage and plasmid strains used are shown in Table 3.1.

Table 3.1

Bacteria, phage and plasmid strains

Strain	Genotype and/or Phenotype	Source or Reference
CG409	C600 *dnaK*$^+$ *thr*::Tn*10*	(10, 11)
CG410	C600 *dnaK*756 *thr*::Tn*10*	(10, 11)
CG799	C600 *dnaK*$^+$ *thr*::Tn*10*	our collection
CG800	C600 *dnaK*103 *thr*::Tn*10*	K. von Meyenberg; this work
CG985	B178/T1 = W3101 *galE*$^-$ T1^r	(10, 11)
CG1064	B178 *ze?*::mini-kan^r near *grpE*$^+$	our collection
DA15	B178 *pheA*::Tn*10*	(2)
DA16	B178 *grpE*280 *pheA*::Tn*10*	(2)
DA130	CG1064 *grpE*$^+$::Ω-cam^r (pDA1)	this work
DA131	" " "	"
DA132	" " "	"
DA133	CG1064 *grpE*$^\Delta$::Ω-cam^r (pDA1)	"
DA134	" " "	"
DA135	" " "	"
DA239	CG800 *thr*::Tn*10* (λ*imm*^{21}*dnaK*$^+$)	"
Phage		
λ*c*I$^-$	λ*c*I60	our collection
λ*grpE*$^+$	λ *imm*$^\lambda$ *int*$^-$ *xis*$^-$*att*λ$^-$*c*I857 *grpE*22	(25)
λ*grpE*$^\Delta$::Ω-cam^r	λ *grpE*22 *grpE*$^-$ cam^r	this work
λ*grpE*$^+$::Ω-cam^r	λ *grpE*22 *grpE*$^+$ cam^r	"
λ*imm*^{21}*dnaK*$^+$	λ *imm*^{21} *att*λ$^+$ *int*$^+$ *xis*$^+$ *c*I$^+$ *dnaK*$^+$	(10)
λ*imm*^{434}*dnaK*$^+$	λ*imm*^{434} *c*I$^-$ *dnaK*$^+$	our collection
P1L4	clear plaque former	L. Caro
Plasmid		
pJK23	pEMBL8$^+$ *grpE*$^+$ amp^r	(19)
pDA1	pBR322 *grpE*$^+$ amp^r	this work
pDA4	pBR322 *grpE*$^\Delta$::Ω-cam^r, amp^r	"
pDA5	pBR322 *grpE*$^+$::Ω-cam^r, amp^r	"
pHP45Ω-cam^r	pBR322 amp^r cam^r	(9)
pBR322	amp^r tet^r	(3)
pEMBL8$^+$	amp^r tet^r	(7)

Media

L-broth and L-agar (2) were used for most of the genetic manipulations. When appropriate, media were supplemented with 20 μg/ml chloramphenicol (cam), 50 μg/ml kanamycin (kan), 15 μg/ml tetracycline (tet) or 50 μg/ml ampicillin (amp) to select and maintain drug resistance.

Transformation

Transformations were done essentially according to the method of Hanahan (12).

Phage lysates

Donor strains were grown in L-broth to A_{595nm} = 1.0. One ml of cells was combined with 40 μl of a P1*L4* lysate (9 x 10^7 pfu/ml), 50 μl 0.1M $CaCl_2$ and 20 ml of top agar (0.6% agar in L-broth). This mixture was poured onto 3 fresh L-agar plates containing $CaCl_2$ at a final concentration of 5 x 10^{-3} M, and the plates were incubated at 30°C overnight . The next day, the top agar was scraped into a 30-ml glass Corex tube, 0.5 ml chloroform was added, and the tube gently vortexed. The tubes were incubated at 37°C for 15 min prior to centrifugation in a Beckman JA-20 rotor at 3,000 x g for 15 min at 4°C. The supernatant was used for subsequent transductions.

P1 transduction

P1-mediated transductions were carried out essentially according to Miller (21). At least 30 min was allowed for expression of Kan^r before selection on L-kan plates.

Construction of plasmids

All DNA fragments were purified from agarose gels by electroelution prior to ligation. Plasmid pDA1 (Fig. 3.1a), which carries the intact *grpE*$^+$ gene and its promoter, was derived from pJK23 (19) by digesting pJK23 with AvaI and AccI to excise the approximately 1700-bp *E. coli* DNA fragment from the pEMBL8$^+$ vector (7), and ligating the fragment to the 2962-bp AvaI-Cla I fragment of pBR322 (3), encoding resistance to ampicillin. The desired clone was selected by transforming DA16 and isolating temperature-resistant (Tr$^+$) colonies at 43°C. Plasmid pDA4 (Fig. 3.1b) was constructed by digesting pDA1 with HindIII and ligating the 4162-bp fragment to the 3700-bp HindIII Ω-cam^r fragment derived from pHP45Ω-cam^r (9). The desired plasmid was selected for chloramphenicol resistance and then screened for inability to complement the *grpE*280 mutation in DA16. This plasmid does not express wild type grpE protein. Plasmid pDA5 (Fig. 3.1c) was constructed by digesting pDA1 with EcoRV to linearize the plasmid. The 3700-bp Ω-cam^r fragment was gel-purified after partial digestion of pHP45Ω-cam^r with SmaI and then ligated to the linearized pDA1. This plasmid confers chloramphenicol resistance and overproduces the grpE protein.

Selection for λ*grpE*::Ω-cam^r

The λ*grpE*22 transducing phage (referred to here as λ*grpE*$^+$) was obtained from Dr. Hisao Uchida (25). This phage carries a 12-kbp fragment of *E. coli* DNA which complements the *E. coli grpE*280 mutation, allowing the phage to propagate in the mutant host. This phage is *attλ*$^-$ and possesses the temperature-sensitive allele, *c*I857. To isolate the λ*grpE*$^\Delta$::Ω-cam^r and λ*grpE*$^+$::Ω-cam^r phage, λ*grpE*$^+$ was propagated for one cycle on *E. coli* B178 carrying plasmids pDA4 and pDA5, respectively.

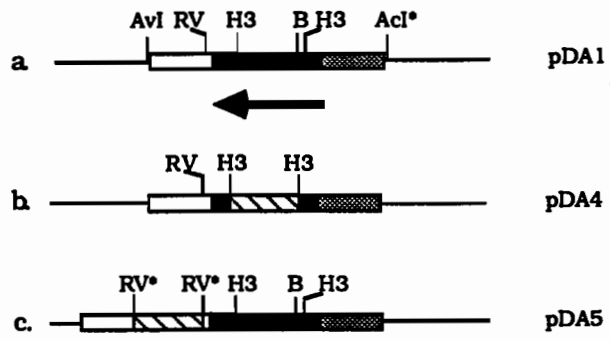


Figure 3.1. Partial restriction maps of *grpE* plasmids. Horizontal lines represent flanking vector sequences; black bar indicates *grpE* sequences; open and stippled bars indicate downstream and upstream flanking sequences, respectively, of the *grpE* gene; bar with diagonal lines indicates Ω-cam^r cassette. Arrow shows direction of transcription of *grpE*. AcI*, AccI-ClaI hybrid site; AvI, AvaI; B, BamHI; H3, HindIII; RV, EcoRV; RV*, EcoRV-SmaI hybrid site. a) pDA1, b) pDA4, c) pDA5. Lengths of bars are not drawn to scale.

During growth of λ*grpE*$^+$ on these two strains, it is expected that at some frequency a recombinational event will occur between the phage and the host sequences on the plasmid. The lysates were screened for phage carrying the Ω-cam^r marker by selecting for Cam^r lysogens upon infection of wild type bacteria at 30°C. Depending on the strain grown on, the phage were confirmed for either the GrpE$^-$ or GrpE$^+$ phenotype.

Western blot analysis

Approximately 8 x 10^7 cells, growing exponentially in L-broth, were pelleted and resuspended in SDS loading dye (2). After boiling for 5 min, the cell lysates were electrophoresed on a denaturing SDS-polyacrylamide (12.5%) gel. The proteins were transferred onto a nitrocellulose membrane filter (Millipore) in a Trans-blot apparatus (BioRad) in 25 mM Tris base, pH 8.3, 192 mM glycine, 20% (v/v) methanol at 50 V for 2 hr (29). The filter was treated with rabbit anti-grpE antisera, followed by incubation with ^{125}I-protein A (*Staphylococcus aureus* protein A; ICN).

Results

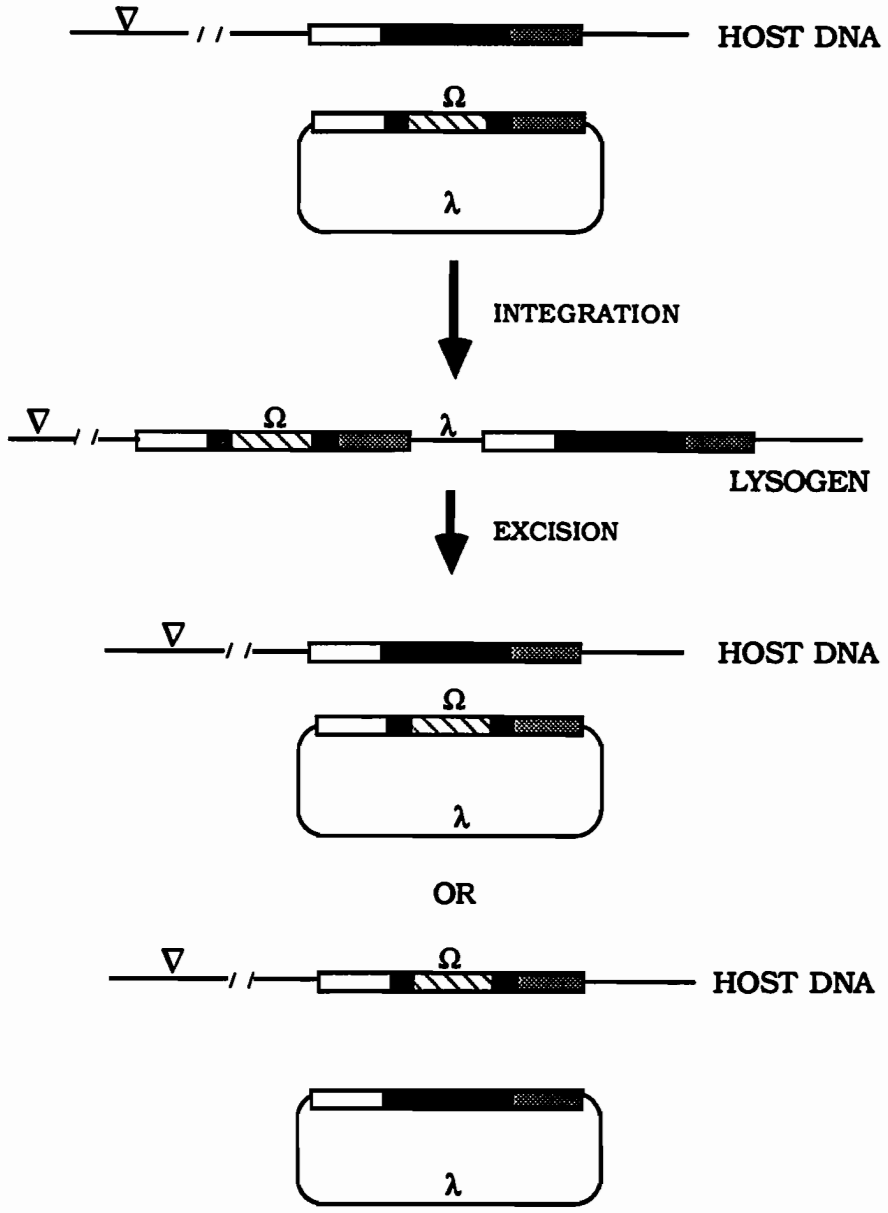
Our approach for constructing a null mutant of the *grpE* gene in *E. coli* required integration of a disrupted version of the *grpE* gene at the *grpE*$^+$ locus on the chromosome, excision of one of the two copies, and selection for the disrupted copy on the chromosome. Since we did not know whether the *grpE*$^+$ gene product is essential for bacterial growth at low temperatures, the construction was done in the presence of extrachromosomal copies of the *grpE*$^+$ gene on a plasmid. The *grpE* region on the *E. coli* chromosome was then transduced into a wild type background to test the lethality of the deletion.

Construction of a *grpE* deletion on the *E. coli* chromosome

The λ*grpE*22 transducing phage (hereafter referred to as λ*grpE*$^+$) (25), was used to introduce a second copy of the *grpE* gene onto the chromosome. The λ*grpE*$^+$ phage was first propagated on *E. coli* carrying plasmid pDA4 or pDA5, as described in **Materials and Methods.** In pDA4, the 500-bp HindIII fragment internal to the *grpE*$^+$ gene DNA coding sequence has been removed and replaced with the Ω-cam^r cassette (Fig. 3.1b). *E. coli grpE*280 mutant bacteria transformed with the pDA4 plasmid are Cam^r but are still temperature-sensitive for growth at 43°C and, furthermore, do not support λ plaque formation (data not shown). *E. coli grpE*280 mutant bacteria transformed with pDA5 (Fig. 3.1c), which carries an intact *grpE*$^+$ gene and the Ω-cam^r cassette inserted in nearby downstream sequences, however, are no longer temperature-sensitive for growth at high temperatures and allow λ plaque formation (data not shown). Through double cross-over recombination events between plasmid- and phage-borne bacterial DNA sequences, transducing phage which carried either the deleted (*grpE*$^\Delta$) or the intact version (*grpE*$^+$) were identified because they conferred Cam^r upon lysogenization.

The next step of the construction required integration of the λ*grpE*::Ω-cam^r transducing phages at the *grpE*$^+$ locus on the chromosome. Because the original λ*grpE*$^+$ transducing phage carries the temperature-sensitive allele, *c*I857 (25), the lysogenization was done at 30°C. The phage is also *attλ*$^-$, favoring integration by homologous recombination at the *grpE*$^+$ locus (Fig. 3.2). Such lysogens are expected to be unstable because of the duplicated sequences on the chromosome. Our strategy was to lysogenize a wild type host, CG1064, with our transducing phage and then select for candidates which had lost the prophage by a homologous recombination event, leaving behind in the bacterial chromosome the Ω-cam^r cassette (Fig. 3.2).

Figure 3.2. Replacement of *grpE*$^{+}$ by *grpE*$^{\Delta}$ on the *E. coli* chromosome. Lines represent flanking chromosomal or λ DNA sequences. The bars are as in Fig. 3.1. The recipient chromosome is marked by a mini-kan^{r} insertion 70-80% cotransducible with the *grpE* locus. See text for details.


HOST DNA
Ω
λ
INTEGRATION
Ω
λ
LYSOGEN
EXCISION
HOST DNA
Ω
λ
OR
Ω
HOST DNA
λ

These candidates could be selected as Cam^r survivors at 43°C since cells still harboring the prophage would die upon *c*I857 repressor denaturation and induction of the prophage at the high temperature. However, because previous results had shown that the *grpE*$^+$ gene product is required at high temperatures (1, 2), the *grpE*$^\Delta$ candidates would not be expected to survive the selection. To overcome this obstacle, prior to selection at the high temperature, the *grpE*+/λ*grpE*::Ω-cam^r lysogens were transformed first with a plasmid, pDA1 (Fig. 3.1a), which carries an intact *grpE*$^+$ gene. Loss of the *grpE*$^+$ gene on the chromosome would then be complemented by the extrachromosomal *grpE*$^+$ copies on the pDA1 plasmid.

Table 3.2 shows the frequency of isolating Cam^r survivors at 43°C in the presence and absence of the pDA1 plasmid. The low frequency of isolating Cam^r *grpE*$^\Delta$ survivors in the absence of the plasmid reaffirms the earlier data that *grpE*+ is necessary for bacterial survival at high temperatures (1, 2). These rare survivors may be due to *c*I857 reversion mutations which now allow repression of the prophage at high temperatures, or deletions of prophage genes, whose expression is lethal to the host. They may also be due to nonhomologous recombination or duplication events which leave behind both an intact *grpE*$^+$ gene copy and the disrupted version marked by the Ω-cam^r cassette. The frequency of obtaining Cam^r *grpE*$^\Delta$ survivors at 43°C increases at least 100-fold in the presence of the pDA1 plasmid, similar to that of the control situation in which Ω is inserted downstream of *grpE*+. It is also important to note that in the control situation, the frequency of obtaining Cam^r survivors at 43°C is the same in the presence and absence of pDA1. This result demonstrates that the insertions do not have any lethal polar effects on the host, at least at 43°C. Six of these heat-resistant "survivors," DA130, DA131, and DA132, which carry the intact *grpE*$^+$ gene cotransducible with the Ω-cam^r cassette at a frequency of > 98%, and DA133, DA134, and DA135 which carry the deleted version, *grpE*$^\Delta$, which is 100%

Table 3.2

Frequency of chloramphenicol-resistant survivors at 43°C

	No. of Kan^r Cam^r survivors at 43°C / no. of Kan^r cells at 30°C
CG1064 ($\lambda grpE^+$::Ω-cam^r) (pDA1)	2.4×10^{-5}
CG1064 ($\lambda grpE^{\Delta}$::Ω-cam^r) (pDA1)	8.5×10^{-5}
CG1064 ($\lambda grpE^+$::Ω-cam^r)	1.9×10^{-4}
CG1064 ($\lambda grpE^{\Delta}$::Ω-cam^r)	2.4×10^{-7}

The cells were grown in L-broth at 30°C to saturation in the presence of kanamycin (50 μg/ml) and ampicillin (50 μg/ml), where appropriate. Dilutions of the cultures were spread on L-plates containing kanamycin (50 μg/ml), chloramphenicol (20 μg/ml), and ampicillin (50 μg/ml), as appropriate. The plates were incubated at 30°C or 43°C.

cotransducible with the Ω-cam^r cassette, were used as donors in the P1 transduction experiments outlined below.

Lethality of *grpE*$^{\Delta}$ in a wild type background

The availability of a *grpE* gene deletion on the chromosome provided a means of testing host viability at lower temperatures in the absence of grpE function. The original strain, CG1064, used to isolate the lysogens, carries a mini-kan^r cassette (31) which is 70-80% cotransducible with the *grpE*$^+$ gene. This marker was used to select for transduction of this region of the chromosome into a wild type background without directly selecting for the *grpE*$^{\Delta}$ itself. Thus, there was no selective pressure for bacterial growth in the absence of grpE function. The bacterial recipients used in these transductions were B178/T1 and B178/T1 (pDA1). In the case where B178/T1 (pDA1) is the recipient, normal cotransduction frequencies between the kan^r and cam^r cassettes is expected with either DA130-132 or DA133-135 as the donors, since loss of *grpE*$^+$ on the chromosome would be compensated by the extrachromosomal *grpE*$^+$ gene copies provided by pDA1. In addition, when DA130-132 are the donors and B178/T1 is the recipient, normal cotransduction frequency between the two markers is also expected since the *grpE*$^+$ gene remains intact on the chromosome. However, when DA133-135 are the donors and B178/T1 is the recipient, normal cotransduction frequency is expected only if loss of grpE function is not lethal to the cell at the selected temperature. Otherwise, the cotransduction frequency should be 0%.

Table 3.3 shows the results of these transductions. While all the controls show normal cotransduction frequencies, it is apparent that when DA133, 134, or 135 are the donors, and the recipient is haploid for the wild type *grpE*$^+$ gene, the probability of obtaining transductants at 30°C which are simultaneously Kan^r and Cam^r is extremely low. This supports the idea that the *grpE*$^+$ gene product, which is essential at 43°C (1,

Table 3.3

Frequency of transduction of *grpE*$^{\Delta}$ at 30°C in the presence or absence of extrachromosomal copies of *grpE*$^{+}$

	Donors					
	grpE^{+}::Ω-cam^{r} kan^{r}			*grpE*$^{\Delta}$::Ω-cam^{r} kan^{r}		
Recipients	DA130	DA131	DA132	DA133	DA134	DA135
B178/T1 (pDA1)	62[a] (41/66)[b]	72 (53/74)	79 (54/68)	71 (44/62)	77 (57/74)	71 (41/58)
B178/T1	74 (46/62)	74 (53/72)	75 (45/60)	3 (2/64)	2 (1/68)	- (0/58)

[a] Percent cotransduction frequency between the kan^{r} and cam^{r} markers.

[b] Actual number of Cam^{r} transductants / actual number of Kan^{r} transductants.

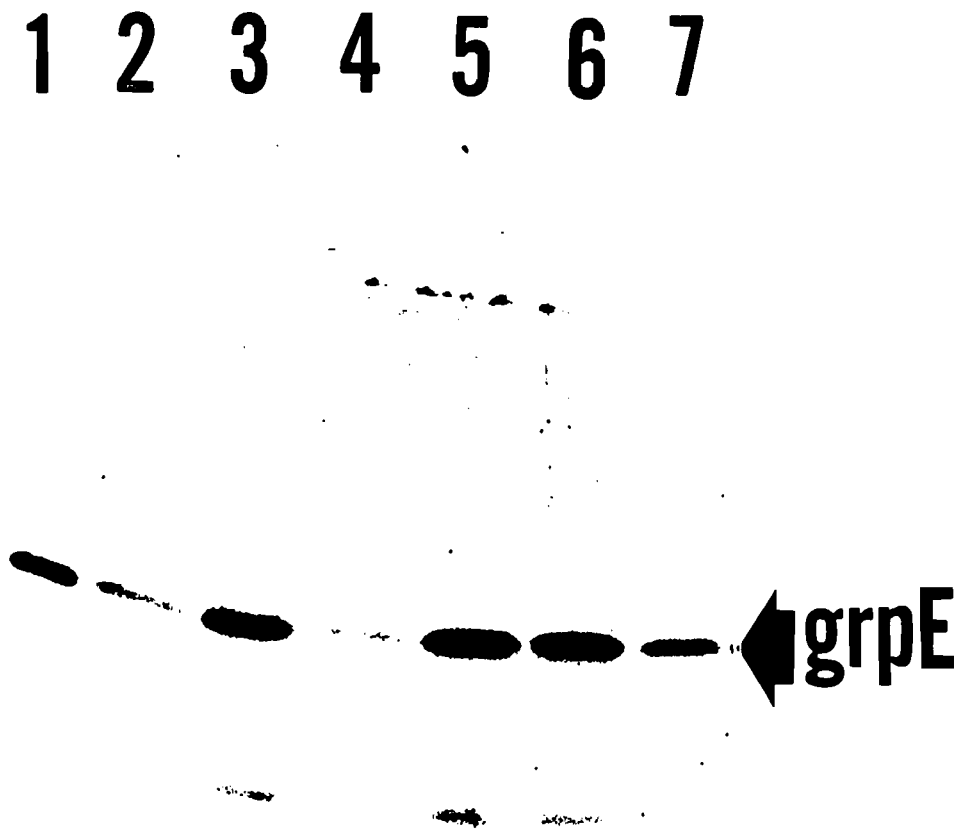
2), is also required for viability at 30°C. Similar results were obtained when thetransductions were carried out at 18°C (data not shown), demonstrating that the grpE protein is also needed for host viability even at this low temperature.

The fact that at a low yet detectable frequency, Kan^r Cam^r transductants do arise when *grpE*$^{\Delta}$ is transduced into a wild type background (Table 3.3), suggests that it may be possible to introduce a deletion in the absence of extrachromosomal copies of a functional *grpE*$^{+}$ gene, perhaps due to accumulation of extragenic suppressor mutation(s). To check for expression of the *grpE*$^{+}$ gene, cell extracts of these rare, exceptional candidates were electrophoresed on denaturing SDS-polyacrylamide gels, and assayed for the presence of grpE protein by Western analysis (39). As shown in Fig. 3.3, these exceptional transductants all express grpE at single copy or higher levels (Fig. 3.3, lanes 4-6). Those which express the higher levels of grpE (lane 5, 6) are also Amp^r, suggesting that the pDA1 plasmid in the donor may have transiently integrated at the chromosomal *grpE*$^{+}$ locus, thus facilitating its transduction into the recipient cell. The candidate which expresses only single copy levels of grpE (lane 4) may have undergone a duplication event, resulting in two copies of *grpE*, an intact version and the deleted version marked by the Ω-cam^r cassette. Whatever the nature of the recombinational event which gave rise to these rare, "unusual" transductants, it is clear that they all express grpE protein. As expected, these exceptional transductants support λ growth and are viable at 43°C (data not shown). We conclude that the grpE protein is essential for *E. coli* viability at temperatures as low as 18°C.

E. coli grpE$^{\Delta}$ is viable in a *dnaK*103 background

The dnaK protein, which is the prokaryotic analogue of Hsp70 in higher organisms, is implicated in several cellular processes. It is a negative regulator of the heat shock response (28); it is postulated to prevent denaturation of proteins during

Figure 3.3. Western analysis of potential *E. coli* $grpE^{\Delta}$ candidates. The filter was treated with rabbit anti-grpE antisera, followed by incubation with ^{125}I-protein A. 1) purified grpE protein; 2) B178/T1 $grpE^{+}$::Ω-cam^{r}; 3) B178/T1 $grpE^{+}$::Ω-cam^{r} (pDA1); 4) B178/T1 $grpE^{\Delta}$::Ω-cam^{r} amp^{s}, #1; 5) B178/T1 $grpE^{\Delta}$::Ω-cam^{r} amp^{r}, #2; 6) B178/T1 $grpE^{\Delta}$::Ω-cam^{r} amp^{r}, #3; 7) purified grpE protein. Lanes 4, 5, and 6 represent extracts from the rare, unexpected Kan^{r} Cam^{r} transductants which carry the $grpE^{\Delta}$ but still express grpE protein. See text for details.


1 2 3 4 5 6 7
grpE

stress or facilitate refolding of denatured proteins following stress (24); it is involved in both host and bacteriophage λ DNA replication (10, 13, 26); and, it may be involved in transport of proteins across a membrane (5, 8). Because grpE and dnaK are known to physically form a complex *in vitro*, which is stable under conditions of high salt concentration but which is specifically disrupted in the presence of ATP (33), one possible role of grpE is *solely* as a regulator of dnaK, i. e., lack of grpE may be lethal because dnaK activity would be uncontrolled. In the absence of dnaK activity, grpE would no longer be required and loss of grpE function would be tolerated. Based on this premise, $grpE^{\Delta}$ was transduced into an *E. coli dnaK*103 strain, CG800, which makes very small amounts of full-length dnaK protein and greater amounts of a truncated 50 kD protein. This strain is also temperature-sensitive for growth, exhibits an abnormal heat shock response, and does not support λ DNA replication (G. N. Chandrasekhar, K. von Meyenburg and C.G., unpublished data). As shown in Table 3.4, when CG800 is used as a recipient in P1 transductions, $grpE^{\Delta}$ can replace $grpE^{+}$ at the expected cotransduction frequency. The ability to delete the $grpE^{+}$ gene in the *E. coli dnaK*103 background is not a general property among all strains with mutated alleles of the *dnaK* gene. As shown in Table 3.4, if *E. coli dnaK*756 (CG410) is used as the recipient, normal cotransduction frequencies are seen between the kan^{r} and cam^{r} markers when the donor is $grpE^{+}$, but very low cotransduction frequencies are seen when the donor is $grpE^{\Delta}$. As expected, the rare Kan^{r} Cam^{r} transductants were shown to express grpE antigen (data not shown).

Two possibilities were considered to explain the ability to transduce $grpE^{\Delta}$ into a *dnaK*103 mutant background: a) loss of dnaK activity obviates the need for grpE, or b) there is an extragenic suppressor mutation(s) in *dnaK*103 bacteria which also allows bypass of grpE function. To test these two possibilities, $grpE^{\Delta}$ was transduced into DA239, which is a $\lambda imm^{21}dnaK^{+}$ lysogen of CG800. This strain, in which the

Table 3.4

Frequency of transduction of *grpE*$^{\Delta}$ into *E. coli dnaK*$^{-}$ strains at 30°C

Recipients	Donors	
	grpE^{+}::Ω-cam^{r} DA130	*grpE*$^{\Delta}$::Ω-cam^{r} DA133
CG800	74[a] (62/84)[b]	75 (88/117)
DA239	76 (52/68)	80 (101/127)
CG799	74 (70/94)	78 (81/104)
CG409	74 (53/72)	3 (1/36)
CG410	74 (59/80)	2 (1/58)

[a] Percent cotransduction frequency between the kan^{r} and cam^{r} markers.

[b] Actual number of Cam^{r} transductants / actual number of Kan^{r} transductants.

*dnaK*103 mutation is complemented by the *dnaK*$^+$ gene on the transducing phage, is phenotypically Tr$^+$ at 43°C and supports λ growth (data not shown). In the case of possibility a), the expression of *dnaK* in DA239 should prevent replacement of the wild type gene with the *grpE*$^\Delta$ gene. In the case of possibility b), it is expected that the presence of an extragenic suppressor may allow replacement by the *grpE*$^\Delta$ gene. The data, presented in Table 3.4, show that *grpE*$^\Delta$ can be transduced into DA239 at the same frequency as the control, even though the dnaK protein is being produced normally. To further support the idea that an extragenic suppressor allows bypass of grpE function, strain CG799, which is isogenic to CG800 except that the *dnaK*103 mutant allele has been replaced by the *dnaK*$^+$ allele through P1 transduction, can also accept *grpE*$^\Delta$ at normal frequencies (Table 3.4). This result further suggests that the suppressor is not in or closely linked to *dnaK* (e.g., it is not in the adjacent *dnaJ* heat shock gene).

Table 3.5 shows the λ plating and temperature-sensitive phenotypes of the Kan^r Cam^r transductants. Each strain exhibits phenotypes consistent with the combinations of wild type and mutant *dnaK* and *grpE* alleles expected to be present. For example, CG800 *grpE*$^\Delta$::Ω-cam^r, which is *dnaK*$^-$ and presumably *grpE*$^-$, is temperature-sensitive for growth at 43°C and does not allow λ growth, typical of either a *dnaK*$^-$ or *grpE*$^-$ mutant (2, 10, 11, 13, 26, 30). However, it also does not allow growth of the λ*imm*^{434}*dnaK*$^+$ transducing phage, which can complement the lack of dnaK function in the host, but cannot complement the lack of grpE function. Likewise, the λ*grpE*$^+$ transducing phage does not grow on this strain since it cannot complement the lack of dnaK function. Strain CG800 *grpE*$^+$::Ω-cam^r behaves as though only the dnaK function is missing. As expected, it is temperature-sensitive for growth at 43°C but allows λ*imm*^{434}*dnaK*$^+$ to grow. DA239 *grpE*$^\Delta$::Ω-cam^r and CG799 *grpE*$^\Delta$::Ω-cam^r do not support λ*imm*^{434}*dnaK*$^+$ growth but do support λ*grpE*$^+$, demonstrating the loss

Table 3.5

Plating properties of *E. coli dnaK*103 Kan^r Cam^r transductants

	30°C	43°C	λ*cI*$^-$	λ*imm*^{434}*dnaK*$^+$	λ*grpE*$^+$
DA15	+[a]	+	+	+	+
DA16	+	-[b]	-	-	+
CG800 *grpE*$^+$::Ω-cam^r	+	-	-	+	-
CG800 *grpE*$^\Delta$::Ω-cam^r	+	-	-	-	-
DA239 *grpE*$^+$::Ω-cam^r	+	+	+	+	+
DA239 *grpE*$^\Delta$::Ω-cam^r	+	-	-	-	+
CG799 *grpE*$^+$::Ω-cam^r	+	+	+	+	+
CG799 *grpE*$^\Delta$::Ω-cam^r	+	-	-	-	+

[a] + indicates large colony or plaque size; efficiencies of plating > 0.5.

[b] - indicates no visible plaques; no visible colony formation; efficiencies of plating $<10^{-4}$.

of grpE function, even in the presence of dnaK.

Fig. 3.4 shows the results of a Western analysis of extracts of the Kan^r Cam^r transductants. Total cell lysates were assayed for the presence of grpE antigen. In each of the three cases where $grpE^{\Delta}$ was transduced into either CG800, DA239 [=CG800 ($\lambda imm^{21} dnaK^+$)], or CG799, none synthesized grpE antigen at levels detectable by this assay. We conclude that maintainance of $grpE^{\Delta}$ on the chromosome in these strains is due to the presence of an extragenic suppressor(s) of the mutant $dnaK^-$ allele.

Discussion

The dnaK, dnaJ, grpE, groEL and groES proteins of *E. coli*, which are necessary for bacteriophage λ growth at all temperatures, are also required for bacterial colony formation at high temperatures (1, 2, 10, 11, 13, 26, 30), as shown by the temperature-sensitive nature of mutations in each of the respective genes. Interestingly, these genes are now known to be members of the heat shock regulon, in which expression is regulated by stress, e.g., a sudden shift-up in temperature (22). Because mutations in these genes show no gross phenotype at normal temperatures, i.e., 30°-37°C, except for the inability to support plaque formation upon infection by λ, it was not clear whether expression of these genes is essential only at high temperatures or upon exposure to other forms of stress, and not during normal growth of the cell. Attempts to delete the $dnaK^+$ and $dnaJ^+$ genes at 30°C were successful (23; S. Sell and C. G., unpublished data), while deletion of either the $groES^+$ or $groEL^+$ gene was found to be lethal (O. Fayet, T. Ziegelhoffer, and C. G., submitted for publication). It has since been shown that the *dnaK* and *dnaJ* null mutations in a wild type background result in cells which grow extremely poorly (4; S. Sell and C.G., unpublished data). However, fast-growing derivatives are easily isolated. It

Figure 3.4. Western blot analysis of *E. coli* $grpE^{\Delta}$ *dnaK*103 transductants. The filter was treated with rabbit anti-grpE antisera, followed by incubation with ^{125}I-protein A. 1) purified grpE protein, 2) CG800 $grpE^{+}$::Ω-cam^{r}; 3) CG800 $grpE^{\Delta}$::Ω-cam^{r}; 4) DA239 $grpE^{+}$::Ω-cam^{r}; 5) DA239 $grpE^{\Delta}$::Ω-cam^{r}; 6) CG799 $grpE^{+}$::Ω-cam^{r}; 7) CG799 $grpE^{\Delta}$::Ω-cam^{r}.


1 2 3 4 5 6 7
grpE

was shown that these derivatives have extragenic suppressor(s) whose identities are, as yet, unknown (4, S. Sell and C.G., unpublished data).

The overwhelming evidence that the dnaK and grpE proteins interact both *in vitro* and *in vivo* (2, 33; C. Johnson, G. N. Chandrasekhar, and C. G., submitted for publication) suggested that deletion of *grpE*$^+$ from the chromosome should also be possible. However, as shown here, deletion of *grpE*$^+$ in a wild type background is lethal in a temperature range of 18°-44°C, since it can be retained on the chromosome if, and only if, a wild type copy of the gene is simultaneously present elsewhere. Recently, Zhou et al. (32) have shown that the *rpoH* gene, coding for σ^{32} which is a subunit of core RNA polymerase that recognizes heat shock promoters, can be deleted at temperatures below 20°C. Since we have shown that the *grpE*$^+$ gene is under σ^{32} regulation (19), and is essential for bacterial growth at 18°C (this work), we made an attempt to reconcile these apparently contradictory results. In preliminary Western analysis experiments, we have shown that in extracts from the *rpoH*$^\Delta$ strain of Zhou et al. (32) detectable levels of grpE antigen are found. We presume that the *grpE*$^+$ gene is being transcribed in the *rpoH*$^\Delta$ background, by an as yet unknown, σ^{32}-independent mechanism. In this respect, it is interesting to note that a σ^{70} consensus promoter sequence is found superimposed on the σ^{32} consensus promoter sequence of the *grpE* gene (19). Transcription from this σ^{70} promoter could be masked in an *rpoH*$^+$ background. An analogous situation has, in fact, been seen with the *groE*$^+$ genes (32).

Surprisingly, the wild type *grpE*$^+$ gene was readily replaced by *grpE*$^\Delta$ in an *E. coli dnaK*103 mutant, as shown by transductional analysis and by the inability to detect grpE antigen in these cell extracts with anti-grpE antibody. In addition, we have shown that a Fraction II extract, prepared as described previously (34), from CG799 *grpE*$^\Delta$::Ω-cam^r does not support λdv DNA replication *in vitro* unless it is

supplemented with purified grpE, λO and λP proteins (J. Skorko, M. Zylicz, D. A., and C. G., unpublished data). A Fraction II prepared from a wild type strain, however, allows λdv replication without the addition of grpE protein. Thus, it appears that a suppressor mutation(s) outside of the *dnaK*$^{+}$ gene permits loss of grpE function. The data reported here support, but in no way prove, that the grpE and dnaK proteins may perform analogous functions *in vivo*, inasmuch as a suppressor(s) of the *dnaK*103 mutation also suppresses the loss of grpE function. It is not understood, however, why it is not possible to select for an extragenic suppressor of *grpE*$^{\Delta}$ directly. Perhaps the frequency of selecting for such suppressors is much lower for *grpE*$^{\Delta}$ than for *dnaK*103.

We have attempted to transduce *grpE*$^{\Delta}$ into several other *E. coli dnaK* mutants, some of which are able to accept the deletion while others are not (unpublished data). Those which are unable to accept the deletion of *grpE*$^{+}$, e.g., *E. coli dnaK*756, may be of two classes: a) those in which the altered amino acid residue(s) of the mutant dnaK protein does not interfere with its interaction with the wild type grpE protein (these mutant bacterial strains may or may not possess extragenic suppressors of the *dnaK*$^{-}$ mutation), or b) those in which the mutation in *dnaK* affects a function of the dnaK protein which does not require the presence of the grpE protein. In this case, those functions which require both dnaK and grpE are unaffected. Again, these *E. coli dnaK* mutants may or may not possess an extragenic suppressor(s) of the mutant *dnaK* allele.

There is evidence that the dnaK, dnaJ and grpE proteins are required for *E. coli* replication (1, 2, 13, 26, 27, 30). Though not understood, their role in *E. coli* replication may be similar to their role in initiation of λ DNA replication. *In vitro* experiments using a purified protein system suggest that they are needed to disrupt the interaction between the λP protein and the host dnaB protein in the preprimosomal

complex. Once free of λP, the helicase activity of dnaB is no longer inhibited and can thus unwind the DNA at *oriλ*, initiating the subsequent series of events leading to DNA replication (17). The disruption of the λP-dnaB complex may be occurring through hydrophobic interactions between λP and dnaK.

As stated earlier, expression of *dnaK*$^+$, *dnaJ*$^+$, and *grpE*$^+$ is induced by heat shock. The dnaK protein has been shown to be a negative regulator of the heat shock response (28). In addition, it may be responsible for protecting susceptible proteins from denaturation or facilitating refolding of denatured proteins by protecting hydrophobic regions in a manner analogous to its behavior in the λ preprimosomal complex. It is not clear what the roles of dnaJ and grpE are in the heat shock response. In principle, they could assist the dnaK protein in regulating the heat shock response in a manner analogous to that in λ DNA replication , e. g., assisting in the release of the σ^{32} polypeptide from the RNA polymerase holoenzyme. The identity of extragenic suppressors of null mutations in these genes should assist in understanding the functions of these proteins, and perhaps uncovering other pathways in which these proteins are involved.

Acknowledgments

This work was supported by grants from the National Institutes of Health (to C. G.) and an NIH training grant (to D. A.). We thank Dr. Kaspar von Meyenberg for providing *E. coli dnaK*103 bacteria. We thank B. for good humor and for typing the manuscript.

Literature Cited

1. **Ang, D., G. N. Chandrasekhar, C. Johnson, M. Zylicz and C. Georgopoulos.** 1987. A genetic and biochemical analysis of the *Escherichia*

coli grpE gene and its product. *In* R. McMacken and T. Kelly (eds.), UCLA Symp. Mol. Cell. Biol. New Series, **47**:521-532, Alan R. Liss, Inc., New York.

2. **Ang, D., G. N. Chandrasekhar, M. Zylicz and C. Georgopoulos.** 1986. *Escherichia coli grpE* gene codes for heat shock protein B25.3, essential for both λ DNA replication at all temperatures and host growth at high temperature. J. Bacteriol. **167**:25-29.

3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95-104.

4. **Bukau, B., C. E. Donnelly and G. C. Walker.** 1988. DnaK and GroE proteins play roles in *E. coli* metabolism at low and intermediate temperatures as well as at high temperatures. *In* M. L. Pardue, J. Feramisco, and S. Lindquist (eds.), Stress-induced proteins, Alan R. Liss, Inc., New York, *in press*.

5. **Chirico, W., M. Waters and G. Blobel.** 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. Nature **332**:805-810.

6. **Craig, E. A.** 1985. The heat shock response. Crit. Rev. Biochem. **18**:239-280.

7. **Dente, L., G. Cesareni and R. Cortese.** 1983. pEMBL: a new family of single-stranded plasmids. Nucleic Acids Res. **11**:1645-1655.

8. **Deshaies, R. J., B. D. Koch, M. Werner-Washburn, E. A. Craig and R. Schekman.** 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature **332**:800-805.

9. **Fellay, R., J. Frey and H. Krisch.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of Gram-negative bacteria. Gene **52**:147-154.

10. **Georgopoulos, C. P.** 1977. A new bacterial gene (*groPC*) which affects λ DNA replication. Mol. Gen. Genet. **151**:35-39.

11. **Georgopoulos, C. P. and I. Herskowitz.** 1971. *Escherichia coli* mutants blocked in lambda DNA synthesis. *In* A. D. Hershey (ed.), The bacteriophage lambda, pp. 553-564, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

12. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557-580.

13. **Itikawa, H. and J.-I. Ryu.** 1979. Isolation and characterization of a temperature-sensitive *dnaK* mutant of *Escherichia coli* B. J. Bacteriol. **138**:339-344.

14. **Jasin, M. and P. Schimmel.** 1984. Deletion of an essential gene in *Escherichia coli* by site-specific recombination with linear DNA fragments. J. Bacteriol. **159**:783-786.

15. **Joyce, C. M. and N. D. F. Grindley.** 1984. Method for determining whether a gene of *Escherichia coli* is essential: application to the *polA* gene. J. Bacteriol. **158**:636-643.

16. **Kanaya, S. and R. J. Crouch.** 1984. The *rnh* gene is essential for growth of *Escherichia coli*.. Proc. Natl. Acad. Sci. U. S. A. **81**:3447-3651.

17. **Liberek, K., C. Georgopoulos and M. Zylicz.** 1988. The role of the *Escherichia coli* DnaK and DnaJ heat shock proteins in the initiation of bacteriophage λ DNA replication. Proc. Natl. Acad. Sci. U. S. A.. **85**:6632-6636.

18. **Lindquist, S.** 1986. The heat shock response. Ann. Rev. Biochem. **55**:1151-1191.

19. **Lipinska, B., J. King, D. Ang and C. Georgopoulos.** 1988. Sequence analysis and transcriptional regulation of the *Escherichia coli grpE* gene, encoding a heat shock protein. Nucleic Acids Res., **16**:7545-7562.

20. **Maurizi, M. R., P. Trisler and S. Gottesman.** 1985. Insertional mutagenesis of the *lon* gene in *E. coli*: *lon* is dispensable. J. Bacteriol. **164**:1124-1135.

21. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

22. **Neidhardt, F. C., and R. A. VanBogelen.** 1987. Heat shock response. *In* F. C. Neidhardt et al. (eds.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology, pp. 1334-1345, American Society of Microbiology, Washington, D. C.

23. **Paek, K.-H. and G. C. Walker.** 1987. *Escherichia coli dnaK* null mutants are inviable at high temperature. J. Bacteriol. **169**:283-290.

24. **Pelham, H. R. B.** 1986. Speculations on the functions of the major heat shock and glucose-regulated proteins. Cell **46**:959-961.

25. **Saito, H., Y. Nakamura and H. Uchida.** 1978. A transducing λ phage carrying *grpE*, a bacterial gene necessary for λ DNA replication, and two ribosomal protein genes, *rpsP* (S16) and *rplS* (L19). Mol. Gen. Genet. **165**:247-256.

26. **Saito, H. and H. Uchida.** 1977. Initiation of the DNA replication of bacteriophage lambda in *Escherichia coli* K-12. J. Mol. Biol. **113**:1-25.

27. **Sakakibara, Y.** 1988. The *dnaK* gene of *Escherichia coli* functions in initiation of chromosome replication. J. Bacteriol. **170**:972-979.

28. **Tilly, K., N. McKittrick, M. Zylicz and C. Georgopoulos.** 1983. The *dnaK* protein modulates the heat-shock response of *Escherichia coli*. Cell **34**:641-646.

29. **Towbin, H., T. Staehelin and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. **76**:4350-4354.

30. **Wada, M., Y. Kadokami and H. Itikawa.** 1982. Thermosensitive synthesis of DNA and RNA in *dnaJ* mutants of *Escherichia coli* K-12. Jpn. J. Genet. **57**:407-413.

31. **Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts and N. Kleckner.** 1986. New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene **32**:369-379.

32. **Zhou, Y.-N., N. Kusukawa, J. W. Erickson, C. A. Gross and T. Yura.** 1988. Isolation and characterization of *Escherichia coli* mutants that lack the heat shock sigma factor (σ^{32}). J. Bacteriol. **170**:3640-3649.

33. **Zylicz, M., D. Ang and C. Georgopoulos.** 1987. The grpE protein of *Escherichia coli:* Purification and properties. J. Biol. Chem. **262**:17437-17442.

34. **Zylicz, M., J. H. LeBowitz, R. M. McMacken and C. Georgopoulos.** 1983. The dnaK protein of *Escherichia coli* possesses an ATPase and autophosphorylating activity and is essential in an *in vitro* DNA replication system. Proc. Natl. Acad. Sci. U. S. A. **80**:6431-6435.

CHAPTER 4

PURIFICATION AND PROPERTIES OF THE *Escherichia coli* grpE280 MUTANT PROTEIN

Abstract

The *Escherichia coli grpE* gene product is essential for both bacteriophage λ DNA replication and host growth at all temperatures. To better understand the mechanism of grpE action, the *grpE*280 mutant gene was cloned and the overproduced protein purified. *E. coli grpE*280 cells which are overproducing grpE280 protein at least ten-fold partially regain the wild type phenotype, i. e., they allow intermediate levels of λ production and are no longer temperature-sensitive for growth at 43.5°C. The purified grpE280 protein has lost many of the properties exhibited by its wild type counterpart, such as a) the ability to support *in vitro* λ DNA replication, b) the ability to function in the λ DNA unwinding assay, c) efficient binding to a dnaK-affinity column, and d) the ability to cosediment with wild type dnaK in glycerol gradients. However, grpE280 does crosslink to wild type dnaK in the presence of glutaraldehyde more efficiently than its wild type counterpart. Nevertheless, this ability to crosslink is very sensitive to a dilution effect, suggesting that the dnaK-grpE280 complex is qualitatively different from that of the wild type complex.

Introduction

A common method of studying a gene function is to alter the gene and then observe the consequences as phenotypic changes *in vivo*. In most cases, the function is performed by an isolatable macromolecule which can be purified and studied in further detail *in vitro*. The system to be discussed here, initiation of bacteriophage λ DNA replication, is particularly amenable to this approach. This chapter will focus on the purification of the *E. coli* grpE280 mutant protein whose wild type counterpart has been shown to be essential for both λ DNA replication and host growth at all temperatures (2, 3, 22).

Lambda is a temperate phage of *E. coli* which relies heavily on host proteins for replication of its DNA. Lambda encodes two "initiator" proteins, λO and λP, which are necessary for *ori*λ-specific initiation and for attracting host proteins to the site of initiation. The other proteins required for initiation *in vivo* - dnaB, dnaK, dnaJ, grpE, dnaG (primase), RNA polymerase and ssb (reviewed in ref. 9) - are all encoded by *E. coli*. The genes for a subset of these proteins were initially identified by indirect or direct selection of mutations in *E. coli* which do not support λ DNA replication (10, 22). Many of these mutations, e. g., *dnaB*558, *dnaK*756, *dnaJ*259 and *grpE*280, also confer a temperature-sensitive phenotype on the host (1, 2, 10, 26). Subsequently it was shown that the *dnaK*756, *dnaJ*259 and *grpE*280 mutations affect both RNA and DNA syntheses in the host at the nonpermissive temperature (14, 22, 23, 27). The implication that the wild type products of these three genes operate in the same pathway in *E. coli* is further supported by the fact that they are members of the heat shock regulon (2, 11; reviewed in ref. 20) and hence, subject to regulation by sudden changes in the environment. Lambda itself has taken advantage of this host regulatory mechanism by evolving the means to induce expression of these proteins upon infection (4, 28).

Physical interactions between many of the proteins required for initiation of λ DNA replication have been established (reviewed in Ch. 1). One prominent interaction is that between the *dnaK* and *grpE* gene products, whose genes encode polypeptides of 69,246 M_r and 21,668-M_r, respectively (6, 17). A variety of evidence suggests that the two proteins intimately interact: a) The two proteins can be co-precipitated in the presence of antibody against one or the other (1, 15), b) the grpE protein was purified on the basis of its retention on a dnaK-affinity column, even in the presence of high concentrations of salt, and eluted specifically by ATP (30), and c)

extragenic suppressors of the *grpE*280 mutation have been shown to map in *dnaK*, providing *in vivo* evidence that these two gene products functionally interact (15).

The mutant dnaK756 protein has been purified and studied to some extent (32, 33). Both the wild type and dnaK756 proteins bind to ATP-agarose and may also be purified on this basis (S. Sell, Ph.D. dissertation, University of Utah, 1987; A. Cegielska, personal comm.). The apparent size of the mutant protein under denaturing conditions is identical to the wild type, but its isoelectric point is more acidic than the wild type (31, 34). Not only are the ATPase and autophosphorylation activities of the mutant protein lower than those of the wild type, but they are also more thermolabile (34). Besides having decreased ATPase and autophosphorylation activities, dnaK756 has decreased affinity for wild type grpE protein (1). Any combination of these three alterations in activity may account for its mutant phenotypes and inability to complement the *in vitro* single-stranded M13 DNA and double-stranded λdv DNA replication systems (16, 34).

In this chapter, the cloning of the *grpE*280 mutant gene into a multicopy plasmid is presented. Overproduction of grpE280 in *E. coli grpE*280 cells reverses the temperature-sensitive phenotype and the inability to allow λ growth. The purified mutant protein, unlike wild type grpE (30), does not form a complex with wild type dnaK protein in a glycerol gradient or on a dnaK-affinity column. Surprisingly, in the presence of glutaraldehyde, grpE280 is crosslinked to dnaK more efficiently than wild type grpE. Substitution of wild type grpE by grpE280 protein in both the *in vitro* λdv DNA replication assay (30) and the λdv DNA unwinding assay (31) results in insignificant levels of nucleotide incorporation and unwinding, respectively, demonstrating that the mutant grpE280 protein is inactive for λ DNA replication *in vitro*.

Materials and Methods

Bacteria, bacteriophage and plasmids

The bacteria, bacteriophage and plasmid strains used in the course of this work are shown in Table 4.1.

Media

L-broth and L-agar were as previously described (2). Chloramphenicol (cam) was added to media, when necessary, at a final concentration of 20 μg/ml.

Lysogenization and marker rescue

Lysogenization and marker rescue were done essentially as described by Miller (19).

DNA manipulations

Phage and plasmid DNAs were purified as described by Maniatis et al. (18). Restriction enzymes were purchased from New England Biolabs and used according to the manufacturer's recommendations. T4 DNA ligase (Bethesda Research Laboratories) was used according to Maniatis et al. (18). Cells were transformed according to Hanahan (12).

Preparation of crude cell extracts

Overnight, stationary cultures (7 ml) of strains DA16 (pDA8) and DA15 (pDA5) were each diluted into 6 l of L-broth containing 20 μg/ml cam, 0.2% glucose and 50 μg/ml thymine. Each culture was evenly distributed among 7 2-l flasks and aerated at 37°C until it reached $A_{595nm} = 0.85$. The cells were pelleted (10.5 g and 11.2 g,

Table 4.1

Bacteria, bacteriophage and plasmids

Strain	Genotype and/or Phenotype	Source or Reference
DA15	B178 *phe*A::Tn*10*, Tet^r	(2)
DA16	B178 *phe*A::Tn*10* *grpE*280, Tet^r	(2)
Phages		
λ*grpE*$^+$	λ*grpE*22 = λ *imm*$^{\lambda}$ *int*$^-$ *xis*$^-$ *att*λ$^-$ *c*I857 *grpE*$^+$	H. Uchida (24)
λ*grpE*$^+$::Ω-cam^r	λ*grpE*$^+$, cam^r cassette downstream of *grpE*$^+$	(3)
Plasmids		
pBR322	Amp^r Tet^r	(7)
pDA5	pBR322 *grpE*$^+$::Ω-cam^r, Amp^r	(3)
pDA8	pBR322 *grpE*280::Ω-cam^r, Amp^r	this work

respectively) and frozen as described previously (35). Fraction II (FII) extracts were prepared as previously described (35).

Crude *in vitro* λdv DNA replication

Oriλ-specific *in vitro* replication assays were done as previously described, using a FII extract derived from strain DA16 (2, 30).

Purification of grpE280 protein

Approximately 100 mls of an overnight, stationary culture of DA16 (pDA8) was diluted into 800 ml L-broth supplemented with chloramphenicol and divided among 12 2-l flasks. The cultures were grown at 37°C with aeration until they reached an A_{595nm} = 1.0 (approx. 4 hr). The cells were pelleted in a Beckman JA-10 rotor, 4400 x g (5000 rpm) for 15 min at 4°C. Following a brief rinse with Buffer A to dilute residual L-broth, the pellets were combined and weighed (18.3 g). They were resuspended in 13 ml Buffer A and frozen in liquid nitrogen before storing at -70°C.

The frozen cells were thawed and lysed essentially as described previously (8, 30). The cells were thawed in a 2-4°C ice/water bath. Lysis buffer (13.2 ml) followed by cold Buffer A was added to the cells to a final volume of 96 ml. As the mixture was gently swirled, 3 ml of 10 mg/ml chicken egg white lysozyme (Sigma) was added in a dropwise fashion. The cells were kept on ice for 45 min, being gently inverted every 10 min. Subsequently, they were transferred to a 37°C waterbath and gently swirled for 5 min. Following this, they were returned to ice for 5 min, followed by centrifugation in a Beckman Type 35 rotor at 104,000 x g (30,000 rpm) for 30 min at 2°C. The supernatant ("cleared lysate," 77 ml) was kept on ice and stirred while 0.35 g crushed ammonium sulfate per ml of supernatant (26.95 g) was added over a period of 15 min. The mixture was stirred for an additional 30 min

before centrifugation in a Beckman Type 35 rotor at 46,600 x g (20,000 rpm) for 20 min at 2°C. The pellet was resuspended in 3 ml Buffer B and dialyzed against 1 l Buffer B and 20 mM KCl for 12 hr. The dialysate (approx. 10 ml) was diluted to 45 ml with Buffer B, 20 mM KCl and applied to a DE52 column (Whatman; 2.5 cm x 19 cm) at 5.5 ml/10 min. The column was washed with 5 x V_t of Buffer B, 20 mM KCl followed by a linear gradient (1 l, 20 mM to 300 mM KCl). GrpE280 eluted from the column at approximately 125 mM KCl. Relevant fractions (52.5 ml) were dialyzed against 2 l Buffer C and 1 M KCl for 6 hr and then applied to a phenyl-Sepharose CL-4B column (Sigma; 1.5 cm x 3.5 cm) at 5 ml/hr. The column was washed with Buffer C containing 1 M KCl at 2.5 ml/15 min. The void fractions (30 ml) were collected and dialyzed against 700 ml Buffer Y, 20 mM KCl for 6 hr. The protein was concentrated (for other purposes) by precipitating in 0.35 g/ml ammonium sulfate. After centrifugation in a Beckman SW 27.1 rotor at 77,000 x g (20,000 rpm) for 30 min at 4°C, the 2 pellets were resuspended in 2 ml Buffer Y, 20 mM KCl, then dialyzed against 350 ml Buffer Y, 20 mM KCl. One-fifth of the dialysate (600 µl) was diluted 10-fold with Buffer Y, 20 mM KCl and applied to a Q-Sepharose column (Pharmacia; 1 cm x 3.2 cm) at 3 ml/10 min. The column was washed with 7 x V_t of Buffer Y, 20 mM KCl. GrpE280 protein was eluted with a linear gradient (200 ml, 20 mM to 500 mM KCl) at 3 ml/10 min. GrpE280 eluted from the column at approximately 140 mM KCl. Relevant fractions (7.5 ml) were pooled and dialyzed against 1 l Buffer Y, 50 mM KCl and frozen in 50-µl aliquots at -70°C. The final concentration was approximately 0.1 mg/ml.

Buffers

Buffer A contains 10% sucrose (Mallinckrodt) and 50 mM Tris/HCl (Sigma), pH $8_{4°C}$; Buffer B contains 25 mM Tris/HCl, pH $8_{4°C}$, 1 mM EDTA (Mallinckrodt), 10%

(v/v) glycerol (Mallinckrodt), and 10 mM 2-mercaptoethanol (Sigma); Buffer C is identical to Buffer B except it has 5% (v/v) glycerol instead of 10%; Buffer D contains 25 mM HEPES (Sigma)/KOH, pH $8_{25°C}$, 1 mM EDTA, 10% (v/v) glycerol, and 10 mM 2-mercaptoethanol; Buffer Y contains 25 mM HEPES/KOH, pH 8, 1mM EDTA, 10% (v/v) glycerol, and 10 mM 2-mercaptoethanol.

Sedimentation in glycerol gradients

DnaK and grpE proteins were sedimented in 20-40% (v/v) glycerol gradients (3.0 ml) as described by Zylicz et al. (30).

DNA unwinding assay

The unwinding assay described for *ori*C-specific plasmids (5) was carried out exactly in the manner adapted for *ori*λ-specific plasmids (31).

Crosslinking of proteins with glutaraldehyde

The crosslinking reaction was done essentially as described by Schlossman et al. (25). Protein was incubated at 30°C for 10 min in a 25-µl reaction mixture containing 40 mM HEPES/KOH, pH 7.6 (Research Organics), 0.5 mM dithiothreitol (Research Organics) and 14.4 mM magnesium acetate (Mallinckrodt). Glutaraldehyde (0.5 µl of an 8% solution) was added to each reaction, which was then incubated for an additional 2 min at 30°C. To reduce any remaining active groups, 2 µl of 1 M sodium borohydride were added, followed by incubation at 4°C for 20 min. Tris, pH 7.5 (6 µl of a 1 M solution) was added and the reactions were further incubated for 5 min at 4°C. Samples were electrophoresed in 5% polyacrylamide/SDS gels as described below.

Gel electrophoresis

One-dimensional gel electrophoresis was performed essentially as described by O'Farrell (21). Slab gels of 12.5% (w/v) polyacrylamide / SDS were used. For crosslinking reactions, 10 µl of SDS sample buffer was added to each prior to a 5 min incubation in a boiling water bath. The samples were electrophoresed in a 5% polyacrylamide gel as described by Weber and Osborn (29) at 110 mA for approximately 5 hr. The running buffer was composed of 0.1 M $NaHPO_4$, pH 7.2, and 0.1% SDS.

Immunoblot analysis

The procedure described in Ref. 3 was used here. Production and processing of rabbit anti-dnaK and -grpE antibodies has been described (3). [^{125}I]-protein A (*Staphylococcus aureus*) was purchased from ICN.

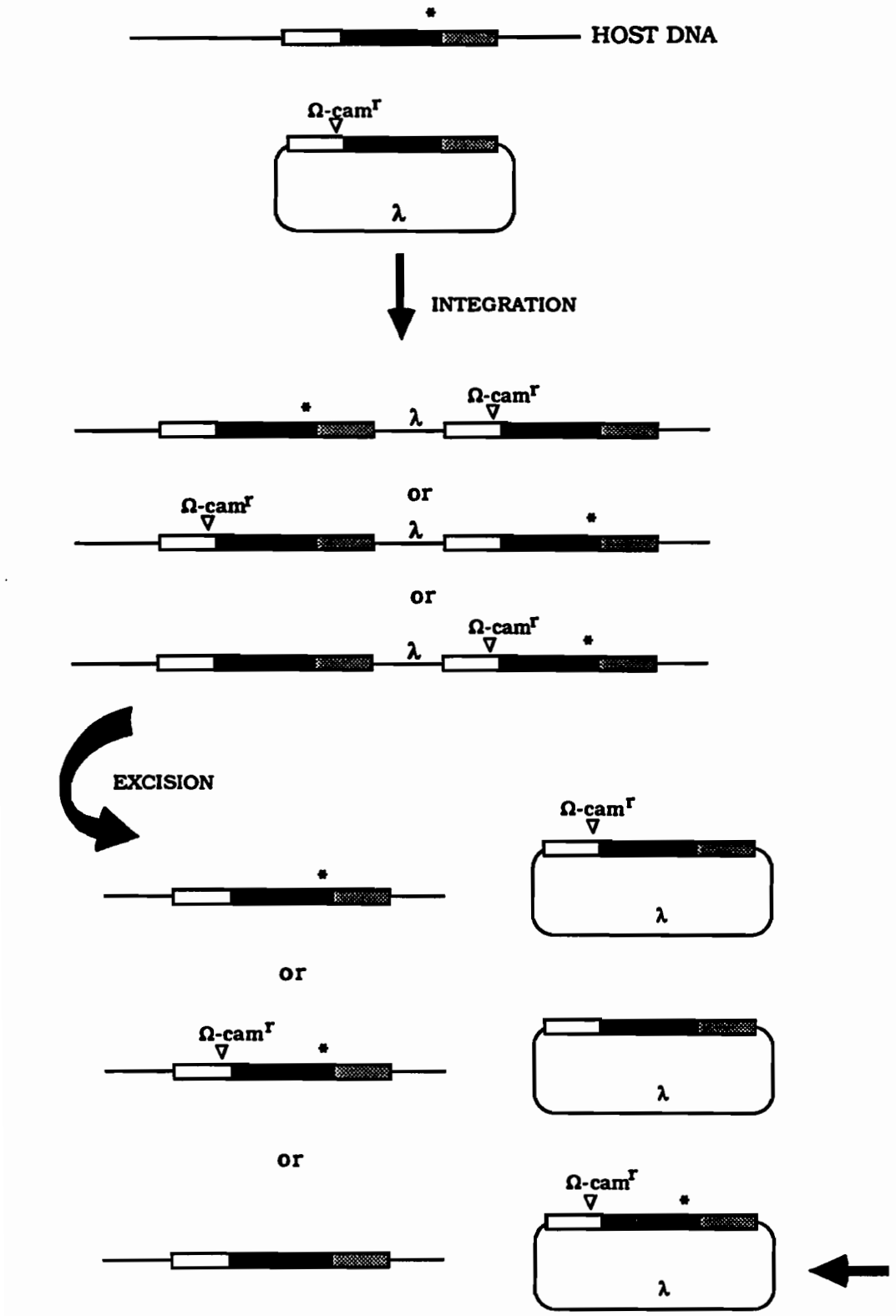
Results

Cloning the *grpE*280 gene

The overall strategy used to clone the *grpE*280 gene required integration of the λ*grpE*$^+$::Ω-cam^r transducing phage at the *grpE*280 locus in *E. coli*. Excision of the prophage was expected to yield several classes of transducing phage, depending on the exact point of the crossover event (Fig. 4.1). Following excision, the recombinant phages were screened and those which were phenotypically GrpE$^-$ Cam^r were purified and the putative *grpE*280 gene was subcloned into an appropriate vector, using Cam^r as a positive selection. Candidates were screened for overproduction of grpE protein prior to further testing.

Strain DA16 (Table 4.1) was infected with λ*grpE*$^+$::Ω-cam^r (3; Table 4.1). Chloramphenicol-resistant lysogens were selected at 30°C, with the majority of

Figure 4.1. Rescue of the *grpE*280 mutation onto a λ *grpE*$^{+}$::Ω-cam^{r} transducing phage by homologous recombination. Depending on the point of crossover relative to the cam^{r} cassette and the *grpE*280 mutation (whose position in the gene is unknown), several integration and excision products are possible, some of which are shown here. Thin lines represent outlying host or bacteriophage sequences. The filled bar represents the *grpE* gene. The open and stippled bars represent nearby flanking sequences. The inverted triangle shows the position of the Ω-cam^{r} cassette which was inserted at the Eco RV site downstream of the *grpE* gene (3). The asterisk represents a hypothetical location of the *grpE*280 mutation. The bold arrow at the bottom of the figure points to the schematic of the transducing phage used to clone the *grpE*280 mutation (see text).


HOST DNA
Ω-cam^r
λ
INTEGRATION
Ω-cam^r
λ
or
Ω-cam^r
λ
or
Ω-cam^r
λ
EXCISION
Ω-cam^r
λ
or
Ω-cam^r
λ
or
Ω-cam^r
λ

integration events expected to occur by homologous recombination at the *grpE*280 locus on the chromosome, due to the *att*⁻ *int*⁻ nature of the transducing phage. The presence of duplicated sequences on the chromosome favors excision of the prophage, again by homologous recombination. At a measurable frequency, crossover events occur in which both the *grpE*280 allele and the Ω-cam^r cassette are found on the same excised phage progeny (Fig. 4.1). These phage were identified by their inability to propagate on *E. coli grpE*280 and by their ability to confer Cam^r to a wild type host upon lysogenization at 30°C. DNA purified from these phage was partially digested with Sau 3A restriction enzyme such that the bulk of the fragments was 6-8 kb in length. The mixture of fragments was ligated to the vector, pBR322 (7), linearized with Bam HI. *E. coli grpE*280 was used as the recipient of this ligation mixture for two reasons: 1) homologous recombination between *grpE*280 candidate plasmids and the chromosome would not result in conversion of the *grpE*280 allele to *grpE*$^+$ on the plasmid, and 2) it might be possible to screen for *grpE*280 plasmids by their inability to complement *E. coli grpE*280 mutant phenotypes (i. e., the cells remain Tr$^-$ at 43°C and do not allow λ growth).

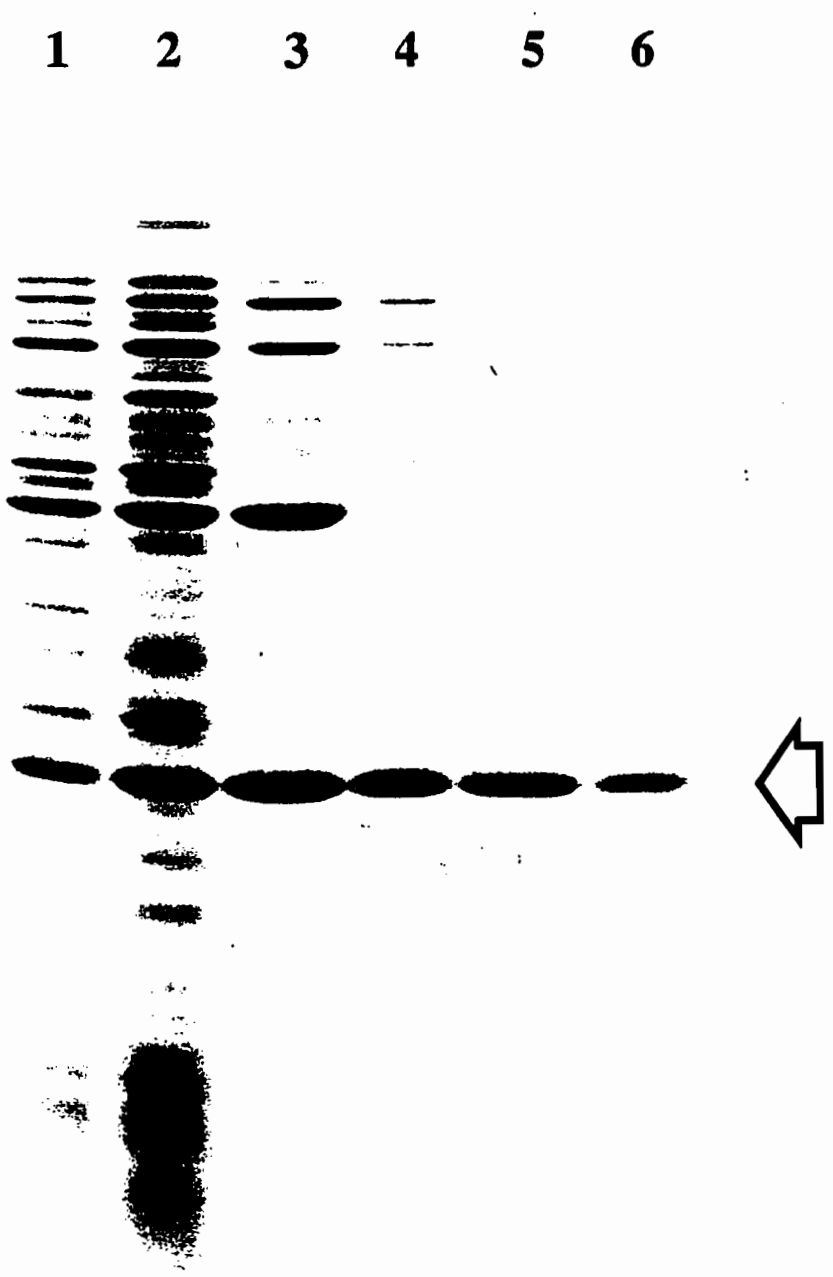
Transformants were selected for Cam^r at 30°C and then screened for overproduction of grpE protein by SDS-polyacrylamide gel electrophoresis of cell lysates. Candidates overproducing grpE protein were subsequently shown to allow some λ growth and to be Tr$^+$ for growth at 43°C. These results suggested that either wild type grpE was being expressed or that overproduction of the mutant grpE280 protein confers a wild type phenotype to *E. coli grpE*280. To test these two possibilities, both genetic and biochemical tests were done. Candidates were infected with λ*grpE*$^+$ at 37°C to rescue the putative cloned *grpE*280 gene onto the transducing phage. Bacteriophage which confer Cam^r were selected on B178 (λ *imm*$^\lambda$) lysogens. Approximately 50% of these λ Cam^r recombinant phage were subsequently shown as

unable to propagate on *E. coli grpE*280 bacteria. The frequency of loss of the GrpE$^+$ phenotype is so great that it is unlikely that another mutation conferring the GrpE$^-$ phenotype was simultaneously selected. A cell extract of one of the grpE280-overproducing candidates was prepared (30) and dilutions, which still contain significant amounts of grpE protein, were used to complement a Fraction II extract prepared from *E. coli grpE*280 cells in the crude *in vitro* λdv DNA replication system (30). An extract from a strain overproducing wild type grpE protein stimulated λ-specific replication in this system. The extract derived from the putative grpE280-overproducing candidate, DA16 (pDA8), stimulated only slightly (data not shown). The amount of grpE protein was comparable in crude extracts analysed on SDS-polyacrylamide gels (the two genes were cloned into the same parent vector and transcribed from their own promoters; however, potential autoregulatory effects have not been studied as yet). The kinetics of the replication reaction were also examined. Addition of grpE$^+$ extract resulted in a sigmoidal curve as normally seen (30). Addition of the grpE280 extract resulted in a lower rate of nucleotide incorporation (data not shown). This strain was used for the purification of the grpE280 protein.

Purification of the grpE protein

The detailed purification procedure is described under **Materials and Methods.** Fig. 4.2 is a photograph of a gel showing fractions from successive steps in the purification scheme. Unlike wild type grpE protein (30), grpE280 binds poorly to a dnaK-affinity column (data not shown). In preliminary trials, it was found that grpE280 binds well to hydrophobic matrices (data not shown). However, in the final scheme, the observation that grpE280 does not bind to a phenyl-Sepharose column in 1 M KCl (although it does bind in 3 M KCl) and that a prominent contaminant does, established the major purification step. The purity of grpE280 at the final step was

Figure 4.2. Successive steps in the purification of the mutant *grpE*280 protein. Lane 1, crude lysate; lane 2, pellet following ammonium sulfate precipitation; lane 3, following DE52 column; lane 4, following phenyl-Sepharose column; lane 5, following Q-Sepharose column; lane 6, purified wild type grpE protein, 1 μg (30). The gel (12.5% polyacrylamide/SDS) was stained with Coomassie Brilliant Blue.


1
2
3
4
5
6

approximately 90% (Table 4.2). As seen in Fig. 4.2 (lanes 5 and 6), the mobility of the mutant protein under denaturing conditions is identical to that of the wild type protein. The isoelectric points (pI) of the mutant and wild type proteins are also the same (data not shown). In addition, rabbit polyclonal anti-grpE antibody binds to the purified mutant protein under immunoblotting conditions. Thus, superficially, the mutant and wild type grpE proteins are alike.

Interaction of grpE280 with wild type dnaK

The wild type grpE protein has been shown to interact with the dnaK protein, both *in vivo* and *in vitro* (1, 2, 15). Wild type grpE protein, under conditions described previously (30), sediments as an approximately 24,000-M_r monomer in a 20-40% glycerol gradient, but sediments at an apparently higher molecular weight in the presence of wild type dnaK protein (30; Fig. 4.3A). To determine whether grpE280 protein also forms a complex with wild type dnaK, these two purified proteins were sedimented together in a 20-40% glycerol gradient. Under these conditions, grpE280 does not form a complex with dnaK (Fig. 4.3B). This is in agreement with immunoprecipitation experiments in which crude extracts are treated with anti-dnaK antibody. In the case of an extract from wild type cells, dnaK and grpE are co-precipitated by anti-dnaK antibody. Treatment of a crude extract derived from an *E. coli grpE*280 strain precipitates a comparable amount of dnaK protein but much less grpE protein (1, 15). In addition, grpE280 protein binds weakly to a dnaK-affinity column (data not shown) compared to wild type grpE protein (30).

The above observations suggest that grpE280 does not form an isolatable complex with wild type dnaK protein. However, in crosslinking experiments, complex formation between grpE280 and dnaK appears to be more efficient, even in the presence of high salt, than between the two wild type proteins (Fig. 4.4). The

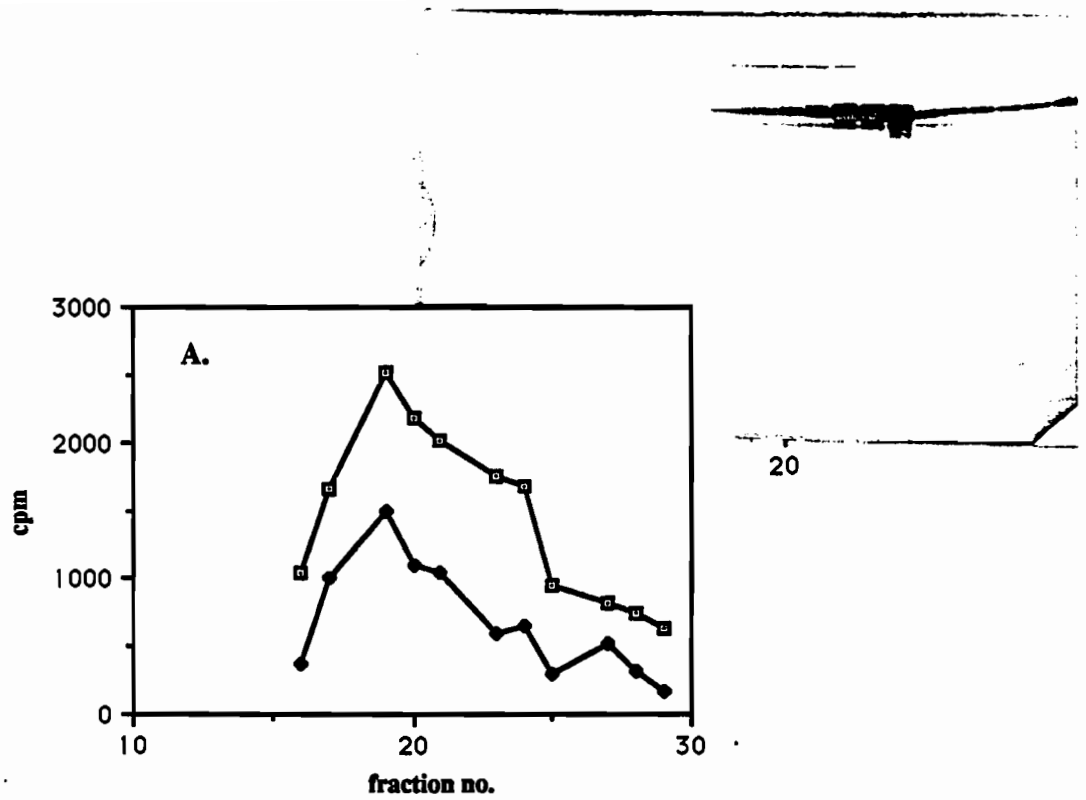
Table 4.2

Purification of grpE280 protein

Step of purification	grpE280*	total protein*	%(grpE280/total)
crude lysate	.0186	.2206	8.4
ammonium sulfate	.0275	.3291	8.4
DE52	.0228	.0905	25.2
phenyl-Sepharose	.0201	.0267	75.3
Q-Sepharose	.0145	.0163	89.0

* The gel shown in Fig. 3 was scanned with a Joyce-Loebl densitometer. The traces of the peaks were cut out from paper and weighed on a Mettler AC100 balance. The values shown in these two columns are the weights of the paper in grams.

Figure 4.3. Sedimentation of wild type grpE and mutant grpE280 proteins in the presence of dnaK protein. Purified wild type grpE and mutant grpE280 proteins (1 μg per gradient) were sedimented in 20-40% glycerol gradients in the presence of purified wild type dnaK protein (2 μg per gradient). Two-drop fractions (approx. 80 μl) were collected and electrophoresed on 12.5% polyacrylamide/SDS gels. Both anti-grpE and anti-dnaK antibodies were used for immunoblot analysis. Bands were cut from the filters and counted in Beckman ReadySafe scintillation fluid in a Beckman LS 333 scintillation counter. Corresponding silver stained gels are shown above each graph. A) wild type dnaK and grpE; B) wild type dnaK and mutant grpE280. (⧈), dnaK; (◆), grpE or grpE280.


A.
3000
2000
1000
0
cpm
10
20
30
fraction no.
20

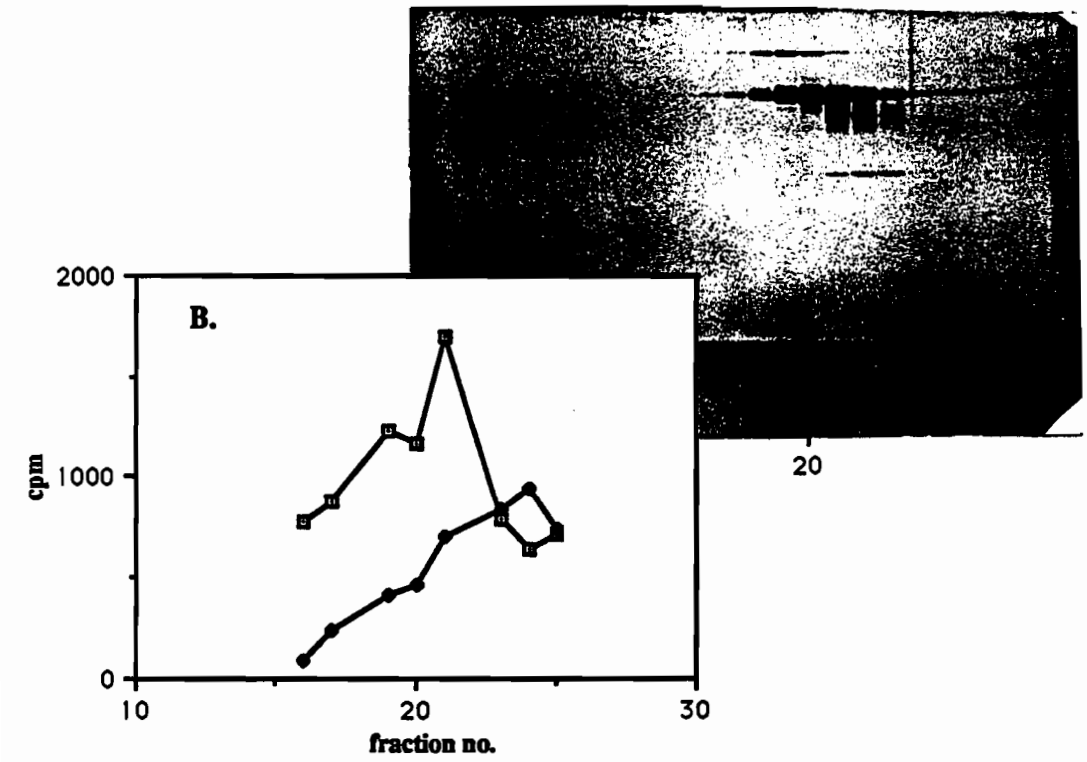
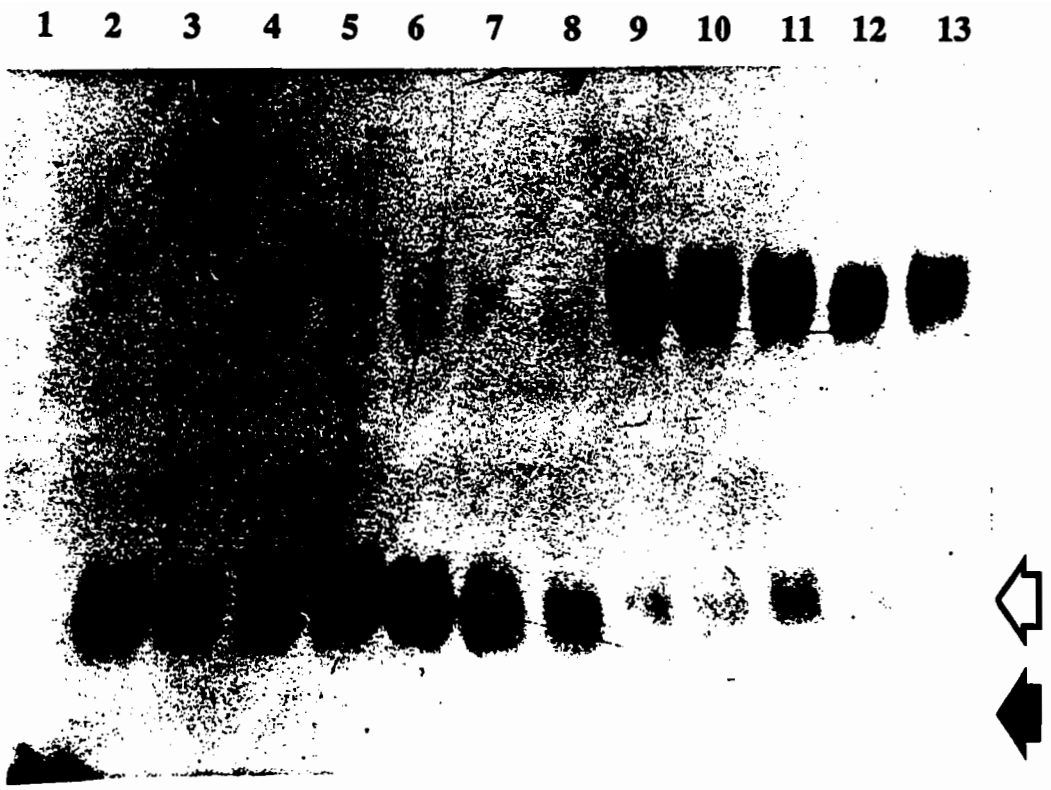

B.
2000
1000
0
cpm
10
20
30
fraction no.
20

Figure 4.4. Crosslinking of purified grpE and dnaK proteins in solutions of increasing ionic strength. Wild type grpE or mutant grpE280 protein, 0.5 μg/reaction; wild type dnaK protein, 1 μg/reaction. After transfer to nitrocellulose, the filter was treated with anti-grpE antibody. Lane 1, dnaK; lane 2, wild type grpE; lane 3, grpE280; lanes 4-8, wild type grpE and dnaK in 0.012 M KCl, 0.05 M KCl, 0.1 M KCl, 0.25 M KCl, 0.5 M KCl, respectively; lanes 9-13, grpE280 and dnaK; same as lanes 4-8. Filled arrow indicates position of grpE monomer (not seen in this experiment) and open arrow indicates position of grpE dimer.


1 2 3 4 5 6 7 8 9 10 11 12 13

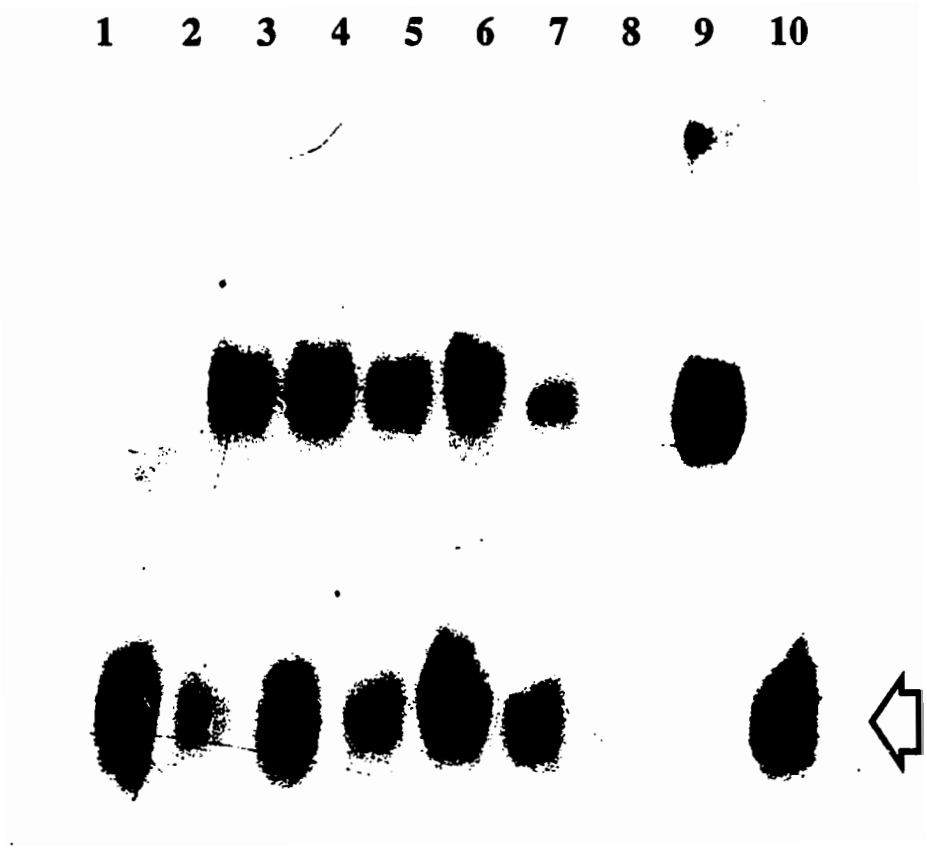
mobility of the dnaK-grpE280 complex is identical to that of the wild type proteins, suggesting that the ratio of dnaK to grpE280 is the same as in the wild type complex and that some specificity in the interaction may still be preserved despite the mutation in *grpE*280. This is supported somewhat by the fact that a complex is not seen between grpE280 and bovine serum albumin, i. e., the *grpE*280 mutation does not confer a nonspecific "stickiness" to the protein.

To determine whether the dnaK-grpE280 complex is as stable as the wild type dnaK-grpE complex under our crosslinking conditions, the proteins were allowed to form complexes in the usual reaction volume (25-µl) and then diluted, the rationale being that if the complexes dissociate, the rate of re-association would be negligible in relation to the time of crosslinking. After an incubation period of ten minutes, glutaraldehyde was added to crosslink any remaining complexes. As shown in Fig. 4.5 (lanes 2-6), a wild type dnaK-grpE complex is seen at all dilutions, suggesting that the complex is stable, even following a 50-fold dilution. Substantial amounts of the dnaK-grpE280 complex, however, are seen only in the undiluted reaction. Upon 50-fold dilution, very little high molecular weight complex is seen.

Inability of purified grpE280 to complement *in vitro* λdv DNA replication

As shown in Fig. 4.6, addition of purified grpE280 protein in quantities known to be optimal for wild type grpE does not stimulate λdv DNA synthesis in the crude *in vitro* system using a FII derived from *E. coli grpE*280 cells. This is in agreement with the *in vivo* observation that wild type λ does not grow on *E. coli grpE*280 cells but λ*grpE*$^+$ does (2, 22, 24). In the steps leading to initiation of λ DNA replication, grpE is thought to act prior to synthesis of primers by dnaG primase, but after assembly of the λO-λP-dnaB complex at *ori*λ (31). It has been shown that for both *ori*C-specific

Figure 4.5. Stability of dnaK-grpE complex following dilution. Purified wild type dnaK protein (1 μg) was preincubated for 10 min at 30°C with either wild type grpE (0.5 μg) or mutant grpE280 (0.5 μg) protein in a 25-μl reaction volume to allow complex formation. Prior to addition of glutaraldehyde, the reactions were diluted and incubated for another 10 min at 30°C. After crosslinking, buffer was added as necessary to bring all reactions to a uniform volume. Trichloroacetic acid (100%) was added to a final concentration of 20%. The precipitated proteins were analysed as described in Materials and Methods, using anti-grpE antibody to visualize complexes containing grpE protein. Lane 1, dnaK; lane 2, wild type grpE. DnaK and wild type grpE: lane 3, no dilution prior to crosslinking; lane 4, 5-fold dilution; lane 5, 10-fold dilution; lane 6, 25-fold dilution; lane 7, 50-fold dilution. Lane 8, diluted 50-fold prior to *preincubation*. DnaK and mutant grpE280: lane 9, no dilution; lane 10, 50-fold dilution. Open arrow indicates position of grpE dimer.


1 2 3 4 5 6 7 8 9 10

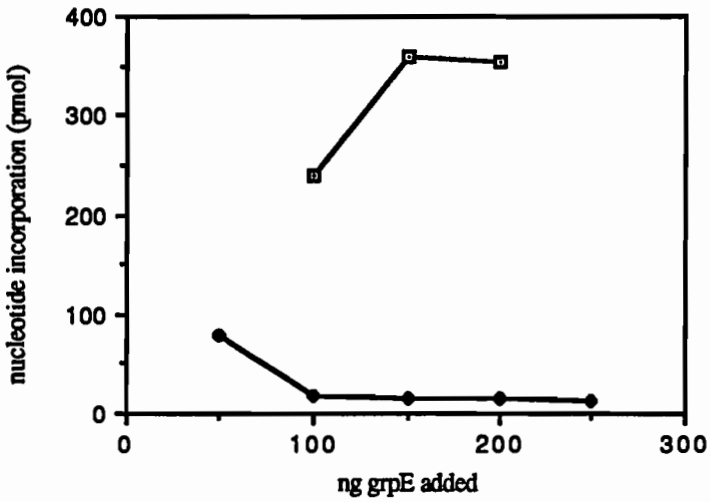


Figure 4.6. Nucleotide incorporation in crude *in vitro* λdv DNA replication system. Purified grpE proteins were used to complement an *E. coli grpE*280 FII. (⊡), wild type grpE; (◆), grpE280.

and *ori*λ-specific replication, extensive unwinding of the duplex DNA flanking the origin precedes initiation (5, 31). In the case of *ori*λ-specific replication, it has been shown that addition of grpE to the purified *in vitro* replication system allows the concentration of dnaK required for either replication or unwinding of the template to be reduced by at least ten-fold (31) Addition of grpE280, which does not stimulate replication of λdv, is not expected to allow unwinding of the template, thus inhibiting λ replication. Fig. 4.7 shows that this is the case.

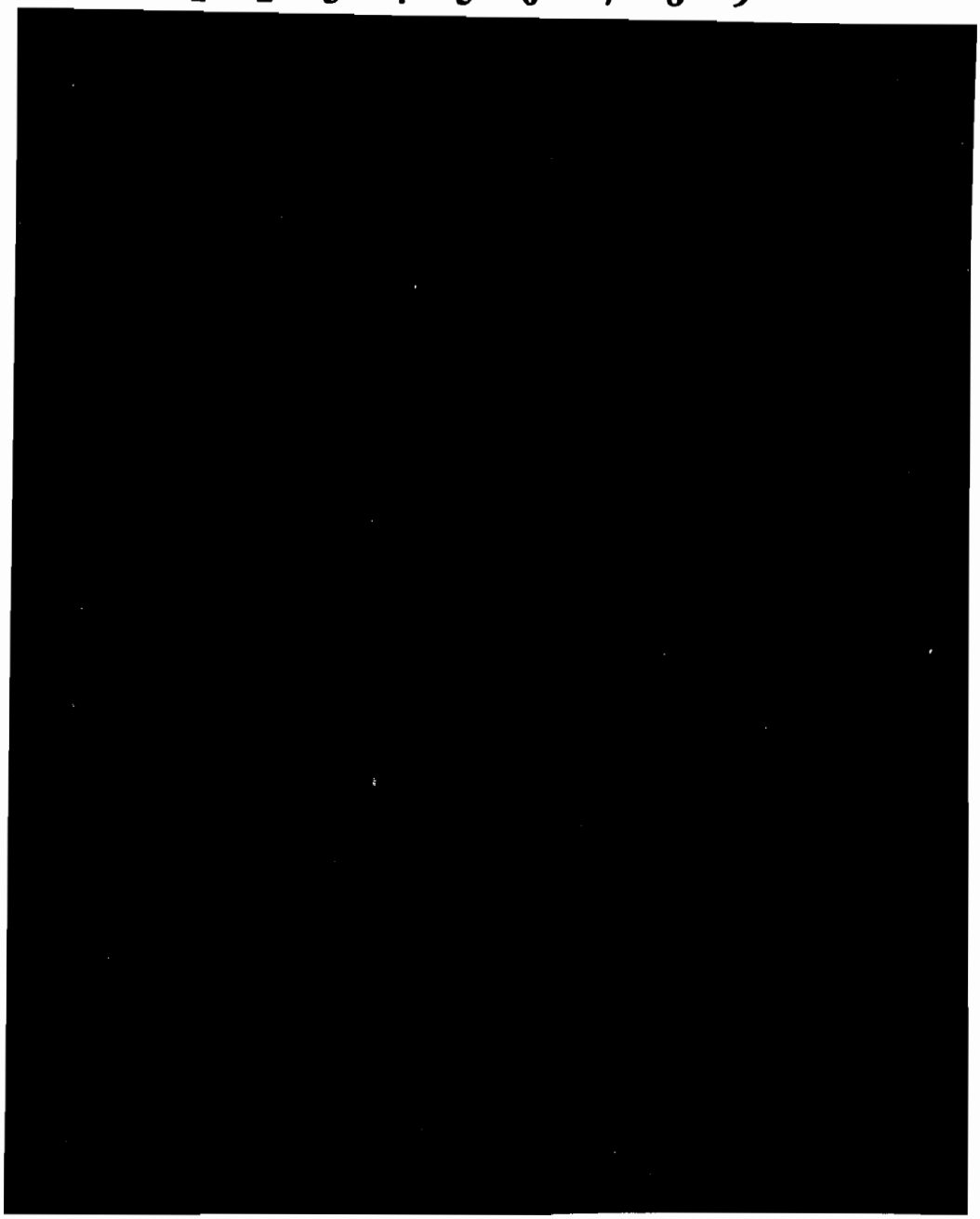
Discussion

The *E. coli grpE*280 mutant was originally isolated by its inability to allow bacteriophage λ DNA replication at 42°C (22). Further testing revealed that host growth is affected in this mutant at temperatures above 43°C (2). Most recently, it has been shown that total absence of *grpE* gene product results in inviability at all temperatures, although extragenic suppressors can be isolated which allow deletion of the *grpE* gene (3).

The mutant *grpE*280 gene was cloned and overexpressed in *E. coli grpE*280 cells. These cells exhibit a partial mutant phenotype; that is, they form small colonies at 43°C and allow some growth of λ. The reversal in phenotype may be due to a greater frequency of interaction of the mutant grpE280 protein with wild type dnaK protein (or other host components). The nature of the interaction between grpE280 and dnaK must be different from the wild type complex since a crosslinked complex between grpE280 and dnaK can be isolated even in the presence of 0.5 M KCl, conditions which do not allow crosslinking of a wild type grpE-dnaK complex. It is a more transient interaction relative to the wild type complex since, upon dilution, only a minor amount of complex is found after crosslinking. The fact that a grpE280-dnaK

Figure 4.7. Unwinding of λdv plasmid in the purified *in vitro* replication system. Varying amounts of wild type grpE or mutant grpE280 protein were added to the reaction mixture described in (31). DnaK protein (0.2 μg/reaction) is present as indicated. Unwound DNA is seen as the high mobility species in lanes 3-5. Lane 1, dnaK only; lane 2, wild type grpE only (0.2 μg). DnaK, wild type grpE: lane 3, 25 ng; lane 4, 0.1 μg; lane 5, 0.2 μg. Lane 6, grpE280 only (0.2 μg). DnaK, grpE280: lane 7, 25 ng; lane 8, 0.1 μg; lane 9, 0.2 μg.

1 2 3 4 5 6 7 8 9



complex is not stable in glycerol gradients, as opposed to the wild type complex, also suggests that the nature of the interaction has changed.

The ability to form a complex in the presence of high salt concentrations but not in the presence of glycerol suggests that the *grpE*280 mutation has altered grpE280 towards a more hydrophobic interaction with dnaK. It is not clear why crosslinking between grpE280 and dnaK is more efficient than between the wild type proteins. Although the grpE280-dnaK complex is more transient, it is apparently of sufficient stability to allow crosslinking within the time course of the reaction. A trivial explanation is that the *grpE*280 mutation may have resulted in substitution of an amino acid more reactive with glutaraldehyde. Alternatively, the mutation may have changed the conformation of the protein such that interacting amino acids are more advantageously juxtaposed for crosslinking by glutaraldehyde.

There is abundant evidence that the interaction between grpE and dnaK in *E. coli* is biologically significant. Immunoprecipitation of dnaK protein by anti-dnaK antibody from crude cell extracts derived from a wild type strain results in co-precipitation of an approximately 24,000-M_r protein subsequently identified as grpE. Immunoprecipitation of dnaK from crude extracts of *E. coli grpE*280 cells, however, does not result in co-precipitation of the grpE280 mutant polypeptide (1). In addition, an extragenic suppressor of *grpE*280 which was shown to map in the *dnaK* gene restores complex formation as shown by immunoprecipitation (15). The facts that a) in the absence of extragenic suppressors, the *grpE* gene product is absolutely required by *E. coli* at all temperatures and *E. coli grpE*280 cells are phenotypically wild type for growth at 30°C (2, 3), b) overproduction of the *grpE*280 gene product partially reverses the mutant phenotypes, and c) a grpE280-dnaK complex can be isolated by crosslinking, suggest that residual interaction between grpE280 and dnaK is responsible for the conditional growth phenotype of the mutant. The fact that λ

growth is inhibited over a wide temperature range in the *grpE*280 host (2, 22) suggests that the requirement for dnaK-grpE interaction in λ DNA replication is more stringent with respect to either conformation or stability of the complex. Another possibility is that grpE interacts with a protein other than dnaK in the prepriming complex shown to initiate λ DNA replication (31) and that the *grpE*280 mutation affects this interaction. Extragenic suppressors of *grpE*280 which restore λ's ability to propagate on *E. coli grpE*280 have been shown to map in the λ*P* gene (π or *reg* mutations; 10, 22), suggesting that grpE interacts with λP. Preliminary evidence shows that a wild type grpE-λP complex can be identified following glutaraldehyde crosslinking (M. Zylicz, D. A. and C. Georgopoulos, unpublished data). Disruption of this complex in *E. coli grpE*280 may account for the more stringent inhibition of λ growth.

Literature Cited

1. **Ang, D., G. N. Chandrasekhar, C. Johnson, M. Zylicz and C. Georgopoulos.** 1987. A genetic and biochemical analysis of the *Escherichia coli grpE* gene and its product. *In* R. McMacken and T. Kelly (eds.), UCLA Symp. Mol. Cell. Biol. New Series, **47**:521-532, Alan R. Liss, Inc., New York.

2. **Ang, D., G. N. Chandrasekhar, M. Zylicz and C. Georgopoulos.** 1986. *Escherichia coli grpE* gene codes for heat shock protein B25.3, essential for both λ DNA replication at all temperatures and host growth at high temperature. J. Bacteriol. **167**:25-29.

3. **Ang, D. and C. Georgopoulos.** The heat shock-regulated *grpE* gene of *Escherichia coli* is required for bacterial growth at all temperatures, but is dispensable in certain mutant backgrounds. Submitted to J. Bacteriol.

4. **Bahl, H., H. Echols, D. B. Straus, D. Court, R. Crowl and C. P. Georgopoulos.** 1987. Induction of the heat shock response of *E. coli* through stabilization of σ^{32} by the phage λcIII protein. Genes Dev. **1**:57-64.

5. **Baker, T. A., K. Sekimizu, B. W. Funnell and A. Kornberg.** 1986. Extensive unwinding of the plasmid template during staged enzymatic initiation of DNA replication from the origin of the *Escherichia coli* chromosome. Cell **45**:53-64.

6. **Bardwell, J. C. A. and E. A. Craig.** 1984. Major heat shock gene of Drosophila and the *Escherichia coli* heat-inducible *dnaK* gene are homologous. Proc. Natl. Acad. Sci. U. S. A. **81**:848-852.

7. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95-104.

8. **Fuller, R. S., J. M. Kaguni and A. Kornberg.** 1981. Enzymatic replication of the origin of the *Escherichia coli* chromosome. Proc. Natl. Acad. Scil. U. S. A. **78**:7370-7374.

9. **Furth, M. E. and S. H. Wickner.** 1983. Lambda DNA replication. *In* R. W. Hendrix, J. W. Roberts, F. W. Stahl and R. A. Weisberg (eds.), Lambda II, pp. 145-173, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

10. **Georgopoulos, C. P. and I. Herskowitz.** 1971. *Escherichia coli* mutants blocked in lambda DNA synthesis. *In* A. D. Hershey (ed.), The bacteriophage lambda, pp. 553-564, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

11. **Georgopoulos, C. P., K. Tilly, D. Drahos and R. Hendrix.** 1982. The B66.0 protein of *Escherichia coli* is the product of the *dnaK* gene. J. Bacteriol. **149**:1175-1177.

12. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557-580.

13. **Hohn, T., B. Hohn, A. Engel, M. Wurtz and P. R. Smith.** 1979. Isolation and characterization of the host protein GroE involved in bacteriophage lambda assembly. J. Mol. Biol. **129**:359-373.

14. **Itikawa, H. and J.-I. Ryu.** 1979. Isolation and characterization of a temperature-sensitive *dnaK* mutant of *Escherichia coli* B. J. Bacteriol. **138**:339-344.

15. **Johnson, C., G. N. Chandrasekhar and C. Georgopoulos.** The dnaK and grpE heat shock proteins in *Escherichia coli* interact both *in vivo* and *in vitro*. Submitted to J. Bacteriol.

16. **LeBowitz, J. H., M. Zylicz, C. Georgopoulos and R. McMacken.** 1985. Initiation of DNA replication on single-stranded DNA templates catalyzed by purified replication proteins of bacteriophage λ and *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **82**:3988-3992.

17. **Lipinska, B., J. King, D. Ang and C. Georgopoulos.** 1988. Sequence analysis and transcriptional regulation of the *Escherichia coli grpE*gene, encoding a heat shock protein. Nucleic Acids Res. **16**:7545-7562.

18. **Maniatis, T., E. F. Fritsch and J. Sambrook.** 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

19. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

20. **Neidhardt, F. C., R. A. VanBogelen and V. Vaughn.** 1984. The genetics and regulation of heat shock proteins. Ann. Rev. Genet. **18**:295-329.

21. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007-4021.

22. **Saito, H. and H. Uchida.** 1977. Initiation of the DNA replication of bacteriophage lambda in *Escherichia coli* K12. J. Mol. Biol. **113**:1-25.

23. **Saito, H. and H. Uchida.** 1978. Organization and expression of the *dnaJ* and *dnaK* genes of *Escherichia coli* K12. Mol. Gen. Genet. **164**:1-8.

24. **Saito, H., Y. Nakamura and H. Uchida.** 1978. A transducing λ phage carrying *grpE*, a bacterial gene necessary for λ DNA replication, and two ribosomal protein genes, *rpsP* (S16) and *rplS* (L19). Mol. Gen. Genet. **165**:247-256.

25. **Schlossman, D. M., S. L. Schmid, W. A. Braell and J. E. Rothman.** 1984. An enzyme that removes clathrin coats: Purification of an uncoating ATPase. J. Cell Biol. **99**:723-733.

26. **Sunshine, M., M. Feiss, J. Stuart and J. Yochem.** 1977. A new host gene (*groPC*) necessary for lambda DNA replication. Mol. Gen. Genet. **151**:27-34.

27. **Wada, M., Y. Kadokami and H. Itikawa.** 1982. Thermosensitive synthesis of DNA and RNA in *dnaJ* mutants of *Escherichia coli* K-12. Jpn. J. Genet. **57**:407-413.

28. **Waghorne, C. and C. R. Fuerst.** 1985. Involvement of the *htpR* gene product of *Escherichia coli* in phage lambda development. Virology **141**:51-64.

29. **Weber, K. and M. Osborn.** 1979. The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406-4412.

30. **Zylicz, M., D. Ang and C. Georgopoulos.** 1987. The grpE protein of *Escherichia coli*: purification and properties. J. Biol. Chem. **262**:17437-17442.

31. **Zylicz, M., D. Ang, K. Liberek and C. Georgopoulos.** Initiation of λ DNA replication with purified host- and bacteriophage-encoded proteins: The role of the dnaK, dnaJ and grpE heat shock proteins. Submitted to EMBO J.

32. **Zylicz, M. and C. Georgopoulos.** 1983. Properties of the purified *dnaK* gene product of *Escherichia coli*. *In* N. R. Cozzarelli (ed.), Mechanisms of DNA replication and recombination, pp. 317-326, Alan R. Liss, Inc., New York, N. Y.

33. **Zylicz, M. and C. Georgopoulos.** 1984. Purification and properties of the *Escherichia coli* dnaK replication protein. J. Biol. Chem **259**:8820-8825.

34. **Zylicz, M., J. H. LeBowitz, R. McMacken and C. Georgopoulos.** 1983. The dnaK protein of *Escherichia coli* possesses an ATPase and autophosphorylating activity and is essential in an *in vitro* DNA replication system. Proc. Natl. Acad. Sci. U. S. A. **80**:6431-6435.

35. **Zylicz, M., T. Yamamoto, N. McKittrick, S. Sell and C. Georgopoulos.** 1985. Purification and properties of the DnaJ replication protein of *Escherichia coli*. J. Biol. Chem. **260**:7591-7598.

CHAPTER 5

EXTRAGENIC SUPPRESSORS OF THE *grpE*280 MUTATION

Abstract

Although the role of grpE in initiation of λ DNA replication has been well studied, little is known of its role in *Escherichia coli* growth. To identify *E. coli* functions which grpE may interact with, Tr^+ Cs^- revertants of *E. coli grpE*280 were identified and shown to form several complementation groups. Putative clones of the suppressor genes (called "*sog*" for suppressor of *grpE*) were isolated from mini-Mu and λ cosmid *E. coli* DNA libraries. In three cases, the locations of the suppressor genes on the *E. coli* genetic map were found using the Kohara *E. coli* DNA library. One was shown to map near but not in the *dnaKJ* operon. The other two were shown to map at 93.4 and 38 min. Thus far, the suppressor genes have not been identified.

Introduction

Regulation of gene expression and function in *E. coli* often occurs through the interactions of proteins -- with each other, with nucleic acids, with cofactors, or with a combination. In the case of the *E. coli grpE* gene product, which was originally identified as one of a group of host functions necessary for initiation of λ DNA replication (27), it has been shown to interact with another member of this group, dnaK. There is abundant biochemical evidence demonstrating that these two proteins physically interact and that this interaction is functional (1, 12, 30, 31). There is also genetic evidence which shows that extragenic suppressors of the *grpE*280 mutation can be selected which reverse the temperature-sensitive phenotype of *grpE*280 and which map in the *dnaK* gene (12). Recently, it has been shown that an *E. coli* *dnaK*103 strain, which synthesizes an unstable, truncated dnaK polypeptide, is viable only in the presence of extragenic suppressor(s) (C. Georgopoulos, unpublished data). These extragenic suppressors allow deletion of *grpE* (3), again suggesting a functional interaction between dnaK and grpE.

Besides initiation of λ DNA replication, grpE has been shown to be essential for *E. coli* growth at all temperatures (2, 3). GrpE affects the rates of RNA and DNA syntheses, and its own synthesis is induced during the heat shock response of *E. coli* (2, 19, 22). It may well be involved in other cellular functions not yet elucidated. Therefore, to better understand the mechanism of grpE action and to identify other *E. coli* functions which interact with grpE, suppressors of the *E. coli grpE*280 mutation were isolated by selecting for temperature-resistant (Tr^+) revertants. These Tr^+ colonies were subsequently screened to identify those which do not map in *grpE*. Several classes of extragenic suppressors may be obtained: a) those altering a protein which normally interacts with wild type grpE, allowing it to interact with the grpE280 mutant protein (some of these are expected to be in *dnaK*), (b) those altering a protein such that its need for interaction with grpE is bypassed, c) those altering a protein that normally does or does not interact with the dnaK - grpE complex such that it can now stabilize the dnaK - grpE280 complex, and d) mutations which suppress in an indirect, nonspecific manner. The positions of three of the suppressors (called "*sog*" for suppressor of *grpE*) have been mapped although the identities of the genes in which they are found have not been determined yet.

Materials and Methods

Bacteria, phage and plasmids

Strains and their relevant genotype / phenotype are listed in Table 5.1.

Media and antibiotics

L-broth and minimal media were prepared as described (2). L-plates and minimal plates are the same as the respective liquid media except for the addition of 1% agar. Where appropriate, tetracycline (tet), ampicillin (amp), chloramphenicol (cam) and/or

Table 5.1

Bacteria, phage and plasmid strains

Strain	Genotype / phenotype	References
DA16	B178 *grpE*280 *pheA*::Tn*10*	(2)
DA127	B178 *grpE*280 *ze?*::Ω - cam^r	(Ch. 4)
B178	W3101 *galE*$^-$	(4)
Phage		
mini-Mu	MudII4042	(10)
λ*c*I$^-$	λ*c*I60	our collection
λ*grpE*$^+$	λ*imm*^{λ} *int- xis*$^-$ *attλ*$^-$ *c*I857 *grpE*22	H. Uchida (28)
λ*grpE*280	λ*imm*^{λ} *int- xis*$^-$ *attλ*$^-$ *c*I857 *grpE*280 *ze?*::Ω - cam^r	(Ch. 4)
Plasmid		
λ cosmid	pREG152 amp^r	(13)

kanamycin (kan) were added to media at a final concentration of 15 μg/ml, 50 μg/ml, 20 μg/ml and 50 μg/ml, respectively.

Selection for Tr$^+$ revertants of *E. coli grpE280*

DA16 and DA127 were grown overnight at 30°C in the presence of the appropriate antibiotic. Approximately 3 x 10^6 cells from each saturated culture were spread on an L-plate with antibiotic and incubated at 43°C. Temperature-resistant survivors were purified through single colony isolation and tested again for their ability to grow at various temperatures.

Mini-Mu and λ cosmid libraries

Mini-Mu and λ cosmid libraries were constructed as described by Groisman and Casadaban (10) and Koomey et al. (15), respectively. *E. coli* B178, which is a wild type strain, was used for both libraries.

Transductions

Transductions with P1 were done essentially as described by Miller (21).

Mapping of extragenic suppressors

Using the *E. coli* library established by Kohara et al. (14), the map positions of *sog*4, *sog*11 and *sog*22 were determined as described by Lipinska et al. (18). Essentially, lysates of each λ transducing phage were spotted in an orderly manner on a lawn of *E. coli* B178 in a single 100 mm petri dish. Plaque lifts onto nylon filters (Hybond-N, Amersham) were probed with the appropriate cloned mini-Mu DNA nick-translated in the presence of ^{32}P-dCTP (20). Filters were exposed to Kodak XAR film.

Results and Discussion

Selection for Tr^+ revertants

*E. coli grp*E280 was originally isolated at 42°C as part of a selection for host mutants which do not allow replication of λ (27). However, further analysis has shown that this strain is temperature-sensitive for growth above 43°C (2). Because the grpE280 protein is identical in size and isoelectric point to the wild type protein (Ch. 4), the mutation is most likely a missense mutation. In addition, the grpE280 protein most likely retains some of the wild type protein's activities since a) grpE is essential for *E. coli* growth at all temperatures (2, 3), and b) *E. coli grpE*280 appears phenotypically wild type for growth at 30°C, although it does not allow λ growth (2, 27). To begin isolating extragenic suppressors of *grpE*280, temperature-resistant (Tr^+) revertants of *E. coli grpE*280 cells marked with an antibiotic resistance marker, either Tet^r (DA16) or Cam^r (DA127) near *grpE*280, were selected at 43°C as described in **Materials and Methods** (Fig. 5.1). Survivors were isolated at a frequency of 10^{-5} - 10^{-4}. Among the survivors expected were true revertants, intragenic suppressors which do not correct the *grpE*280 mutation, and extragenic suppressors. Fifty candidates isolated in three separate selections were kept for further screening.

Occasionally, when a suppressor mutation is selected for reversal of a Tr^- phenotype to Tr^+, it simultaneously endows the bacteria with a Cs^- phenotype, i. e., the mutation interferes with the ability of the cell to grow at lower temperatures (Fig. 5.1). This gives the geneticist an advantage because it provides a direct selection for cloning the wild type allele of the suppressor gene (see below). Twenty-three of the fifty candidates displayed this Cs^- phenotype, and some of these were examined further. The majority of Cs^- revertants listed in Table 5.2 do not support λ growth but do support λ*grpE*$^+$ growth. This suggests that these candidates still possess the

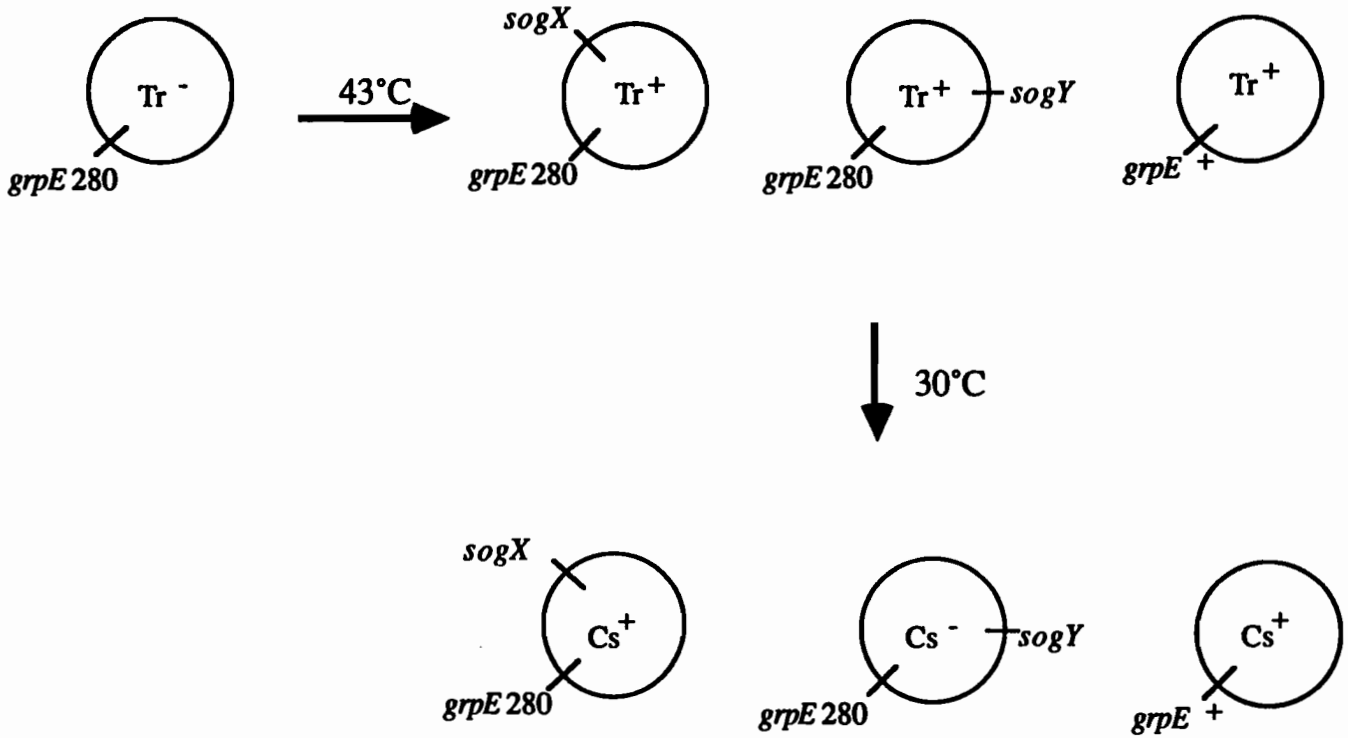


Figure 5.1. Selection for suppressor mutations of *grpE*280. The schematic diagram shows three possible classes of suppressor mutations. Circles represent the *E. coli* chromosome. "Tr⁻" indicates temperature-sensitive phenotype for growth 43°C and above, "Tr⁺" indicates temperature-resistance, "Cs⁻" indicates cold-sensitive phenotype for growth at 30°C, and "Cs⁺ indicates cold-resistance.

Table 5.2

Properties of suppressor alleles in *E. coli grpE*280

Strain[a]	suppressor allele no.	mini-Mu	λ cosmid	map position	accepts grpE$^{\Delta}$?[b]
DA142	*sog*4	**Mu4^{+}**[c]	not found[d]	93.4'	no
DA144, 149, 150	*sog*11, 19, 20	**Mu11^{+}**,19^{+},20^{+},11***e**	pAM49, 50,51[e]	38'	yes
DA147, 165, 186, 188, 191	*sog*16, 31-2, 53-2, 54-2, 56	Mu16^{+}	pCG1530, 1531		no
DA151	*sog*22	not found	pCG1263	0.4'	no
DA163	*sog*30-3	ND[f]	pCG1533		ND
DA184	*sog*52	ND	ND		ND
DA190	*sog*55-2	ND	ND		ND

[a] *E. coli grpE*280 *sog*$^{-}$ are phenotypically Tr^{+} at 43°C and Cs^{-} at 30°C. Those listed together are in the same complementation group, i.e., mini-Mu or λ cosmid plasmids isolated in one strain also suppress the Cs^{-} phenotype in the other strains of the same group.

[b] The *grpE* deletion construct (Ch. 3) was transduced (by P1) into Tet^{r} strains. The presence of grpE protein was ascertained by immunoblotting of lysates of transductants which are both Kan^{r} and Cm^{r} (see text).

[c] Bold type indicates that the mini-Mu isolated has been shown to carry sequences homologous to the region on the chromosome carrying the original *sog* mutation, and that it is not a "pseudo" suppressor (see text).

[d] The strain was infected with the library but no complementing plasmid was isolated.

[e] pAM49, 50, 51 and Mu11* were all shown to be "pseudo" suppressors of *sog*11, 19, and 20. They all carry *E. coli* DNA located at 84' on the genetic map (see text).

[f] Not determined.

*grpE*280 mutation and that the suppressor either does not compensate for an aspect of grpE function involved in λ growth, or that the suppression is insufficient to allow initiation of λ DNA replication. To show that the *grpE*280 mutation is still present and that the suppressor maps away from this region of the chromosome, P1 was grown on 13 of these candidates and used to transduce either the Tet^r or Cam^r marker into a wild type background. Drug-resistant transductants were isolated at 30°C and then checked for Tr^+ at 43°C. In no case was the frequency of cotransduction between the drug-resistance marker and the Tr^- phenotype lower than normally expected. In other words, if a suppressor mutation had been located near the *grpE*280 mutation, a much higher number of Tr^+ transductants would have been observed.

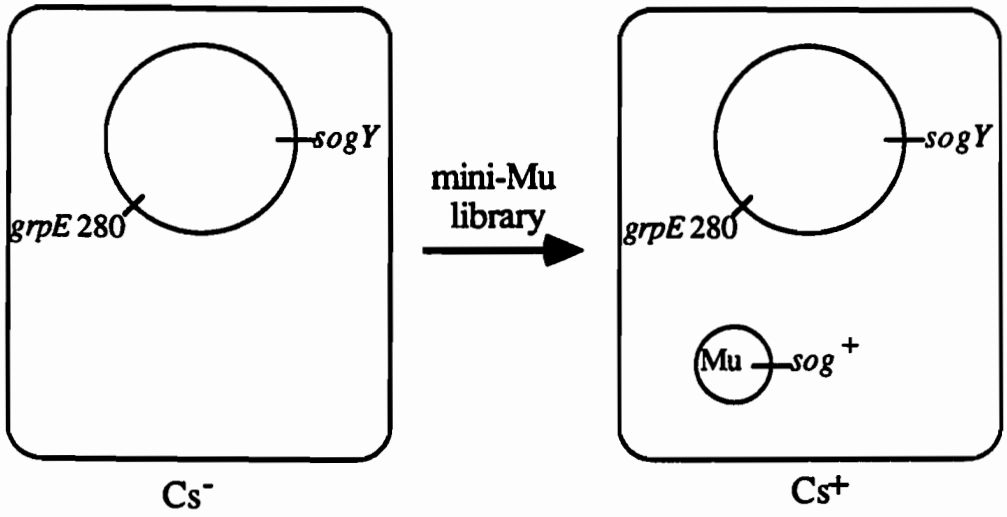
Cloning the wild type version of the suppressor genes

As alluded to above, the Cs^- phenotype of the Tr^+ revertants can be used to select for clones of the wild type version of these extragenic suppressors. As diagrammed in Fig. 5.2, the *E. coli grpE*280 *sog*$^-$ strains DA142, 144, 149 and 150 were infected with a lysate of the Mu-replicon plasmid MudII4042 (10) previously grown on wild type *E. coli*. The DNAs in these plasmids comprise random fragments of wild type *E. coli* DNA (approximately 10 kb) flanked on either side by MudII4042 sequences. If the Mu sequences are in direct orientation with each other, a single homologous recombination event can occur upon infection of a recipient cell which results in an autonomously replicating plasmid (referred to as "mini-Mu") which confers Cam^r and carries random *E. coli* DNA sequences. Thus, by infecting *E. coli grpE*280 *sog*$^-$ with this library of *E. coli* DNA and selecting for Cam^r at 30°C, it is possible to isolate putative clones of the wild type version of the suppressor gene based on the ability of the corresponding mini-Mu plasmid to reverse the Cs^- phenotype. This type of selection assumes that the original *sog*$^-$ mutation is not dominant, or if it is dominant,

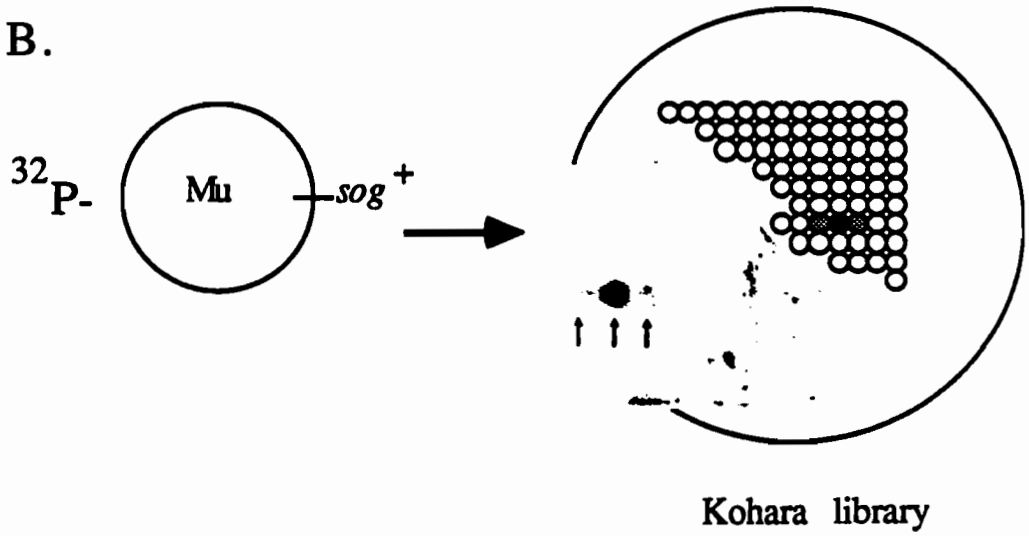
Figure 5.2A. Selection for mini-Mu plasmids which carry putative genes of the suppressor mutations. Tr^+ Cs^- revertants of *E. coli grpE*280 were infected with a mini-Mu library, selecting for Cs^+ revertants at 30°C.

Figure 5.2B. Schematic representation of method to identify λ transducing phage in Kohara *E. coli* DNA library. "^{32}P - Mu" represents radioactively-labeled mini-Mu plasmid DNA with putative wild type suppressor gene which is hybridized to filter (large circle on right) of plaque lift of the entire Kohara library, each small circle representing cleared area due to killing of the *E. coli* lawn by one of the transducing phage. Shading of small circles represents hybridization of mini-Mu to transducing phage DNA. Inset shows photograph of part of actual autoradiogram after hybridization of ^{32}P-Mu4^+ to Kohara library. λ645, 646 and 647 are indicated by arrows.

A.


sogY
grpE 280
mini-Mu
library
sogY
grpE 280
Mu
sog +
Cs-
Cs+

B.


32P-
Mu
sog +
Kohara library

that the copy number of the mini-Mu plasmid (30 - 50 copies/cell) is sufficient to overcome this dominance. Mini-Mu plasmids were isolated for each of the four *E. coli grpE*280 *sog*$^-$ strains DA142, 144, 149 and 150, based on their ability to reverse the Cs$^-$ phenotype to Cs$^+$. Upon subsequent testing it was shown that these mini-Mu plasmids also reverse the Tr$^+$ phenotype of the suppressor mutation. This last observation suggests that the wild type version of each suppressor gene was indeed cloned. Transduction of the four plasmids into each of the four strains defines at least two complementation groups.

In the same manner as for the mini-Mu library, a pREG152 λ cosmid library (15) encompassing the wild type *E. coli* genome was used to infect some of the strains (Table 5.2). A pREG152 λ cosmid exists in only 3 - 5 copies per cell; thus, most likely only recessive *sog*$^-$ mutations can be complemented by a λ cosmid clone. The inability of *sog*4, which can be complemented by mini-Mu plasmids, to be complemented by a λ cosmid suggests that *sog*4 may be dominant over the wild type gene product.

The complementation group defined by *sog*16 is noteworthy in that the five strains which comprise this group, isolated in three independent selections, are all suppressed by λ cosmids originally selected on a Cs$^-$Tr$^+$ revertant of an *E. coli dnaK*25 mutant (C. Georgopoulos, unpublished data). A possibility is that two independent genes on the same plasmid are responsible for reversing the Cs$^-$ phenotype in the two strains. However, because of the large amount of data supporting a dnaK-grpE interaction, it is quite plausible that a single protein interacts with both grpE and dnaK, either as a complex or separately. Although the map position of this putative suppressor has not been determined yet, it is known that it does not map in the *dnaKJ* region (A. Maddock and C. Georgopoulos, unpublished data).

The group represented by DA144 has simultaneously acquired an unusual phenotype for λ growth. As shown in Table 5.3, when *E. coli grpE*280 *sog*11 is transduced to *grpE*$^+$, the strain, which is now Cs$^-$Tr$^+$, allows λ to grow but not λ*grpE*$^+$. At first glance, this observation is consistent with a model in which the presence of excess amounts of wild type grpE protein blocks λ growth in a *sog*11 strain unless *sog*11 is corrected by overproduction of *sog*11$^+$. Assuming this model is correct, when an *E. coli grpE*280 *sog*11 strain is transformed with a high copy plasmid encoding the wild type *grpE* gene, one would expect that neither λ nor λ*grpE*$^+$ will grow on this strain. On the contrary, λ is observed to grow on this strain (Table 5.3). Clearly, more experiments are required to understand the mechanism by which the *sog*11 mutation results specifically in the inability to propagate λ*grpE*$^+$. Nevertheless, the *E. coli grpE*$^+$*sog*11 strain may be useful because it allows direct selection for λ*grpE*$^-$ transducing phage.

Mapping of suppressor genes

The approximate locations of the suppressor mutations on the *E. coli* chromosome were determined essentially as described by Lipinska et al. (18). The mini-Mu plasmids were used as probes for the detection of λ transducing phages in the Kohara *E. coli* library (14) which have sequences complementary to the mini-Mu plasmids. As shown in Fig. 5.2, three suppressors were mapped following this procedure. The Mu4$^+$ plasmid hybridizes to the adjacent phages λ645, λ646 and λ647 of the Kohara library. Likewise, Mu11$^+$ hybridizes to λ324, λ325 and λ318 (although λ318 is located 40 kbp away from the other two phages, it has been shown to rescue the *sog*11 mutation, suggesting there may be an error in the Kohara library). In addition, the restriction map of the DNA cloned into the Mu4$^+$ plasmid was shown to match the restriction map of the *E. coli* chromosome in the region represented by

Table 5.3

Efficiency of λ growth in *E. coli* strains with the *sog*11 mutation

Strain genotype	**λ**	**λ*grpE*$^+$**	**λ*grpE*280**
grpE$^+$ *sog*$^+$	+	+	+
*grpE*280 *sog*$^+$	+/-	+	-
grpE$^+$ *sog*11	+	-	+
*grpE*280 *sog*11	+/-	+/-	+/-
grpE$^+$ *sog*11 / p*sog*11$^+$	+	+	+
*grpE*280 *sog*11 / p*sog*11$^+$	-	+	-
*grpE*280*sog*11 / p*grpE*$^+$	+	-	+

\+ indicates an efficiency of plating (e. o. p.) of 1.0 with large plaque size.

+/- indicates an e. o. p. of 0.1 - 1.0 with small plaque size.

\- indicates an e. o. p. of $< 10^{-4}$.

λ645-647 (A. Maddock, unpublished data). Kan^r markers have been placed near *sog*4 and *sog*11 (98% and 88% cotransduction frequency, respectively) and it has been possible to recombine the Kan^r markers onto the appropriate Kohara transducing phage, further demonstrating that indeed the locations of the *sog*4 and *sog*11 mutations have been determined (A. Maddock, unpublished data). By means of cotransduction with the nearby Kan^r marker, *sog*4 and 11 have also been transduced into a wild type background. In the absence of *grpE*280, the Cs$^-$ phenotype is still observed, demonstrating that it is due to the *sog* mutations and that they do not require the *grpE*280 mutation for their expression. Another possibility, however, is that the Cs$^-$ phenotype is due to a second mutation closely linked to the *sog* mutations and that the phenotype would not be observed in the absence of this second mutation.

Occasionally, it is possible to isolate plasmids which suppress the Cs$^-$ phenotype but do not restore the Tr$^-$ phenotype of the original *grpE*280 mutation, suggesting that they are "pseudo" suppressors of the phenotype. That is, overexpression of a gene different from the *sog*11$^+$ gene can also suppress the Cs$^-$ phenotype. Mu11* is a mini-Mu which suppresses the *sog*11 Cs$^-$ phenotype without simultaneously reversing the Tr$^+$ phenotype. This mini-Mu hybridizes to λ570, 571 and 572 of the Kohara library (14) which map at 84 min on the *E. coli* genetic map. As expected, these phage do not rescue the Kan^r marker near *sog*11. In addition, the λ cosmids pAM49, 50 and 51, which suppress the *sog*11 Cs$^-$ phenotype, have also been shown to map at 84 min and may represent the same "pseudo" suppressor as Mu11*. The identity of this "pseudo" suppressor is not known at this time.

In the case of *sog*22, the Cs$^-$ phenotype in DA151 is reversed by a λ cosmid pCG1263 which carries *E. coli* DNA encompassing the *dnaKJ* operon (C. Georgopoulos, unpublished data). By transforming *E. coli dnaK*$^\Delta$ :: cam^r [in which the *dnaK* gene has been deleted (24)] with pCG1263, it was possible to obtain a

cosmid derivative which no longer complements *dnaK*$^-$ or *dnaJ*$^-$ mutations. However, this plasmid still complements the Cs$^-$ phenotype in DA151. By isolating other λ cosmids, it was possible to show that the region of interest is at approximately 0.4 min on the *E. coli* genetic map, approximately 5 kb away from the *dnaKJ* operon. The overlapping phage λ102 and 103 of the Kohara library (14) complement the *sog*22 Cs$^-$ phenotype as prophage in DA151. λ102 and 101, which is adjacent to λ102, complement *dnaK*$^-$ and *dnaJ*$^-$ mutations (A. Maddock, unpublished results). Two open reading frames at 0.4 min are candidates for genes with the *sog*22 mutation. One encodes *ileS* which is a tRNA synthetase for isoleucine (13). The putative product of the other open reading frame has not yet been identified (13).

To summarize, mutations which suppress the Tr$^-$ phenotype of *E. coli grpE*280 cells have been isolated which themselves confer a Cs$^-$ phenotype to the bacteria. These mutations map outside of the *grpE* gene, and in four cases, it has been shown that they do not map in the *dnaKJ* operon.

What are the identities of these suppressor genes and what are their functions in *E. coli*? Recently, attention has been focused on proteins which assist other proteins to fold, assemble into complexes, or translocate across membranes, but which are not found as part of the final product (11, 25). The common feature of these proteins seems to be the ability to interact with a variety of proteins in a nonspecific manner, guiding them in activities common to all proteins, i. e., folding, formation of quaternary structure. DnaK, which is the bacterial analog of the eukaryotic heat shock protein Hsp70 (5), has been postulated to a) assist in the protection and refolding of denatured proteins following heat shock (25; C. Johnson, Ph.D. dissertation, University of Utah, 1988), b) facilitate the translocation of proteins across membranes (8, 9), and c) participate in initiation of λ DNA replication, by either releasing dnaB from λP or by altering the interaction between λP and dnaB (17). Because of the

known interaction between dnaK and grpE, it is inferred that grpE performs analogous functions or participates indirectly by modulating dnaK protein activity. The suppressors of the *dnaK*$^-$ or *grpE*$^-$ mutations may be located in genes whose products can now substitute in the folding or translocation processes, or stabilize the dnaK-grpE complex to allow function. Because dnaK and grpE are members of the heat shock regulon (22) and because dnaK, which is a negative regulator of the heat shock response (29), has been shown to have a sequence similar to that defined as a "transmitter module" (26; E. Kofoid and S. Parkinson, personal communication) seen in proteins which allow communication between different regulatory networks, e. g., the Ntr regulon and the chemotaxis regulon (23), perhaps mutations in a protein involved in sending intracellular signals can suppress mutations in dnaK and grpE. In fact, two of the suppressors reported here, *sog*11 and *sog*22, are found in regions encoding threonyl tRNA synthetase and isoleucine tRNA synthetase, respectively. These enzymes are capable of synthesizing unusual nucleotides, like AppppA, whose level increases during the heat shock response and which has been shown to inhibit the 5'-nucleotidase activity of dnaK (6, 7, 16, 32). In fact, the *lysU* gene product, the lysyl tRNA synthetase, has been shown to be a heat shock protein (reviewed in ref. 22).

Literature Cited

1. **Ang, D., G. N. Chandrasekhar, C. Johnson, M. Zylicz and C. Georgopoulos.** 1987. A genetic and biochemical analysis of the *Escherichia coli grpE* gene and its product. *In* R. McMacken and T. Kelly (eds.), UCLA Symp. Mol. Cell. Biol. New Series, 47:521-532, Alan R. Liss, Inc., New York.

2. **Ang, D., G. N. Chandrasekhar, M. Zylicz and C. Georgopoulos.** 1986. *Escherichia coli grpE* gene codes for heat shock protein B25.3, essential for both λ DNA replication at all temperatures and host growth at high temperature. J. Bacteriol. **167**:25-29.

3. **Ang, D. and C. Georgopoulos.** The heat shock-regulated *grpE* gene of *Escherichia coli* is required for bacterial growth at all temperatures, but is dispensable in certain mutant backgrounds. Submitted to J. Bacteriol.

4. **Bachmann, B. J.** 1987. Derivations and genotypes of some mutant derivatives of *Eschericha coli* K-12. *In* F. C. Neidhardt et al. (eds.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology, pp. 807-876, American Society for Microbiology, Washington D. C.

5. **Bardwell, J. C. A. and E. A. Craig.** 1984. Major heat shock gene of Drosophila and the *Escherichia coli* heat-inducible *dnaK* gene are homologous. Proc. Natl. Acad. Sci. U. S. A. **81**:848-852.

6. **Bochner, B. R., M. Zylicz and C. Georgopoulos.** 1986. *Escherichia coli* dnaK protein possesses a 5'-nucleotidase activity that is inhibited by AppppA. J. Bacteriol. **168**:931-935.

7. **Bochner, B. R., P. C. Lee, S. W. Wilson, C. W. Cutler and B. N. Ames.** 1984. AppppA and related adenylylated nucleotides are synthesized as a consequence of oxidation stress. Cell 37:225-232.

8. **Chirico, W. J., M. G. Waters and G. Blobel.** 1988. 70 K heat shock related proteins stimulate protein translocation into microsomes. Nature **332**:805-810.

9. **Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig and R. Schekman.** 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature **332**:800-805.

10. **Groisman, E. A. and Casadaban, M. J.** 1986. Mini-Mu bacteriophage with plasmid replicons for *in vivo* cloning and *lac* gene fusing. J. Bacteriol. **168**:357-364.

11. **Hemmingsen, S. M., C. Woolford, S. M. van der Vies, K. Tilly, D. T. Dennis, C. P. Georgopoulos, R. W. Hendrix and R.J. Ellis.** 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. Nature **333**:330-334.

12. **Johnson, C., G. N. Chandrasekhar and C. Georgopoulos.** The dnaK and grpE heat shock proteins in *Escherichia coli* interact both *in vivo* and *in vitro*. Submitted to J. Bacteriol.

13. **Kamio, Y. C.-K. Lin, M. Regue and H. C. Wu.** 1985. Characterization of the *ileS - lsp* operon in *Escherichia coli*: Identification of an open reading frame upstream of the *ileS* gene and potential promoter(s) for the *ileS - lsp* operon. J. Biol. Chem. **260**:5616-5620.

14. **Kohara, Y., K. Akiyama and K. Isono.** 1987. The physical map of the whole *E. coli* chromosome: Application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50**:495-508.

15. **Koomey, J. M., R. E. Gill and S. Falkow.** 1982. Genetic and biochemical analysis of gonococcal IgA1 protease: Cloning in *Escherichia coli* and construction of mutants of gonococci that fail to produce the activity. Proc. Natl. Acad. Sci. U. S. A. **79**:7881-7885.

16. **Lee, P. C., B. R. Bochner and B. N. Ames.** 1983. AppppA, heat-shock stress, and cell oxidation. Proc. Natl. Acad. Sci. U. S. A. **80**:7496-7500.

17. **Liberek, K., C. Georgopoulos and M. Zylicz.** 1988. The role of the *Escherichia coli* DnaK and DnaJ heat shock proteins in the initiation of bacteriophage λ DNA replication. Proc. Natl. Acad. Sci. U. S. A. **85**:6632-6636.

18. **Lipinska, B., O. Fayet, L. Baird and C. Georgopoulos.** 1988. Identification, characterization and mapping of the *Escherichia coli htrA* gene, whose product is essential for bacterial viability only at elevated temperatures. J. Bacteriol., *in press*.

19. **Lipinska, B., J. King, D. Ang and C. Georgopoulos.** 1988. Sequence analysis and transcriptional regulation of the *Escherichia coli grpE* gene, encoding a heat shock protein. Nucleic Acids Res. **16**:7545-7562.

20. **Maniatis, T., E. F. Fritsch and J. Sambrook.** 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

21. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

22. **Neidhardt, F. C., R. A. VanBogelen and V. Vaughn.** 1984. The genetics and regulation of heat shock proteins. Ann. Rev. Genet. **18**:295-329.

23. **Ninfa, A. J., E. G. Ninfa, A. N. Lupas, A. Stock, B. Magasanik and J. Stock.** 1988. Crosstalk between bacterial chemotaxis signal transduction proteins and regulators of transcription of the Ntr regulon: Evidence that nitrogen assimilation and chemotaxis are controlled by a common phosphotransfer mechanism. Proc. Natl. Acad. Sci. U. S. A. **85**:5492-5496.

24. **Paek, K. and G. C. Walker.** 1987. *Escherichia coli dnaK* null mutants are inviable at high temperature. J. Bacteriol. **169**:283-290.

25. **Pelham, H. R. B.** 1986. Speculations on the functions of the major heat shock and glucose-regulated proteins. Cell **46**:959-961.

26. **Ronson, C. W., B. T. Nixon and F. M. Ausubel.** 1987. Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell **49**:579-581.

27. **Saito, H. and H. Uchida.** 1977. Initiation of the DNA replication of bacteriophage lambda in *Escherichia coli* K12. J. Mol. Biol. **113**:1-25.

28. **Saito, H., Y. Nakamura and H. Uchida.** 1978. A transducing λ phage carrying *grpE*, a bacterial gene necessary for λ DNA replication, and two ribosomal protein genes, *rpsP* (S16) and *rplS* (L19). Mol. Gen. Genet. **165**:247-256.

29. **Tilly, K., N. McKittrick, M. Zylicz and C. Georgopoulos.** 1983. The dnaK protein modulates the heat-shock response of *Escherichia coli.* Cell **34**:641-646.

30. **Zylicz, M., D. Ang and C. Georgopoulos.** 1987. The grpE protein of *Escherichia coli*: Purification and properties. J. Biol. Chem. **262**:17437-17442.

31. **Zylicz, M., D. Ang, K. Liberek and C. Georgopoulos.** Initiation of λ DNA replication with purified host- and bacteriophage-encoded proteins: The role of the dnaK, dnaJ and grpE heat shock proteins. Submitted to EMBO J.

32. **Zylicz, M., J. H. LeBowitz, R. McMacken and C. Georgopoulos.** 1983. The dnaK protein of *Escherichia coli* possesses an ATPase and autophosphorylating activity and is essential in an *in vitro* DNA replication system. Proc. Natl. Acad. Sci. U. S. A. **80**:6431-6435.

CHAPTER 6

OVERVIEW AND PERSPECTIVES

In the last few years, a group of proteins has been identified whose role is to "chaperone" or assist other proteins -- to fold, to unfold, to assemble into larger complexes. Interestingly, many of these proteins have been shown to belong to the so-called stress or heat shock group of proteins. In *E. coli* , these include the dnaK protein, which is 48% identical to Hsp70 in eukaryotes, htpG which is 38% identical to the eukaryotic Hsp83 protein, *groEL* which is 46% identical to Hsp58 in mammalian organisms and the Rubisco binding protein of plants, and dnaJ and grpE. I have shown that a protein antigenically related to grpE is present in Chlamydomonas, suggesting that this protein has also been conserved in evolution. Mutations in the *dnaK*, *dnaJ*, *grpE*, *groES* and *groEL* genes of *E. coli* have been shown to result in pleiotropic defects including a decrease in both DNA and RNA syntheses and a block to cell division. These observations and the possible involvement of these proteins in assembly or disassembly of diverse polypeptides or their complexes exemplifies their fundamental role in *E. coli* growth.

An example of "survival of the fittest" is seen in the case of the temperate phage λ. Lambda has ensured itself against the loss of host functions vital for its own propagation by evolving to depend on this group of highly conserved *E. coli* heat shock proteins. GroEL and groES are required by λ for proper morphogenesis of its head. DnaB, dnaK, dnaJ and grpE are all required for initiation of λ DNA replication. As the ultimate example of a "survivor," λ has even evolved the means to exploit the host's own mechanism of transiently increasing expression of these genes. The cIII protein of λ has been shown to stabilize the σ^{32} subunit of RNA polymerase, allowing increased transcription from heat shock promoters even under non-heat shock conditions.

The goals of this dissertation have been to better understand the role of the grpE protein in both λ and *E. coli* growth, and to better understand the nature of its

interaction with the dnaK protein. The last four years have delivered their share of predicted results as well as several surprises. Included in this latter group:

a) A complex of dnaK and grpE protein can be isolated which, once formed, is remarkably stable even in a high concentration of salt, but which is easily disrupted by ATP.

b) Null mutants of *dnaK* and *dnaJ* had been isolated previously in wild type *E. coli*, but null mutants of *grpE* could not be obtained using analogous procedures. Unexpectedly, deletion of the *grpE* gene is readily accepted in some *E. coli dnaK*$^-$ mutants. Additional work has demonstrated the existence of an extragenic suppressor(s) which can compensate for the loss of both the dnaK and grpE functions.

c) Contrary to the *in vivo* situation, in the *in vitro* purified λdv replication system, *ori*λ-specific replication can occur in the absence of grpE protein. The addition of grpE, however, allows at least a ten-fold reduction in the amount of dnaK required for initiation of replication.

In Ch. 1 as well as in App. C, a model for initiation of replication at *ori*λ was presented. The accumulated evidence suggests that the order of events is a) binding of λO to *ori*λ, which results in melting of the AT-rich region adjacent to *ori*λ, b) localization of a λP-dnaB complex at the *ori*λ-λO structure, c) action by dnaK, dnaJ and grpE, resulting in the release of λP from dnaB, which is now activated as a helicase, unwinding the duplex as it migrates along the lagging strand template, and d) at this stage, dnaG can synthesize primers which are elongated into DNA by DNA polymerase III.

Many protein-protein interactions have been established among the protein participants, which are postulated to result in the series of events leading to initiation

of λ DNA replication. The details of these interactions and their importance have been discussed in Ch. 1 and App. C. Here, other possible mechanisms of grpE action are highlighted.

One of the complexities of the *in vitro* λ DNA replication system is that the λP protein can inhibit the dnaB helicase activity, not only at the *oriλ* site, but also subsequent to its release, by binding and inhibiting the dnaB helicase at the replication fork. GrpE could function by releasing λP from the *oriλ* complex, thus liberating dnaB and sequestering λP so that it can no longer inhibit "free" dnaB at the replication fork. The postulated role of grpE in effectively "sequestering" λP away from dnaB following its release from *oriλ* is suggested by the fact that the λP-grpE interaction, unlike that of λP-dnaK and grpE-dnaK, is resistant to ATP which must be present in the reaction for dnaB helicase activity and DNA replication. Hence, *in vivo*, where the estimated intracellular concentration of ATP is 3-5 mM, the λP-grpE interaction may be more biologically significant than that of λP-dnaK. The ability of grpE to effectively lower the amount of dnaK needed in the *in vitro* DNA replication system may be due solely to its effective sequestration of λP following release from *oriλ*, thus preventing significant λP-dnaK interaction under the experimental conditions.

Although mutations in the *dnaK*, *dnaJ*, *grpE*, *groES* and *groEL* genes affect *E. coli* DNA replication, the steps at which their products are involved have not been elucidated yet. It has been shown that a) elevated levels of groE proteins allow correct DNA initiation in certain *dnaA*$^-$ temperature-sensitive mutations, and b) that the *dnaK*$^-$ imposed block can be bypassed by mutations in the *rnh* (RNAse H) gene, suggesting that dnaK is needed *in vivo* for *ori*C-dependent replication. The demonstrated interaction between dnaK and grpE, and the fact that a *grpE*$^\Delta$ can be tolerated in the presence of mutations which suppress the lack of dnaK function, suggest that grpE

may also be involved in *E. coli* DNA replication, perhaps at the same step as dnaK. Its exact role, though, remains elusive for now.

Another common feature of mutations in the *dnaK*, *dnaJ* and *grpE* genes is the overproduction of heat shock proteins at all temperatures. This ability has been traced to the stabilization of the σ^{32} polypeptide. The σ^{32} polypeptide when complexed with the RNA polymerase core allows RNA polymerase to specifically transcribe from heat shock promoters. A model to explain the role of these heat shock proteins in regulating their own synthesis can be derived from knowledge of the steps leading to λ DNA replication *in vitro*. The model assumes that the σ^{32} polypeptide is stable when complexed with the RNA polymerase core, but unstable when released from the core. It could be that the dnaK, dnaJ and grpE proteins interact with the σ^{32} polypeptide as well as the RNA polymerase core, and that this interaction leads to σ^{32} release from RNA polymerase, analogous to the release of λP from the *ori*λ complex. This release may result in more accessibility of σ^{32} to host proteases, leading to its instability and simultaneous replacement by the σ^{70} polypeptide, required for transcription from promoters of normal housekeeping genes. The requirement for all three heat shock polypeptides is consistent with the phenotype of mutations in any of the three genes. This model is not only economical but also explains the autoregulatory nature of the heat shock response in *E. coli*.

Further *in vitro* experiments with purified components may shed more light on the exact roles of grpE in *E. coli* physiology and growth. The elucidation of the nature of the suppressors which allow the deletion of *grpE* from the *E. coli* genome is also necessary for our complete understanding of the roles of this small yet biologically important heat shock protein.

APPENDIX A

THE grpE PROTEIN OF *Escherichia coli:* PURIFICATION AND PROPERTIES

J. Biol. Chem. **262**:17437-17442

Abstract

The *grpE* gene of *Escherichia coli* was first identified because a mutation in it, *grpE*280, prevented bacteriophage λ DNA replication *in vivo*. Subsequent work resulted in the identification of the grpE protein in two-dimensional gels and its classification as a great shock protein. Here we report the purification of the grpE protein. We show that overproduction of grpE occurs in *dnaK*103 bacteria which do not produce a functional M_r 72,000 dnaK protein. The grpE protein was purified from this strain primarily by its specific retention on a dnaK-affinity column. The interaction between these two proteins, which is stable in the presence of 2 M KCl, allowed other proteins to be washed from this column. grpE was then eluted by ATP, which disrupts the interaction. During purification, grpE activity was monitored by its ability to complement an *in vitro* λ*dv* DNA replication system dependent on the λO and λP proteins.

The effect of ATP on the dnaK-grpE complex was also observed during sedimentation of the two proteins in glycerol gradients. Purified grpE protein has a M_r of approximately 23,000 under both denaturing and native conditions, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and sedimentation, respectively. However, in the presence of dnaK under native conditions, grpE cosediments with dnaK. When ATP is added to the gradient, the complex is disrupted, and the two proteins sediment independently as monomers.

Introduction

Bacteriophage λ DNA replication is a prime example of viral dependence on the host replication proteins, both at the level of initiation and elongation (reviewed in refs. 10 and 12). Bacteriophage λ encodes two replication proteins, λO and λP, which are responsible for accurate initiation at the *cis*-acting *oriλ* site on the λ genome. In

addition, several *Escherichia coli* proteins are required for λ DNA initiation. These genes encoding the dnaK, dnaJ, and grpE proteins were originally identified by host mutations which prevented λ DNA replication at any temperature (10). Subsequently, these genes were also shown to be essential for host growth at high temperature (3, 13, 14, 29, 33). Bacteria carrying mutations in these three genes exhibit similar pleiotropic effects at the nonpermissive temperature, which include a decrease in the rate of synthesis of both DNA and RNA (3, 17, 30, 40). An additional interesting feature of dnaK, dnaJ, and grpE is that they belong to the so-called heat shock class of proteins (3, 4, 15; reviewed in refs. 10 and 23). That is, their rate of synthesis is transiently increased upon a shift-up in temperature.

In this article, we describe the purification of the grpE protein. This procedure is dependent upon the fortuitous discovery of an extremely stable interaction between the grpE and dnaK proteins under conditions of high salt which can be disrupted in the presence of ATP. As a consequence, a one-column purification scheme using a dnaK-affinity column was employed.

GrpE activity was monitored using an *in vitro* λ DNA replication assay, as described previously (44). A crude cell extract, Fraction II (11), has been shown to support *ori*λ-specific DNA replication if supplemented with the λO and λP proteins (1, 37, 43). GrpE activity was assayed by complementation of a Fraction II extract prepared from *grpE*280 bacteria.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains C600, C600 *grpE*280, and C600 *dnaK*756, have been described previously (3, 13, 15, 44). Strain C600 *dnaK*103 was obtained from Dr. Kaspar von Meyenburg (Department of Microbiology, The Technical University of

Denmark) and shown by us to be an allele of the *dnaK* gene (G. N. Chandrasekhar and C. G., unpublished results). Strain C600 (*tac*12H *htpR*$^+$) overproduces htpR protein under the control of the *tac* promoter (K. Tilly and C. G., unpublished results).

Media, growth, and storage of cells

Bacteria were grown in L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.4) supplemented with 0.2% glucose. The bacterial cultures were centrifuged in a Beckman JA-10 rotor at 7000 rpm for 10 min at 0°C; the bacterial pellets were rinsed with 10% (w/v) sucrose in 50 mM Tris/HCl (Sigma), pH 8, and stored at -70°C.

Nucleic acids and λ DNA replication enzymes

DNA from plasmid pRLM4 (which carries *oriλ*) (kindly provided by Dr. Roger McMacken, Department of Biochemistry, The Johns Hopkins University School of Hygiene and Public Health) was prepared as previously described (45) and used as the substrate for *in vitro* λ DNA replication.

The λO and λP proteins were purified from a strain carrying the plasmid pMY17-3 (39), which overproduces both proteins (kindly provided by Dr. Kenichi Matsubara, Laboratory of Molecular Genetics, Osaka University Medical School, Osaka, Japan). The purification of the two proteins has been previously described (26, 45).

Preparation of crude cell extracts

Fraction II extracts were prepared from C600 and C600 *grpE*280 bacteria grown at 37°C to $A_{595\ nm}$ = 1.0. The Fraction II extracts were prepared as previously described (47).

Replication assay

The λ*dv* replication assay was a modified version of that described in Ref. 22. The 25-μl (final volume) assay mixture contained 1.5 μl of 12 mg/ml bovine serum albumin (Sigma) in 1 M Hepes (Sigma)/KOH, pH 8, 0.3 μl of 2 M magnesium acetate, 1.8 μl of 25 mM each CTP, GTP, and UTP (Sigma), 0.6 μl of 100 mM ATP (Sigma), 2.5 μl of 2 M creatine phosphate, pH 7.6 (Boehringer Mannheim), 1 μl of 2.5 mg/ml creatine kinase (Boehringer Mannheim), 1 μl of 2.5 mM each dATP, dCTP, dGTP, and [methyl-^{3}H]dTTP (Amersham Corp.) (54 dpm/pmol dNTP), 1 μl of pRLM4 plasmid DNA $A_{260\ nm}$ = 4.54, 940 ng of λO protein, 60 ng of λP protein, 6 μl of 25% (w/v) polyvinyl alcohol (Sigma), and 1.5 μl of Fraction II (300 μg of protein from C600 *grpE*280 bacteria). Reactions were incubated at 30°C for 20 min, stopped, and counted as previously described (44, 46). One unit of activity is defined as the incorporation of 1 pmol of nucleotides in 1 min at 30°C under conditions in which the rate of incorporation is linear with respect to the amount of protein added.

Buffers

Buffer A contained 10% (w/v) sucrose (Mallinckrodt Chemical Works), 50 mM Tris/HCl (Sigma), pH 8; Buffer B contained 25 mM Hepes (Sigma)/KOH, pH 8, 1 mM EDTA, 10 mM 2-mercaptoethanol (Sigma), 10% (v/v) glycerol; Buffer C contained 25 mM Hepes/KOH, pH 8, 50 mM KCl, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 20% (v/v) glycerol; Buffer D contained 20 mM Tris/HCl, pH 7.5, 20 mM NaCl, 0.1 mM EDTA, 15 mM 2-mercaptoethanol, 3 mM $MgCl_2$; and Buffer E contained 25 mM Hepes/KOH, pH 8, 100 mM NaCl, 5 mM 2-mercaptoethanol.

Protein determination

The protein concentration was estimated using the BioRad protein assay. A solution of a known concentration of bovine serum albumin was used as a standard.

Gel electrophoresis

One-dimensional gel electrophoresis was performed essentially as described by O'Farrell (24). Slab gels of 12.5% (w/v) SDS-polyacrylamide were used.

Preparation of a dnaK-affinity column

DnaK protein was purified in a manner analogous to that described for its eukaryotic analog, Hsp70 (41). Essentially, C600 bacteria which bear the dnaK-overproducing plasmid pMOB45 *dnaK*$^+$ were grown and lysed as described previously (14). Following a preliminary separation on a DE52-cellulose column (Whatman), relevant fractions were passed over a 5-ml ATP-agarose affinity column (1 x 7 cm) (C-8 linkage, Sigma A-2767) equilibrated with Buffer D. The column was washed with 500 mM NaCl in Buffer D. After re-equilibration of the column with Buffer D, the dnaK protein was eluted with 10 mM ATP in Buffer D, adjusted to pH 7.6. The protein was then dialyzed against Buffer E (S. Sell and M. Z., unpublished results).

Affi-Gel 10 affinity support (BioRad) was cross-linked to purified dnaK protein according to the procedure described by Formosa et al. (9). Approximately 100 mg of dnaK protein (20 ml) was combined with 20 ml of Affi-Gel 10. The protein was bound with an efficiency of approximately 85%.

Purification of grpE protein from C600 *dnaK*103 bacteria

E. coli dnaK103 bacteria were grown in L-broth supplemented with 0.2% glucose at 37°C to A_{595nm} = 1.0. The culture was centrifuged for 10 min at 7,000 rpm at 0°C in a Beckman JA-10 rotor. The pellets (15.4 g total) were rinsed with Buffer A, resuspended in a minimal volume of Buffer A, and frozen at -70°C. The cells were thawed slowly in a 4°C waterbath. Ten ml of a solution of 46 µg/ml spermidine (Sigma), 50 mM dithiothreitol (Sigma), 50 mM EDTA, and 0.2 g/ml ammonium sulfate was added, followed by 2.2 ml of a fresh 20 mg/ml lysozyme (from chicken egg white; Sigma) solution. The volume of the mixture was brought to 74 ml by adding Buffer A. The resuspended cells were kept on ice for 45 min and then transferred to 37°C for 5 min with gentle shaking. The lysate was cooled on ice for 5 min and then centrifuged for 30 min at 30,000 rpm at 0°C in a Beckman fixed angle 35 rotor. After decanting the supernatant, the pellet was transferred to a dialysis bag (Spectrapor, M_r cutoff: 12,000-14,000) and resuspended in approximately 2 ml of Buffer B. The suspension was dialyzed three times against 1 liter of Buffer B at 4°C for 30 min each time. The dialyzed fraction (approximately 5 ml) was diluted with Buffer B (no KCl) to a conductivity equivalent to that of 50 mM KCl in Buffer B (fraction 2, 19 ml). A 20-ml dnaK-affinity column (1.5 cm x 12 cm) (Bio-Rad Affi-Gel 10) was equilibrated with Buffer B prior to loading. Fraction 2 was applied to the column at flow rate of 3.5 ml/30 min. The column was washed successively with 200 ml of 50 mM KCl in Buffer B, 70 ml of 0.5 M KCl in Buffer B, and 70 ml of 2 M KCl in Buffer B. The column was re-equilibrated with 100 ml of 50 mM KCl in Buffer B, followed by elution of the grpE protein with 50 ml of 50 mM KCl, 20 mM $MgCl_2$, and 10 mM ATP in Buffer B (pH adjusted to 7.6). The presence of the grpE protein was monitored by the λ*dv* DNA replication assay described under **Materials**

and Methods and in the legend of Fig A.1. The grpE protein was dialyzed three times against 2 liters of Buffer C at 4°C for 10 h each time and stored at -70°C.

Amino-terminal analysis

The amino-terminal sequencing of purified grpE protein was done in the laboratory of Dr. W. Gray (Department of Biology, University of Utah). The stepwise degradation (8) was carried out in a Beckman 890 D spinning cup instrument using a 1 M Quadrol program. The 3-phenyl-2-thiohydantoin derivatives were identified by high performance liquid chromatography on a column (0.46 x 15 cm) of Ultrasphere ODS using a gradient of acetonitrile in 0.05 M sodium acetate, pH 4.5.

Results

Overproduction of grpE protein. We had shown earlier that the grpE protein of *E. coli* is a heat shock protein (3) since a shift-up in temperature from 30° to 43°C results in a transient increase in its rate of synthesis. We had also shown that the dnaK protein, which is the *E. coli* analog of the Hsp70 protein of *Drosophila* (4), is a negative modulator of the heat shock response (34) because (a) *dnaK*756 bacteria overproduced heat shock proteins at 30°C and failed to turn off the heat shock response at 43°C, and (b) overproduction of dnaK protein dampened the magnitude of the heat shock response. We took advantage of this information and asked whether various *dnaK*$^-$ mutants overproduced grpE activity at 30°C. To do this, we assayed ammonium sulfate fractions prepared from various mutant strains for λ*dv in vitro* replication as described previously (47). As a control, we also assayed a wild type strain transformed with an *htpR*$^+$-bearing plasmid (K. Tilly and C. G., unpublished results) in which the *htpR*$^+$ (*rpoH*) gene is under *tac*12H promoter control. The *htpR*

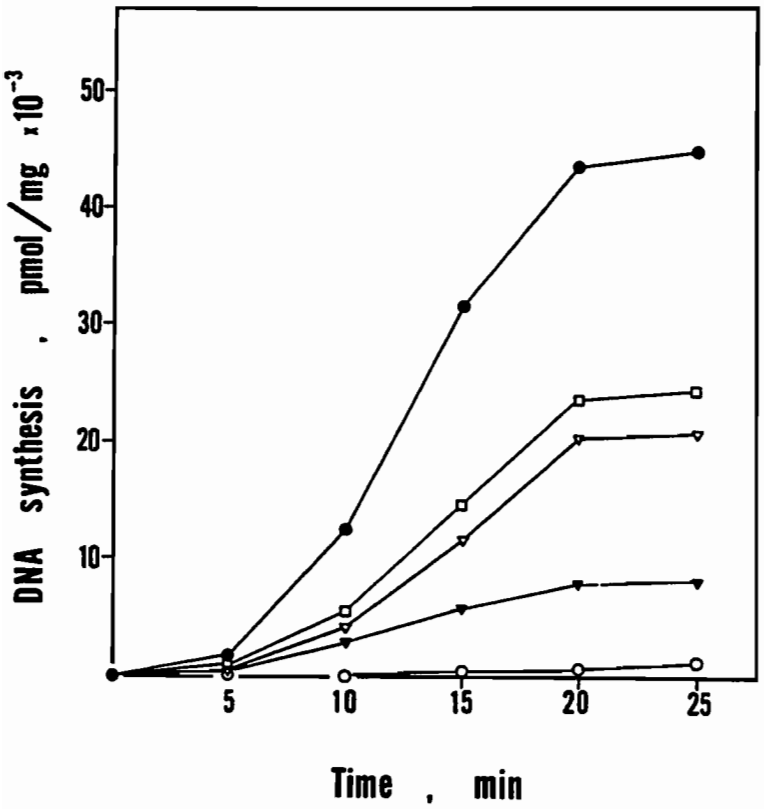
gene encodes a σ factor of RNA polymerase which is required for transcription of heat shock promoters (16).

As shown in Fig. A.1, a Fraction II prepared from a *grpE*280 mutant did not support λ*dv* replication. Crude ammonium sulfate fractions from the various bacterial strains mentioned above were used to complement the *grpE*280 Fraction II extracts for λ*dv* DNA replication. From the data presented in Fig. A.1, it is clear that extracts from the two *dnaK*$^-$ mutants and the htpR-overproducing strain contain substantially more grpE activity than that of the corresponding wild-type strain, confirming and extending our previous observations (34). We chose to purify the grpE activity from the *dnaK*103 strain for two reasons. (a) Of the four strains assayed, *dnaK*103 showed the greatest overexpression of grpE activity, and (b) immunoprecipitation of a *dnaK*103 cell extract with rabbit anti-dnaK antibody resulted in precipitation of only low levels of an unstable, truncated protein (G. N. Chandrasekhar and C. G., unpublished results), suggesting that no full-length dnaK protein is made in *dnaK*103 bacteria. As developed below, this is important because the purification of the grpE protein depends upon its ability to bind strongly and specifically to a dnaK affinity column. Thus, in the *dnaK*103 mutant, no functional dnaK protein is present to compete with the dnaK affinity column for binding of the grpE protein.

Purification of the grpE protein

Initially, we tested to see whether the grpE protein could be specifically retained on a variety of columns including phenyl-agarose, L-leucine-agarose, octyl-agarose, hydroxylapatite, and Affi-Gel blue. The presence of the grpE protein was monitored by (a) its ability to complement a Fraction II extract prepared from *grpE*280 bacteria in the *in vitro* λ*dv* replication assay, and (b) its presence following electrophoresis on SDS-polyacrylamide gels. None of these columns provided us with a good

Figure A.1. GrpE activity in extracts from various *E. coli* bacterial strains. An *in vitro* λdv DNA replication system utilizing a Fraction II prepared from C600 *grpE*280 mutant bacteria was carried out as described under **Materials and Methods.** Crude ammonium sulfate fractions from the different mutant strains listed below were prepared as described in **Materials and Methods.** These were used to complement λdv DNA replication for the missing grpE activity. The protein concentration of these fractions was in the range where DNA synthesis is proportional to the protein concentration of the complementing fraction. Aliquots of 25 µl were removed at the indicated times and immediately precipitated with 10% trichloroacetic acid and 50 µl of saturated sodium pyrophosphate as described previously (44, 46). Incorporation of [^{3}H]dNTP precursors into λdv DNA was approximately 10 pmol/20 min when the extract was not supplemented with wild type grpE protein. ●, C600 *dnaK*103; □, C600 *dnaK*756; ▽, C600 (*tac*12*htpR*$^+$ plasmid); ▼, C600; ○, C600 *grpE*280.


DNA synthesis , pmol/mg x10⁻³
50
40
30
20
10
5
10
15
20
25
Time , min

purification step (data not shown). We had shown earlier that immunoprecipitation of [^{35}S]methionine-labeled proteins from extracts of heat-shocked cells with rabbit anti-dnaK antibody results in precipitation of the M_r 72,000 dnaK protein and an approximately M_r 23,000 protein (2). Immunoprecipitation of extracts from *dnaK*103 mutants did not result in precipitation of either the M_r 72,000 dnaK or M_r 23,000 protein (G. N. Chandrasekhar and C. G., unpublished results). The M_r 23,000 protein was subsequently shown to possess the same isoelectric point as a protein encoded by a λ *grpE*$^+$ transducing phage which complements *in vivo* the *grpE*280 mutation (3, 28). These observations provided strong evidence that the dnaK and grpE proteins physically interact. We took advantage of these observations by cross-linking dnaK protein to an agarose matrix, as described under **Materials and Methods**, and used this as a major purification step for the grpE protein.

The exact purification procedure employed is detailed in **Materials and Methods**. Briefly, cells were lysed and pelleted. Ammonium sulfate was added to the supernatant to a final concentration of 0.35 g/ml. The precipitated proteins were resuspended and dialyzed prior to loading onto the dnaK-affinity column. Fig. A.2 shows the overall protein and grpE activity profiles of fractions from various KCl elution steps from the dnaK affinity column. The binding of grpE to the column appears to be highly resistant to salt as evidenced by its retention even in the presence of 2 M KCl (Fig. A.2). The fact that the dnaK protein exhibits a weak 5'-nucleotidase and autophosphorylating activities (5, 46) suggested to us that the dnaK-grpE complex may be disrupted by ATP. This turned out to be the case. As seen in Fig. A.2, a 10 mM ATP solution eluted grpE quantitatively from the dnaK-affinity column. The protein preparation was at least 95% pure as judged by staining (Fig. A.3). Its specific activity increased at least 650-fold over that of the crude ammonium sulfate fraction in the *in vitro* replication assay (Table A.1).

Figure A.2. Purification of the grpE protein on a dnaK-affinity column. The dnaK-agarose affinity column was prepared as described under **Materials and Methods**. The ammonium sulfate fraction prepared from C600 *dnaK*103 bacterial extracts was passed through this column and washed with successively higher concentrations of KCl. GrpE was eluted with ATP. Two-ml fractions were collected, and 1 μl of each fraction was used in DNA complementation assays for grpE activity (●). The protein concentration (○) was determined with the Bio-Rad protein assay, using bovine serum albumin as the standard.

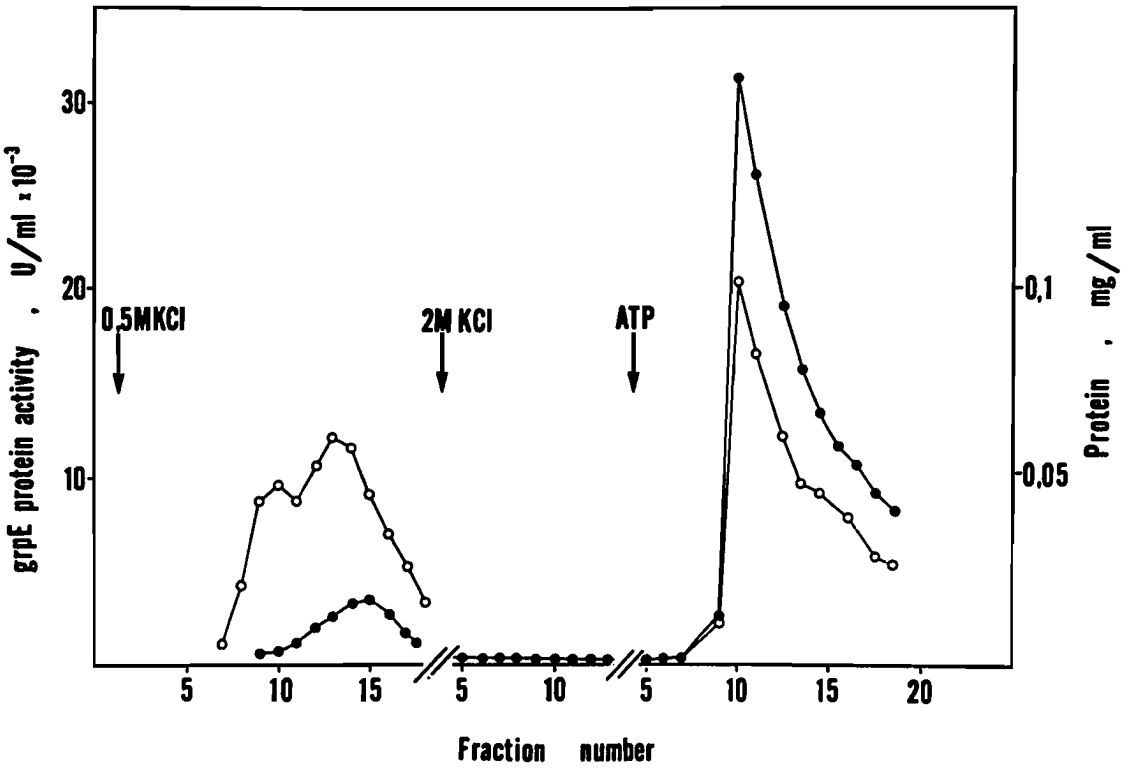

30
20
10
0,5MKCl
2M KCl
ATP
grpE protein activity , U/ml x 10⁻³
0,1
0,05
Protein , mg/ml
5
10
15
5
10
5
10
15
20
Fraction number

Figure A.3. Polyacrylamide gel electrophoresis of various protein fractions from the purification of the grpE protein. Lanes 1 and 5, protein molecular weight standards (phosphorylase b from rabbit muscle, M_r 97,400; bovine serum albumin, M_r 66,000; ovalbumin, M_r 45,000; glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle, M_r 29,000; trypsinogen from bovine pancreas, M_r 24,000; trypsin inhibitor from soybeans, M_r 20,100; β-lactoglobulin from bovine milk, M_r 18,400; α-lactalbumin from bovine milk, M_r 14,200); lane 2, crude lysate, 56 μg; lane 3, ammonium sulfate fraction, 43 μg; lane 4, grpE protein fraction 11 from ATP wash of dnaK-affinity column, 5 μg. The 12.5% SDS-polyacrylamide gel was electrophoresed and silver stained as described previously (22).

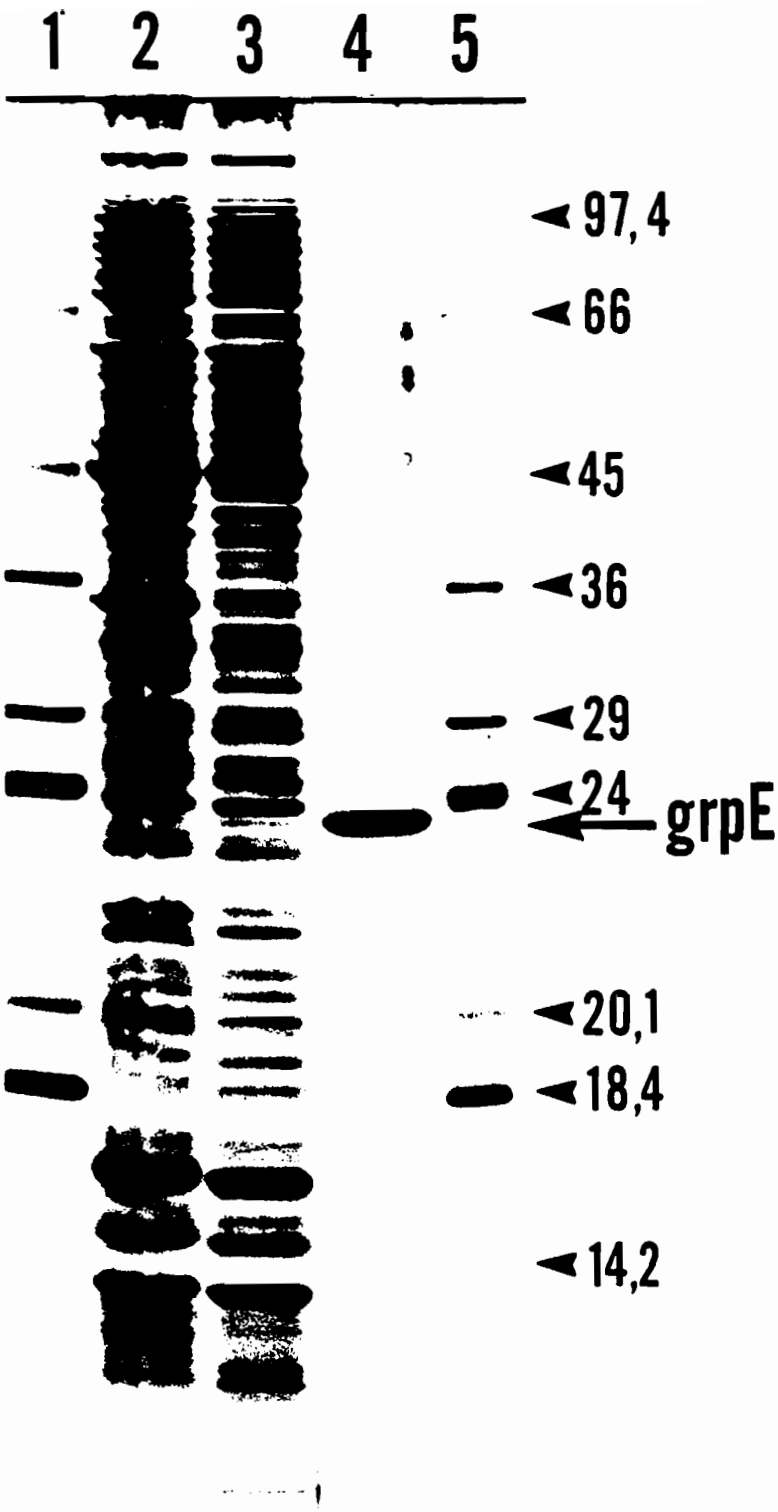

1
2
3
4
5
97,4
66
45
36
29
24
grpE
20,1
18,4
14,2

Table A.1

Purification of grpE protein from C600 *dnaK*103 bacteria

Purification step	Protein	Volume	Activity	Specific Activity
	mg	*ml*	*units x 10^{-3}*	*units x 10^{-3}/mg*
Lysate[a]	984	73		
Ammonium sulfate	820	19	401.8	0.49
dnaK affinity chromatography	1.7	50	545.5	319

[a] Starting from 15.4 g of wet cell paste.

The grpE protein is required for λ*dv* replication

Early genetic studies identified various host functions necessary for λ propagation *in vivo* (reviewed in refs. 10 and 12). These include the *dnaB*, *dnaJ*, *dnaK*, and *grpE* gene products. Additional genetic and biochemical studies have demonstrated a requirement for transcription by the *E. coli* RNA polymerase holoenzyme and for the dnaG (primase), gyrA, gyrB, single-stranded binding, and DNA polymerase III holoenzyme proteins both *in vivo* and *in vitro* (reviewed in ref. 21).

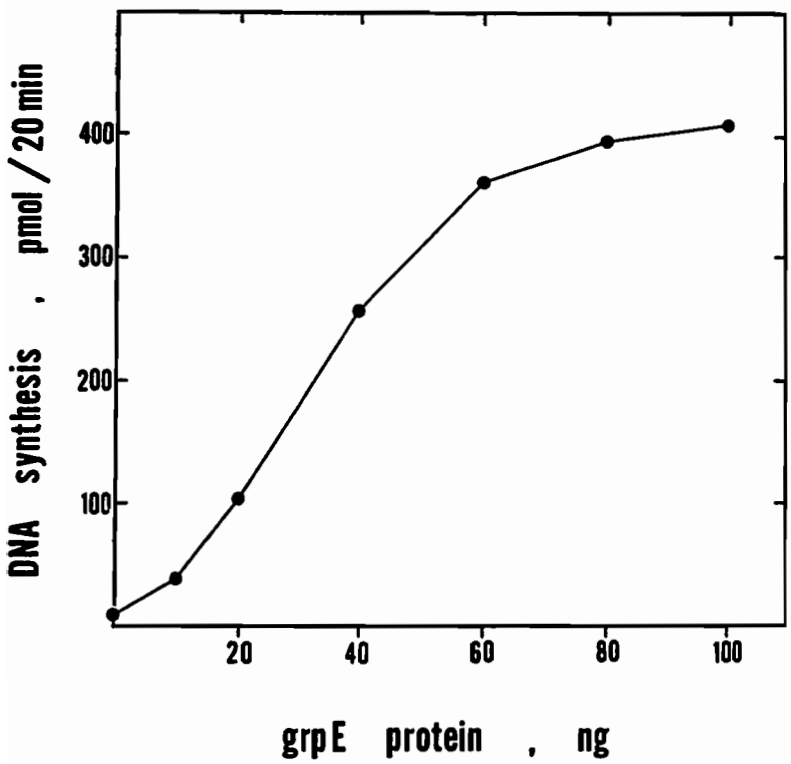
Using the purified protein, we were able to demonstrate that grpE is required in the λ*dv in vitro* replication assay. The addition of the purified protein to a Fraction II prepared from *grpE*280 mutant bacteria allowed levels of λ DNA replication comparable to those seen with a Fraction II from wild-type cells (Fig. A.4).

Biochemical and physical properties of the grpE protein

We have previously shown that the synthesis of an acidic protein with M_r of approximately 23,000 correlated with the ability of a λ*grpE*$^+$ transducing phage (28) to complement the *grpE*280 mutation (3). Co-electrophoresis of the purified protein and a lysate of UV-irradiated cells infected with the λ*grpE*$^+$ transducing phage demonstrated that the two proteins migrate to the same position on a two-dimensional gel (data not shown).

To support further the contention that the grpE protein had indeed been purified, the amino terminus of the purified protein was sequenced and compared to that predicted by preliminary DNA sequence data (D. A., J. King and C. G., unpublished results). The first 15 amino-terminal residues determined were Ser-Ser-Lys-Glu-Gln-Lys-Thr-Pro-Glu-Glu-Gln-Ala-Pro-Glu-Glu, in perfect agreement with the predicted sequence except that the initial methionine residue is not found in the purified protein.

Figure A.4. Ability of the purified grpE protein to complement Fraction II extracts lacking grpE activity. The λ*dv* DNA replication reaction, using a Fraction II prepared from *E. coli* C600*grpE*280, was carried out as described under **Materials and Methods.** Purified grpE protein from fraction 11 of the ATP wash was added before initiation of the reaction. Incorporation of dNTPs into λ*dv* DNA was measured in the presence (●) of λ DNA replication proteins, λO and λP. In the absence of grpE protein, a background of 10 pmol/20 min of incorporation was observed.


DNA synthesis , pmol / 20 min
400
300
200
100
20
40
60
80
100
grpE protein , ng

The molecular weight of the purified protein was estimated by SDS-polyacrylamide gel electrophoresis under denaturing conditions (Fig. A.5). The molecular weight standards are those listed in the legend to Fig. A.3. Under these conditions, the molecular weight of the grpE monomer appears to be approximately 23,000.

The purified protein was also sedimented under nondenaturing conditions on a 20-40% glycerol gradient. Its position in the gradient was determined both by assaying its activity in λ*dv in vitro* DNA replication and by SDS-polyacrylamide gel electrophoresis. The protein behaved as a monomer, with a sedimentation coefficient slightly less that $s_{20,w} = 2.5$ (Fig. A.6). However, in the presence of purified dnaK protein, grpE sedimented with the leading fractions of dnaK, ahead of ovalbumin (Fig. A.7a). Cosedimentation of dnaK and grpE is not seen in the presence of 10 mM ATP. Instead, they migrate separately as monomers (Fig. A.7b), with grpE at a position similar to that seen in Fig. A.6 relative to ovalbumin. These are the results expected from the behavior of grpE on the dnaK-affinity column.

Discussion

Bacteriophage λ has evolved various mechanisms of utilizing *E. coli* functions to promote its own growth (reviewed in ref. 2). One example of this is the replication of its DNA. Previous work has established that bacteriophage λ relies heavily on host-coded proteins for replication of its DNA. These include the dnaJ, dnaK, and grpE proteins which (a) are essential for λ DNA replication at all temperatures, (b) are heat shock proteins, and (c) are essential for host viability at 43°C. Interestingly, mutations in these three genes result in the same host phenotype at high temperature. That is, they block both RNA and DNA synthesis. Null or insertional mutants have been isolated in the *dnaK*and *dnaJ* genes (25; S. Sell, P. Kang, E. A. Craig and C. G., unpublished results). Such mutants are viable at 30°C and 37°C under laboratory

Figure A.5. Estimation of the molecular weight of the grpE protein under denaturing conditions. Five μg of grpE protein was combined with proteins of various molecular weights (listed in the legend of Fig. A.3) and electrophoresed on a 12.5% SDS-polyacrylamide gel. The mobilities of the molecular weight standards were calculated relative to the dye front. The slope and *y* intercept of the line were determined by linear regression.

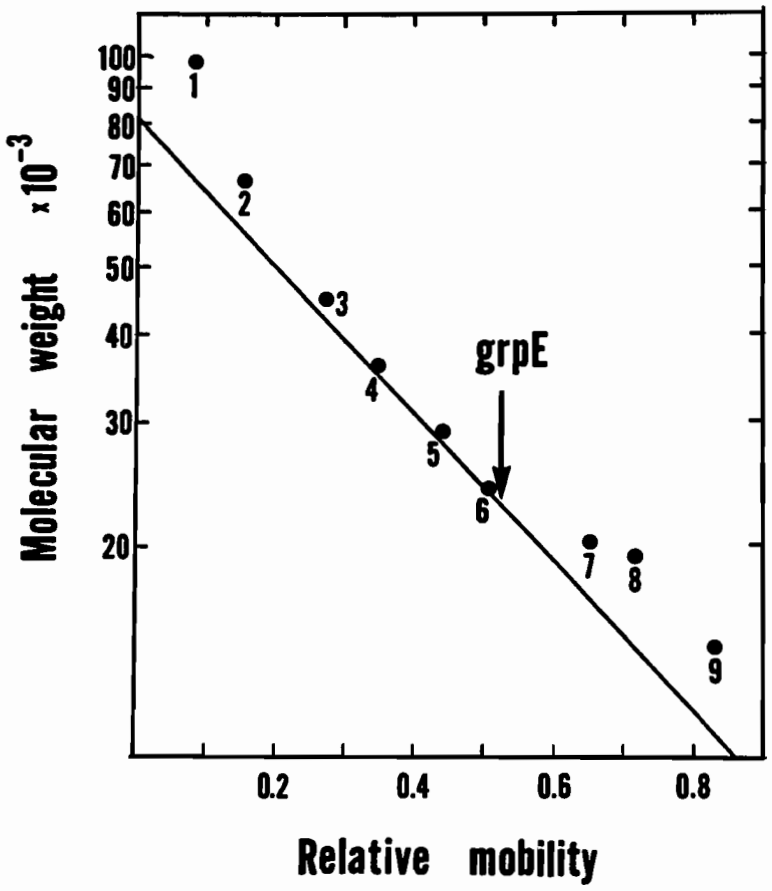

Molecular weight x10^{-3}
100
90
80
70
60
50
40
30
20
1
2
3
4
5
6
7
8
9
grpE
0.2
0.4
0.6
0.8
Relative mobility

Figure A.6. Sedimentation coefficient ($s_{20,w}$) of the purified grpE protein. One-hundred µl of grpE protein (approximately 1µg) in 25 mM Hepes/KOH, pH 8, 0.1 mM EDTA, 10 mM dithiothreitol, 0.1 M KCl was loaded onto a 20-40% glycerol gradient. Sedimentation proceeded for 28 h at 45,000 rpm at 0°C in a Beckman SW 50.1 rotor. Two-drop fractions (approximately 100 µl) were collected from the bottom of the tube. One-µl aliquots for each fraction were used to assay for replication activity of the grpE protein. In a parallel experiment, grpE protein was loaded onto a gradient in the presence of proteins with known sedimentation coefficients as standards: 1, transferrin, $s_{20,w} = 5.1$; 2, bovine serum albumin, $s_{20,w} = 4.3$; 3, ovalbumin, $s_{20,w} = 3.6$; and 4, trypsinogen, $s_{20,w} = 2.5$. The sedimentation position of each protein was determined following SDS-polyacrylamide gel electrophoresis of the gradient fractions.

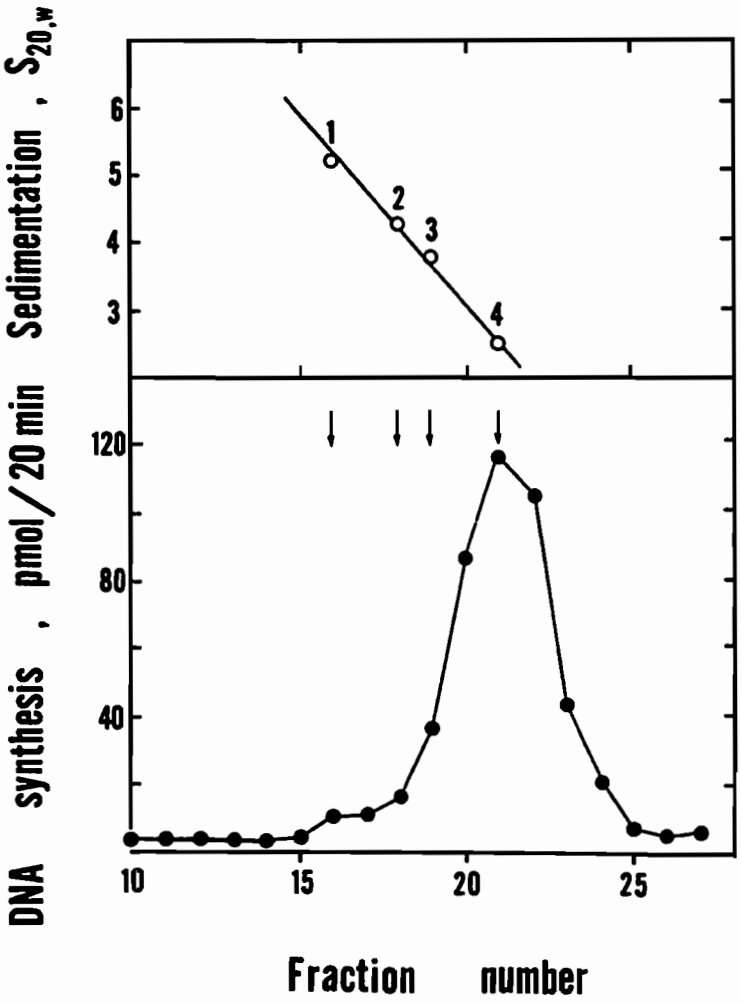
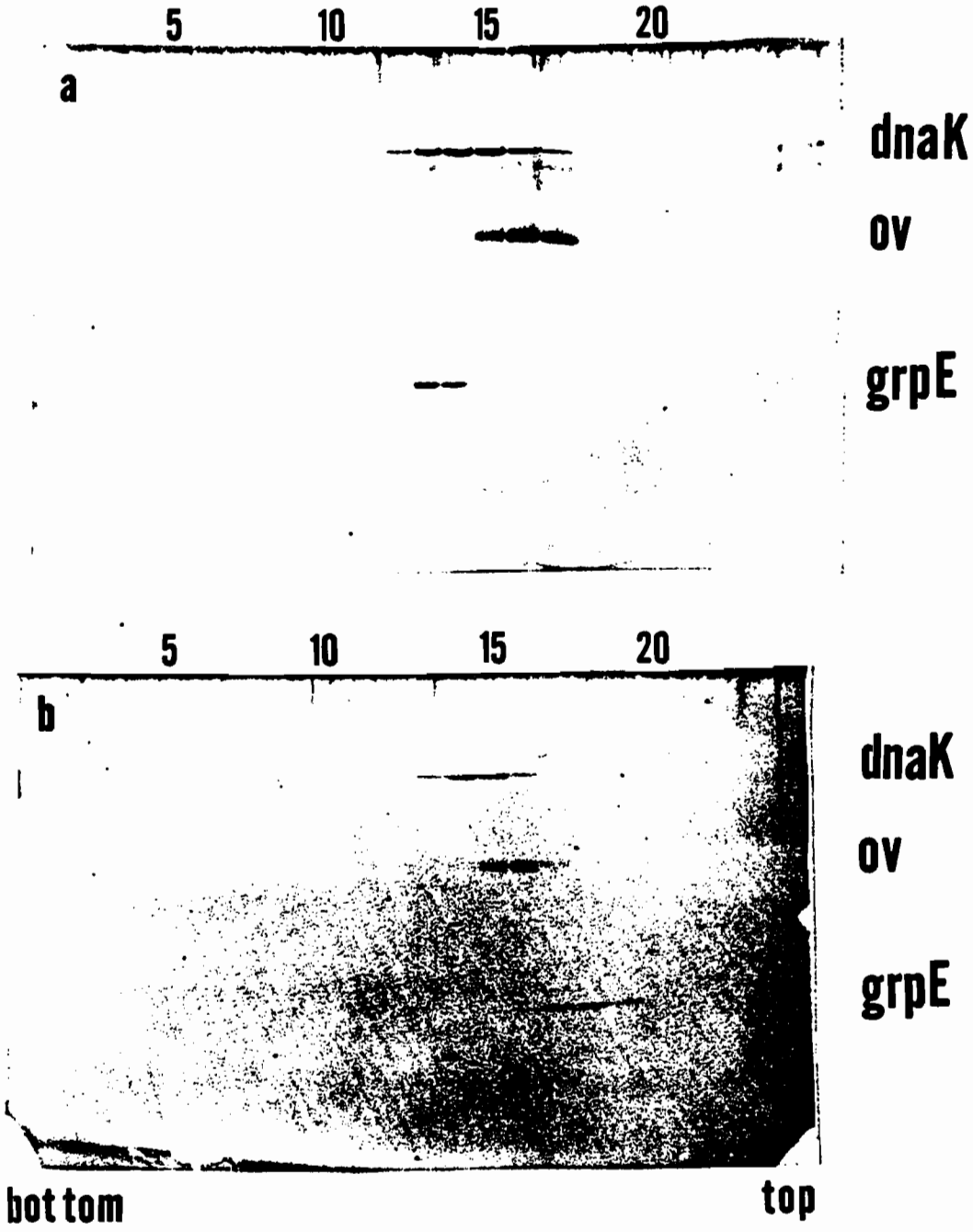

DNA synthesis , pmol/20 min Sedimentation , $S_{20,w}$
6
5
4
3
1
2
3
4
120
80
40
10
15
20
25
Fraction number

Figure A.7. Cosedimentation of the dnaK and grpE proteins in the presence and absence of ATP. Four μg of dnaK and 1.2 μg of grpE proteins were loaded onto 20-40% glycerol gradients (3.0 ml). Ovalbumin (ov) was included as a standard. The composition of the buffer was 25 mM Hepes/KOH, pH 8, 20 mM KCl, 10 mM dithiothreitol, 0.2 mM EDTA, 5 mM $MgCl_2$ and a) no ATP or b) 10 mM ATP throughout the gradient. The two proteins were incubated together at 30°C for 10 min prior to loading. The gradients were centrifuged in a Beckman SW 60.1 rotor at 50,000 rpm at 2°C for 24 h. Two-drop fractions (approximately 100 μl) were collected, and aliquots were electrophoresed on 12.5% SDS-polyacrylamide gels. Gels were silver-stained (22) to visualize protein.


5
10
15
20
a
dnaK
ov
grpE
5
10
15
20
b
dnaK
ov
grpE
bottom
top

conditions, but not at 43°C. However, growth of the mutant strains at the permissive temperature is slower than wild-type, with the concomitant selection for extragenic suppressors (S. Sell and C. G., unpublished results).

We have purified the grpE protein of *E. coli* to at least 95% homogeneity using a strain which overproduces heat shock proteins. This protein has the same isoelectric point as a protein encoded by a λ*grpE*$^+$ transducing phage (28). The one-column purification scheme was devised based on earlier immunoprecipitation results suggesting a physical interaction between the grpE and dnaK proteins. Recently, extragenic suppressors of the *grpE*280 mutation have been mapped in the region of the *dnaK* gene (C. Johnson and C. G., unpublished results), further supporting the existence of functional interactions between the two proteins *in vivo*. The grpE protein was eluted from the column in the presence of 10 mM ATP. This may be due to an ATP-induced conformational change in the dnaK protein which allows the release of the grpE protein. DnaK has both a weak 5'-nucleotidase and autophosphorylating activities (5, 46). Alternatively, ATP may induce a conformational change in both dnaK and grpE or in grpE alone. The interaction between grpE and dnaK has been verified by glycerol gradient centrifugation. Alone, the grpE protein sediments as expected of a M_r 23,000 monomer. Together with dnaK protein, however, grpE sediments with the leading fractions of dnaK, a position consistent with a 1:1 complex of the two proteins.

Previous work from this and other laboratories has established interesting protein-protein interactions in λ DNA replication. These include an interaction between λO and λP (35, 35), λP and dnaB (37, 18, 20, 36, 42), λP and dnaK (46), dnaJ and dnaB (K. Liberek, M. Z. and C. G., unpublished results), and grpE and dnaK (this work) proteins. All of these interactions appear to be important in *in vivo* λ DNA replication and together suggest the following model. The λO protein is known to

bind specifically to *oriλ* DNA sequences (38) and to form an "O-some" structure with the DNA wrapped around it (7). The λP protein binds to both dnaB and λO and, in so doing, localizes dnaB to the origin of λ DNA. This results in the formation of an even larger protein-DNA aggregate at *oriλ* (7). At this stage, the dnaB protein, which is a helicase (19), is thought to be tightly bound to λP. *In vitro* studies have shown that interaction of dnaB with λP partially inhibits the dnaB protein's NTPase activity (18). Additional multiple protein-protein interactions and the known DNA-dnaJ interaction (47) could result in a "loosening up" of the tight λP-dnaB interaction and the subsequent positioning of dnaB onto a single-stranded region near *oriλ*. This single-stranded region can be created *in vivo* as a consequence of the protein complex binding onto DNA and/or through transcription from p_R. Once the dnaB protein has gained access onto single-stranded DNA, it could then unwind the double-stranded DNA through its helicase and NTPase activities (19). In agreement with this model is the observation that, in the complete system of Dodson et al. (6), DNA unwinds from *oriλ*, specifically in the rightward direction. This system includes supercoiled λ*dv* plasmid, λO, λP, dnaB, dnaK, dnaJ, and single-stranded binding proteins. It is not clear why only unidirectional unwinding is observed *in vitro*. *In vivo*, early λ DNA replication is usually bidirectional (32); although occasionally unidirectionality, with rightward preference, has also been observed *in vivo* (31). It could be that a host factor, such as grpE, missing from this system is important in conferring bidirectionality to the system.

Recently, McMacken et al. (21) have reported that the addition of gyrase and DNA polymerase III to the above purified system results in λ*dv* replication *in vitro*. Two interesting observations were made. First, RNA polymerase transcription is not necessary in this system. It turns out that the HU DNA-binding protein (27) is an inhibitor of this system. On the basis of their results, McMacken et al. (21) propose

that RNA transcription *in vivo* and *in vitro* is necessary to antagonize the inhibitory role of HU protein. In the purified system, no HU protein is present; hence, there is no need for RNA polymerase transcription. Second, grpE is not essential in this system, as is also the case in the M13 single-stranded system. What, then, is the role of grpE in λ DNA replication? A key observation made by Alfano and McMacken (C. Alfano and R. McMacken, personal communication) is that, in the presence of grpE, the requirement for high levels of dnaK protein in the *in vitro* system is drastically reduced. Several possibilities can be considered to explain this result. It could be (a) that the dnaK-grpE complex is more active than the dnaK protein alone in performing the required dnaK function at *ori*λ, (b) that the only role of grpE protein is to allow for oligomerization of dnaK protein and that an oligomerized dnaK protein is the active species in λ DNA replication, or (c) that grpE "modifies" or stabilizes the dnaK protein into an active form and that such a modified dnaK form is the active species in λ DNA replication. Now that both purified grpE and dnaK proteins are available, these possibilities can be tested.

Literature Cited

1. **Anderl, A. and A. Klein.** 1982. Replication of λdv DNA in vitro. Nucleic Acids Res. **10**:1733-1740.

2. **Ang, D., G. N. Chandrasekhar, C. Johnson, M. Zylicz and C. Georgopoulos.** 1986. A genetic and biochemical analysis of the *Escherichia coli grpE* gene and its product. *In* T. Kelly and R. McMacken (eds.), UCLA Symp. Mol. Cell. Biol. New Series, **47**:521-532, Alan R. Liss, Inc., N. Y.

3. **Ang, D., G. N. Chandrasekhar, M. Zylicz and C. Georgopoulos.** 1986. The *grpE* gene of *Escherichia coli* codes for heat shock protein B25.3, essential for λ DNA replication at all temperatures and host viability at high temperature. J. Bacteriol. **167**:25-29.

4. **Bardwell, J. C. A. and E. A. Craig.** 1984. Major heat shock gene of *Drosophila* and the *Escherichia coli* heat inducible *dnaK* gene are homologous. Proc. Natl. Acad. Sci. U. S. A. **81**:848-852.

5. **Bochner, B. R., M. Zylicz and C. Georgopoulos.** 1986. *Escherichia coli* DnaK protein possesses a 5'-nucleotidase activity that is inhibited by AppppA. J. Bacteriol. **168**:931-935.

6. **Dodson, M., H. Echols, S. Wickner, C. Alfano, K. Mensa-Wilmot, B. Gomes, J. LeBowitz, J. D. Roberts and R. McMacken.** 1986. Specialized nucleoprotein structures at the origin of replication of bacteriophage λ: localized unwinding of duplex DNA by a six-protein reaction. Proc. Natl. Acad. Sci. U. S. A. **83**:7638-7642.

7. **Dodson, M., J. Roberts, R. McMacken and H. Echols.** 1985. Specialized nucleoprotein structures at the origin of replication of bacteriophage λ: complexes with λ O, λ P and *Escherichia coli* DnaB proteins. Proc. Natl. Acad. Sci. U. S. A. **82**:4678-4682.

8. **Edman, P. and G. Begg.** 1967. A protein sequenator. Eur. J. Biochem. **1**:80-91.

9. **Formosa, T., R. L. Burke and B. M. Alberts.** 1983. Affinity purification of bacteriophage T4 proteins essential for DNA replication and genetic recombination. Proc. Natl. Acad. Sci. U. S. A. **80**:2442-2446.

10. **Friedman, D. I., E. R. Olson, K. Tilly, C. Georgopoulos, I. Herskowitz and F. Banuett.** 1984. Interactions of bacteriophage λ and host macromolecules in the growth of bacteriophage λ. Microbiol. Rev. **48**:299-325.

11. **Fuller, R. S., J. M. Kaguni and A. Kornberg.** 1981. Enzymatic replication of the origin of the *Escherichia coli* chromosome. Proc. Natl. Acad. Sci. U. S. A. **78**:7370-7374.

12. **Furth, M. E. and S. H. Wickner.** 1983. Lambda DNA replication. *In* R. W. Hendrix, J. W. Roberts, F. W. Stahl and R. A. Weisberg (ed.), Lambda II, p. 145-173, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

13. **Georgopoulos, C. P.** 1977. A new bacterial gene (*groPC*) which affects λ DNA replication. Mol. Gen. Genet. **151**:35-39.

14. **Georgopoulos, C. P. and I. Herskowitz.** 1971. *Escherichia coli* mutants blocked in lambda DNA synthesis. *In* A.D. Hershey (ed.), The bacteriophage lambda, p. 553-564, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

15. **Georgopoulos, C. P., K. Tilly, D. Drahos and R. Hendrix.** 1982. The B66.0 protein of *Escherichia coli* is the *dnaK*$^+$ gene product. J. Bacteriol. **149**:1175-1177.

16. **Grossman, A. D., J. W. Erickson and C. A. Gross.** 1984. The *htpR* gene product of *E. coli* is a sigma factor for heat shock promoters. Cell **38**:383-390.

17. **Itikawa, H. and J.-I. Ryu.** 1979. Isolation and characterization of a temperature-sensitive *dnaK* mutant of *Escherichia coli* B. J. Bacteriol. **138**:339-344.

18. **Klein, A., E. Lanka and E. Schuster.** 1980. Isolation of a complex between the *P* protein of phage λ and *dnaB* protein of *Escherichia coli*. Eur. J. Biochem. **105**:1-6.

19. **LeBowitz, J. H. and R. McMacken.** 1986. The *Escherichia coli* DnaB replication protein is a DNA helicase. J. Biol. Chem. **261**:4738-4748.

20. **McMacken, R., M. S. Wold, J. H. LeBowitz, J. D. Roberts, J. B. Mallory, J. A. K. Wilkinson and C. Loehrlein.** 1983. Initiation of DNA replication *in vitro* promoted by the bacteriophage λO and P replication proteins. *In* N. R. Cozzarelli (ed.), UCLA Symp Mol. Cell. Biol. Ser., **10**:819-848, Alan R. Liss, Inc., N. Y.

21. **McMacken, R., C. Alfano, B. Gomes, J. H. LeBowitz, K. Mensa-Wilmot, J. D. Roberts and M. Wold.** 1986. Biochemical mechanisms in the initiation of bacteriophage λ DNA replication. *In* T. Kelly and R. McMacken (eds.), UCLA Symp. Mol. Cell. Biol. New Series, **47**:227-246, Alan R. Liss, Inc., N. Y.

22. **Morrissey, J. H.** 1981. Silver stain for protein in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. **117**:307-310.

23. **Neidhardt, F. C., R. A. VanBogelen and V. Vaughn.** 1984. The genetics and regulation of heat-shock proteins. Annu. Rev. Genet. **18**:295-329.

24. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007-4021.

25. **Paek, K.-H. and G. C. Walker.** 1987. *Escherichia coli dnaK* null mutants are inviable at high temperature. J. Bacteriol. **169**:283-290.

26. **Roberts, J. D. and R. McMacken.** 1983. The bacteriophage λ O replication protein: isolation and characterization of the amplified initiator. Nucleic Acids Res. **11**:7435-7452.

27. **Rouviere-Yaniv, J. and F. Gros.** 1975. Characterization of a novel, low-molecular-weight DNA-binding protein from *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **72**:3428-3432.

28. **Saito, H., Y. Nakamura and H. Uchida.** 1978. A transducing λ phage carrying *grpE*, a bacterial gene necessary for λ DNA replication, and two ribosomal protein genes, *rpsP* (S16) and *rplS* (L19). Mol. Gen. Genet. **165**:247-256.

29. **Saito, H. and H. Uchida.** 1977. Initiation of the DNA replication of bacteriophage lambda in *Escherichia coli* K-12. J. Mol.Biol. **113**:1-25.

30. **Saito, H. and H. Uchida.** 1978. Organization and expression of the *dnaJ* and *dnaK* genes of *Escherichia coli* K-12. Mol. Gen. Genet. **164**:1-8.

31. **Schnos, M., K. J. Denniston, F. R. Blattner and R. B. Inman.** 1982. Replication of bacteriophage λ DNA. Examination of variants containing double origins and observation of a bias in directionality. J. Mol. Biol. **159**:441-455.

32. **Schnos, M. and R. B. Inman.** 1970. Position of branch points in replicating λ DNA. J. Mol. Biol. **51**:61-73.

33. **Sunshine, M., M. Feiss, J. Stuart and J. Yochem.** 1977. A new host gene (*groPC*) necessary for lambda DNA replication. Mol. Gen. Genet. **151**:27-34.

34. **Tilly, K., N. McKittrick, M. Zylicz and C. Georgopoulos.** 1983. The DnaK protein modulates the heat shock response of *Escherichia coli*. Cell **34**:641-646.

35. **Tomizawa, J.-I.** 1971. Functional cooperation of genes *O* and *P*. *In* A. D. Hershey (ed.), The bacteriophage lambda, pp. 549-552. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

36. **Tsurimoto, T. and K. Matsubara.** 1982. Replication of bacteriophage λ DNA. Cold Spring Harbor Symp. Quant. Biol. **47**:681-691.

37. **Tsurimoto, T. and K. Matsubara.** 1982. Replication of λdv plasmid *in vitro* by purified λ O and P proteins. Proc. Natl. Acad. Sci. U. S. A. **79**:7639-7643.

38. **Tsurimoto, T. and K. Matsubara.** 1981. Purified bacteriophage λ O protein binds to four repeating sequences at the λ replication origin. Nucleic Acids Res. **9**:1789-1799.

39. **Tsurimoto, T., T. Hase, H. Matsubara and K. Matsubara.** 1982. Bacteriophage lambda initiators: preparation from a strain that overproduces the O and P proteins. Mol. Gen. Genet. **187**:79-86.

40. **Wada, M., Y. Kadokami and H. Itikawa.** 1982. Thermosensitive synthesis of DNA and RNA in *dnaJ* mutants of *Escherichia coli* K-12. Jpn. J. Genet. **57**:407-413.

41. **Welch, W. J. and J. R. Feramisco.** 1985. Rapid purification of mammalian 70,000-dalton stress proteins: affinity of the proteins for nucleotides. Mol. Cell. Biol. **5**:1229-1237.

42. **Wickner, S.** 1978. DNA replication proteins of *Escherichia coli* and phage λ. Cold Spring Harbor Symp. Quant. Biol. **43**:303-310.

43. **Wold, M., J. Mallory, J. Roberts, J. LeBowitz and R. McMacken.** 1982. Initiation of bacteriophage λ DNA replication in vitro with purified λ replication proteins. Proc. Natl. Acad. Sci. U. S. A. **79**:6176-6180.

44. **Zylicz, M. and C. Georgopoulos.** 1984. Purification and properties of the *Escherichia coli* dnaK replication protein. J. Biol. Chem. **259**:8820-8825.

45. **Zylicz, M, I. Gorska, K. Taylor and C. Georgopoulos.** 1984. Bacteriophage λ replication proteins: formation of a mixed oligomer and binding to the origin of λ DNA. Mol. Gen. Genet. **196**:401-406.

46. **Zylicz, M., J. H. LeBowitz, R. M. McMacken and C. Georgopoulos.** 1983. The dnaK protein of *Escherichia coli* possesses an ATPase and autophosphorylating activity and is essential in an *in vitro* DNA replication system. Proc. Natl. Acad. Sci. U. S. A. **80**:6431-6435.

47. **Zylicz, M., T. Yamamoto, N. McKittrick, S. Sell and C. Georgopoulos.** 1985. Purification and properties of the dnaJ replication protein of *Escherichia coli*. J. Biol. Chem. **260**:7591-7598.

APPENDIX B

SEQUENCE ANALYSIS AND TRANSCRIPTIONAL REGULATION OF THE *Escherichia coli grpE* GENE, ENCODING A HEAT SHOCK PROTEIN

Nucleic Acids Res. **16**:7545-7562

Abstract

We have sequenced the *Escherichia coli grpE* gene and shown that it encodes a 197-amino acid residue protein of 21,668-Mr. The predicted N-terminal amino acid sequence, as well as the overall amino acid composition agree well with that of the purified protein. From Northern analysis, we have shown that transcription of the *grpE* gene is under heat shock regulation, i.e., there is a rapid and transient increase in the rate of synthesis of *grpE* mRNA upon a shift-up in temperature. Forty-six bases upstream of the structural gene is a sequence closely related to the consensus heat shock promoter identified by Cowing et al. [Proc. Natl. Acad. Sci. U. S. A., 82:2679-2683]. We have shown by S1 mapping and RNA sequencing that this is indeed the promoter for the *grpE* mRNA. It appears that all discernible transcription initiates only from this promoter, even under non-heat shock conditions.

Introduction

The *grpE* gene of *E. coli* was initially identified by a mutation, *grpE*280, which inhibits replication of bacteriophage λ DNA at all temperatures (15). In the presence of this mutation, *E. coli* itself is temperature-sensitive for growth. Both host RNA and DNA syntheses are inhibited at the non-permissive temperature (1). The phenotypes exhibited by the *E. coli grpE*280 mutant are shared by strains with mutations in the *dnaK*, *dnaJ*, *groEL* and *groES* genes, whose products are all required for λ growth (reviewed in ref. 5).

It was shown earlier that the grpE protein is identical to the heat shock protein, B25.3, of *E. coli* (1). The *dnaK*, *dnaJ*, *groEL* and *groES* gene products are also members of the so-called heat shock regulon of *E. coli* (for a review of the heat shock response in *E. coli*, see ref. 14). When cells experience stressful conditions - for example, a sudden shift-up in temperature - these genes are rapidly and transiently

expressed at a higher rate (14). Although the function of these gene products during stressful periods is not clear, it could be that some are required to protect the integrity of the cell and that others are expressed in anticipation of recovery. Regulation of those genes examined is due to the product of the *htpR* (*rpoH*) gene, which is a sigma factor (σ^{32}) of the RNA polymerase and which allows specific recognition of heat shock promoters (7). In this paper, we present the nucleotide sequence of the *grpE* gene. We show that its transcription is under heat shock regulation and, more specifically, under *htpR* (*rpoH*) control. We show that the promoter sequence for the *grpE* gene agrees with the previously published consensus heat shock promoter sequence (3).

Materials and Methods

Bacterial and phage strains

The isogenic B178 and B178 *grpE*280 strains have been previously described (1). The origin of transducing bacteriophage $\lambda grpE^{+}\Delta 7$ has been described (16). Strain NM522 71/18 $hsdR^{-}hsdM^{+}$ (4), used for growing male-specific phages, was obtained from Dr. Noreen Murray, Department of Molecular Biology, University of Edinburgh, Edinburgh, Scotland. The bacteriophage M13 IR-1 has been described (4). The isogenic $htpR^{+}$ and *htpR*165 strains (13) were originally obtained from Dr. Fred Neidhardt, Department of Microbiology, University of Michigan Medical School. The *htpR*6 and *htpR*15 strains (25) were obtained from Dr. Takashi Yura, University of Kyoto, Japan. The *htpR*6 and *htpR*15 alleles were subsequently moved into the B178 background by P1 transduction (12) using the nearby tet^{r} marker for selection and subsequently screening for the HtpR phenotype.

Plasmids

The pEMBL8+ plasmid vector has been described (4).

Media

The media for propagation of bacteria and phage were as described previously (1). Twenty μg/ml of either ampicillin or tetracycline was added to media used for selection of plasmids or transductants.

Plasmid DNA extraction and transformations

The procedures followed were essentially those outlined by Maniatis et al. (10). Plasmid and phage DNAs were purified at least once through a CsCl equilibrium gradient before use (10).

Restriction enzyme digestions

The procedures followed were essentially those recommended by the manufacturers. Restriction endonucleases were purchased from Bethesda Research Laboratories and New England Biolabs.

Ligations

DNA ligations were carried out with T4 DNA ligase (New England Biolabs) as described by Maniatis et al. (10).

DNA sequencing procedures

The techniques for the chain-termination method (19) were as described by Messing (11) except that the pEMBL vector/phage system was used (4). Dideoxynucleotide triphosphates and DNA primers were purchased from Pharmacia/P-

L Biochemicals. Klenow enzyme was purchased from Boehringer Mannheim. Synthetic oligomers used as primers were purchased from Dr. Kirk Thomas, Department of Biology, University of Utah, Salt Lake City, Utah. The synthetic oligomers were complementary or identical to nucleotides 582-595, 948-967 and 986-1006 of the coding sequence of the *grpE* gene.

S1 mapping

Plasmid pJK23 was digested with Ava II, and the 561-nt fragment was purified following electrophoresis in low melting agarose (10). This fragment was subsequently digested with Bam HI. The Ava II-Bam HI 226-nt fragment was recovered from a 5% polyacrylamide gel (10), purified on a NENSORB column (New England Nuclear) according to the manufacturer's protocol and end-labeled at the 5' ends with [γ-^{32}P]-ATP (5000 Ci/mmol; Amersham) using T4 polynucleotide kinase (IBI) in a forward reaction (2). The labeled probe was hybridized to total RNA preparations in the presence of 80% formamide at 51°C, according to a procedure described for double-stranded probes which favors formation of RNA-DNA hybrids (24). The hybrids were treated with S1 nuclease (Boehringer-Mannheim) at a final concentration of 600 U/ml unless stated otherwise.

RNA sequencing

RNA sequencing was carried out according to a method utilizing dideoxynucleotides (Pharmacia/P-L Biochemicals) (8). Approximately 1.5 µg of total RNA, prepared as described below, and reverse transcriptase (IBI) at a final concentration of 750 U/ml were used per set of sequencing reactions. A 20-nt oligomer complementary to the nucleotides 669-688 of the *grpE* sequence was used as the primer.

Electrophoresis of DNA

Samples from Sl mapping and RNA sequencing experiments were resolved on 6% polyacrylamide sequencing gels (18). DNA fragments after digestion with restriction enzymes were electrophoresed on agarose or acrylamide gels according to methods described by Maniatis et al. (10).

Preparation of RNA

RNA was extracted in a hot SDS solution, as described previously (23), from cells grown at 30°C (30° RNA) and from cells which were shifted to 44°C at the desired time before extraction (44° RNA).

Northern blotting

Gels of total RNA samples were blotted to nitrocellulose (Schleicher and Schuell) as described before (23). The Bam HI-Hind III 480-nt fragment of plasmid pJK23, which carries most of the *grpE* coding sequence, or the Pst I-EcoRI fragment of plasmid pJK23, which carries the entire *grpE* gene and flanking sequences, was labeled with [α-^{32}P]-dCTP (3000 Ci/mmol; NEN Research Products) by nick-translation (10).

Results

Cloning of the DNA fragment encoding the *grpE* gene.

The *grpE* gene was originally isolated from a λ library of *E. coli* DNA by selecting for λ growth on an *E. coli* strain with the *grpE*280 mutation (16). Deletions of this λ transducing phage were selected, some of which still maintained the ability to complement the *grpE*280 mutation (16). We used one of these, $\lambda grpE^{+}\Delta 7$ as the

substrate for a partial Sau 3A digest. The digested DNA was cloned into the pEMBL8+ vector (8) at the unique Bam HI site. Plasmids encoding the *grpE* gene were selected by transforming the *grpE*280 mutant, which is temperature-sensitive for growth (1), and isolating colonies at 43°C. The plasmid, pJK23, which has an approximately 1700-bp insert of *E. coli* DNA, was used to derive various subclones. The modified chain-termination method described by Messing (11) was used to generate the overlapping sequences shown in Fig. B.1. In three cases, synthetic oligomers were used to prime chain synthesis in regions lacking convenient restriction sites. Subclones of the pJK23 insert carrying sequences only to the right or only to the left of the internal Bam HI site no longer rescued the GrpE phenotype, suggesting that this site lay either within the structural gene or within the control regions of the gene. Completion of the sequence showed that the Bam HI site indeed lies 115 bp downstream of the initiation codon.

The sequence of the *grpE* gene is shown in Fig. B.2. It encodes a 197-amino acid residue protein with a predicted molecular weight of 21,668. Its apparent molecular weight on a denaturing SDS-polyacrylamide gel is approximately 23,000 daltons (1, 16). Table B.1 compares the predicted amino acid composition with that determined by acid hydrolysis of the purified protein (21, 26). From its composition, the grpE protein appears to be acidic. This is in agreement with its migration in an isoelectric focusing gel, which predicts an apparent pI of 4.9 (1, 16). Amino-terminal analysis of the purified protein showed that the sequence of the first 15 residues, Ser-Ser-Lys-Glu-Gln-Lys-Thr-Pro-Glu-Gly-Gln-Ala-Pro-Glu-Glu, agrees with that predicted by the nucleotide sequence, except that the initial methionine residue is missing in the *in vivo* product. These results demonstrate that the correct gene has been cloned and sequenced.

Figure B.1. Sequencing strategy of the *grpE* gene. The *E. coli* DNA fragment cloned into pEMBL8+, resulting in pJK23, is shown by the shaded bar with nearby flanking vector sequences shown by a line. Pertinent restriction sites are shown above. The coding sequence of the *grpE* gene is shown by the boldface arrow just below the shaded bar. The other arrows indicate the extent and orientation of the sequencing. In most cases, convenient restriction sites allowed the use of a universal primer complementary to a sequence found in the vector. However, in three cases, the absence of any convenient restriction sites required the use of synthetic primers (see **Materials and Methods**). B, Bam HI; B2, Bgl II; C, Cla I; H, Hind III; P, Pst I; P2, Pvu II; S, Sal I; S3, Sau 3A; X, Xma I.

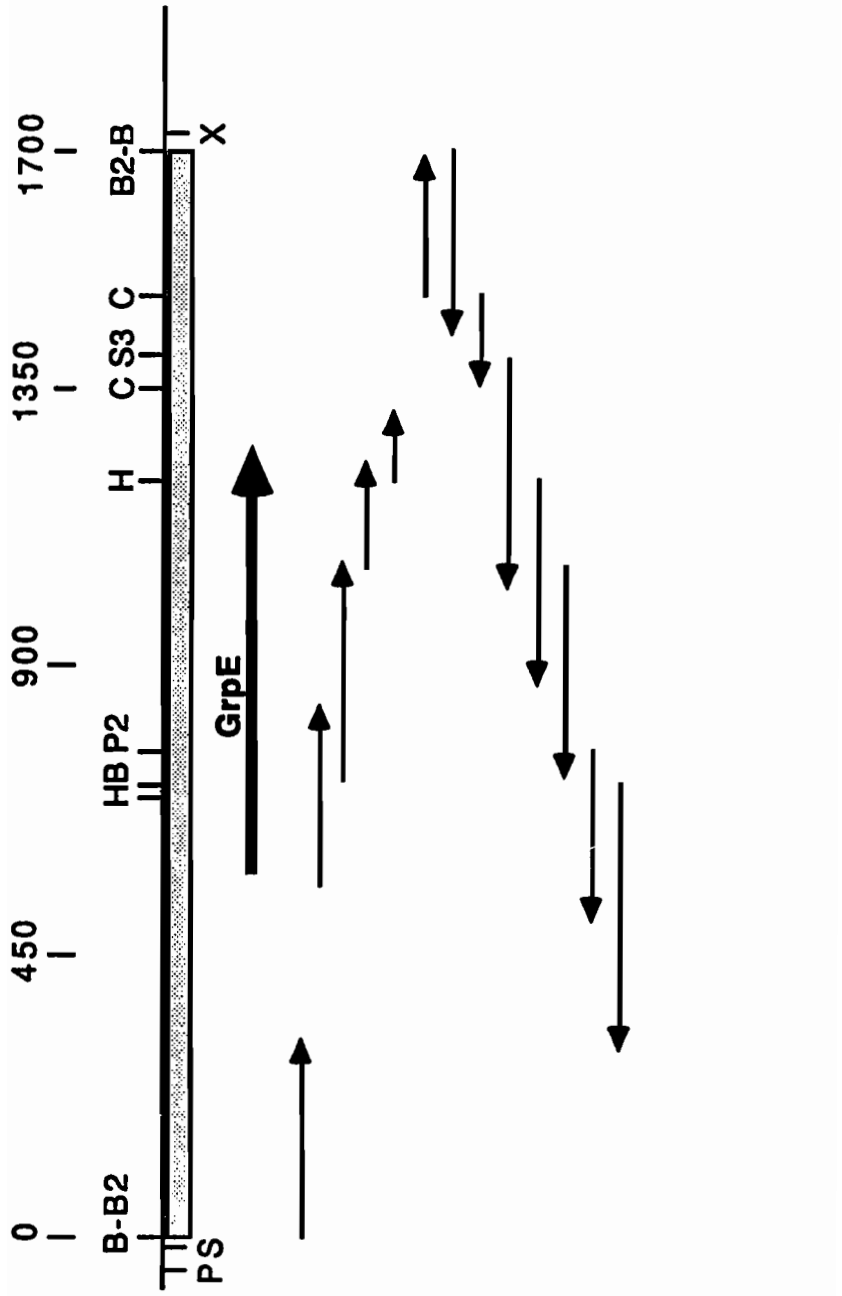

0
450
900
1350
1700
B-B2
HB P2
H
C S3 C
B2-B
X
PS
GrpE

Figure B.2. Nucleotide sequence of the *grpE* gene. The *grpE* DNA sequence is shown in capital letters. The amino acid sequence of the grpE protein is shown in italics, starting with the AUG initiation codon at nucleotide position 616.

```
1     TTCGAACTAAATCATATGCGCCAGCTTTGCCTGGAATGAAGCACCACTTCATTTATCGCGGTGCTGATGCGTTTC

76    TGGCCAATCTTGCTAGACAGACTTGCGCTTCCAGCAAAAAACGTTTCTCGCTGATGTAGTGGCCCTTCCAGCACA

151   TCGGCTAACTGTTGCTGGGCGTTATCGGGGTCAAGGTCAGTCAGGAAACCCAGGTTGCCACGGTTGATTCCAATA

226   ACTTTAATATCGTAACGGGCGAGTGTGCGTGCCGCGCCCAGCATATTACCGTCGCCACCAACGACTACCGCGAGA

301   TCAGCTAGTTGCCCAATCTCCGCGAGCGTGCCAGTTTTCACATTCTTCAGTTGCAGTTCGTGAGCGATTTGTTGC

376   TCAACGATGACCTCGTAACCTTTTGTGCACAGCCAGCGGTAGAGCATTTCATGTGTTGTCAGTGCAGTGGGGTGC

451   CGTGGTGTCCCACAATGCCAATACACTTGAAATGATTATTCATTTTTCCGAGGTCCTTGTTGCGAAGATTGATGA

526   CAATGTGAGTGCTTCCCTTGAAACCCTGAAACTGATCCCCATAATAAGCGAAGTTAGCGAGATGAATGCGAAAAA

601   AACGCGGAGAAATTC ATG AGT AGT AAA GAA CAG AAA ACG CCT GAG GGG CAA GCC CCG GAA
                      f-met ser ser lys glu gln lys thr pro glu gly gln ala pro glu

661   GAA ATT ATC ATG GAT CAG CAC GAA GAG ATT GAG GCA GTT GAG CCA GAA GCT TCT GCT
      glu ile ile met asp gln his glu glu ile glu ala val glu pro glu ala ser ala

718   GAG CAG GTG GAT CCG CGC GAT GAA AAA GTT GCG AAT CTC GAA GCT CAG CTG GCT GAA
      glu gln val asp pro arg asp glu lys val ala asn leu glu ala gln leu ala glu

775   GCC CAG ACC CGT GAA CGT GAC GGC ATT TTG CGT GTA AAA GCC GAA ATG GAA AAC CTG
      ala gln thr arg glu arg asp gly ile leu arg val lys ala glu met glu asn leu

832   CGT CGT CGT ACT GAA CTG GAT ATT GAA AAA GCC CAC AAA TTC GCG CTG GAG AAA TTC
      arg arg arg thr glu leu asp ile glu lys ala his lys phe ala leu glu lys phe

889   ATC AAC GAA TTG CTG CCG GTG ATT GAT AGC CTG GAT CGT GCG CTG GAA GTG GCT GAT
      ile asn glu leu leu pro val ile asp ser leu asp arg ala leu glu val ala asp

946   AAA GCT AAC CCG GAT ATG TCT GCG ATG GTT GAA GGC ATT GAG CTG ACG CTG AAG TCG
      lys ala asn pro asp met ser ala met val glu gly ile glu leu thr leu lys ser

1003  ATG CTG GAT GTT GTG CGT AAG TTT GGC GTT GAA GTG ATC GCC GAA ACT AAC GTC CCA
      met leu asp val val arg lys phe gly val glu val ile ala glu thr asn val pro

1060  CTG GAC CCG AAT GTG CAT CAG GCC ATC GCA ATG GTG GAA TCT GAT GAC GTT GCG CCA
      leu asp pro asn val his gln ala ile ala met val glu ser asp asp val ala pro

1117  GGT AAC GTA CTG GGC ATT ATG CAG AAG GGT TAT ACG CTG AAT GGT CGT ACG ATT CGT
      gly asn val leu gly ile met gln lys gly tyr thr leu asn gly arg thr ile arg

1174  GCG GCG ATG GTT ACT GTA GCG AAA GCA AAA GCT TAATTTCTGCTTTCGTAATAATTCACGGCCC
      ala ala met val thr val ala lys ala lys ala

1238  TGCATGCGAATGCCGGGCCGTTTTCGTTACTCCGCCACACTCTCGCGCAGCGGTTTCACAGGAAAAACTTTTACC

1313  TGCTTAATCATATTGTCCTGTACGTCGAGAATATCGATATCGTACTCGCCAATACGCACGCGGGTGCCTGCGACA

1388  GGGATCTCTTCCATGCCTCAAGAATGACGCCATTAACCGTGCGGGCATCATCTTCCGGTAGATGCCAGTTAAAGG

1463  CTTTGTTGATTTCCGCACGTTGCGGTGCCATCGATAATCACCGAACCGTCGTTTTGCGGCGTGACCTCTTGGCAA

1538  GTGTTGGCGCATCGACGTAGTGAAATCGCCGACAATCTCTTCCAG
```

Table B.1

Amino acid composition of the grpE protein

	Actual*	Predicted
Ala	24.4	24
Arg	12	11
Asn]	20.5	8
Asp]		13
Cys	0	0
Glu]	31.4	26
Gln]		8
Gly	9.2	8
His	3.1	3
Ile	9.7	12
Leu	16.9	16
Lys	14.1	13
Met	8.1	9
Phe	2.9	3
Pro	10.3	9
Ser	7.4	7
Thr	8.5	8
Trp	ND**	0
Tyr	1.4	1
Val	17.3	18

* The amino acid analysis was done in the laboratory of Dr. W. Gray, Department of Biology, University of Utah. The purified grpE protein (26) was hydrolyzed with redistilled 6 N HCl *in vacuo* for 20 h at 105°C. Analysis was carried out on a Beckman 121C amino acid analyzer (21).

**Not determined. The acid hydrolysis treatment destroys the amino acid tryptophan.

In Fig. B.3A, the consensus heat shock promoter sequence (3) is compared with a sequence located 46 nucleotides upstream of the coding sequence of the *grpE* gene. In Fig. B.3A, this sequence is also compared with the consensus sequence recognized by the canonical RNA polymerase-σ^{70} holoenzyme (3). The data shown below support the assignment of this sequence as the promoter of the *grpE* transcript.

The sequence of the *grpE* gene was also analyzed for possible termination sites. Using the Perceptron program (22), a site 17 bases downstream of the termination codon was found which may form a stem-loop structure (Fig. B.3B) and cause the RNA polymerase enzyme to terminate transcription *in vivo*.

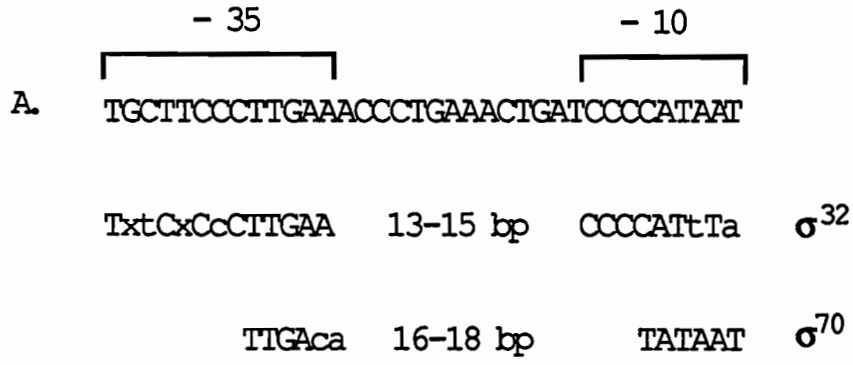
Positive regulation of *grpE* gene transcription by temperature shift and by the *htpR* (*rpoH*) gene product

It had been shown previously that the *grpE* gene product is identical to the heat shock protein B25.3, whose level increases dramatically upon a temperature shift (1). The heat shock response, which is universal to most cells examined (14), involves a rapid and transient induction of a small set of proteins thought to be required for protection and recovery of the cell during periods of stress (14). Of the genes examined, it has been shown that their transcription is positively regulated by the *htpR* (*rpoH*) gene product, which is a sigma factor of RNA polymerase (7), and is required for the recognition of heat shock promoters (7). To see if the rate of synthesis of the *grpE* gene is transcriptionally regulated, we examined by Northern analysis the levels of *grpE* mRNA in *E. coli* cells before and after heat shock. We found that the level of *grpE* mRNA increases rapidly between 2.5 and 4 minutes after shift from 30°C to 44°C, reaches a maximum at about 5 minutes and slowly decreases to return to a steady low level after approximately 20 minutes at the elevated temperature (Fig. B.4A). The estimated size of this heat-inducible *grpE* mRNA is 700 (±50) nucleotides

Figure B.3. Putative promoter and termination sequences found near the *grpE* coding region.

A. The first line shows the heat shock promoter-like sequence found 46 nucleotides upstream of the structural gene. The following two lines show the consensus promoter sequences recognized by the *htpR* (*rpoH*) and the *rpoD* gene products, the σ^{32} and σ^{70} subunits of RNA polymerase, respectively (3). Nucleotides shown in lower case are less conserved. See text for discussion.

B. The Perceptron program (22) was used to search the *grpE* sequence for potential stem-loop structures which may serve as termination sites for RNA polymerase *in vivo*. This figure shows one such possible structure located 17 nucleotides downstream of the termination codon. G-U base pairing was not allowed in this particular search.


- 35
- 10
A.
TGCTTCCCTTGAAACCCTGAAACTGATCCCCATAAT
TxtCxCcCTTGAA 13-15 bp CCCCATtTa σ32
TTGAca 16-18 bp TATAAT σ70

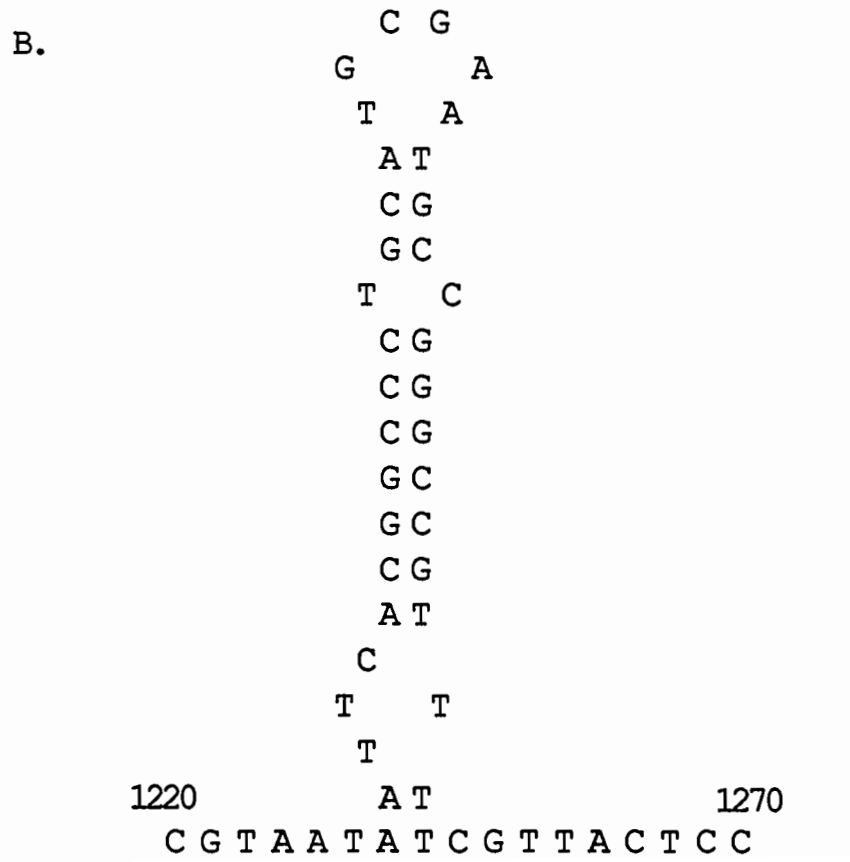
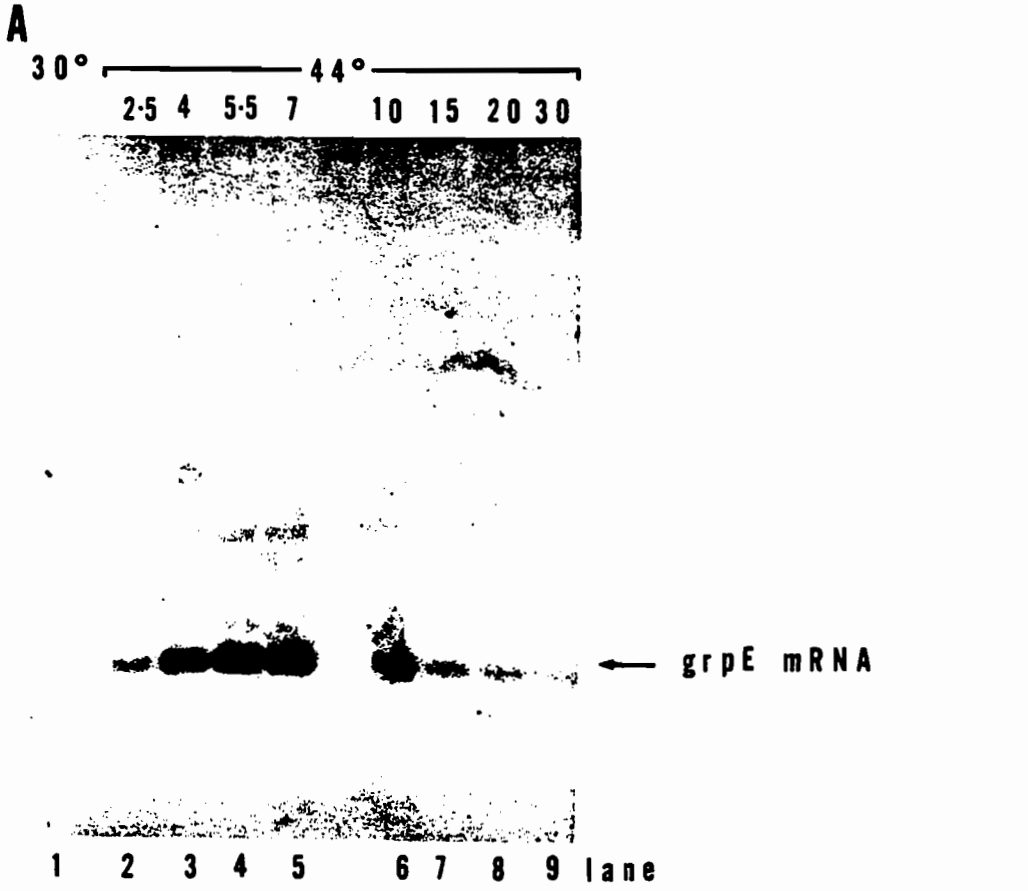
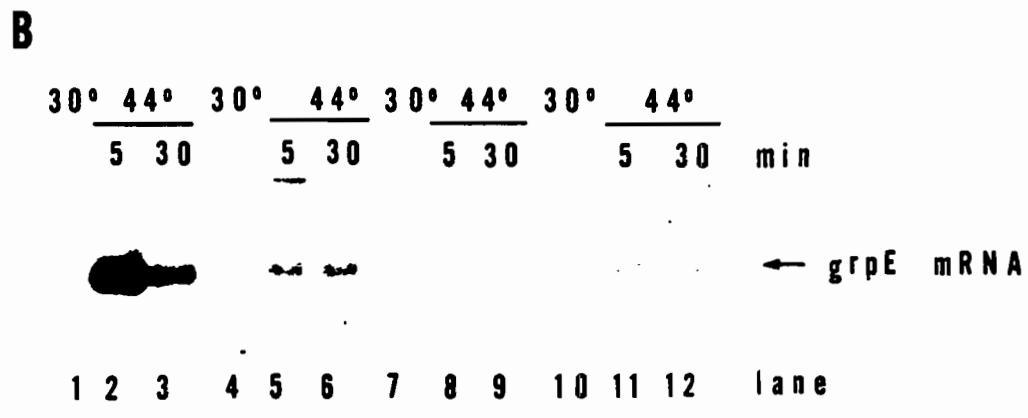

B.
C G
G A
T A
A T
C G
G C
T C
C G
C G
C G
G C
G C
C G
A T
C
T T
T
1220 A T 1270
C G T A A T A T C G T T A C T C C

Figure B.4. Regulation of *grpE* gene transcription by temperature shift and the *htpR* gene product. *E. coli* cells were grown at 30°C and subsequently transferred to 44°C. RNA was extracted from the 30° cells and the 44° cells at various times after the temperature shift, and electrophoresed on 1% denaturing agarose gels.

A. RNA extracted from *E. coli* B178 cells grown at 30°C (lane 1) and at 2.5, 4, 5.5, 7, 10, 15, 20 and 30 min after temperature shift to 44°C (lanes 2-9). The filter was probed with the nick-translated 480-bp Bam HI-Hind III fragment of pJK23 containing most of the coding sequence of the *grpE* gene.

B. RNA extracted from *E. coli* B178 (lanes 1-3), *E. coli htpR*15 (lanes 4-6), *E. coli htpR*6 (lanes 7-9) and *E. coli htpR*165 (lanes 10-12). Cells were grown at 30°C (lanes 1, 4, 7, 10) or shifted to 44°C for 5 min (lanes 2, 5, 8, 11) or 30 min (lanes 3, 6, 9, 12). The filter was probed with nick-translated DNA containing the entire *grpE* gene (the Pst I-EcoRI fragment of pJK23). It was also probed with *htpR* sequences, accounting for the doublet seen just above the *grpE* mRNA.


A
30°
44°
2·5 4 5·5 7 10 15 20 30
grpE mRNA
1 2 3 4 5 6 7 8 9 lane


B
30° 44° 30° 44° 30° 44° 30° 44°
5 30 5 30 5 30 5 30 min
grpE mRNA
1 2 3 4 5 6 7 8 9 10 11 12 lane

(data not shown). This would correspond to a protein with a maximum size of approximately 230 amino acid residues. In all probability then, the *grpE* gene is monocistronic.

Since the *htpR* (*rpoH*) gene product, σ^{32}, has been shown to positively regulate transcription of other heat shock genes (14, 7), we determined whether the *htpR* (*rpoH*) gene has an effect on *grpE* mRNA synthesis. We isolated RNA from different *htpR* mutants during growth at 30°C and following a shift to 44°C. The RNA was examined by Northern blot analysis (Fig. B.4B). Previously, it had been shown that at 30°C, the relative rate of basal level expression of heat shock proteins in *htpR* mutants is similar when compared to the wild type strain. However, upon heat shock, these mutant strains show no significant induction of heat shock protein synthesis, in contrast to the wild type strain (25). This effect is also seen at the level of transcription. Although it is not apparent in Fig. B.4B, longer exposures of the Northern blot show that the basal level of *grpE* mRNA in the *htpR*15 and *htpR*165 mutants is almost identical to the basal level found in the wild type strain. However, upon heat induction, the level of *grpE* mRNA increased to a much lesser extent in these two mutants (lanes 4-6 and 10-12) than in the wild-type cells (lanes 1-3). The fact that there is some induction in the *htpR*15 and 165 mutants may be due to residual activity of the htpR protein [the mutant alleles encode a missense mutation near the C-terminus and an amber mutation approximately midway through the structural gene, respectively (25)]. In the case of the *htpR*6 amber mutant, it is not understood why the basal level of *grpE* mRNA is higher than that of wild-type, but there is almost no increase of the *grpE* mRNA upon heat shock.

In the autoradiogram shown in Fig. B.4A, a higher molecular weight mRNA species is present. We are not certain of the significance of this putative mRNA. Its presence is not always reproducible and its position seems to coincide with that of 16S

ribosomal RNA. In principle, it could represent a *grpE*-specific read-through transcript or a transcript initiated at an upstream promoter.

The filter used in Fig. B.4B had been simultaneously probed with *htpR* sequences. The two bands seen above the *grpE* mRNA are due to *htpR* transcripts.

Identification of the *grpE* promoter

Because of the putative heat shock promoter sequence upstream of the *grpE* gene (Fig. B.3A), we determined the position of the 5' end of the *in vivo* transcript using S1 mapping and RNA sequencing techniques.

S1 mapping. As described in **Materials and Methods**, we used RNA extracted from cells harboring plasmid pJK23, which contains the entire *grpE* gene, and found two protected fragments: a major one, which is 154 nucleotides long, and a minor one, which is 150 nucleotides in length (Fig. B.5A and B).

The 5' ends of these putative transcripts correspond to nucleotide positions -39 and -35 from the AUG initiation codon. Both transcripts increased in abundance after heat shock (Fig. B.5A, lanes 4, 10). Similar results were obtained when RNA from cells containing no plasmid were used, although the 150-nt moiety was detected only under heat shock conditions (Fig. B.5A, lanes 1, 2). When we increased the concentration of S1 nuclease, the ratio of the protected fragments became reversed: the 154-nt fragment almost disappeared while the smaller one was still present (Fig. B.5A, lanes 5, 6). This suggests that the shorter fragment may be a degradation product, resulting from S1 nuclease action at the ends of the DNA:RNA hybrid moiety.

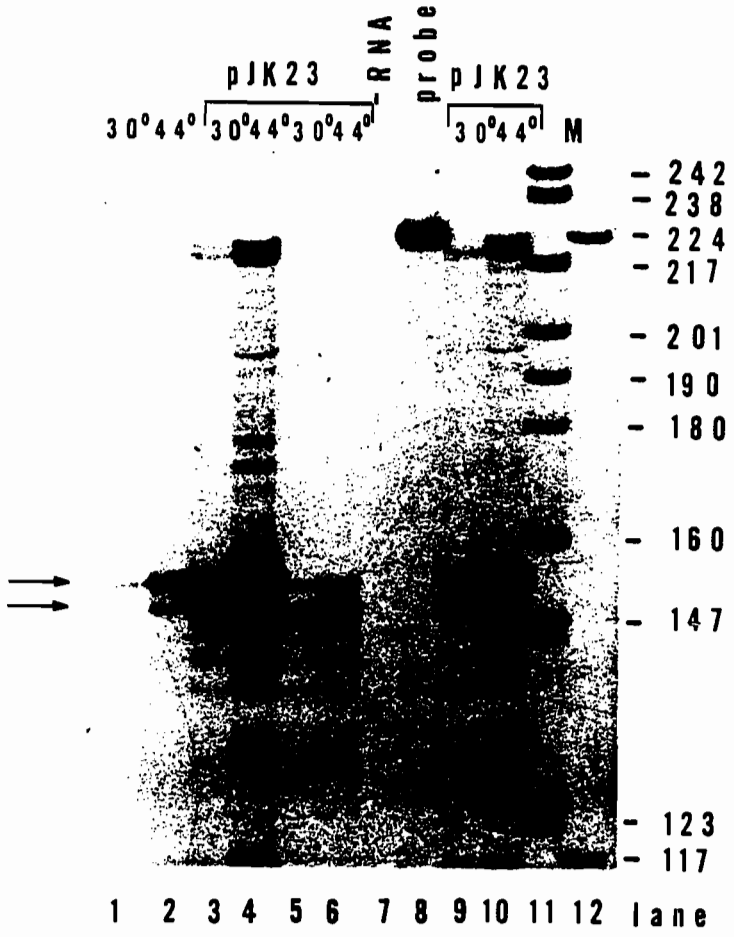
In the gel shown in Fig. B.5A, there is a doublet of bands which migrate as approximately 220-bp fragments (lanes 4, 10), which suggests the presence of an additional promoter further upstream, active under heat shock conditions. However,

Figure B.5. S1 mapping of the 5' end of the *grpE* transcript.

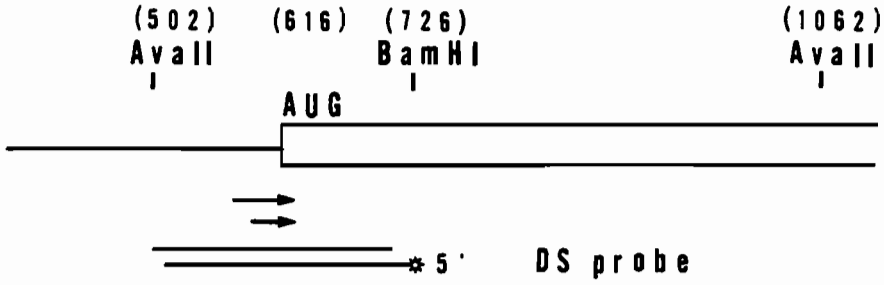
A. Gel showing size of protected fragment. 44° RNA was extracted from cells 5 min after a temperature shift from 30°C to 44°C. Lanes 1 and 2, ten μg of 30° RNA and 44° RNA respectively, extracted from *E. coli* B178; lanes 3, 9 and 4, 10, two μg of 30°RNA and 44° RNA, respectively, extracted from *E. coli* B178 (pJK23); lanes 5 and 6, two μg of 30° RNA and 44° RNA, respectively, extracted from *E. coli* B178 (pJK23) and treated with a 5-fold higher concentration of S1 nuclease; lane 7, control reaction without RNA; lane 8, untreated probe; lanes 11 and 12, pBR322 DNA digested with Msp I and bacteriophage λ DNA digested with BstE II, respectively, end-labeled and used as size standards.

B. Schematic drawing of 5' region of the *grpE* gene. The open bar represents the coding region of the *grpE* gene. The two bottom lines show the double-stranded probe. The star indicates the ^{32}P-labeled 5' end of the strand which is complementary to the coding strand. The other strand is also labeled but the position of ^{32}P has not been marked. The arrows indicate the start positions of two potential transcripts.

A


pJK23
RNA probe
pJK23
30°44°30°44°30°44°30°44°
30°44°
M
242
238
224
217
201
190
180
160
147
123
117
1 2 3 4 5 6 7 8 9 10 11 12 lane

B


(502)
AvaII
(616)
(726)
BamHI
(1062)
AvaII
AUG
5'
DS probe

in our RNA sequencing experiments (see text below) in which RNA extracted from heat-shocked *htpR*$^+$ cells was also used, no termination bands above those shown in Fig. B.6A, even on highly overexposed autoradiograms, were seen. Identical results were obtained in primer extension experiments where the reactions with reverse transcriptase were incubated for a longer period of time than in the RNA sequencing experiments, thus ensuring completion of the reaction (data not shown). The doublet of bands may be the result of nonspecific protection of the probe.

***grpE* mRNA sequencing.** To determine precisely the transcriptional start, we sequenced the 5' terminal portion of *grpE* mRNA synthesized in cells carrying the pJK23 plasmid. RNA isolated from cells grown at 30°C and cells grown at 30°C followed by a shift to 44°C was used as template. The *grpE* mRNA starts at nucleotide position -39 (shown as position +1 in Fig. B.6B) from the AUG initiation codon both at 30°C and after a shift to 44°C (Fig. B.6A and B). An additional termination band at nucleotide position -38 from the initiation codon (Fig. B.6A, lanes 7, 8) was detected. This may be the result of premature termination by the reverse transcriptase enzyme.

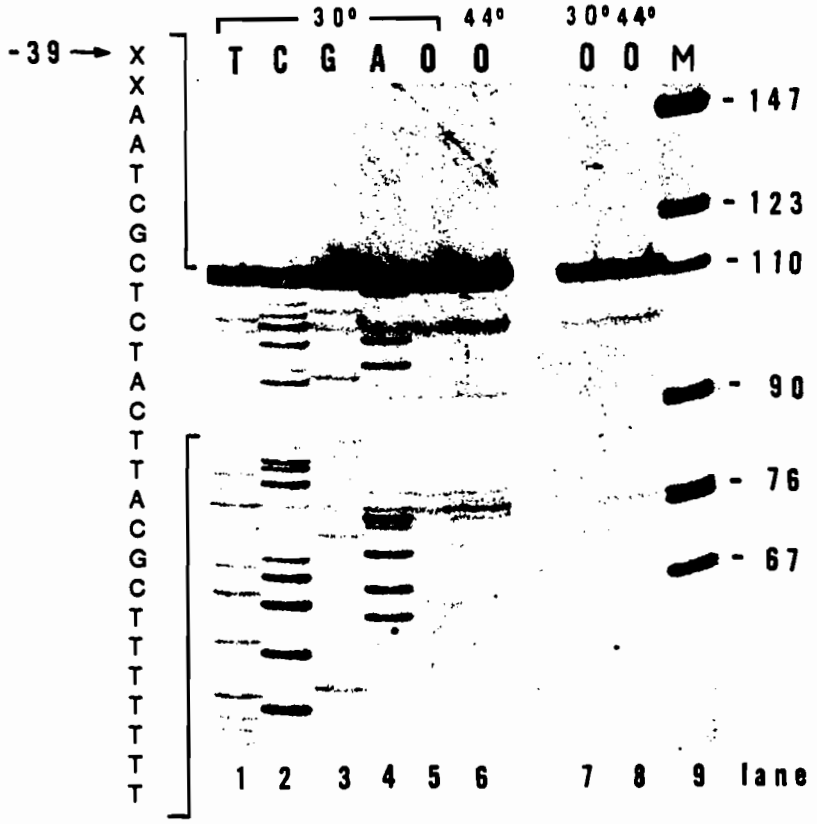
From the S1 mapping and RNA sequencing data, we conclude that the *grpE* mRNA starts at nucleotide -39 from the AUG initiation codon both at 30°C and 44°C. The shorter protected fragment in the S1 experiments suggests initiation at nucleotide position -35, but this possibility is ruled out by the RNA sequencing results. This fragment is most probably a degradation product of the full length mRNA-DNA hybrid. On the other hand, RNA sequencing data suggest the existence of a transcript starting at nucleotide position -38, but such a transcript has not been detected in the S1 protection experiments.

Figure B.6. Sequencing of the *grpE* transcript.

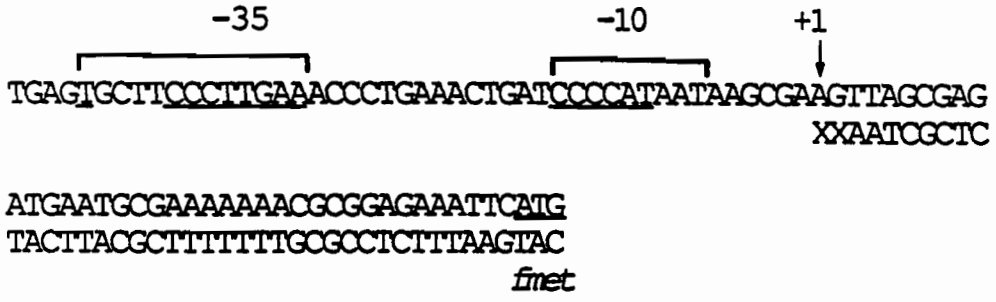
A. Gel showing sequence of the 5' end of the *grpE* transcript. RNA extracted from *E. coli* B178 (pJK23) was sequenced, using a 20-nt primer complementary to the nucleotides 669-688 of the *grpE* sequence (Fig. B.2). Cells, prior to extraction, were grown at 30°C (lanes 1-5, 7) or were shifted to 44°C for 5 min (only a reaction with no dideoxynucleotides is shown, lanes 6, 8). pBR322 DNA digested with Msp I and end-labeled was used as a size standard (lane 9). The arrow indicates position -39 from the AUG initiation codon.

B. *grpE* gene promoter sequence. Brackets indicate the -35 and -10 regions of the promoter. The arrow indicates the 5' end of the mRNA. The bottom line shows the complementary sequence read from the mRNA sequencing gels. The underlined nucleotides are identical to the consensus sequence for heat shock promoters (3).

A


30°
44°
30°44°
-39
T C G A 0 0
0 0 M
X
X
A
A
T
C
G
C
T
C
T
A
C
T
T
A
C
G
C
T
T
T
T
T
T
T
- 147
- 123
- 110
- 90
- 76
- 67
1 2 3 4 5 6 7 8 9 lane

B


-35
-10
+1
TGAGTGCTTCCCTTGAAACCCTGAAACTGATCCCCATAATAAGCGAAGTTAGCGAG
XXAATCGCTC
ATGAATGCGAAAAAAACGCGGAGAAATTCATG
TACTTACGCTTTTTTTGCGCCTCTTTAAGTAC
fmet

Discussion

The *grpE* gene sequence presented here predicts a 197-amino acid residue protein with a calculated molecular weight of 21,668 daltons. Its theoretical approximate charge at pH 7.0 is 15. These data are in good agreement with the previous findings that the *grpE* gene product is a mildly acidic protein of about 23,000-M_r (1, 26). The proof that the *grpE* sequence presented here is the correct one is reflected by the facts that (a) the 15 N-terminal amino acids of the purified grpE protein match perfectly those predicted by the DNA sequence, (b) the overall amino acid composition of the purified grpE protein matches that predicted by the DNA sequence, (c) the minimal calculated size of the *grpE* mRNA (630 nt of coding sequence plus 39 nt transcribed before the AUG initiation codon) matches well the experimentally estimated size of *grpE* mRNA (700 ± 50 nt), (d) the discussed ORF is preceded by a consensus sequence for heat shock promoters and transcription of the *grpE* gene was shown by S1 mapping and RNA sequencing to actually start there, and (e) GGAG, a sequence matching part of the consensus Shine-Dalgarno ribosomal binding domain AGGAGGU (20), is found 10 nt upstream from the AUG initiation codon.

We have identified the heat-inducible promoter for the *grpE* gene *in vivo*. This promoter is positively regulated by the *htpR* (*rpoH*) gene [coding for the σ^{32} subunit of RNA polymerase (14, 7)] and contains regions which are highly homologous to the consensus sequence for heat shock promoters recognized by the RNA polymerase-σ^{32} holoenzyme (14, 3). In addition, the nucleotide spacing between the -35 and -10 promoter regions is also conserved (Fig. B.3A). Typical spacing for the σ^{32}-holoenzyme is 13-15 bp (14, 3) while the *grpE* promoter has a spacing of 14 bp. This is additional evidence that the operons belonging to the heat shock regulon are transcribed by RNA polymerase cooperating with the σ^{32} subunit. However, our results do not exclude the possibility that the *grpE* gene may also be transcribed by

RNA polymerase-σ^{70} holoenzyme. This would explain the seemingly contradictory findings that, on the one hand, bacteria with a deletion of the *htpR* (*rpoH*) gene can survive at low temperature (T. Yura, personal communication), and that, on the other hand, *grpE* is an essential gene both at high and low temperatures (D. A. and C. G., unpublished results). Further support that the σ^{70}-holoenzyme may bind to the *grpE* promoter is that interdigitated with the σ^{32}-holoenzyme consensus recognition sequence is a sequence homologous to the consensus sequence for σ^{70} promoters (3), i.e., the same TTGA sequence in the -35 region and the CATAAT or ATAATA in the 10 region (see Fig. B.3A). Spacing between these putative -35 and Pribnow box sequences is 16-18 bp, similar to that found with other promoters. The fact that only the σ^{32}-holoenzyme-promoted transcript is observed *in vivo* could be explained in either of two ways, not mutually exclusive. It could be that the promoter recognized by the σ^{70}-holoenzyme is intrinsically weak or that the σ^{32}-holoenzyme excludes the σ^{70}-holoenzyme from binding there. In this respect, it would be interesting to analyze *grpE* gene transcription in an *htpR* (*rpoH*) deleted host at various temperatures.

We found that *grpE* transcription starts at the same promoter both at low temperature and following a heat shock. This is in agreement with the results of Cowing et al. (3) who also demonstrated a similar phenomenon with the heat shock promoters of the *groE*, *dnaK* and *C62.5* genes.

The grpE protein has been shown to be essential for λ DNA replication both *in vivo* and *in vitro* at all temperatures (1). Both genetic and biochemical evidence has been accumulated which demonstrates a functional interaction between the grpE and dnaK proteins (1, 5; C. Johnson, G. N. Chandrasekhar, D. A. and C. G., unpublished results). This helps explain the fact that mutations in either the *grpE* or *dnaK* genes exhibit the same pleiotropic effects for either phage or bacterial growth (1, 5). Recent genetic data have been presented suggesting that a functional dnaK protein

is necessary for *E. coli* DNA replication from *oriC in vivo* (17). In agreement with this, excess anti-dnaK protein antibody inhibits *oriC* DNA replication in a Fraction II system *in vitro* (27). However, no dnaK protein participation is necessary in the minimal *in vitro oriC* DNA replication system composed of purified proteins (6). Further experiments are necessary to clarify the role of the dnaK and grpE proteins in *E. coli* DNA replication. In λ DNA replication the role of the dnaK and grpE proteins has been localized to a specific step of the initiation process, namely, the disassembly of the λP-dnaB complex at *oriλ* (K. Liberek, M. Zylicz, D. A. and C. G., unpublished results). Removal of λP "liberates" the dnaB protein, thus enabling it to unwind λ DNA near *oriλ* using its helicase activity (9). The dnaG primase locates the dnaB - single-stranded DNA complex at apparently random sites and makes an RNA primer. This primer is extended into DNA by the DNA polymerase III holoenzyme.

Acknowledgments

We would like to thank Sanjeev Sharma for help with the experiments shown in Fig. B.6, Dr. Bill Gray for N-terminal sequence analysis and amino acid composition and Drs. Sandy Parkinson and Eric Kofoid for assistance using the Perceptron program. This work was supported by grants from the National Institutes of Health.

Literature Cited

1. **Ang, D., G. N. Chandrasekhar, M. Zylicz and C. Georgopoulos.** 1986. *Escherichia coli grpE* gene codes for heat shock protein B25.3, essential for both λ DNA replication at all temperatures and host growth at high temperature. J. Bacteriol. **167**:25-29.

2. **Arrand, J. E.** 1985. Preparation of nucleic acid probes. *In* B. D. Hames, and S. J. Higgins (eds.), Nucleic Acid Hybridization: A Practical Approach, pp. 17-45, IRL Press, Oxford-Washington, D.C.

3. **Cowing, D. W., J. C. A. Bardwell, E. A. Craig, C. Woolford, R. W. Hendrix and C. A. Gross.** 1985. Consensus sequence for *Escherichia coli* heat shock gene promoters. Proc. Natl. Acad. Sci. USA **82**:2679-2683.

4. **Dente, L., A. Cesareni and R. Cortese.** 1983. pEMBL: A new family of single stranded plasmids. Nucleic Acids Res. **11**:1645-1655.

5. **Friedman, D. E., E. R. Olson, C. Georgopoulos, K. Tilly, I. Herskowitz and F. Banuett.** 1984. Interactions of bacteriophage and host macromolecules in the growth of bacteriophage λ. Microbiol. Rev. **48**:299-325.

6. **Funnell, B. E., T. A. Baker and A. Kornberg.** 1986. Complete enzymatic replication of plasmids containing the origin of the *Escherichia coli* chromosome. J. Biol. Chem. **261**:5616-5624.

7. **Grossman, A. D., J. W. Erickson and C. A. Gross.** 1984. The *htpR* gene product of *E. coli* is a sigma factor for heat-shock promoters. Cell **38**:383-390.

8. **Guild, N., M. Gayle, R. Sweeney, T. Hollingsworth, T. Modeer and L. Gold.** 1988. Transcriptional activation of bacteriophage T4 middle promoters by the motA protein. J. Mol. Biol. **199**:241-258.

9. **LeBowitz, J. H. and R. McMacken.** 1986. The *Escherichia coli* DnaB replication protein is a DNA helicase. J. Biol. Chem. **261**:4738-4748.

10. **Maniatis, T., E. F. Fritsch and J. Sambrook.** 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

11. **Messing, J.** 1983. New M13 vectors for cloning. *In* L. Grossman, and K. Moldave (eds.), Methods in Enzymology, **101**:20-78.

12. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

13. **Neidhardt, F. C. and R. A. VanBogelen.** 1981. Positive regulatory gene for temperature-controlled proteins in *Escherichia coli*. Biochem. Biophys. Res. Commun. **100**:894-900.

14. **Neidhardt, F. C. and R. A. VanBogelen.** 1987. Heat shock response. *In* F. C. Neidhardt et al. (eds.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology, pp. 1334-1345, American Society for Microbiology, Washington, D. C.

15. **Saito, H. and H. Uchida.** 1977. Initiation of the DNA replication of bacteriophage lambda in *Escherichia coli* K12. J. Mol. Biol. **113**:1-25.

16. **Saito, H., Y. Nakamura and H. Uchida.** 1978. A transducing λ phage carrying *grpE*, a bacterial gene necessary for λ DNA replication, and two ribosomal protein genes, *rpsP* (S16) and *rplS* (L19). Mol. Gen. Genet. **165**:247-256.

17. **Sakakibara, Y.** 1988. The *dnaK* gene of *Escherichia coli* functions in initiation of chromosome replication. J. Bacteriol. **170**:972-979.18.

18. **Sanger, F. and A. R. Coulson.** 1978. The use of thin acrylamide gels for DNA sequencing. FEBS Letters **87**:107-110.

19. **Sanger, F., S. Nicklen and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. **74**:5463-5467.

20. **Shine, J. and L. Dalgarno.** 1974. The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: Complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. U. S. A. **71**:1342-1346.20.

21. **Spackman, D. H., W. H. Stein and S. Moore.** 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. **30**:1190-1206.

22. **Stormo, G. D., T. D. Schneider, L. Gold and A. Echrenfeucht.** 1982. Use of the 'Perceptron' algorithm to distinguish translational initiation sites in *E. coli*. Nucleic Acids Res. **10**:2997-3011.

23. **Tilly, K., J. Erickson, S. Sharma and C. Georgopoulos.** 1986. Heat shock regulatory gene *rpoH* mRNA level increases after heat shock in *Escherichia coli*. J. Bacteriol. **168**:1155-1158.16.

24. **Williams, J. G. and P. J. Mason.** 1985. Hybridisation in the analysis of RNA. *In* B. D. Hames and S. J. Higgins (eds.), Nucleic Acid Hybridization: A Practical Approach, pp. 139-160, IRL Press, Oxford-Washington, D.C.

25. **Yura, T., T. Tobe, K. Ito and T. Osawa.** 1984. Heat shock regulatory gene (*htpR*) of *Escherichia coli* is required for growth at high temperatures but is dispensable at low temperature. Proc. Natl. Acad. Sci. U. S. A. **81**:6803-6807.

26. **Zylicz, M., D. Ang and C. Georgopoulos.** 1987. The grpE protein of *Escherichia coli*: Purification and properties. J. Biol. Chem. **262**:17437-17442.

27. **Zylicz, M. and C. Georgopoulos.** 1984. Purification and properties of the *Escherichia coli* dnaK replication protein. J. Biol. Chem **259**:8820-8825.

APPENDIX C

INITIATION OF λ DNA REPLICATION WITH PURIFIED HOST- AND BACTERIOPHAGE-ENCODED PROTEINS: THE ROLE OF THE dnaK, dnaJ AND grpE HEAT SHOCK PROTEINS**

** Maciej Zylicz, Debbie Ang, Krzysztof Liberek and Costa Georgopoulos, submitted to EMBO J.

Abstract

Based on previous *in vivo* genetic analysis of bacteriophage λ growth, we have developed two *in vitro* λ DNA replication systems composed entirely of purified proteins. One is termed "grpE-independent" and consists of supercoiled λdv plasmid DNA, the λO and λP proteins, as well as the *E. coli* dnaK, dnaJ, dnaB, dnaG, ssb, DNA gyrase and DNA polymerase III holoenzyme proteins. The second system includes the *E. coli* grpE protein and is termed "grpE-dependent." Both systems are specific for plasmid molecules carrying the *ori*λ DNA initiation site. The major difference in the two systems is that the "grpE-independent" system requires at least a ten-fold higher level of dnaK protein compared to the grpE-dependent one. The λ DNA replication process may be divided into several discernible steps, some of which are defined by the isolation of stable intermediates. The first is the formation of a stable *ori*λ-λO structure. The second is the assembly of a stable *ori*λ-λO-λP-dnaB complex. The addition of dnaJ to this complex also results in an isolatable intermediate. The dnaK, dnaJ and grpE proteins destabilize the λP-dnaB interaction, thus liberating dnaB's helicase activity, resulting in unwinding of the DNA template. At this stage, a stable DNA replication intermediate can be isolated, provided that the grpE protein has acted and/or is present. Following this, the dnaG primase enzyme recognizes the single-stranded DNA-dnaB complex and synthesizes RNA primers. Subsequently, the RNA primers are extended into DNA by DNA polymerase III holoenzyme. The proposed model of the molecular series of events taking place at *ori*λ is substantiated by the many demonstrable protein-protein interactions among the various participants.

Introduction

The recent development of the crude *in vitro* system for *ori*C DNA replication (9), and its subsequent adaptation for *ori*λ-containing plasmids (1, 26, 29), has facilitated the study of the molecular series of events which occur in the initiation and regulation of bacteriophage λ DNA replication. As is the case *in vivo,* λ DNA replication in the crude *in vitro* system starts from a unique sequence, called *ori*λ, and proceeds in a bidirectional fashion through θ-structure intermediates (reviewed in ref. 10). As expected, initiation of the *ori*λ -containing plasmid, λdv, in the crude *in vitro* system is dependent on the presence of the two λ replication proteins, λO and λP. Crude soluble protein extracts prepared from *E. coli* strains carrying mutations in either *dnaB, dnaJ, dnaK, grpE*, or *dnaG*, are not active in the crude *in vitro* λdv DNA replication system unless the respective wild type protein is provided (31, 35). Using the crude *in vitro* system as a complementation assay, we have purified all of these bacteriophage- and host-encoded proteins to homogeneity (31, 32, 34, 35). Three of these proteins - dnaK, dnaJ and grpE - are not only involved in the initiation of λ DNA replication, but belong to the so-called heat shock family of proteins which has been highly conserved throughout evolution (reviewed in ref. 20).

It was shown both *in vivo* and *in vitro* that transcription through the origin is required for initiation of *ori*λ DNA replication, a phenomenon referred to as "transcriptional activation" (8, 30). However, in the purified *in vitro* system no transcriptional activation is required (17, 18). This observation has led McMacken et al. (17, 18) to the discovery that the addition of purified HU protein restores the requirement for transcriptional activation. Thus, transcriptional activation has been explained as a need for the removal of DNA binding proteins, which otherwise inhibit the assembly of the replication complex at *ori*λ (17, 18).

McMacken and his colleagues (in collaboration with us), previously established a reconstituted enzyme system composed of eight purified phage and bacterial proteins [λO, λP, dnaB, dnaK, dnaJ, single-stranded DNA binding protein (ssb), dnaG primase, and DNA polymerase III holoenzyme] that supports the replication of an M13 single-stranded DNA template (14). There is no need for *oriλ* DNA sequences in this system. From the studies on this "λ single-strand replication reaction," we learned that the rate limiting step in the initiation of replication is the formation of a λO - λP - dnaB complex on the single-stranded DNA template (at apparently random sites) and activation of this complex by an ATP-, dnaK-, dnaJ-dependent reaction (14). Once assembled on the template strand, dnaB protein apparently migrates in a processive fashion along the ssb-coated DNA chain, serving as a locus for dnaG-dependent primer synthesis. The observation that dnaB protein migrates on single-stranded DNA in this system led to its identification as a DNA helicase (13).

In this paper, we demonstrate that when the eight highly purified bacteriophage- and host-encoded proteins involved in the "λ single-strand replication reaction" (14) are supplemented with purified *E. coli* DNA gyrase, this set of proteins is sufficient for the initiation of DNA synthesis on double-stranded supercoiled DNA encoding *oriλ*. Because this system functions in the absence of grpE, we have termed it "grpE-independent." An analogous purified system has been established independently by McMacken et al. (17, 18).

Recently, we purified, using a dnaK-affinity column, another host protein required for initiation of λ DNA replication (31). The *E. coli grpE* gene product was previously identified as essential for λ DNA replication both *in vivo* and in the crude *in vitro* system (22, 31). Here, we present evidence that the grpE protein is a prepriming protein required at the same step as dnaK and dnaJ for the activation of dnaB helicase activity, which is inhibited in the prepriming *oriλ* - λO - λP - dnaB complex. Because

this system depends on the presence of grpE protein, we have termed it "grpE-dependent." We show that the presence of grpE protein a) does not abolish the requirement for any of the other replication proteins, b) lowers the concentration of dnaK protein required in the purified *in vitro* system at least ten-fold, and c) results in the formation of a more stable, isolatable prepriming intermediate complex.

Materials and Methods

Proteins and DNA

Highly purified proteins (90% or greater purity) were used; their specific activities were as follows: λO protein, 1 x 10^5 units/mg (21); λP protein, 1.5 x 10^5 units/mg (24); dnaB protein, 1 x 10^5 units/mg [2, except that the fraction obtained following the DE52 column was purified further on an ATP-agarose column according to Lanka *et al.* (12)]; dnaJ protein, 4 x 10^5 units/mg [35, except that the ammonium sulfate step was omitted, and a hydroxylapatite column (2.5 x 4 cm), washed by buffer containing 140 mM $KHPO_4$ before elution of dnaJ activity by 0.5 M $KHPO_4$, was used]; dnaK protein, 3 x 10^3 units/mg (31); gyrA protein subunit, 3 x 10^5 units/mg (19); gyrB protein subunit, 5 x 10^4 units/mg (19); grpE protein, 5 x 10^5 units/mg (31). Ssb was purchased from United States Biochemical Corporation. The dnaK756 mutant protein was purified as described (15). The grpE280 mutant protein was purified from cells carrying a plasmid which overproduces the grpE280 protein (D. A., Ph.D. dissertation, University of Utah, 1988). DNA polymerase III holoenzyme preparations were generous gifts from Drs. C. McHenry (University of Colorado), A. Kornberg (Stanford University) and P. Modrich (Duke University). DnaG protein (5 x 10^5 units/mg) was purified as described (35) although some of the experiments were done using dnaG protein kindly provided by Dr. J. Kaguni (University of Michigan). The units of activity were defined as previously described (35). Supercoiled plasmid

DNA containing the *ori*λ sequence (pRLM4) and *ori*ϕ82 (pRLM5) have been described (29). They were purified by alkali lysis, followed by purification on two consecutive cesium chloride-ethidium bromide gradients as described (16).

Purified *in vitro* DNA replication system

The reaction (25 µl) consisted of 40 mM HEPES/KOH pH 7.6, 7.2 mM magnesium acetate, 2 mM ATP, 300 ng λdv plasmid DNA, 200 ng λO, 150 ng λP, 150 ng dnaB, 860 ng ssb, 200 ng gyrA, 90 ng gyrB, 100 ng dnaJ, 100 ng dnaG, 200 ng DNA polymerase III holoenzyme, 100 µM each dATP, dCTP, TTP, dGTP, with [methyl-^{3}H] TTP at 50 cpm per pmol of total deoxynucleotide, 200 µM each GTP, CTP, and UTP, 20 µM phosphocreatine, 500 ng creatine kinase, and 50 µg/ml bovine serum albumin. "grpE-independent" system, 5 µg dnaK; "grpE-dependent" system, 0.5 µg dnaK and 150 ng grpE. The reactions were incubated 30 min at 32°C and processed as described (30) except that carrier calf thymus DNA (500 µg) and bovine serum albumin (200 µg) were added to each reaction just prior to precipitation with trichloroacetic acid.

Identification of λdv DNA replication products

The products of λdv DNA replication reactions were analysed by equilibrium centrifugation in a cesium chloride gradient in the presence of ethidium bromide, as previously described (29), except that the gradients were centrifuged at 30,000 rpm in a Beckman Ti 50 rotor for 48 hr at 15°C. The analysis of density-labeled λdv DNA after *in vitro* replication in the two purified protein systems was essentially as described (27). The replication reactions (250 µl) were carried out as described above except that *in vivo*-labeled [^{3}H]-λdv DNA was used as the template, 100 µM 5-bromo-2'-deoxyuridine 5'-triphosphate (Sigma) was used instead of TTP, and [α-

^{32}P]-dCTP (500 cpm/pmol of total deoxynucleotides; Amersham) was used instead of [^{3}H]-TTP. The reactions was incubated for 20 min at 32°C, then terminated by addition of EDTA and SDS to final concentrations of 25 mM and 1%, respectively. The reactions were extracted with an equal volume of phenol:chloroform (1:1) and diluted to 3 ml with 10 mm Tris/HCl pH 7.5, 20 mM NaCl, and 1 mM EDTA prior to addition of 4 g of cesium chloride.

Following centrifugation at 35,000 rpm for 48 hr at 15°C in a Beckman SW50.1 rotor, the cesium chloride density gradients were fractionated and assayed for acid-insoluble ^{3}H and ^{32}P radioactivity as described for the DNA replication assays.

DNA unwinding assay

The reaction mixture (25 µl) is similar to the one used for the purified *in vitro* DNA replication assay except that dATP, dCTP, TTP, dGTP, GTP, CTP, UTP, primase and DNA polymerase III holoenzyme were omitted. The reaction was incubated for 30 min at 38° and terminated by the addition of EDTA and SDS to final concentrations of 10 mM and 0.5%, respectively. The reaction was mixed with a sucrose, bromophenol blue solution to give final concentrations of 10% and 0.05%, respectively. This mixture was analysed on a 1% agarose gel containing 25 µg/ml chloroquine phosphate (Sigma) in TPE buffer (80 mM Tris-phosphate, 8 mM EDTA, pH 8.6). The samples were electrophoresed at 30 V for approximately 20 hr. The gel was washed in water for 2 hr (to remove excess chloroquine), followed by staining with ethidium bromide. The percentage of unwinding was estimated by densitometric scanning of individual lanes on photographic negatives of the gels, using a Joyce-Loebl densitometer.

Isolation of DNA replication intermediates

The 120-µl reaction, containing the prepriming proteins listed under "**Proteins and DNA**" (above), λdv DNA, ATP, HEPES, and magnesium acetate in the concentrations described for the DNA replication assay, was incubated for 12 min at 32°C and applied to a Sepharose 4B (Pharmacia) column (0.5 cm x 7.5 cm) which had been equilibrated with 40 mM HEPES/KOH pH 7.6, 1 mM dithiothreitol, 5 mM $MgCl_2$, 50 mM KCl, 4 mM ATP and 0.5 mg/ml bovine serum albumin at room temperature. Fractions (100 µl) were collected and 17 µl of the void volume fraction (fraction #4), containing the DNA and proteins associated with it, was assayed in the purified reconstituted replication system. Missing prepriming proteins, if any, along with dnaG primase and DNA polymerase III holoenzyme, were added at this time. Before applying the 120-µl reaction to the Sepharose 4B column, a 17-µl aliquot was removed and incubated at room temperature. This aliquot served as a control to calculate the recovery of replication activity.

Results

The "grpE-independent" system

When the eight highly purified proteins - λO, λP, dnaB, dnaG, dnaJ, dnaK, ssb, and DNA polymerase III holoenzyme (Fig. C.1) -, known from previous work to support the λ single-strand replication reaction (14), are supplemented with *E. coli* DNA gyrase, efficient *ori*λ-specific DNA replication occurs (Table C.1). This "grpE-independent" DNA replication system no longer requires the presence of RNA polymerase holoenzyme (compared to the crude *in vitro* system), as shown by its insensitivity to rifampicin (Table C.1). The requirement for all proteins used in this *in vitro* reconstitution assay is absolute, because omitting any one of these proteins leads to at least a ten-fold reduction in DNA synthesis (Table C.1). The specificity of the

Figure C.1. Proteins used in the purified *in vitro* λ DNA replication systems. *E. coli* and λ replication proteins were purified as described in **Materials and Methods**, and shown here on a 12.5% SDS-polyacrylamide gel stained with silver. Lane S, molecular weight standards: rabbit phosphorylase b (97,400-M_r), bovine serum albumin (66,200-M_r), hen egg ovalbumin (42,700-M_r), bovine carbonic anhydrase (31,000-M_r), soybean trypsin inhibitor (21,500-M_r), hen egg white lysozyme (14,400-M_r).

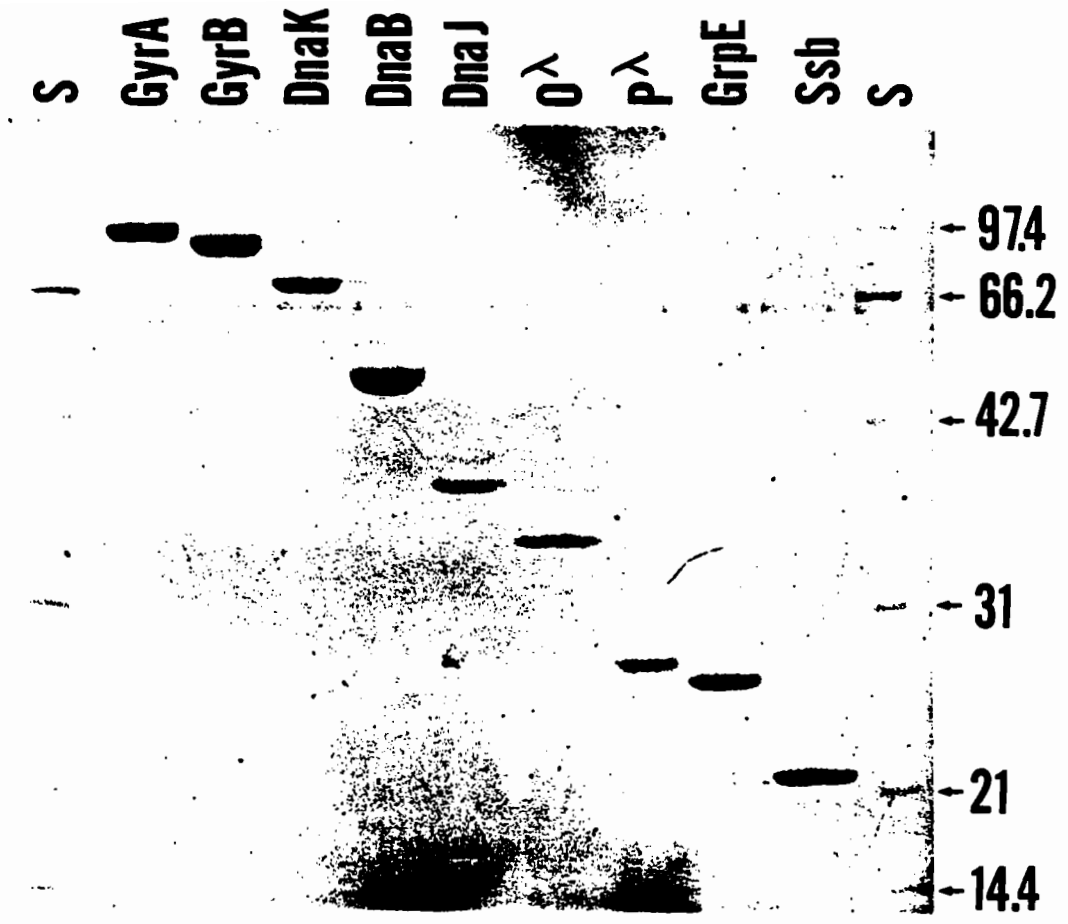

S
GyrA
GyrB
DnaK
DnaB
DnaJ
Oλ
Pλ
GrpE
Ssb
S
97.4
66.2
42.7
31
21
14.4

Table C.1

Reconstitution of *oriλ* plasmid DNA replication with purified proteins

Component omitted or added	DNA synthesis (pmol/30 min/32°C) System A ("grpE-independent")	System B ("grpE-dependent")
None	429	333
-λO	3	2
-λP	6	4
-dnaB	5	4
-dnaJ	22	19
-dnaK	3	3
-ssb	4	4
-dnaG	10	7
-DNA gyrase (+ 50 μM coumermycin)	43	32
-DNA polymerase III holoenzyme	5	4
+grpE	611	-
+rifampicin (40 μg/ml)	398	298
+*oriφ*82 instead of *oriλ* DNA	6	4
-grpE	-	9
-creatine phosphate and creatine kinase	380	280

system is demonstrated by the fact that substitution of *ori*λ-containing supercoiled plasmid with a plasmid DNA containing the *ori* ϕ82 DNA sequence results in total inhibition of DNA synthesis (Table C.1).

The number of copies of each of the replication proteins required in the prepriming stages of this purified *in vitro* system varies between 40-60 molecules per *ori*λ site, with the striking exception of the dnaK protein which, for maximum DNA synthesis, is needed at approximately ten-fold higher concentration (Fig. C.2B). As expected from previous *in vitro* work (32), purified dnaK756 mutant protein is not able to substitute for the wild type dnaK protein (Fig. C.2B).

The "grpE-dependent" system

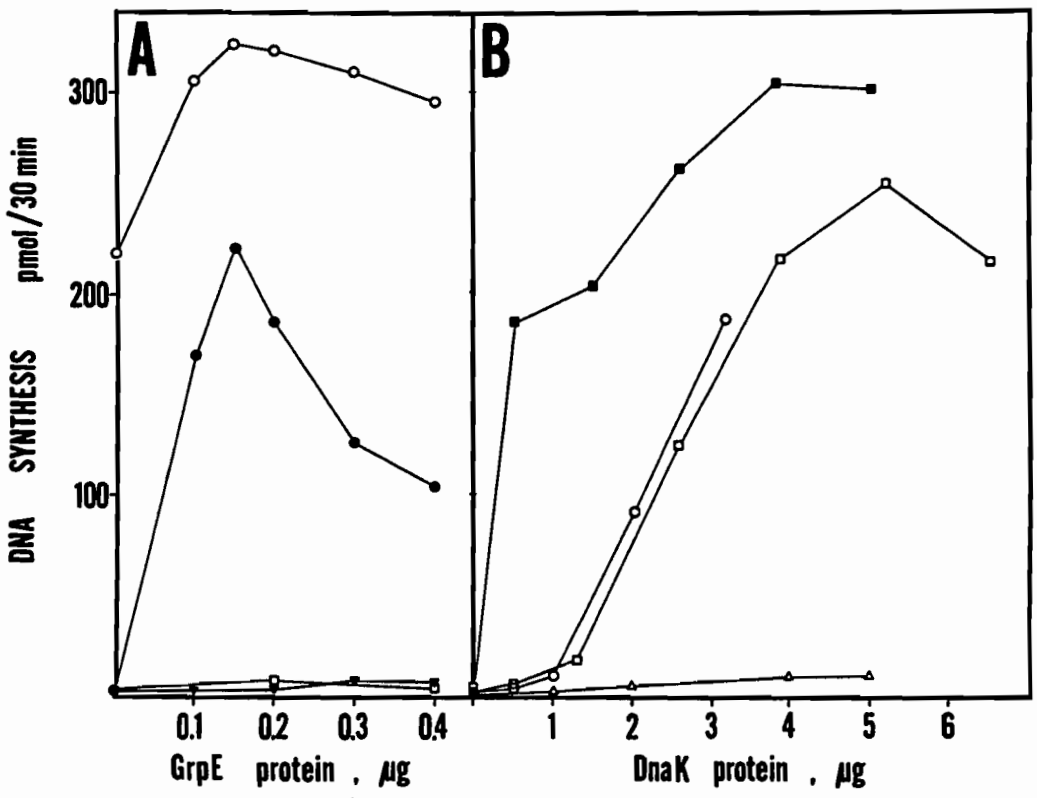
The grpE protein, purified using a dnaK-affinity column (31), efficiently stimulates the purified *in vitro* system for λ DNA replication (Table C.1, Fig. C.2). The presence of grpE protein in the reconstitution assay allows an approximately ten-fold reduction in the molar concentration of dnaK protein required for maximum DNA synthesis (Fig. C.2A). The optimal concentration of grpE used in the "grpE-dependent" assay has a rather narrow range, since the DNA replication reaction is efficiently inhibited when the level of grpE protein exceeds that of dnaK (Fig. C.2A, and unpublished data). As shown in Table C.1, the grpE protein does not substitute for any of the other proteins in the purified *in vitro* λ DNA replication system.

The mutant grpE280 protein, purified from cells transformed with a plasmid which overproduces the grpE280 protein (D. A., Ph.D. dissertation, University of Utah, 1988), does not substitute for the wild type grpE protein in the purified *in vitro* system when added in comparable concentrations (Fig. C.2A). This is consistent with the observation that the *E. coli grpE*280 mutant does not support λ DNA replication *in vivo* at any temperature (22; D. A. and C. G., unpublished data).

Figure C.2. Dependency of the *oriλ*-specific purified *in vitro* DNA replication system on the presence of grpE (panel A) and dnaK (panel B).

A. Reactions (25 µl) were carried out as described in **Materials and Methods** except that the concentration of grpE protein was varied between 0 and 0.4 µg per assay, and the concentrations of dnaK were as follows: (○), 4 µg dnaK; (●), 0.4 µg dnaK; (▼), no dnaK added; (□), 0.4 µg dnaK, but the wild type grpE protein was substituted by the grpE280 mutant protein.

B. Reactions were carried out as described in **Materials and Methods**, except that the concentration of dnaK protein was varied between 0 and 6 µg and the concentrations of grpE protein were as follows: (■), 150 ng grpE; (□), no grpE added; (○), no grpE added and the dnaK protein used was purified from an *E. coli* strain deleted for the *grpE* gene; (△), 150 ng grpE, but the wild type dnaK protein was substituted by the dnaK756 mutant protein.


A
B
300
200
100
DNA SYNTHESIS pmol/30 min
0.1
0.2
0.3
0.4
GrpE protein , µg
1
2
3
4
5
6
DnaK protein , µg

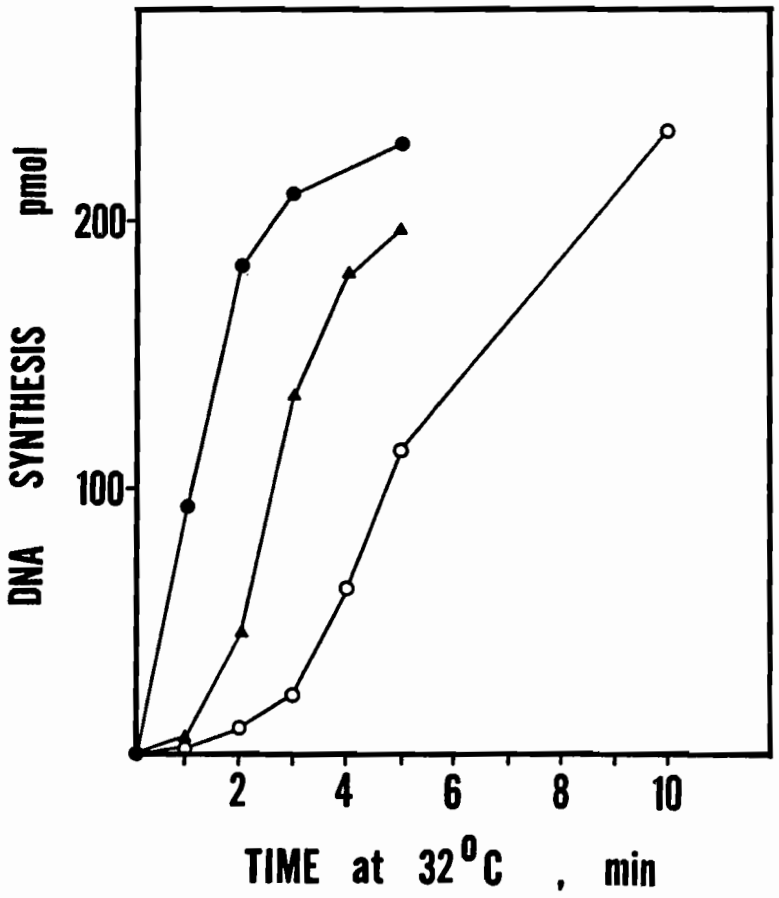
Kinetics of DNA replication

The kinetics of DNA synthesis is similar in both the "grpE-dependent" (Fig. C.3) and "grpE-independent" (unpublished data) systems. When all the prepriming proteins and λdv DNA are preincubated for 10 minutes at 32°C, prior to addition of dnaG primase and DNA polymerase III, efficient DNA synthesis is initiated without lag and is completed in 5 minutes (Fig. C.3). A 2-3 minute lag in commencement of DNA synthesis is observed in both the "grpE-dependent" and "grpE-independent" systems when the prepriming proteins are not preincubated for 10 minutes at 32°C, but instead are added simultaneously with dnaG primase and DNA polymerase III holoenzyme. When grpE is absent during the preincubation period, but instead is added at the same time as primase and DNA polymerase III holoenzyme, the lag in DNA synthesis is reduced, but not entirely (Fig. C.3). Similar results are obtained when either dnaK or dnaJ are not added until after the preincubation period (in either the "grpE-dependent" or "grpE-independent" systems), at the same time as dnaG primase and DNA polymerase III holoenzyme (unpublished data). The observed lag suggests at least two potential rate-limiting steps in the prepriming reaction: a) formation and/or stabilization of the *ori*λ - λO - λP - dnaB complex, and b) activation of this complex by dnaK and dnaJ ("grpE-independent" system) or dnaK, dnaJ and grpE ("grpE-dependent" system).

Products of λdv DNA replication

The products of the "grpE-independent" and "grpE-dependent" λdv DNA replication systems were examined by equilibrium centrifugation in a cesium chloride gradient in the presence of ethidium bromide as described by Wold et al. (29). In both cases, most of the products of DNA replication were identified as being catenated,

Figure C.3. Kinetics of *ori*λ-specific DNA synthesis in the "grpE-dependent" purified *in vitro* λ DNA replication system. A 250-μl replication reaction was assembled on ice as described in **Materials and Methods**. (●), all prepriming proteins (λO, λP, dnaB, dnaK, dnaJ, grpE) as well as ssb and DNA gyrase were preincubated with ATP, magnesium acetate and λdv DNA for 15 min at 32°C before addition of dnaG primase, DNA polymerase III holoenzyme and DNA and RNA synthesis precursors. At various times following the addition of dnaG primase and DNA polymerase III holoenzyme (t = 0), 25-μl aliquots were removed and the replication reaction terminated and processed as described (30); (▲), all prepriming proteins except grpE protein were preincubated for 15 min at 32°C before the addition of grpE protein, dnaG primase and DNA polymerase III holoenzyme; (○), no preincubation of the prepriming proteins. Instead the reaction was assembled in the presence of dnaG primase and DNA polymerase III holoenzyme and immediately transferred from 0° to 32°C.


DNA SYNTHESIS pmol
200
100
2
4
6
8
10
TIME at 32°C , min

nicked λdv DNA circles (60%). In addition, a small amount of other forms [catenated plasmid circles in which half were nicked and half were relaxed but not nicked covalently closed circles (30%), or catenated covalently closed circles (less than 10%)] were also detected (unpublished data).

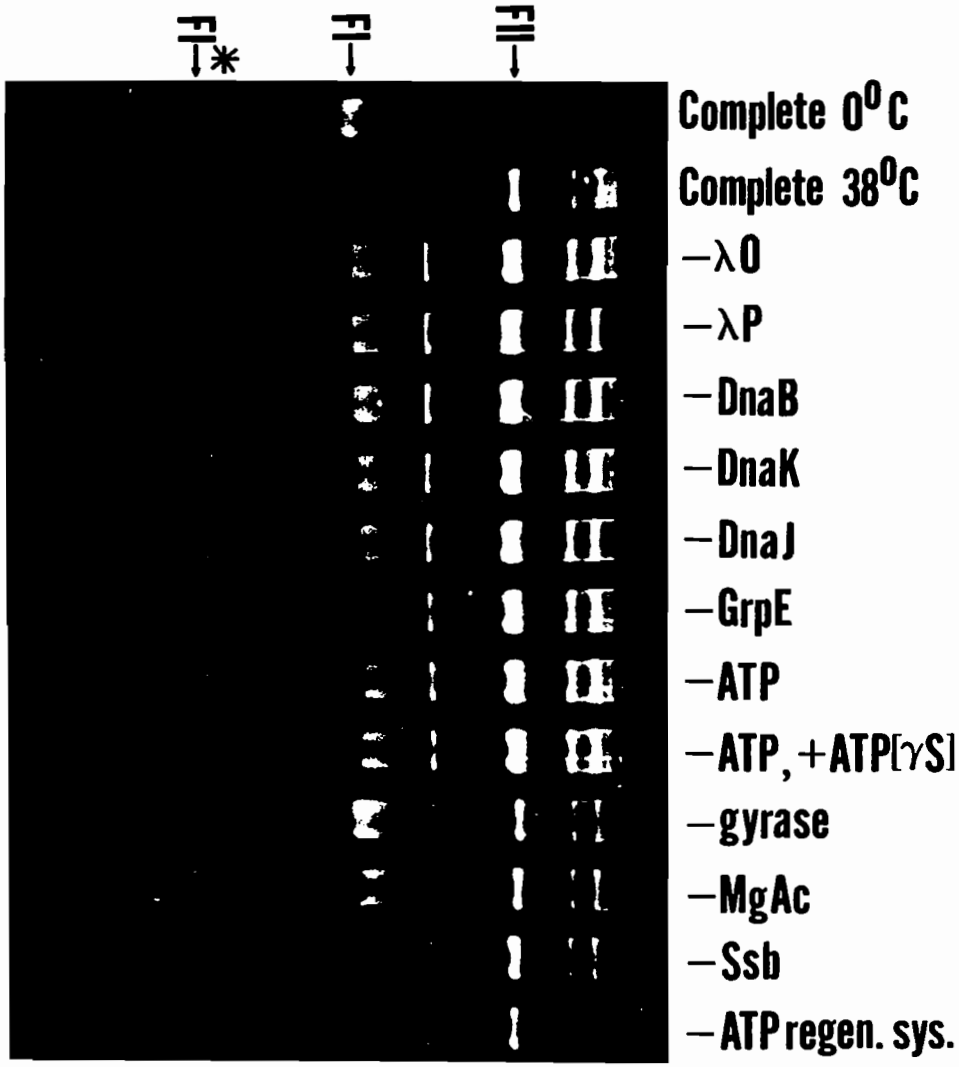
The products of the "grpE-independent" and "grpE-dependent" reactions were also analysed in a density shift experiment as described by Tsurimoto and Matsubara (27). In both the "grpE-independent" and "grpE-dependent" reactions, more than 80% of the parental DNA was recovered in the hybrid density position, indicating that a single, almost complete round of λdv DNA replication had taken place in both systems (unpublished data).

Unwinding of DNA template

In the case of *ori*C-dependent DNA synthesis, extensive unwinding, catalyzed by dnaB helicase, of double-stranded DNA near *ori*C is followed by dnaG primer synthesis (3). To determine whether the grpE protein is involved in the step before or during dnaG primer synthesis in λdv DNA replication, we developed an *ori*λ-dependent unwinding assay based on the conditions described by Baker et al. (3). Bacteriophage λ- and host-encoded prepriming proteins are incubated with supercoiled λdv DNA under the conditions developed for optimal DNA synthesis in the "grpE-dependent" system. It was found that the overall requirements for the unwinding reaction correspond to those for DNA synthesis (Table C.1), i. e., as shown in Fig. C.4, the unwinding reaction is dependent on the presence of λO, λP, dnaB, dnaK, dnaJ, grpE, gyrase, Mg^{++}, and the hydrolysis of ATP, and is efficiently stimulated by ssb protein. In addition, it is only slightly dependent on an ATP regeneration system.

This unwinding assay exhibits a similar relationship between the presence of grpE protein and reduction in the concentration of dnaK protein as does the actual purified

Figure C.4. Unwinding of the λdv plasmid DNA during initiation of λdv DNA replication. The prepriming replication proteins were incubated at 0° or 38°C for 30 min with supercoiled λdv DNA under the conditions described in **Materials and Methods**. The incomplete systems, lacking one of the prepriming factors, were incubated at 38°C for 30 min. The reactions were stopped by addition of EDTA and SDS as described in **Materials and Methods** and the DNA sample analysed on a 1% agarose gel containing 25 μg/ml chloroquine. The gel was stained with ethidium bromide to visualize the DNA. FII represents the position of open, relaxed circular DNA; FI, the position of supercoiled DNA; FI*, the position of covalently closed, highly underwound circular DNA. During incubation of the complete system at 38°C, some of the supercoiled DNA (FI) is converted to open circular DNA (FII) due to the nicking activity of gyrase and potentially some contaminating nuclease activity present in either ssb or the ATP regeneration system.


FI*
FI
FII
Complete 0°C
Complete 38°C
−λO
−λP
−DnaB
−DnaK
−DnaJ
−GrpE
−ATP
−ATP, +ATP[γS]
−gyrase
−MgAc
−Ssb
−ATP regen. sys.

in vitro DNA replication assay (Fig. C.5). The presence of grpE protein reduces the concentration of dnaK protein required for unwinding at least ten-fold. Unwinding of DNA is possible without grpE protein, but, in this case, the concentration of dnaK protein per assay needs to be in the microgram range, similar to the data presented in Fig. C.2 for DNA synthesis. Again, as shown for DNA synthesis (Fig. C.2), high concentrations of grpE protein partially inhibit the unwinding reaction. The mechanism by which this inhibition occurs is unknown. One possibility is that excess grpE protein effectively "sequesters" dnaK protein, thus preventing it from interacting with λP protein. As a control, the substitution of grpE protein by the grpE280 mutant protein does not lead to unwinding of DNA (unpublished data), consistent with the results obtained in the purified *in vitro* replication system.

The results shown in Figs. C.4 and C.5 strongly suggest that grpE protein is required for the unwinding reaction prior to dnaG-dependent primer synthesis, consistent with the idea that grpE protein functions at the same step as dnaK and dnaJ proteins.

Isolation of DNA replication intermediates

The data presented in Table C.2 demonstrate that the prepriming reaction of the initiation of λ DNA replication may be divided into several discernible steps. After each of these steps, it is possible to isolate a stable intermediate by gel filtration, as described by Liberek et al. (15). These intermediates were shown to be capable of replication upon addition of missing prepriming proteins (Table C.2). In the reconstituted, "grpE-independent" system, three stable DNA replication intermediates can be recovered. These are a) the *ori*λ - λO complex, b) the *ori*λ - λO - λP - dnaB complex, and c) an *ori*λ-λO-λP-dnaB-dnaJ complex. Following formation of this third complex, the λP protein is released from the complex in a dnaK-, dnaJ-

Figure C.5. Dependence of unwinding of λdv supercoiled DNA on the concentration of the dnaK and grpE proteins. The unwinding reaction was carried out as described in **Materials and Methods**, except that the dnaK and grpE protein concentrations were changed as indicated at the top of the figure. Lane 1, both dnaK and grpE protein omitted from the unwinding assay; lanes 2-7 ("grpE-independent" reaction), grpE protein is omitted and the concentration of dnaK varied from 0.2 μg to 3.2 μg per 25-μl reaction; lanes 8-13 ("grpE-dependent" reaction), the concentration of dnaK protein per 25-μl assay is 0.2 μg and the concentration of grpE protein varied from 25 ng to 250 ng.

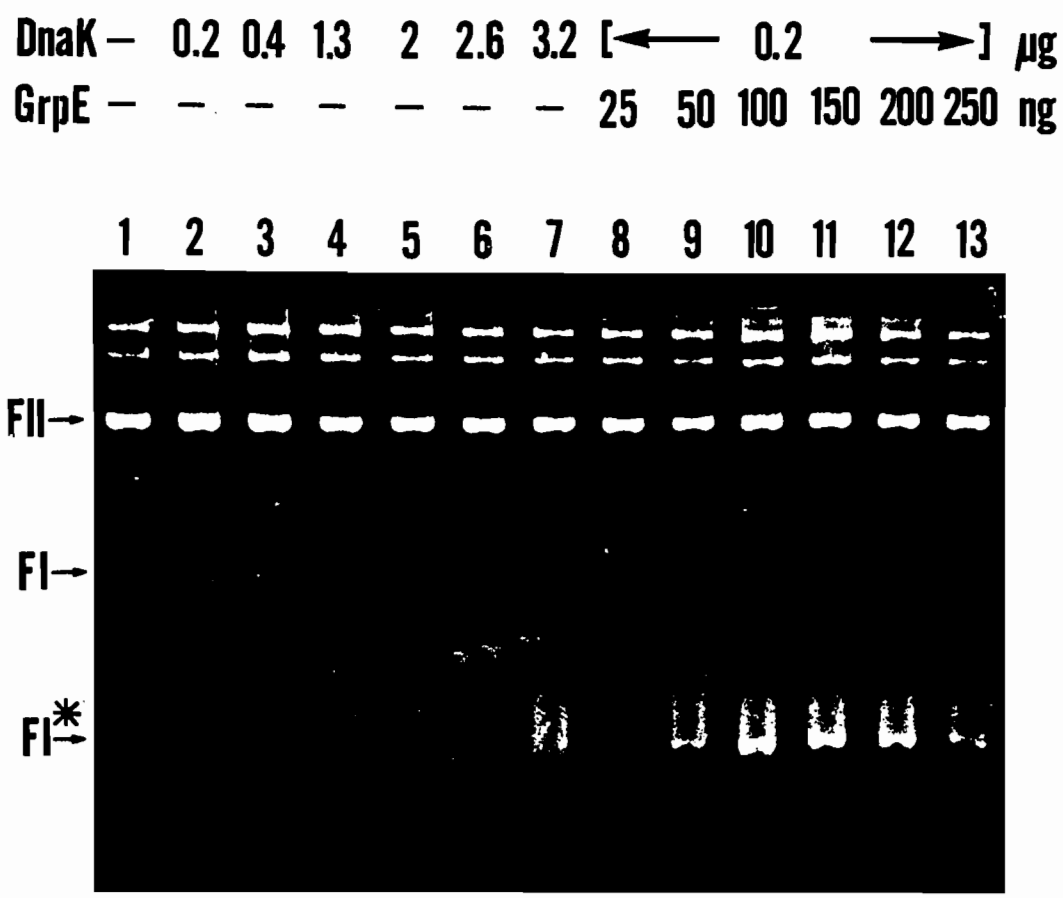

DnaK – 0.2 0.4 1.3 2 2.6 3.2 [← 0.2 →] µg
GrpE – – – – – – – 25 50 100 150 200 250 ng
1 2 3 4 5 6 7 8 9 10 11 12 13
FII→
FI→
FI*→

Table C.2

Isolation of DNA replication intermediates

A. "grpE-independent" system

Prepriming proteins preincubated with λdv DNA before chromatography	% recovery of λ DNA replication activity≠	% of unwound plasmid DNA§
1. λO	70-80%	< 5%
2. λO, λP, dnaB	60-70%	< 5%
3. λO, λP, dnaB, dnaJ	30-40%	< 5%
4. λO, λP, dnaB, dnaJ, dnaK	< 5%	< 5%
4. λO, λP, dnaB, dnaJ, dnaK*	20-30%	< 5%
5. λO, λP, dnaB, dnaJ, dnaK, ssb, gyrase	< 5%	> 80%

B. "grpE-dependent" system

Prepriming proteins preincubated with λdv DNA before chromatography	% recovery of λ DNA replication activity	% of unwound plasmid DNA
1-3. same as for part A, above.		
4. λO, λP, dnaB, dnaJ, dnaK, grpE	60-75%	<5%
5. λO, λP, dnaB, dnaJ, dnaK, grpE, ssb, gyrase	50-60%	> 80%

≠ Missing prepriming proteins, if any, were added to the void volume fractions after gel filtration on a Sepharose 4B column, along with DNA polymerase III holoenzyme and dnaG primase as described in Materials and Methods.

§ A portion of the void volume fraction following gel filtration on a Sepharose 4B column was analysed on a 1% (w/v) agarose gel containing chloroquine. The percentage of unwound DNA was determined as described in Materials and Methods.

* Following gel filtration, grpE protein (150 ng) was added to the void volume fraction at the same time as DNA polymerase III holoenzyme and dnaG primase.

dependent reaction (15). In this step, DNA is unwound if the additional proteins required for unwinding, i. e., ssb and DNA gyrase, are added. However, it is not possible to isolate a stable DNA replication intermediate at this stage. An explanation is that, following gel filtration, the residual λP protein associated with the complex is capable of inhibiting dnaB's helicase activity, especially because most of the dnaK protein is no longer present to inhibit λP action (see Discussion). However, when grpE protein is added, together with DNA polymerase III and dnaG primase following the prepriming reaction, some replication activity can be recovered (Table C.2). An explanation is that grpE prevents residual levels of λP protein from inhibiting dnaB's helicase activity.

Surprisingly, in the "grpE-dependent" system, it is possible to isolate a stable DNA replication intermediate following unwinding of DNA (Table C.2, reaction 5). Addition of extra λO, λP, dnaB, dnaK, dnaJ, grpE or gyrase protein after gel filtration does not significantly increase the recovery of DNA replication activity. However, addition of excess ssb protein after gel filtration of the prepriming intermediate does increase recovery of DNA replication to approximately 75% (unpublished data). At this time, we do not fully understand why the presence of grpE results in the formation of a stable DNA replication intermediate (see Discussion).

The isolated nucleoprotein intermediates shown in Table C.2 represent true prepriming complexes because a) isolation of the complexes depends on the preincubation of the listed proteins with DNA at 32°C (unpublished data), and b) DNA synthesis on the isolated intermediates does not require further addition of any of the prepriming proteins used initially to form the intermediate (Table C.2).

Discussion

Reconstituted from extensively purified proteins, *in vitro* λdv DNA replication, as presented here, is absolutely dependent on the presence of the bacteriophage-encoded replication proteins, λO and λP, as well as the *E. coli* -encoded proteins, dnaB, dnaG, dnaK, dnaJ, ssb, DNA gyrase, and DNA polymerase III holoenzyme. We believe that this "grpE-independent" (high concentration of dnaK protein, no grpE protein), *ori*λ-specific DNA replication system is similar to the purified λdv DNA replication system previously described (17, 18). Both the "λ single-strand replication reaction" (14) as well as the system presented here are capable of initiating *ori*λ-specific DNA synthesis on double-stranded supercoiled template, provided that more than 600 molecules of dnaK protein are used per *ori*λ site. Addition of highly purified grpE protein to the reconstituted "grpE-independent" system further stimulates λdv DNA synthesis. However, in the presence of grpE ("grpE-dependent" system), the amount of dnaK protein actually required for efficient DNA synthesis is reduced by at least ten-fold. One trivial explanation for these results is that our purified dnaK protein, which is known to form a stable, hydrophobic complex with grpE (31), is contaminated to some extent by grpE protein. Three control experiments eliminate this possibility: a) immunoblot assays of our dnaK protein preparation using anti-grpE antibodies do not detect any grpE contamination (unpublished data), b) our dnaK protein preparation fails to stimulate a crude *in vitro* λ DNA replication system derived from *grpE*280 mutant bacteria (31), and c) wild type dnaK protein purified from *E. coli* strain DA259, which carries a deletion of the *grpE* gene (D. A. and C. G., submitted for publication), has the same specific activity in the "grpE-independent" purified system as dnaK protein purified from a wild type dnaK-overproducing strain (Fig. C.2). From these control experiments, we conclude that, in the absence of grpE protein,

dnaK protein is needed in large quantity in the purified system to stimulate *oriλ*-dependent DNA synthesis.

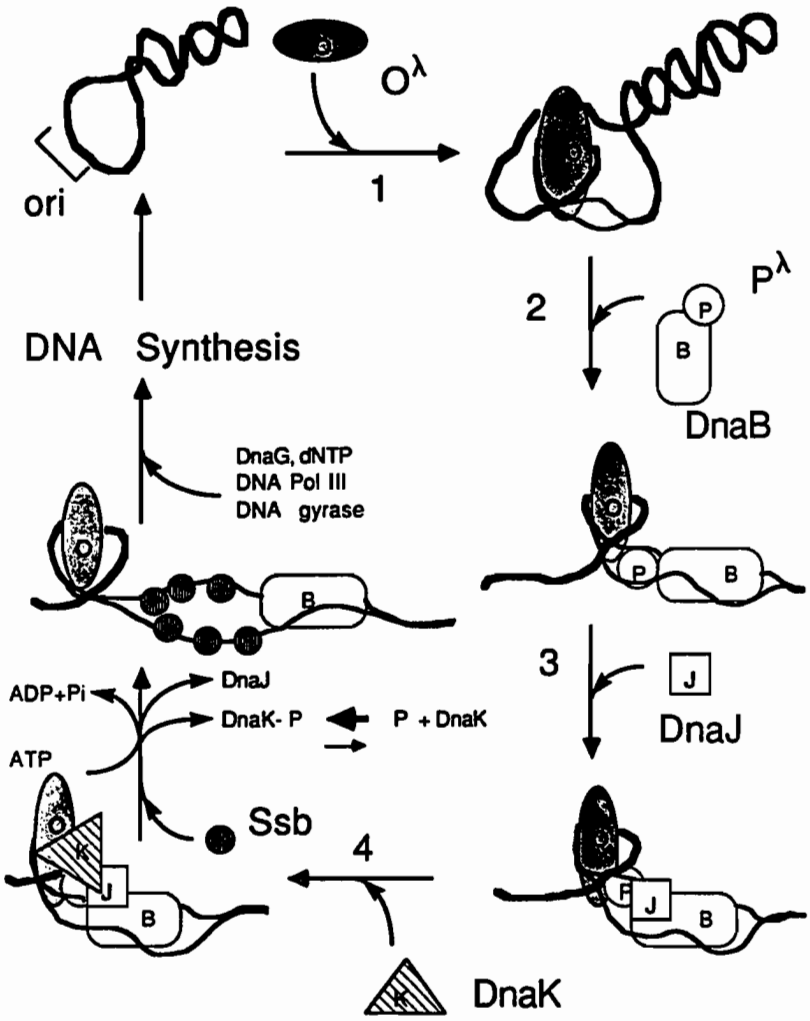
Why is dnaK required in high concentrations in the "grpE-independent" system and how can grpE protein decrease the requirement for dnaK? As supported by genetic experiments, one of the targets for dnaK action is the λP protein (11, 22). We have shown that λP protein binds to a dnaK-affinity column and, in the presence of ATP, is partially eluted (15). There is also evidence of λP - dnaK interaction from gel filtration and crosslinking experiments.[1] In addition, both dnaK and dnaJ proteins are responsible for the release of λP protein from the prepriming *oriλ* - λO -λP - dnaB complex (15). From this as well as other unpublished data [1], we conclude that a) the affinity of dnaK for λP protein is low because high concentrations of dnaK protein must be used in order to bind 1 to 2 molecules of λP to 1 molecule of dnaK protein, b) binding is specific because purified mutant dnaK756 protein is not able to bind to λP protein,[1] and c) the presence of 4 mM ATP only partially inhibits formation of the λP-dnaK complex. The last observation suggests that in the presence of ATP, at the levels used in our DNA replication and DNA unwinding assays, there is a steady state equilibrium between free λP protein and a λP-dnaK complex.

According to the hypothetical model presented in Fig. C.6, following formation of the *oriλ* - λO - λP - dnaB complex, dnaJ protein interacts with dnaB and λP [1] and assists, by additional interaction with dnaK and λP, in positioning dnaK protein near λP protein. In an ATP-dependent reaction, λP protein, complexed with dnaK, is removed from dnaB and a steady state equilibrium results between the λP-dnaK complex and free λP protein. One prediction of this model is that excess free λP protein, not sequestered by dnaK, can reassociate with dnaB protein present at *oriλ* or

---

[1]D. Ang, K. Liberek, C. Georgopoulos and M. Zylicz, manuscript in preparation.

Figure C.6. Model of initiation of λdv DNA replication in the purified *in vitro* system. The series of envisioned events leading to DNA replication can be summarized as follows: the λO protein binds to *oriλ* sequence, and induces bending of the DNA in such a way that single-stranded DNA regions are formed near the *oriλ* sequence (step 1) (23). The λP–dnaB complex (28), through the demonstrated λO - λP interaction (33), recognizes the *oriλ* sequence and binds in such a way that the dnaB helicase is positioned near the single-stranded, double-stranded junction of DNA created by binding of the λO protein. In the *oriλ* - λO - λP - dnaB complex, the dnaB helicase is inhibited by the presence of λP protein (step 2) (6, 15). The dnaJ protein, which exhibits affinity for both dnaB and λP proteins,[1] recognizes the λP protein on the surface of dnaB and binds to both dnaB and λP proteins (step 3). The dnaK protein binds preferentially to those λP protein molecules bound by dnaJ protein molecules (step 4). ATP hydrolysis, catalyzed presumably by the dnaK protein's ATPase activity, triggers partial disassembly of the prepriming complex. The dnaJ and dnaK proteins, in a complex with λP, are released from the prepriming complex (15). The dnaB protein, thusly liberated from λP, unwinds the DNA near the *oriλ* sequence (6). For extensive unwinding of DNA by the dnaB helicase, the presence of DNA gyrase and ssb are required (step 5). The dnaG primase recognizes the proper dnaB-single-stranded DNA complex and proceeds to synthesize RNA primers at apparently random sites. The DNA polymerase III holoenzyme extends the RNA primers into DNA (step 6).


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

the replication fork, thus inhibiting dnaB's helicase activity, and creating an unfavorable situation for the initiation of λdv DNA replication.

We postulate that, in the "grpE-independent" system, a high concentration of dnaK protein is needed to shift the equilibrium of the λP - dnaK $\xrightarrow{ATP}$ λP + dnaK reaction in favor of formation of the λP-dnaK complex. This reaction is important in two stages of the prepriming reaction: a) recognition of λP which is bound to dnaB protein at *oriλ*, and b) sequestration of λP when released from the *oriλ* - λO - λP - dnaB complex. However, sequestration of λP protein by dnaK engages the dnaK molecules, effectively preventing them from releasing additional λP protein complexed with dnaB at *oriλ*. This could justify the need for a high concentration of dnaK in this situation.

In the "grpE-dependent" system, a much lower concentration of dnaK protein is required for efficient *oriλ*-dependent replication. One attractive hypothesis is that grpE protein itself sequesters the λP protein following dnaK action, thus alleviating the requirement for high concentrations of dnaK protein. The dnaK protein, free from its complex with soluble λP protein, can then recycle and help release additional λP protein which may be complexed to dnaB at *oriλ* or which may have "recaptured" dnaB at the replication fork(s). The inhibition of both DNA replication and DNA unwinding by excess grpE protein (this work) could be due to the demonstrable grpE-dnaK interaction (31; C. Johnson, G. N. Chandrasekhar and C. G., submitted for publication). The high levels of grpE could shift the equilibrium towards the formation of a grpE-dnaK complex, thus effectively interfering with dnaK's ability to release λP.

It is not certain which of the two purified *in vitro* λdv DNA replication systems described here mimics more closely the *in vivo* situation. We suggest that, because the *grpE*280 point mutation, as well as the *grpE*$^{\Delta}$ deletion, completely block the initiation

of λ DNA replication *in vivo* , the reconstituted "grpE-dependent" system is the more physiological one. The importance of grpE function *in vivo* is demonstrated by the fact that in the *grpE*$^{\Delta}$ strain, it is not possible to isolate λP mutations ("π"; 11) which can bypass the complete lack of grpE protein (D. A. and C. G., unpublished data). Since dnaK is involved in the heat shock response and, potentially, transport of proteins across membranes (4, 5), the need for high concentrations of dnaK protein in the crude *in vitro* system (32) may be due to binding of dnaK to other proteins absent in the purified λ DNA replication system.

We have not yet examined the obvious possibility that grpE protein may also be required for switching λ DNA replication from a unidirectional to a bidirectional mode. Formally, there is the possibility that both systems are simultaneously involved in the initiation process, the "grpE-dependent" system in the initiation of leading strand synthesis and the "grpE-independent" system in the initiation of lagging strand synthesis. This could explain why the preprimosomal structure including grpE has been shown to be more stable and why, in the presence of a high concentration of dnaK protein, we observe a steady state equilibrium between build-up and destruction of the preprimosomal complex (15).

Acknowledgments

This work was supported by NIH grants to C. G., an NIH training grant to D. A., and a Polish Academy of Sciences grant (CPBR 3.13) to M. Z. We thank Drs. J. Kaguni, A. Kornberg, C. McHenry and P. Modrich for generous gifts of purified enzymes. We would like to acknowledge Dr. R. McMacken for collaboration during the early steps of this work, and the fact that Drs. C. Alfano and R. McMacken were the first to show that our purified grpE preparation greatly reduced the amount of dnaK protein needed in the purified reconstituted λ DNA replication system.

Literature Cited

1. **Anderl, A. and A. Klein.** 1982. Replication of λdv DNA *in vitro*. Nucleic Acids Res. **10**:1733-1740.

2. **Arai, K., S. Yasuda and A. Kornberg.** 1981. Mechanism of DnaB protein action. I. Crystallization and properties of DnaB protein, an essential replication protein in *Escherichia coli*. J. Biol. Chem. **256**:5247-5252.

3. **Baker, T. A., K. Sekimizu, B. W. Funnell and A. Kornberg.** 1986. Extensive unwinding of the plasmid template during staged enzymatic initiation of DNA replication from the origin of the *Escherichia coli* chromosome. Cell **45**:53-64.

4. **Chirico, W. J., M. G. Waters and G. Blobel.** 1988. 70 K heat shock related proteins stimulate protein translocation into microsomes. Nature **332**:805-810.

5. **Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig and R. Schekman.** 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature **332**:800-805.

6. **Dodson, M., H. Echols, S. Wickner, C. Alfano, K. Mensa-Wilmot, B. Gomes, J. H. LeBowitz, J. D. Roberts and R. McMacken.** 1986. Specialized nucleoprotein structures at the origin of replication of bacteriophage λ: Localized unwinding of duplex DNA by six-protein reaction. Proc. Natl. Acad. Sci. U. S. A. **83**:7638.

7. **Dodson, M., J. Roberts, R. McMacken and H. Echols.** 1985. Specialized nucleoprotein structures at the origin of replication of bacteriophage λ: complexes with λO, λP and *Escherichia coli* DnaB proteins. Proc. Natl. Acad. Sci. U. S. A. **82**:4678-4682.

8. **Dove, W. F., H. Inokuchi and W.F. Stevens.** 1971. Replication control in phage lambda. *In* A. D. Hershey (ed.), The bacteriophage lambda, pp. 747-771, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

9. **Fuller, R. S., J. M. Kaguni and A. Kornberg.** 1981. Enzymatic replication of the origin of the *Escherichia coli* chromosome. Proc. Natl. Acad. Sci. U. S. A. **78**:7370-7374.

10. **Furth, M. E. and S. H. Wickner.** 1983. Lambda DNA replication. *In* R. W. Hendrix, J. W. Roberts, F. W. Stahl and R. A. Weisberg (eds.), Lambda II, pp. 145-173, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

11. **Georgopoulos, C. P. and I. Herskowitz.** 1971. *Escherichia coli* mutants blocked in lambda DNA synthesis. *In* A. D. Hershey (ed.), The bacteriophage lambda, pp. 553-564, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

12. **Lanka, E., C. Edelbluth, M. Schlicht and H. Schuster.** 1978. *Escherichia coli* dnaB protein: Affinity chromatography on immobilized nucleotides. J. Biol. Chem. **253**, 5847-5851.

13. **LeBowitz, J. H. and R. McMacken.** 1986. The *Escherichia coli* DnaB replication protein is a DNA helicase. J. Biol. Chem. **261**:4738-4748.

14. **LeBowitz, J. H., M. Zylicz, C. Georgopoulos and R. McMacken.** 1985. Initiation of DNA replication on single-stranded DNA templates catalyzed by purified replication proteins of bacteriophage λ and *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **82**:3988-3992.

15. **Liberek, K., C. Georgopoulos and M. Zylicz.** 1988. The role of the *Escherichia coli* DnaK and DnaJ heat shock proteins in the initiation of bacteriophage λ DNA replication. Proc. Natl. Acad. Sci. U. S. A. **85**:6632-6636.

16. **Maniatis, T., E. F. Fritsch and J. Sambrook.** 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

17. **McMacken, R., C. Alfano, B. Gomes, J. H. LeBowitz, K. Mensa-Wilmot, J. D. Roberts and M. Wold.** 1987. Biochemical mechanisms in the initiation of bacteriophage λ DNA replication. *In* T. J. Kelly and R. McMacken (eds.), DNA replication and recombination, pp. 227-245, Alan R. Liss, Inc., N. Y.

18. **McMacken, R., K. Mensa-Wilmot, C. Alfano, R. Seaby, K. Carroll, B. Gomes and K. Stephens.** 1988. Reconstitution of purified protein systems for the initiation and regulation of bacteriophage λ DNA replication. *In* T. Kelly and B. Stillman (eds.), DNA replication in eukaryotes, **6**:25-34, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (Cancer Series).

19. **Mizuuchi, K., M. Mizuuchi, M. H. O'Dea and M. Gellert.** 1984. Cloning and simplified purification of *Escherichia coli* DNA gyrase A and B proteins. J. Biol. Chem. **259**:9199-9201.

20. **Neidhardt, F. C. and R. A. VanBogelen.** 1987. Heat shock response. *In* F. C. Neidhardt et al. (eds.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology, pp. 1334-1345, American Society for Microbiology, Washington, D. C. .

21. **Roberts, J. D. and R. McMacken.** 1983. The bacteriophage λ O replication protein: Isolation and characterization of the amplified initiator. Nucleic Acids Res. **11**:7435-7452.

22. **Saito, H. and H. Uchida.** 1977. Initiation of the DNA replication of bacteriophage lambda in *Escherichia coli* K12. J. Mol. Biol. **113**:1-25.

23. **Schnos, M., K. Zahn, R. B. Inman and F. R. Blattner.** 1988. Initiation protein induced helix destabilization at the λ origin: A prepriming step in DNA replication. Cell **52**:385-395.

24. **Tsurimoto, T., T. Hase, H. Matsubara and K. Matsubara.** 1982. Bacteriophage lambda initiators: Preparation from a strain that overproduces the O and P proteins. Mol. Gen. Genet. **187**:79-86.

25. **Tsurimoto, T. and K. Matsubara.** 1981. Purified bacteriophage λO protein binds to four repeating sequences at the λ replication origin. Nucleic Acids Res. **9**:1789-1799.

26. **Tsurimoto, T. and K. Matsubara.** 1982a. Replication of λdv plasmid *in vitro* by purified λO and P proteins. Proc. Natl. Acad. Sci. U. S. A. **79**:7639-7643.

27. **Tsurimoto, T. and K. Matsubara.** 1982b. Replication of bacteriophage λ DNA. Cold Spring Harbor Symp. Quant. Biol. **47**:681-691.

28. **Wickner, S.** 1978. DNA replication proteins of *Escherichia coli* and phage λ. Cold Spring Harbor Symp. Quant. Biol. **43**:303-310.

29. **Wold, M., J. Mallory, J. Roberts, J. LeBowitz and R. McMacken.** 1982. Initiation of bacteriophage λ DNA replication *in vitro* with purified λ replication proteins. Proc. Natl. Acad. Sci. U. S. A. **79**:6176-6180.

30. **Yamamoto, T., J. McIntyre, S. M. Sell, C. Georgopoulos, D. Skowyra and M. Zylicz.** 1987. Enzymology of the pre-priming steps in λdv DNA replication *in vitro*. J. Biol. Chem. **262**, 7996-7999.

31. **Zylicz, M., D. Ang and C. Georgopoulos.** 1987. The grpE protein of *Escherichia coli*: Purification and properties. J. Biol. Chem. **262**:17437-17442.

32. **Zylicz, M. and C. Georgopoulos.** 1984. Purification and properties of the *Escherichia coli* dnaK replication protein. J. Biol. Chem **259**:8820-8825.

33. **Zylicz, M., I. Gorska, K. Taylor and C. Georgopoulos.** 1984. Bacteriophage λ replication proteins: Formation of a mixed oligomer and binding to the origin of λ DNA. Mol. Gen. Genet. **196**:401-406.

34. **Zylicz, M., J. H. LeBowitz, R. McMacken and C. Georgopoulos.** 1983. The dnaK protein of *Escherichia coli* possesses an ATPase and autophosphorylating activity and is essential in an *in vitro* DNA replication system. Proc. Natl. Acad. Sci. U. S. A. **80**:6431-6435.

35. **Zylicz, M., T. Yamamoto, N. McKittrick, S. Sell and C. Georgopoulos.** 1985. Purification and properties of the DnaJ replication protein of *Escherichia coli*. J. Biol. Chem. **260**:7591-7598.