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Regulation and function of the heat shock response in *Escherichia coli*

Delaney, John Michael, Ph.D.
The University of Arizona, 1989
Regulation and Function
of the Heat shock Response in \textit{Escherichia coli}

by

John Michael Delaney

A Dissertation Submitted to the Faculty of
MICROBIOLOGY AND IMMUNOLOGY

In Partial Fulfilment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY

In the Graduate College
THE UNIVERSITY OF ARIZONA

1989
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by John M. Delaney entitled Regulation and Function of the Heat Shock Response in Escherichia coli and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Harris Bernstein
June 6, 1989

Dissertation Director
STATEMENT BY AUTHOR

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SIGNED:  

[Signature]
DEDICATION

This dissertation and the accompanying degree are dedicated to my parents, John and Sylvia Delaney. Through their endless kindness, generosity, and encouragement, I was given the opportunity to attempt this endeavor. Through their guidance, I was given the inquisitiveness and self-reliance to succeed in attaining this degree. Through their love, I was given the confidence to achieve any goal.
ACKNOWLEDGEMENTS

Throughout the course of my life as a graduate student I have learned many things. The most physical evidence of that which I have learned is the text which you now hold, my dissertation. This book-length treatise on a small, obscure division of biology is the product of perhaps the most practical, but the least important aspect of my new knowledge. It has been my experience that the most significant bits of wisdom are those which are the least tenable. Such has been the case here, and the ethereal fragments of wisdom imparted to me will influence the rest of my life. The knowledge that allowed me to write a dissertation was supplied by many people. The wisdom that will make it possible for me to be successful was subtly and graciously given to me by one man: Harris Bernstein. Through his words and actions Harris has taught me lessons that are beyond price, and is one of only a handful of the truly kind people I know.

There are several people to whom I would like to express my gratitude. First my family. To Therese Welchert (my wife) for making life in Tucson bearable. To Kris, Bob, and Megan Howard for always being there and for their unconditional love. To my sister Jan for reminding me that there is more to life than sitting inside complaining about the heat. I would like to thank Steve Abadon, Carol Bernstein, Peh Yean Cheah, Vivian Gage, Paul Hyman, Virginia Johns, Risa Kandell, Rolf Kuestner, Pat McCreary, John Obringer, and John Spizizen for their many thoughtful discussions and insight. To Mark Leusch for being my friend. Thank you all very much.
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The heat shock response is a highly conserved genetic mechanism which is induced by a wide range of environmental stimuli. Although intensively studied in both prokaryotes and eukaryotes, no regulatory mechanism has been identified by which the environmental stimuli affect expression of the heat shock genes. In addition, although many inducers of the heat shock response are known to cause DNA damage, the role of heat shock in repair of DNA damage remains unclear.

Mutants of *Escherichia coli* defective in the recA, uvrA, and xthA genes are more sensitive to heat than wild type. However, these mutants are able to develop thermotolerance, suggesting that thermotolerance is an inducible response capable of repairing heat-induced DNA damage independent of recA, uvrA, and xthA. Thermotolerance itself is shown to depend on the dnaK gene, directly linking the *E. coli* heat shock response to thermotolerance. In addition, the dnaK mutant is sensitive to heat and H₂O₂, but not to UV suggesting
that the DnaK protein may function to protect cells from
the specific DNA damage caused by heat and H₂O₂.

An *E. coli grpE* mutant was found to be
substantially more resistant to 50°C heat treatment than
wild type. However, *grpE*− cells have the same H₂O₂ and
UV sensitivity as wild type. This implies that the
conditions, for which a *grpE* mutation is beneficial, are
unique to heat exposure and are not caused by H₂O₂ or UV
exposure. Furthermore, heat shock protein synthesis
occurs sooner in the *grpE* mutant than in wild type,
indicating that the *grpE* gene product of *E. coli* may act
as a negative regulator of the heat shock response.

An adenyl cyclase deletion mutant of *E. coli* (*cya*)
failed to exhibit a heat shock response even after 30
min. at 42°C. Furthermore, a presumptive cyclic AMP
receptor protein (CRP) binding site exists within the
promoter region of the *E. coli htpR* gene. Together,
these results suggest that the *cya* gene may regulate
the heat shock response, through cyclic AMP, by directly
affecting the level of expression of the heat shock
sigma factor.
Chapter I

Introduction

During its lifetime an organism is exposed to a wide range of environmental conditions, many of which are stressful. Through the course of evolution cellular systems have developed which enable organisms to cope with these stresses. The heat shock response is one such system. When cultured cells or whole organisms are exposed to high temperatures, a small, specific set of highly conserved proteins is synthesized. These proteins are called heat shock proteins and are characteristic of the heat shock response.

The heat shock response is universal in nature, being observed in every organism studied to date. It is found in every cell and tissue type of multicellular organisms, in explanted tissues, and in cultured cells (Lindquist 1986). Furthermore, on the basis of their homology and antigenic similarity across the prokaryotic, eukaryotic, and archaeabacterial kingdoms, the heat shock proteins are among the most highly conserved proteins presently known (Neidhardt et al. 1984).
The ubiquity and conservative nature of the heat shock response implies that there is a strong selective advantage for such a response, and that the proteins involved play some fundamental role in the physiological processes that protect a cell or an organism from environmental stress. However, the specific protective mechanisms provided by the heat shock response remain elusive. The functions of only a few of the bacterial heat shock proteins have been established and, so far, these functions only relate to DNA replication in bacteria and bacteriophage (Table 1-1).

The cellular response of an organism to heat shock was first described in Drosophila. It was noted that puffs in specific locations on the polytene chromosomes in the salivary glands of Drosophila buskii could be induced by a brief pulse of heat or treatment with dinitrophenol (Ritossa 1962). Following this discovery, the study of the heat shock response was taken up by several investigators and much information about the response was obtained. In particular, it was found that the chromosomal puffs in Drosophila induced by heat were a) produced within a few minutes (Ashburner 1970),
Table 1-1. The heat shock genes of *Escherichia coli* and their known functions.

<table>
<thead>
<tr>
<th>Number</th>
<th>Name</th>
<th>Gene</th>
<th>Possible function</th>
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<tr>
<td>1</td>
<td>---</td>
<td><em>hpsA</em></td>
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<td>2</td>
<td>GroEL</td>
<td><em>mopA</em></td>
<td>Morphology of coliphage (weak ATPase activity); some role in RNA and DNA synthesis (?)</td>
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<tr>
<td>3</td>
<td>DnaK (groPC)</td>
<td><em>dnaK</em></td>
<td>Phage DNA replication (weak ATPase activity); modulation of heat shock response; necessary for RNA and DNA synthesis</td>
</tr>
<tr>
<td>4</td>
<td>sigma</td>
<td><em>rpoD</em></td>
<td>Promoter recognition; subunit of RNA polymerase</td>
</tr>
<tr>
<td>5</td>
<td>---</td>
<td><em>hpsE</em></td>
<td>---</td>
</tr>
<tr>
<td>6</td>
<td>GroES</td>
<td><em>mopB</em></td>
<td>Morphology of coliphage; some role in RNA and/or DNA synthesis (?)</td>
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<tr>
<td>7</td>
<td>---</td>
<td><em>hpsG</em></td>
<td>---</td>
</tr>
<tr>
<td>8</td>
<td>---</td>
<td><em>lupH</em></td>
<td>---</td>
</tr>
<tr>
<td>9</td>
<td>---</td>
<td><em>hpsI</em></td>
<td>---</td>
</tr>
<tr>
<td>10</td>
<td>lysyl-tRNA synthetase form II</td>
<td><em>lysU</em></td>
<td>Charging of tRNA; synthesis of diadenosine tetraphosphate (?)</td>
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<td>11</td>
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<td><em>hpsK</em></td>
<td>---</td>
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<td>12</td>
<td>---</td>
<td><em>hpsL</em></td>
<td>---</td>
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<td>---</td>
<td><em>hpsM</em></td>
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<td>14</td>
<td>---</td>
<td><em>hpsN</em></td>
<td>---</td>
</tr>
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<td>15</td>
<td>---</td>
<td><em>hpsO</em></td>
<td>---</td>
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<tr>
<td>16</td>
<td>Lon, La</td>
<td><em>lon</em></td>
<td>ATP-dependent protease</td>
</tr>
<tr>
<td>17</td>
<td>DnaJ</td>
<td><em>dnaJ</em></td>
<td>Some role in RNA and DNA synthesis?</td>
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b) associated with newly synthesized RNA (Leenders and Berendes 1972), c) found in other Drosophila species and in different tissues (Ritossa 1964, Berendes 1965), and d) accompanied by the disappearance of previously active puffs (Leenders and Berendes 1972). The analysis of the heat shock response at the molecular level began soon thereafter when it was found that coincidental to the appearance of the chromosomal puffs, a heat shock induced the synthesis of a small number of new proteins. These heat-inducible proteins are now termed the heat shock proteins and have been characterized quite extensively.

Recent research in the prokaryotic heat shock response (elucidated largely through studies in Escherichia coli) has produced much new information about both the induction of the response, and the genetic elements of the response. In E. coli, the heat shock response can be induced by many different agents; from heat to substances which inhibit DNA replication. Further, seventeen heat shock proteins are known in E. coli, with sizes ranging from 10 to 94kd. Some of these proteins are among the most abundant in the cell, while others are undetectable until induced. Seven of these heat shock proteins have been identified as the products
of known genes (Neidhardt et al. 1984). One of these proteins, the product of the dnaK gene is probably one of the most conserved proteins known. Another of these proteins, the product of the htpR gene, has been cloned and expressed in vitro (Neidhardt et al. 1983). Analyses of the htpR gene product by means of sequence determination and in vitro transcription assays with purified protein have led to the discovery that this protein is a novel sigma factor, specific for transcription of the genes involved in the heat shock response (Grossman et al. 1984). The promoter regions of the E. coli heat shock genes dnaK and groE and the gene encoding the heat shock protein C62.5 have a consensus sequence (Cowing et al. 1985) which allows these genes to be specifically transcribed by the heat shock sigma factor modified RNA polymerase.

The eukaryotic heat shock response and the proteins associated with this response have been studied extensively (Tanguay 1983). The response was first described in Drosophila (Ritossa 1962), and much of the present work on the subject in eukaryotes is still being done with that organism (Ashburner and Bonner 1979). The similarities in their heat shock responses are
striking. The eukaryotic response is characterized by a
dramatic and sudden increase in the synthesis of, on
average, 12 proteins (Neidhardt et al. 1984). However,
the number of proteins synthesized in response to heat
varies between organisms, from two in the frog *Xenopus
laevis* (Schlesinger et al. 1982) to ten in mammalian and
avian cells (Carlsson and Lazardes 1983). In Drosophila,
eight heat shock proteins have been reported
(Schlesinger et al. 1982). In yeast the number of heat
shock proteins appears to be nearly identical to the
number found in *E. coli*. In fact, the entire response
in yeast appears to mimic that found in *E. coli*
(Miller et al. 1982). This large number of heat-
inducible proteins seen in *E. coli* and in yeast
contrasts sharply with the smaller number of similar
proteins in higher eukaryotes.

The eukaryotic heat shock response is also
characterized by the suppression of synthesis of most
non-heat shock proteins. As with the number of heat
shock proteins, the degree to which the synthesis of
normal proteins is suppressed following heat treatment
varies among organisms. In Drosophila and human cells
this suppression is quite significant, while in yeast,
*Xenopus*, and chickens it is barely detectable
(Schlesinger et al. 1982). However, at very high, lethal inducing treatments, few proteins other than heat shock proteins are produced in any type of cell (Neidhardt et al. 1984).

In both prokaryotes and eukaryotes, the heat shock response is initiated quickly and is transient. In *E. coli* the response is transcriptionally activated in less than one minute. Within five minutes, synthesis of heat shock messenger RNAs begins to decline (Lindquist 1986). In Drosophila, the first indication of the response is the appearance of new puffs, mostly on the polytene chromosome 3, within one minute following the inducing treatment, and the response reaches its peak between 30 and 60 min (Neidhardt et al. 1984).

**Mechanisms of the Heat Shock Response**

**Induction of the Heat Shock Response**

In *E. coli* the heat shock response can be induced by a temperature shift up, by ethanol, and by some agents that damage DNA, alter the structure of DNA, or inhibit DNA replication. Inducers belonging to this latter group include ultraviolet light, nalidixic acid,
coumermycin, and viral infection (Neidhardt et al. 1984). Other inducers include the extracellular alkalinity (Taglicht et al. 1987) and hydrogen peroxide ($H_2O_2$) (VanBogelen et al. 1987b).

Induction by heat has two characteristic general features: (a) transient induction over a broad range of permissive growth temperatures with increasing inhibition of non-heat shock proteins as the temperature is raised, and (b) long lasting, nearly exclusive, synthesis of heat shock proteins at lethal temperatures of $50^\circ C$ and above (Neidhardt et al. 1984). The primary cellular effect of heat seems to be perturbation of the integrity of DNA including DNA damage and loss of negative superhelicity (Bridges et al. 1969a, Bridges et al. 1969b, Woodcock and Grigg 1972, Pellon et al. 1980).

Induction of the heat shock response by ethanol is similar to the induction by heat. As with heat, induction by ethanol treatment depends upon the E. coli htpR gene. The effects of ethanol on the cell are very diverse, ranging from lowering the internal pH of the cell to affecting the processing of proteins for transmembrane translocation (Neidhardt et al. 1984).

Ultraviolet light (UV) irradiation induces many of the heat shock proteins. This induction is htpR
dependent, but is delayed compared to heat induction. The peak induction occurs 15-20 min after UV treatment. Ultraviolet light irradiation also induces the E. coli SOS response, but the dose required to induce the heat shock response is much greater than that required to induce the SOS response (100 J/m² compared to 10 J/m²). Induction of heat shock by UV is independent of the SOS regulatory genes recA and lexA (Krueger and Walker 1984). Given the high doses of UV required for heat shock induction, it is reasonable to assume that a considerable amount of DNA damage occurs during UV treatment.

Nalidixic acid is another inducer of the E. coli SOS response (Gellert 1981). It is somewhat more effective as an inducer of the heat shock response than UV irradiation, although it is not as effective as heat. The maximum induction of heat shock following naladixic acid treatment is at 10 min (Krueger and Walker 1984). This agent is an inhibitor of DNA gyrase which is essential for DNA replication in E. coli (Drlica 1984). It is assumed that treatment of E. coli with DNA gyrase inhibitors generates a similar inducing signal to that of UV irradiation (Gellert 1981). Coumermycin, another
DNA gyrase inhibitor, also induces the heat shock response to some degree (Schlesinger et al. 1982, p.127).

Infection of E. coli with bacteriophage lambda causes the induction of several heat shock proteins. This increase in protein synthesis begins at 5 minutes and reaches a maximum at 15 min after infection (Drahos and Hendrix 1982). Interestingly, at least four E. coli heat shock proteins, the products of the dnaK, dnaJ, groE, and grpE genes, are essential for the correct assembly and replication of several coliphages such as lambda, T4, and T5 (Drahos and Hendrix 1982). Further, the induction of the heat shock response by bacteriophage infection seems to be htpR dependent, and several stages of the phage life cycle, including prophage excision, head assembly, and processing or assembly of tail fibers, are dependent on htpR gene function (Neidhardt et al. 1984). The exact inducing signal caused by bacteriophage infection, whether a result of the direct action of some phage gene product or some effect of infection on the physiological state of the host cell, is unknown at present (Neidhardt et al. 1984).
An alkaline shift in the pH of the growth media of a culture of *E. coli* also induces the heat shock response in an htpR-dependent fashion. Further, this induction follows the kinetics of the response when induced by heat. The mechanism by which the extracellular pH triggers the heat shock response is not known, but the induction may be due to a hyperpolarization of the cell membrane (Taglicht et al., 1987).

Finally, hydrogen peroxide (H$_2$O$_2$) has been shown to induce the heat shock response, at least partially, in both *E. coli* and *Salmonella typhimurium*. Three heat shock proteins are induced in *E. coli* by H$_2$O$_2$ treatment (VanBogelen et al. 1987b), and five heat shock proteins are induced in *Salmonella* (Morgan et al. 1986). However, no dependence on the htpR gene of *E. coli* has been established for this induction. Hydrogen peroxide has also been shown to induce the *E. coli* SOS response (Imlay and Linn 1987). Although no specific stimulus has been identified for H$_2$O$_2$ induction of the heat shock response, the effects of this treatment on the *E. coli* cell are relatively well known. In metabolically active cells low concentrations (1-3 mM) of H$_2$O$_2$ result in DNA damage (Imlay and Linn 1987). This damage takes the
form of base alterations, single and double strand
breaks, and DNA cross-links (Massie et al. 1972).

Generally, the same agents that induce the heat
shock response in prokaryotes also induce the heat shock
response in eukaryotes. However, there are several
inducing treatments which are either unique to
eukaryotes or have not been tested in prokaryotes
(Neidhardt et al. 1984). These "eukaryotic" inducers
include: sodium arsenite; cadmium; zinc, copper, and
mercury ions; sulphydryl reagents; calcium ionophores;
steroid hormones; chelating agents; pyridoxine;
methylene blue; glucosamine; deoxyglucose; and various
RNA and DNA viruses. Many of these agents are species
specific, or have only been tested in a few cell types
(Lindquist 1986). Anaerobiosis, and recovery from
anaerobiosis induces the heat shock response in many
organisms (Lindquist 1986), but was found to be
ineffective at inducing this response in Salmonella
typhimurium (Spector et al. 1986). Treatments known
not to induce the heat shock response in E. coli include
high salt, high intracellular pH, amino acid analogues,
and shifts between aerobic and anaerobic conditions
(Neidhardt et al. 1984).
Regulation of the Heat Shock Response

A notable feature of heat shock gene expression is that the responses of different organisms, and, in some cases, different cell types within an organism, are regulated in different ways (Lindquist 1986). For example, in E. coli and in yeast the response is controlled at the level of transcription. In Drosophila, both transcriptional and translational regulation is found. In Xenopus, the response in somatic cells is controlled primarily at the transcriptional level, whereas in oocytes the response is regulated at the translational level (Lindquist 1986).

Prokaryotic heat shock gene regulation

In E. coli, as mentioned above, the regulation of the heat shock response takes place primarily at the level of transcription. Induction of this response requires de novo RNA synthesis and the messages (mRNA) produced have half-lives of 1-2 min which is normal for E. coli mRNAs (Yamamori and Yura 1982). The promoter sequences of several heat shock genes have been identified by mapping the 5' ends of their messenger
RNAs (Lindquist 1986). A comparison of these sequences reveals two regions which show consensus elements in the -35 and -10 regions. These consensus sequences are unique to heat shock promoters. Different consensus sequences in the -35 and -10 regions of other *E. coli* genes are essential for transcription initiation by the normal (sigma-70) RNA polymerase (McClure 1985). A small sequence is shared by both heat shock and sigma-70 promoters in the -35 region, but there is no common sequence in the -10 region (Cowing *et al.* 1985).

The transcription activating factor which allows RNA polymerase recognition of heat shock promoters is coded for by the *E. coli* htpR gene, also called rpoH (Grossman *et al.* 1984, Crickmore and Salmond 1986). This transcription factor has been found to be an alternate sigma factor, called sigma-32 (Grossman *et al.* 1984). The sigma-32 protein is regulated in two ways. First, changes in the concentration of htpR mRNA is increased by a heat shock (Lindquist 1986). How this increase in synthesis is brought about is perplexing since expression of htpR does not require sigma-32 (Bloom *et al.* 1986), and since none of the 4-7 htpR promoter elements found is a heat shock promoter (Lindquist 1986; Crickmore and Salmond 1986). Further,
the stability of the sigma-32 protein in *E. coli* is affected by the heat shock gene, dnaK. Bacteria carrying the dnaK756 mutation fail to turn off the heat shock response. Likewise, bacteria carrying the wild type dnaK gene on a multi-copy plasmid which over-produces the DnaK protein have a greatly diminished heat shock response (Neidhardt et al. 1984). Activity of sigma-32 is also affected by changes in the concentration of sigma-70 presumably through competition with sigma-70 for core polymerase or holoenzyme (Lindquist 1986). This competition may also help to explain why increased transcription of heat shock genes is accompanied by decreased transcription of other genes (Lindquist 1986). No evidence has been presented for post translational regulation of the heat shock response in prokaryotes (Neidhardt et al. 1984).

**Function of the Heat Shock Response**

At the most basic level, the function of the response is, most likely, homeostatic. That is, to protect a cell or organism against adverse environmental conditions, and to ensure that a cell is able to continue its normal metabolic processes after the
environmental stress is removed. There are two characteristics of the heat shock response which support this idea. First, the heat shock response is transient (Neidhardt et al. 1984), being expressed only until the stress has passed. Second, cells exposed to one heat treatment are more resistant to a second heat treatment. This phenomenon is called thermotolerance and has been observed in many organisms and cell types (Lindquist 1986).

At least two homeostatic mechanisms have been proposed for the heat shock response. The first of these proposals suggests that certain heat shock proteins play a role in protein folding and assembly. The heat shock response, it is suggested, can be induced in eukaryotic cells by the presence of denatured proteins (Ananthan et al. 1986). At least two eukaryotic heat shock proteins (Hsp70 and Hsp90) are present in the endoplasmic reticulum and in the cytoplasm (Pelham 1986), both sites of protein maturation. Further, a large amount of hsp70 is found in the cell nucleus during heat stress. Here, it presumably functions to sort out protein aggregations that occur during protein maturation (Pelham 1986). On the basis of these considerations, it has been
postulated that heat shock proteins may play a role in protecting the cell from any heat induced protein denaturation that may occur during heat stress.

The second homeostatic mechanism proposed for the heat shock response also involves the activity of heat shock proteins. By this proposal, the heat shock proteins are synthesized in response to an alteration in chromosome structure or to DNA damage. Heat shock is known to cause DNA damage. This damage takes the form of single and double strand breaks (Bridges et al. 1969a, Bridges et al. 1969b, Woodcock and Grigg 1972), but also includes base alterations resulting in apurinic and apyrimidinic sites (Massie et al. 1972, Hagensee and Moses 1986, Ripley 1988). Heat shock is also known to cause major changes in the degree and pattern of methylation of core histones (Neidhardt et al. 1984). Furthermore, nucleoids isolated from E. coli cells heat shocked at 50°C have a lower sedimentation coefficient than non-treated cells. This indicates DNA unfolding, loss of DNA supercoiling, and/or dissociation of the RNA and protein involved in maintenance of the nucleoid structure (Pellon et al. 1980). In addition, during the period following a lethal (50°C) heat treatment, the
*E. coli* nucleoid becomes associated with protein structures which appear to be the same proteins that are synthesized during heat shock (Pellon et al. 1982).

**Repair of DNA Damage in *E. coli***

As mentioned above many agents which induce the heat shock response are known to cause DNA damage. In *E. coli*, DNA damage can be repaired by means of at least two general DNA repair pathways: excision repair and recombinational repair. Both excision repair and recombinational repair processes are part of an inducible repair process in *E. coli* known as the SOS response (Walker 1985).

The SOS system was the first regulatory system to be described which is induced by DNA damaging agents, and is the largest, and best understood DNA damage-inducible system (Walker 1985). When cells of *E. coli* are exposed agents which damage DNA or prevent its replication, the SOS response is induced. The expression of the genes in the SOS regulatory network is under the control of two genes: *lexA*, and *recA* (Little and Mount 1982). The product of the *lexA* gene is a repressor which binds to the promoter of every gene involved with the SOS response, inhibiting its
expression. The product of the recA gene is multifunctional. Besides being essential for homologous recombination, the RecA protein is a protease which, in the presence of DNA damage, cleaves the LexA protein thus allowing expression of SOS genes (Little and Mount 1982). Among the cellular activities which characterize the SOS response are the following: (1) Weigle reactivation, (2) filamentous growth, (3) induction of prophage, (4) a capacity to carry out inducible excision repair, and (5) an increased capacity to repair double stranded breaks (Witkin 1976).

Excision repair is an important strategy for the accurate removal of DNA lesions and takes advantage of the fact that information in a DNA molecule is present in two copies as a consequence of the complementary, double stranded structure of DNA. Agents that damage only one strand of a DNA molecule can be repaired by means of the following operations: (1) the incision of the damaged DNA strand at or near the site of the lesion, (2) the excision of the fragment of DNA containing the damage, (3) resynthesis of the DNA that was removed during the excision step using the complementary DNA strand as a template, and (4) ligation
of the newly synthesized fragment to form an intact DNA molecule (Walker 1985). These steps outline the process of excision repair.

Operationally, excision repair processes can be divided into two categories on the basis of the mechanism by which the incision step is carried out. In excision repair processes of the first category, a glycosylase enzyme first removes the damaged base generating an apurinic or apyrimidinic (AP) site. An AP endonuclease then breaks a phosphodiester bond at the AP site (Lindahl 1982). Excision repair may then proceed. Several glycosylases that recognize damaged bases have been described. In general, most of these glycosylases are very specific and recognize only one damaged base (Lindahl 1982). In excision repair processes of the second category, a protein or protein complex recognizes a lesion in the DNA and then directly incises the DNA without the formation of an AP site (Hanawalt et al. 1979).

Studies of excision repair in *E. coli* has revealed three genes which are required for this repair process. Mutations in the genes *uvrA*, *uvrB*, or *uvrC* prevent excision repair and render cells sensitive to killing by UV light (Howard-Flanders et al. 1964). Another gene,
uvrD, was also identified that interfered with excision repair of pyrimidine dimers. Unlike mutations in uvrA, uvrB, or uvrC, mutations at the uvrD locus can result in a large increase in the rate of spontaneous mutation (Walker 1985). The UvrA, UvrB, and UvrC proteins have been purified and, when combined together in the presence of ATP and Mg$^{2+}$ have an endonucleolytic activity that is capable of incising UV irradiated DNA. Omission of any one of these proteins or ATP or Mg$^{2+}$ results in the loss of incision activity (Yeung et al. 1983). Incision by the UvrABC endonuclease results in the formation of a single-stranded gap 12-13 nucleotides long (Yeung et al. 1983). The incision step of excision repair is followed by DNA synthesis which fills in the excised gaps (Pettyjohn and Hanawalt 1964). A DNA polymerase appears to fill in the gap left after UvrABC action with very little exonucleolytic expansion of the gap (Walker 1985). DNA Polymerase I appears to be the major enzyme which acts during the resynthesis event of excision repair (Walker 1985).

A second type of DNA repair process in E. coli is recombinational repair. Several genes which play a role in homologous recombination have been identified: recA,
recB, recC, recF, recJ, recN, and ruv. Mutation in any one of these genes result in increased sensitivity to killing by DNA damaging agents. Furthermore, the expression of at least three of these genes, the recA, recN, and ruv genes, is induced by DNA damaging agents and is controlled by the SOS regulatory circuit (Walker 1985). This finding indicates that the products of these recombination genes play either direct or indirect roles in the repair of DNA damage in E. coli. At least two repair processes have been described in which a subset of these genes is utilized: (1) daughter strand gap repair, and (2) double strand break repair (Walker 1985).

When DNA replication occurs in cells that have been exposed to UV light, the newly synthesized DNA strand has a lower molecular weight than newly synthesized DNA from unirradiated cells. The lower molecular weight is the result of gaps in the new strand. These gaps are formed when replication is blocked by a DNA lesion such as a pyrimidine dimer and then resumes at a site past the lesion. Daughter strand gap, also known as post-replication recombinational repair, is utilized to repair these gaps and is recA dependent (Hanawalt et al. 1979). The gaps are filled by means of recombinational
strand exchange between parent and daughter strands and results in the covalent attachment of parental DNA to daughter strands (Rupp et al. 1971). In excision repair-deficient mutant, pyrimidine dimers remain in the DNA, and become equally distributed between parental and daughter strands as a consequence of strand exchanges (Ganesan and Seawell 1975). Therefore, daughter strand gap repair fills gaps in DNA generated by replication blocks at the sites of certain DNA damages. However, these damages are not removed by this type of repair. Rather, they persist in the DNA until removed by means of excision repair processes. That is, once the strand exchange has occurred, the damages in the original strand as well as those transferred to the daughter strand can be tolerated by the cell at least temporarily until the damages themselves can be removed.

Double strand break repair in *E. coli* is another recombinational repair process. Double strand breaks in DNA can be formed through the action of certain chemical and physical agents such as mitomycin C or ionizing radiation (Walker 1985). In *E. coli* the ability to repair double strand breaks is an inducible process since pretreatment of cells with either X-rays or UV
light results in increased resistance to killing by either X-rays of gamma rays (Pollard and Achey 1975). This induced resistance is dependent on the recA gene and also requires de novo protein synthesis (Pollard and Achey 1975). Perhaps the most critical requirement for inducible double strand break repair is the presence of another DNA duplex which is homologous to the damaged molecule (Krasin and Hutchinson 1977). In fact, *E. coli* cells grown such that they contain 4-5 genomes per cell are more resistant to killing by ionizing radiation (Pollard et al. 1981).

**Thermotolerance**

There is much data which imply that the development of thermotolerance depends upon the heat shock response. For example, *E. coli htpR* mutants, in which heat shock gene expression is blocked, fail to exhibit thermotolerance (Yamamori and Yura 1982). Likewise, in *Neurospora crassa*, when heat shock protein synthesis is blocked with cycloheximide, thermotolerance is not observed (Plesofsky-Vig and Brambl 1985). Agents other than heat, such as ethanol, which induce the heat shock response also induce thermotolerance, for example: ethanol induces thermotolerance in *Saccharomyces*
cerevisiae (Plesset et al. 1982), anoxia induces thermotolerance in Drosophila (Velazquez and Lindquist 1984), and sodium arsenite and ethanol induce thermotolerance in Chinese hamster fibroblasts (Li 1983). In mammalian cells, the accumulation and degradation of heat shock proteins directly coincides with the degree to which thermotolerance is expressed (Landry et al. 1982, Subjeck and Sciandra 1982, Li and Hahn 1987). Of all heat shock proteins, the concentration of the 70 kilodalton heat shock protein, Hsp70, best correlates with the development of thermotolerance (Lindquist 1986).

The *E. coli* DnaK protein

The *E. coli* dnaK gene product has been shown to share 48% amino acid sequence homology with the Hsp70 protein of Drosophila (Bardwell and Craig 1984), and 47% homology with the Hsp70 protein of humans (Hunt and Morimoto 1985). This high degree of homology implies that the function of the DnaK protein also may have been conserved as well. This protein has been shown to have several activities. These include: (1) a weak DNA-independent ATPase activity; (2) *in vitro* and *in vivo*
autophosphorylation (Zylicz et al. 1983); (3) initiation of DNA replication in bacteriophage lambda (Dodson et al. 1986); (4) a 5'-nucleotidase activity that is inhibited by AppppA (Bochner et al. 1986); and (5) is active during initiation of DNA replication in E. coli (Sakakibara 1988). Another characteristic of the dnaK gene product is that it is one of only a few heat shock proteins induced by both heat and hydrogen peroxide in E. coli and Salmonella typhimurium (VanBogelen et al. 1987b, Morgan et al. 1986). As mentioned, many inducers of heat shock in E. coli, particularly heat, H₂O₂, and UV are DNA damaging agents (Neidhardt et al. 1984; Woodcock and Grigg 1972; Bridges et al. 1969; Massie et al. 1972; Ananthaswamy and Eisenstark 1977). This finding together with the known activities of the E. coli DnaK protein suggest that a function of the heat shock response, and thermotolerance, may be to protect against the lethal effects of certain environmental agents, possibly by repairing DNA damage induced by those agents.

Effect of a dnaK mutation on the heat sensitivity of Escherichia coli

In order to understand the nature of the heat shock
response, I studied the inactivating effect of heat, H$_2$O$_2$ and UV on repair-deficient mutants and a dnaK mutant of E. coli. I found that the E. coli dnaK mutant is sensitive to heat and H$_2$O$_2$ but not to UV. This implies that the dnaK gene is needed to protect cells from the lethal effects of heat and H$_2$O$_2$ or to repair DNA damaged by these agents. In addition, I studied the ability of these mutants to develop thermotolerance and found that the dnaK mutant failed to develop thermotolerance even though other mutants involved in repair (recA, uvrA, and xthA) retained this capacity. This implies that thermotolerance in E. coli depends on the dnaK gene.

Effect of a grpE mutation on the heat sensitivity of Escherichia coli

The grpE gene of E. coli was originally identified because grpE mutants failed to support bacteriophage DNA replication (Saito and Uchida 1977). The grpE gene product is, in fact, essential for $\lambda$ DNA replication (Furth and Wickner 1983). Recently, the grpE gene product has been shown to be a heat shock protein (Ang et al. 1986). In addition, it was shown that bacteria
carrying the grpE280 mutation fail to form colonies above 43.5°C and do not allow phage λ replication at any temperature (Ang et al. 1986). Although these characteristics of grpE mutants establish the role of the grpE gene product in λ DNA replication, no function of the GrpE protein has been correlated to the heat shock response itself. In order to determine the role of grpE in the development of thermotolerance, the inactivating effect of heat, H₂O₂, and UV on a grpE mutant was studied. The results of these experiments revealed that the grpE mutant was more resistant to heat than wild type, and when induced for thermotolerance, this strain became even more resistant to heat. These results indicated that the grpE gene may function as a negative regulator of thermotolerance in E. coli.

Interestingly, the grpE mutant is not more resistant than wild type to H₂O₂ or UV. This suggests that the conditions for which the grpE gene product is required, exist during high temperature but not during H₂O₂ or UV exposure.

Effect of a cya mutation on heat shock gene expression in Escherichia coli

As mentioned above, the htpR gene of E. coli is
essential for the induction of a heat shock response. This gene encodes a sigma factor that allows RNA polymerase to specifically recognize heat shock promoters.

The intracellular level of heat shock sigma factor correlates well with the expression of heat shock genes (Straus et al. 1987, Skelly et al. 1987). Thus, increased expression of heat shock genes is accompanied by an increase in the level of heat shock sigma factor present within a cell. Interestingly, transcription of htpR depends on the primary sigma factor of E. coli, sigma-70 (Bloom et al. 1986, Erickson et al. 1987). Exactly what the signal is that directs $\sigma^{70}$ RNA polymerase to the htpR promoter during heat shock is unknown. Cyclic AMP may provide such a signal.

The product of the cya gene is the enzyme adenyl cyclase which converts adenosine triphosphate (ATP) to adenosine 3', 5'-cyclic phosphate (cyclic AMP or cAMP) (Botsford 1981). Cyclic AMP regulates gene expression through the binding of the cyclic AMP receptor protein (CRP or CAP) to specific promoters (de Crombrugghe et al. 1984). I studied the effect of a cya deletion mutation on heat shock gene expression. The cya mutant failed to
exhibit a heat shock response in that heat shock protein synthesis was not induced. Therefore, cyclic AMP appears to be essential for heat shock gene expression.

All known effects of cyclic AMP in bacteria are mediated through the cyclic AMP receptor protein (CRP) (de Crombrugghe et al. 1984). The CRP protein binds to specific sequences in the promoter regions of cyclic AMP-regulated genes. When I searched the published DNA sequence of the htpR gene for such a sequence, I found a presumptive CRP binding site which supports the idea that cyclic AMP could, in fact, regulate the E. coli heat shock response. Regulation of the heat shock response by cyclic AMP would provide the cell with a means to relay information about the temperature of the extracellular environment to the positive control element of the heat shock response (the htpR gene). Induction of the heat shock response would then protect the cell from the lethal effects of heat.
Bacterial strains

The bacterial strains used in this study are described in Table 2-1. Strains AB2463, AB2480, GW4813, and BW9101 will be henceforth referred to as recA-, recA- uvrA-, dnaK-, and xthA- respectively. The first three strains are derived from AB1157, is designated wild type, whereas the fourth strain, BW9101, is derived from KL16.

Strain DA16 carries a grpE280 missense mutation. It was derived from the wild type strain DA15. Both of these were the generous gift of Dr. Debbie Ang (Ang et al. 1986). These strains have a Tn10 (tet') insertion in the pheA gene which is 50-60% cotransducible with grpE (D. Ang, personal communication). A grpE' revertant derived from DA16 was also used in this study. The isolation of this strain is described in the Results (Chapter III).

E. coli strain CA8306.41 is cya+, and served as wild type. Strain CA8306 is a cya deletion mutant
derived from CA8306.41, and will henceforth be referred to as cya- (Brickman et al. 1973, Kiely and O'Gara 1983). Both of these strains were the generous gift of Dr. Richard Friedman.

Heat treatment

Overnight cultures of bacteria were grown at 30°C in Hershey broth (Steinberg and Edgar 1962) prior to each experiment. Hershey broth was prepared according to the following recipe: Bacto Nutrient Broth 8.0g; Bacto Peptone 5.0g; NaCl 5.0g glucose 1.0g; distilled deionized water, one liter. The media was adjusted to pH 7.2 - 7.4 with 8N NaOH prior to autoclaving. The cultures were diluted into fresh Hershey broth and incubated with shaking at 30°C to a log phase concentration of about 1x10⁸ cells/ml. One ml. of this culture was then pipetted into each of seven Eppendorf tubes, one tube for each time point of treatment being studied. The tubes were placed in a 50°C waterbath until the desired time. At each time of treatment, a tube was withdrawn from the waterbath. The bacterial suspensions were serially diluted in M-9 salts solution (Adams 1959, p.446) and plated on Hershey agar plates using the agar overlay method (Adams 1959, p.450).
Table 2-1. Characteristics and sources of *E. coli* strains used.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>Source</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1157</td>
<td>wild type</td>
<td>B. Bachman</td>
<td>Howard-Flanders <em>et al.</em> (1969)</td>
</tr>
<tr>
<td>AB2463</td>
<td>recA13</td>
<td>B. Bachman</td>
<td>Howard-Flanders and Theriot (1968)</td>
</tr>
<tr>
<td>AB2480</td>
<td>recA13 uvrA6</td>
<td>B. Bachman</td>
<td>Howard-Flanders <em>et al.</em> (1969)</td>
</tr>
<tr>
<td>BW9101</td>
<td>xthA90 (ΔxthA)</td>
<td>B. Bachman</td>
<td>White <em>et al.</em> (1976)</td>
</tr>
<tr>
<td>GW4813</td>
<td>dnaK52 (ΔdnaK)</td>
<td>G. Walker</td>
<td>Paek and Walker (1987)</td>
</tr>
<tr>
<td>DA15</td>
<td>wild type</td>
<td>D. Ang</td>
<td>Ang <em>et al.</em> (1986)</td>
</tr>
<tr>
<td>DA16</td>
<td>grpE280</td>
<td>D. Ang</td>
<td>Ang <em>et al.</em> (1986)</td>
</tr>
<tr>
<td>DA16-1</td>
<td>grpE' (revert.)</td>
<td>This lab</td>
<td></td>
</tr>
<tr>
<td>CA8306.41</td>
<td>wild type</td>
<td>R. Friedman</td>
<td>Kiely and O'Gara (1983)</td>
</tr>
<tr>
<td>CA8306</td>
<td>Δcya</td>
<td>R. Friedman</td>
<td>Kiely and O'Gara (1983)</td>
</tr>
</tbody>
</table>
M-9 salts solution was prepared according to the following recipe: NH₄Cl 1.0g; KH₂PO₄ 3.0g; Na₂HPO₄ 6.0g; distilled deionized water, one liter. Following preparation, the solution was adjusted to pH 6.8 with 10M HCl and autoclaved. Hershey plates consist of: Bacto agar 10.0g; Bacto tryptone 13g; NaCl 8.0g; sodium citrate 1.0g; glucose 3.0g; distilled deionized water, one liter. Soft (top) agar was prepared with the same concentration of ingredients as the plate agar except that the agar content was decreased to 6.5g/l, and the glucose content was reduced to 1.3g/l. The bacterial suspensions at all time points were diluted in duplicate except those at the zero dose time point, which were diluted in triplicate. Plates were incubated for 36-48 hrs. at 30°C. Possible photoreactivation was avoided during these experiments and during plate incubation by maintaining darkened conditions. Survival at any given time of heat treatment was determined as the ratio of colony forming units (cfus) after treatment to the number of colony forming units at the zero time point.

Thermotolerance

Thermotolerance experiments were carried out as described above for heat treatment except that prior to
the 50°C exposure, the bacterial suspensions were placed at 42°C for 15 min. The cell concentration was not seen to decline during this treatment. Following this 42°C heat treatment, the bacteria were immediately transferred to 50°C. Survival was determined as above.

**H$_2$O$_2$ treatment**

Bacteria were grown and distributed into Eppendorf tubes as described above. CuSO$_4$ was added to each tube of bacterial suspension to a final concentration of 0.1mM to increase the rate of hydroxyl radical formation. Next, H$_2$O$_2$ (Mallinckrodt 30%) was added to a final concentration indicated for each H$_2$O$_2$ killing experiment detailed in the Results section (chapter III). After addition of H$_2$O$_2$, the bacterial suspensions were either placed on ice (as with strains AB1157, GW4813, and BW9101) or kept at room temperature (as with strains DA15 and DA16) for the desired time of reaction. At each time point, 2000 units of catalase (Sigma) was added to the appropriate tube. The bacterial suspension was then serially diluted, plated, and incubated as above. Survival was determined as indicated above.
UV treatment

Bacterial cultures were grown as described above. When the culture reached a concentration of about $1 \times 10^8$ cells/ml, the culture was centrifuged at 1000xg for 20 min. The media was poured off and the cell pellet was resuspended in an equal volume of M-9 salts solution. The suspension was centrifuged again, as above, and the cell pellet resuspended in an equal volume of M-9 salts solution a second time. The suspension was then allowed to stand at room temperature for at least 20 min. One ml of the suspension was placed into each of seven small dishes (35x10mm), one dish for each time point being studied. The dishes were placed individually under a UV lamp (General Electric) emitting a dose of 1.0 J/m²/sec, for a specific length of time. After exposure, the dish was removed from the UV light, and the bacteria were serially diluted, plated, and incubated as described above. Photoreactivation was avoided, and survival was determined as above.
Preparation of bacterial cells for sodium dodecyl sulfate polyacrylamide gel electrophoresis

For labeling of proteins, bacteria were grown as described above, and serially diluted in M-9 salts solution and plated to determine cell concentration. 100μl of the bacterial culture was pipetted into Eppendorf tubes and placed into a 42°C waterbath for the desired time of heat shock protein induction. Five minutes prior to each time point, 10μCi [35S] methionine (1162 Ci/mM) (ICN) was added to the cell suspensions. This allowed 5 min for radiolabel incorporation. At each time point, the corresponding tube was withdrawn from the waterbath and rapidly cooled and kept on ice for at least five minutes. Cells were collected in a microcentrifuge and resuspended in 50μl of lysing buffer and 50μl SDS-PAGE loading buffer. Lysing buffer contains 10mg lysozyme/ml (Sigma, eggwhite lysozyme, 40,000 units/mg) and 0.12M EDTA. SDS-PAGE loading buffer contains: 20% glycerol; 10% SDS; 25% 1M Tris-HCl (pH 6.8); and 0.02% bromophenol blue as a tracking dye. The suspension was boiled for five minutes and allowed to cool. Prior to electrophoresis, each suspension was run through a Hamilton syringe several times to
homogenize the suspensions.

SDS-PAGE electrophoresis

Electrophoretic separation of proteins was carried out using a modification of the method described by Laemmli (1970). A Bio-Rad SDS-PAGE Protean II apparatus was used with a discontinuous buffer system and a 0.5mm slab gel composed of a 4.0% polyacrylamide stacking gel and a 12.5% polyacrylamide separating gel. The stacking gel was made up of: 0.62ml 1M Tris-HCl (pH 6.8); 0.15ml 10% SDS; 0.6ml 30% acrylamide (Sigma) with 0.8% bis-acrylamide (Bio-Rad); and 3.1ml sterile distilled and deionized water. The separating gel contained: 5.4ml 1M Tris-HCl (pH 8.8); 0.2ml 10% SDS; 8.33ml 30% acrylamide with 0.8% bis-acrylamide; and 6.07ml sterile distilled and deionized water. Polymerization of acrylamide was achieved by adding ammonium persulfate (15mg/ml) and TEMED to the acrylamide solutions. Equal numbers of cells from each sample (approximately 20µl) were loaded into each well of the gel using a Hamilton syringe. Molecular weight standards (Sigma SDS-7B) of 116kd to 26.6kd were loaded in the two outside wells.

Electrophoresis was conducted at constant voltage for 85 volt-hours/cm² (usually 100 volts). After
electrophoresis, the proteins were fixed in a solution containing 45% methanol and 10% acetic acid for at least two hours. The gel was then dried at 80°C with vacuum for 45 minutes. After drying, the gel was exposed to diagnostic film (Kodak X-OMAT RP) for 24 - 48 hours at room temperature. Intensifying screens (Kodak X-omatic regular) were used to diminish background and enhance protein band clarity. Following exposure, the film was developed with a Kodak OMAT processor.
CHAPTER III

RESULTS

Heat is a known DNA damaging agent, and the heat shock response may be involved in protecting a cell from heat induced DNA damage. The dnaK gene is a major heat shock gene in E. coli and may play a role in such a protective process. By studying the effect of heat on a dnaK mutant, I hoped to gain understanding of the role of the heat shock response in protecting against DNA damage. I also tested mutants defective in other known DNA repair pathways to determine if these are employed in repairing heat induced DNA damage.

Heat sensitivity of DNA repair deficient mutants

The sensitivities of wild type and mutant strains of E. coli to heat are shown in Figure 3-1. Repair defective mutants, recA and recA uvrA are more sensitive to heat than their wild type parent (Figs. 3-1a and 3-1b). These results are in agreement with previous reports (Bridges et al. 1969a, Bridges et al. 1969b), and confirm the conclusion that heat causes DNA damage. Furthermore, the recA uvrA double mutant is more sensitive to heat than the recA strain. This indicates
that both excision repair and recombinational repair pathways are employed in eliminating heat-induced DNA damages.

An xthA deletion mutant is also more sensitive to heat than the wild type (Fig. 3-1c). Because xthA mutants are also sensitive to H$_2$O$_2$, a known DNA damaging agent (Ames et al. 1985, Demple et al. 1983, Demple et al. 1986), this result implies that the DNA damage caused by heat may be similar to that caused by H$_2$O$_2$. This also implies that exonuclease III, the product of the xthA gene, may be used to repair heat-induced DNA damage.

Heat sensitivity of a dnaK mutant

The DnaK protein is a major heat shock protein in E. coli. As shown in Figure 3-1d, the dnaK deletion mutant GW4813 is very sensitive to heat. The inactivation curves of the wild type and the dnaK mutant were replotted with best fit straight lines obtained through linear regression analysis (data not shown). Comparison of the slopes of these curves reveals that the slope of the dnaK inactivation curve is 3.6 times greater than the slope of the wild type inactivation
Figure 3-1. Sensitivity of E. coli strains to 50°C. Each point is a mean value based on determinations from at least three experiments. Wild type is AB1157.
curve. These results indicate that the dnaK gene is important in protecting E. coli from the lethal effect of heat. Because the known activities of the DnaK protein are at the DNA level (see Chapter I) the protective action of the DnaK protein in wild type cells may act directly, or indirectly to repair heat-induced DNA damage.

Effect of DNA repair deficient mutants on thermotolerance development

The survival of E. coli strains subjected to 50°C following a heat shock of 42°C for 15 minutes is shown in Figure 3-2. The three mutants recA, recA uvrA, and xthA with defects in known repair pathways, and wild type exhibit strong thermotolerance. In other words, after heat shock at 42°C for 15 minutes there is almost no killing from subsequent treatment at 50°C (compare Figs. 3-2a-c to Figs. 3-1a-c). These DNA repair defective mutants, therefore, acquire tolerance to heat-induced damages when pretreated at 42°C, even though these mutants are sensitive to direct 50°C treatment (Figs. 3-1a-c). This implies that, after establishment of thermotolerance, there is little or no need to repair heat-induced DNA damage by means of repair pathways.
Figure 3-2. Sensitivity of *E. coli* strains to 50°C following an exposure to 42°C for 15 minutes. Each point is a mean value based on determinations from at least three experiments. Wild type is AB1157.
employing the recA, uvrA, or xthA gene products.

Effect of a dnaK mutation on the development of thermotolerance

The dnaK deletion mutant was also given a heat shock at 42°C for 15 minutes, and then heat treated at 50°C. It was inactivated very rapidly (Fig. 3-2d). A comparison of the inactivation curves of wild type and the dnaK mutant shows that slope of the dnaK mutant inactivation is 19.6 times greater than the slope of the wild type inactivation curve. The rate of inactivation of the dnaK mutant was essentially the same whether pretreated at 42°C (Fig. 3-2d) or not (Fig. 3-1d). Thus the dnaK gene appears to be essential for the development of thermotolerance, as well as for protecting against or repairing heat-induced DNA damages during 50°C heat treatment.

The dnaK deletion mutant is sensitive to H₂O₂

Since the dnaK gene is induced by H₂O₂ treatment (VanBogelen et al. 1987b), I studied the effect of H₂O₂ on the survival of the dnaK mutant. An xthA mutant, previously reported to be sensitive to H₂O₂ (Demple et al. 1983), was also tested for comparison. The results
Figure 3-3. Sensitivity of E. coli strains to 4.4 mM H$_2$O$_2$. Each point is a mean value based on determinations from at least three experiments. Wild type is AB1157.
of these experiments are shown for two concentrations of \( \text{H}_2\text{O}_2 \) in Figures 3-3 and 3-4. When subjected to 4.4mM \( \text{H}_2\text{O}_2 \), both the \text{dna}K and \text{xth}A mutants are more sensitive than wild type (Figs. 3-3a-b). Wild type is relatively insensitive to \( \text{H}_2\text{O}_2 \) up to 60 minutes of exposure, and then survival declines more rapidly. Both the \text{dna}K and \text{xth}A mutants are inactivated by \( \text{H}_2\text{O}_2 \) at approximately the same rate. At the higher \( \text{H}_2\text{O}_2 \) concentration of 8.8mM, wild type cells are killed more rapidly (Fig. 3-4). Here, wild type cells are relatively resistant to \( \text{H}_2\text{O}_2 \) until 30 minutes of exposure, at which time survival declines rapidly. The \text{dna}K and \text{xth}A strains are initially much more sensitive to this concentration of \( \text{H}_2\text{O}_2 \) than the lower one. Killing is most rapid in the \text{dna}K mutant during the early treatment times so that only 20% of the cells survive after 5 minutes of exposure (Fig. 3-4a). For the \text{xth}A mutant, killing is also most rapid during the first 5 minutes of treatment. After this initial steep inactivation, the rate of killing of both \text{dna}K and \text{xth}A mutants slows somewhat. These results indicate that the \text{dna}K gene is needed to protect \text{E. coli} from \( \text{H}_2\text{O}_2 \) as well as heat. Through a mechanism that remains unknown.
Figure 3-4. Sensitivity of *E. coli* strains to 8.8mM H$_2$O$_2$. Each point is a mean value based on determinations from at least three experiments. Wild type is AB1157.
Figure 3-5. Sensitivity of E. coli strains to UV irradiation. Each point is a mean value based on determinations from at least three experiments. Wild type is AB1157.
The sensitivity of dnaK- cells to UV

Another agent which turns on the heat shock response and induces the dnaK gene is UV light (Krueger and Walker 1984). To determine if the dnaK gene is involved in DNA repair generally or if it is limited to the repair of specific types of DNA damages, the effect of UV on the dnaK deletion mutant was studied. As shown in Figure 5, the dnaK mutant is not sensitive to UV compared to wild type. This implies that the dnaK gene is not involved in protection against, or repairing UV-induced DNA damage.

Studies involving the grpE gene

Since a major effect of heat on a cell is the loss of DNA structural integrity, some of the genes whose known functions are at the DNA level may be important in heat shock protection. The grpE gene of E. coli is a heat shock gene whose known functions include involvement in the initiation of replication in both bacteriophage lambda and E. coli. To better characterize the role of the heat shock response in protecting a cell from lethal heat exposure, the effect of heat on a grpE mutant of E. coli was studied.
Figure 3-6. Sensitivity of wild type (DA15), $grpE^-$, and $grpE^+$ revertant E. coli to 50°C exposure. Each point is a mean value based on determinations from at least four experiments.
Heat sensitivity of a *grpE* mutant

The heat sensitivities of the wild type and the *grpE* mutant are shown in Figure 3-6a. As can be seen, the *grpE* mutant is much more heat resistant than wild type. These inactivation curves were replotted with best fit straight lines obtained through linear regression analysis (data not shown). Comparison of the slopes of these killing curves reveals that the slope of the wild type curve is 2.2 fold greater than the slope of the *grpE* mutant. Indeed, at the higher dose points, the percent survival of the mutant is on the order of 100-fold greater than that of wild type.

Isolation and heat sensitivity of *grpE*+ revertant

In order to confirm that the greater heat resistance of the *grpE* mutant is associated with the *grpE* mutation itself, a *grpE*+ revertant was isolated. This was achieved by taking advantage of the inability of the *grpE*280 mutant to form colonies above 43.5°C or to support a phage λ infection at any temperature. Therefore, the *grpE* mutant was grown to a cell density of 1x10⁸ cells/ml in Hershey's broth at 30°C, plated on Hershey's agar plates, and incubated overnight at 45°C.
Colonies which formed at this temperature were picked and grown again at 30°C in Hershey's broth. These cultures were then passed through another 45°C selection as above. Cultures retaining the capacity to form colonies at 45°C were tested for their ability to support phage λ replication at 37°C. Bacterial isolates on which phage λ plaques could form at this temperature were tested for their ability to support λ growth at 45°C. The bacterial isolate which had the best efficiency of plating (i.e. that nearest wild type) was chosen and used as a grpE+ revertant. I observed that the wild type strain was quite motile, while the grpE mutant was not. The grpE+ revertant was also motile.

The above revertant was subjected to 50°C heat treatment and its relative heat sensitivity was determined (Fig. 3-6b). As can be seen, the grpE+ revertant is more sensitive to heat than the grpE mutant from which it was derived. A comparison of the revertant and grpE mutant heat inactivation curves obtained through linear regression analysis reveals that the slope of the revertant inactivation curve is 2.1 fold greater than that of the grpE mutant. In fact, a comparison of the slopes of wild type and revertant inactivation curves (-0.144 and -0.136 respectively)
Figure 3-7. Sensitivity of wild type (DA15), \( \text{grpE}^- \), and \( \text{grpE}^+ \) revertant \( E. \text{coli} \) to 50°C following a treatment at 42°C for 15 minutes. Each point is a mean value based on determinations from at least four experiments.
reveals that the rates of inactivation are similar for these strains.

Effect of a \textit{grpE} mutation on the development of thermotolerance

To test for thermotolerance, the wild type, \textit{grpE}^-, and revertant strains were subjected to 50°C following a 15 min exposure to 42°C. As shown in Figure 3-7a, both the wild type and the \textit{grpE} mutant exhibit thermotolerance. That is, following exposure to 42°C for 15 min, both of these strains become more resistant to heat. The wild type strain had the most pronounced increase in heat resistance. However, the \textit{grpE} mutant also became more heat-tolerant (compare Fig.s 3-6a and 3-7a). The \textit{grpE}^+ revertant also exhibited thermotolerance, again behaving similarly to wild type (Fig. 3-7b). It appears as though \textit{grpE}^+ and \textit{grpE}^- cells are similar in their ability to survive high temperature (50°C) if they receive a prior thermotolerance inducing treatment. However, if such a treatment is not administered, \textit{grpE}^- cells are more heat resistant than cells without such a mutation.
Figure 3-8. Sensitivity of wild type (DA15) and grpE− E. coli to 44mM H₂O₂ exposure. Each point is a mean value based on determinations from at least five experiments.
Figure 3-9. Sensitivity of wild type (DA15) and \textit{grpE}^{-} \textit{E. coli} to UV irradiation. Each point is a mean value based on determinations from at least three experiments.
H₂O₂ and UV sensitivity of a grpE mutant

In order to determine if grpE- cells are generally resistant to agents which induce the heat shock response, the wild type and grpE mutant strains were subjected to hydrogen peroxide and UV treatments. Both H₂O₂ and UV induce the heat shock response in E. coli (VanBogelen et al. 1987b; Krueger and Walker 1984). In addition, H₂O₂ has been shown to induce thermotolerance in E. coli (VanBogelen et al. 1987a). Interestingly, both the wild type and the grpE mutant are equal in their sensitivities to H₂O₂ and UV (Figs. 3-8 and 3-9). Therefore, the resistance to heat conferred upon a cell by the virtue of a grpE280 mutation does not extend to H₂O₂ and UV.

Effect of a grpE mutation on heat shock protein synthesis

These results imply that the grpE gene product may be a negative regulator which, when defective allows a cell to become more resistant to heat. In order to determine if heat shock protein synthesis is altered in the grpE mutant relative to wild type, both these strains were heat treated at 42°C and their proteins
Figure 3-10. Comparison of heat shock protein synthesis in wild type (DA15) and \textit{grpE}\textsuperscript{-} \textit{E. coli} treated at 42°C. Equal numbers of cells were added to each lane. Odd numbered lanes represent wild type. Even numbered lanes represent \textit{grpE}\textsuperscript{-} cells. Lanes and times of treatment are as follows: lanes 1 and 2, controls (no exposure to 42°C); lanes 3 and 4, 5 min.; lanes 5 and 6, 10 min.; lanes 7 and 8, 20 min.; lanes 9 and 10, 30 min.
visualized on a denaturing polyacrylamide gel (Fig. 3-10). Both strains, as expected, show an induction of heat shock proteins. However, in the grpE mutant, induction seems to occur earlier than in wild type (compare lane 3 to lane 4). This early induction is especially pronounced for the DnaK and GroEL proteins and also for a protein with a molecular weight of about 84kd. After 5 min at 42°C, these proteins are quite visible in the grpE mutant (lane 4). However, in the wild type strain, the DnaK and GroEL proteins are not readily apparent until after 10 min of heat treatment (lane 5). This implies that grpE230 mutants are able to elicit a heat shock response more quickly than wild type cells, and this may explain the relatively high heat resistance seen in the grpE mutant (Fig. 3-6).

The role of cyclic AMP in regulation of the heat shock response

In order for a heat shock response to be elicited in E. coli, information about a cell's external environment must be relayed from the cell membrane to the heat shock genes themselves. Precisely how this information is transferred is unclear.
Two lines of evidence are presented here indicating that cyclic AMP is involved in the regulation of the heat shock response in *E. coli*: (a) An *E. coli* mutant defective in the cya gene, which is necessary for cyclic AMP-mediated gene regulation, is shown to be deficient in the heat shock response; and (b) a CRP binding site is identified in the promoter region of the *E. coli* gene encoding the heat shock sigma factor.

Two genes are essential for cyclic AMP-mediated gene regulation in *E. coli*. These are the cya and crp genes. The *E. coli* crp gene codes for the CRP protein which binds to gene promoters and effects gene expression. The cya gene codes for the enzyme adenylate cyclase which converts ATP to cyclic AMP thereby controlling the intracellular level of cyclic AMP. If a cell contains a mutant cya gene resulting in a non-functional adenylate cyclase enzyme, then the intracellular concentration of cyclic AMP cannot be changed and, therefore, cyclic AMP-mediated gene expression cannot take place.

Experiments were performed to test the idea that if the *E. coli* heat shock response depends on the cya gene, if this is indeed the case, then cells with a mutation in this gene will not exhibit the protein band pattern...
on polyacrylamide gels which is characteristic of a normal heat shock response, or the intensity of heat shock protein bands will be diminished. Either of these results would indicate that efficient synthesis of heat shock proteins in E. coli depends upon cyclic AMP-mediated gene regulation.

Effect of a cyA mutation on heat shock gene expression

Wild type (CA8306.41) and a cyA- mutant were exposed to 42°C heat for up to 30 min. The pattern of protein synthesis in these cells prior to and during heat treatment was then visualized with SDS-polyacrylamide gel electrophoresis (Fig. 3-11). Prior to heat exposure, the pattern of protein synthesis and the absolute amounts of each protein in wild type and the cyA mutant are similar, upon heat exposure, protein synthesis is drastically changed in wild type cells. These cells exhibit a classic heat shock response. Most protein bands are completely absent from heat treated cells, while some proteins become more pronounced. Two proteins are particularly evident in their heat induction. These proteins have molecular weights of 70 and 63 kilodaltons (kd), and are most likely the DnaK
Figure 3-11. Comparison of heat shock protein synthesis in wild type and cya\textsuperscript{−} E. coli treated at 42°C. Equal numbers of cells were added to each lane. Odd numbered lanes represent wild type. Even numbered lanes represent cya\textsuperscript{−} cells. Lanes and times of treatment at 42°C are as follows: lanes 1 and 2, controls (no 42°C treatment); lanes 3 and 4, 10 min.; lanes 5 and 6, 20 min.; lanes 7 and 8, 30 min.
and GroEL proteins respectively. They are quite apparent after 10 min at 42°C and are still induced at 30 min of heat treatment (Fig. 3-11, lanes 3 and 7. Protein synthesis is also reduced during heat treatment in the cya mutant. However, unlike wild type, the cya mutant does not exhibit a heat shock response even after 30 min at 42°C. The 70kd and 63kd proteins are not induced, nor are other proteins whose synthesis is induced in wild type. The 70kd protein (DnaK) appears to be maintained at a basal level during heat treatment. These results imply that the cya gene of E. coli is involved in regulation of heat shock protein synthesis during heat (42°C) exposure.

Heat sensitivity of a cya mutant

In order to determine if a cya deletion mutation effects the degree to which cells are able to survive a heat treatment, wild type and cya- strains were exposed to 50°C and the relative survival was determined (Fig. 3-12). Surprisingly, the cya mutant was more resistant to heat than wild type. The cya mutant was killed steadily for 60 min at which point about 1% of the cells were able to form colonies. The wild type strain, however, was killed quite rapidly for 40 min at which
Figure 3-12. Sensitivity of wild type and cya- E. coli to 50°C heat exposure. Each point is a mean value based on determinations from at least three experiments for wild type and from at least seven experiments for the cya- strain.
point only 0.2% of cells were able to form colonies. After 40 min of 50°C exposure survival of wild type cells leveled off. This decreased rate of killing may be due to a small population of heat-resistant cells. The heat inactivation curves for wild type and cya- strains were redrawn with best fit straight lines obtained through linear regression analysis (data not shown). Comparison of the slopes of these lines (excluding the resistant tail of the wild type) reveals that the slope of the wild type inactivation curve is 2.7 fold greater than that of the cya mutant. In fact, the survival of wild type cells after 40 min of heat treatment is about 100 fold less than the survival of cya- cells.

Effect of a cya mutation on the development of thermotolerance

Since heat shock protein synthesis is not induced by heat in the cya deletion mutant, and because these cells are more resistant to heat than wild type, the ability of wild type and cya- strains to become thermotolerant was studied. Both the wild type and the cya mutant, when pretreated at 42°C for 15 min, became more resistant to 50°C exposure (Fig. 3-13). The cya
Figure 3-13. Sensitivity of wild type and cya- E. coli to 50°C heat exposure following a treatment at 42°C for 15 min. Each point is a mean value based on determinations from at least seven experiments.
mutant is strikingly resistant to heat. The wild type strain, on the other hand, is killed more quickly. A comparison of the slopes of the inactivation curves, obtained through linear regression analysis, reveals that the slope of the wild type curve is 6.2 fold greater than that of the cya mutant. It is quite evident that the cya deletion mutant is able to become thermotolerant. Indeed, the cya mutant becomes much more resistant to heat than wild type. These results imply that, although heat shock protein synthesis is absent, cya- cells are still able to withstand prolonged exposure to high temperature (50°C). This suggests that heat shock protein synthesis is not necessary for the development of thermotolerance.

A presumptive CRP binding sequence exists in the promoter of the htpR gene

In bacteria, all known effects of cyclic AMP are mediated through the binding of its receptor protein, CRP, to cAMP-regulated promoters (deCrombrugghe et al. 1984, Emmer et al. 1970). Therefore, if cyclic AMP does indeed regulate heat shock protein synthesis in E. coli, a CRP binding site must be present in the promoter region of the htpR gene. The requirements for a CRP
binding sequence as described by deCrombrugghe et al. (1984) are as follows: (1) the sequence can be on either the "transcribed" or "non-transcribed" strand of the promoter DNA; (2) the sequence 3' AGTGT 5', which is highly conserved among CRP binding sites, must be present; (3) a six-base pair (bp) block that shows some sequence preference following the AGTGT motif may be present; and (4) a second sequence that follows the 6bp block may also be present.

The DNA sequence of the htpR promoter region (Crickmore and Salmond 1986) was analyzed for the presence of a CRP binding site. A sequence, centered around 160bp upstream of the start site of htpR transcription, meets the requirements for a CRP binding sequence (Fig. 3-14). This presumptive CRP binding site contains, most importantly, a perfect homology to the 3' AGTGT 5' sequence. The six-base pair block 3' to the AGTGT motif matches well (4 of 6 bases) to the most prevalent bases seen in this region (deCrombrugghe et al. 1984). The five bases at the 3' end of the presumptive CRP binding site (AGTAG) do not show great homology (1 of 5 bases) to the most commonly observed bases (ACACT) in this region of defined CRP binding.
sites. However, the bases in this presumptive CRP binding site are not forbidden since these same bases can occur in sites to which CRP is known to bind (deCrombrugghe et al. 1984). One other characteristic of promoter regions of cAMP-regulated genes in E. coli is a partial correspondence with the -35 and -10 consensus sequences (deCrombrugghe et al. 1984). As can be seen in Figure 3-14, these sequences (Crickmore and Salmond 1986) exhibit only partial homology to these consensus sequences. The presence of a CRP binding site upstream of the htpR transcription start site suggests that cyclic AMP and, therefore, the cya gene regulates htpR transcription, and thus, expression of the heat shock genes.
Figure 3-14. Sequence of the promoter region of the htpR gene of *E. coli* (Crickmore and Salmond 1986) showing a presumptive CRP binding sequence centered around 160bp upstream of the start site of transcription. The *E. coli* CRP binding consensus sequence is shown above the presumptive binding site with homologies indicated. The transcription start site is indicated with an arrow. The -35 and -10 promoter sequences are underlined and deviations from the consensus are indicated by lower case letters. A presumptive DnaA binding site (Crickmore and Salmond 1986) is also shown. The coding strand is presented.
CHAPTER IV

DISCUSSION

The data presented suggest several conclusions concerning the *E. coli* heat shock response. These are:

(1) genes *xthA* and *dnaK* are involved in overcoming the lethal effect of heat; (2) thermotolerance, a potent protective mechanism against the lethal effect of heat, is defective in a *dnaK* mutant; (3) a *dnaK* mutant is very sensitive to H$_2$O$_2$; and (4) a *dnaK* mutant is not sensitive to UV compared to wild type; (5) a *grpE* mutant is more resistant to heat than wild type; (6) although already relatively heat resistant, a *grpE* mutant can be induced to become even more thermotolerant when given a brief, sub-lethal heat treatment; (7) a *grpE* mutant is not sensitive to either H$_2$O$_2$ or UV relative to wild type; (8) heat shock protein synthesis, in response to heat, occurs earlier in a *grpE* mutant than in wild type; (9) a *cya* deletion mutant does not exhibit a heat shock response, but is still able to develop thermotolerance; (10) a presumptive CRP binding site is present within the *htpR* promoter region.
Involvement of dnaK and xthA in repair of heat induced DNA damage

It is likely that the requirement for xthA and dnaK in overcoming the lethal effect of heat is due to their involvement in repair of DNA damage. It has been shown that high temperature (50°C) causes DNA damage (Bridges et al. 1969a, Bridges et al. 1969b, Neidhardt et al. 1984). This damage may include single- and double-strand breaks (Woodcock and Grigg 1972). Some heat-induced damages can be repaired in E. coli by recombinational or excision repair pathways as indicated by the heat sensitivities of the recA single mutant and recA uvrA double mutant (Figs. 1a-1b). The xthA gene product, exonuclease III, appears to be required for repair of DNA damages induced by heat since an xthA mutant is sensitive to heat (Fig. 3-1c). This enzyme has several activities which could be involved in DNA repair including endonuclease and exonuclease activities (Demple et al. 1983).

Mackey and Seymour (1987), and Williams-Hill and Grecz (1983), while not studying the heat shock response directly, presented evidence that xthA mutants are not sensitive to heat, in contrast to the results reported
here. In both of these reports, however, heat treatment was performed on *E. coli* suspended in a minimal salts solution, which would not allow cell growth or protein synthesis. In the experiments presented here, all strains were heat treated in a rich medium (see Materials and Methods) consistent with the procedures used in most studies presented in the literature (e.g. Privalle and Fridovich 1987, VanBogelen *et al.* 1987, Ramsay 1988). This difference in treatment media could explain the contradictory results.

The *dnaK* deletion mutant is very sensitive to heat (Fig. 3-1d). The *dnaK* gene, therefore, is important in protecting *E. coli* from the lethal effect of heat, and could possibly be involved in repairing the specific DNA damages caused by heat.

**Thermotolerance depends on the *dnaK* gene**

The need for *recA*, *uvrA*, and *xthA* in repairing heat-induced damage can be dispensed with if thermotolerance has been induced (Fig. 3-2a-c). In other words, once thermotolerance has been induced, *recA*, *recA uvrA*, or *xthA* mutants are no longer heat sensitive. Therefore, an inducible protective pathway appears to abrogate the need for *recA*, *uvrA*, and *xthA*
repair pathways upon induction of thermotolerance. In yeast, thermotolerance can be acquired in excision repair or recombinational repair deficient mutants as well (Mitchel and Morrison 1984). These results imply that thermotolerance may involve an inducible system which either repairs or protects against heat-induced DNA damage in both prokaryotes and eukaryotes.

Thermotolerance is closely related to the induction of heat shock proteins (Subjeck and Sciandra 1982), but a direct link between these two phenomena has never been demonstrated. Prior to this study, a particular dnaK mutant (dnaK756) was shown to exhibit thermotolerance (Ramsay 1988). However, this mutant is not a deletion, and while defective, does not completely lack all DnaK activities (Ramsay 1988). Because the dnaK mutant used in the present investigation was a deletion, DnaK function was completely lacking (Paek and Walker 1987). As shown in Figure 3-2d, the dnaK deletion mutant is very sensitive to heat even under conditions in which thermotolerance should be induced. This result indicates that the dnaK gene is essential for the production of thermotolerance, and establishes a direct association between thermotolerance and a specific gene
of the heat shock regulon in E. coli. Furthermore, these data suggest that not only is an uninduced level of dnaK involved in protecting a cell from heat induced DNA damage (Fig. 3-1d), but that heat shock-induced DNA protection (thermotolerance) requires the dnaK gene.

The DnaK protein may be a repair enzyme

The dnaK gene is induced by H₂O₂ treatment (VanBogelen et al. 1987b). Therefore, one might expect the dnaK mutant to be sensitive to H₂O₂. As can be seen in Figures 3-3 and 3-4, uninduced levels of dnaK are important in protecting E. coli from the lethal effects of H₂O₂. In fact, the dnaK mutant is similar in its striking H₂O₂ sensitivity to the xthA mutant, which is even more sensitive to H₂O₂ than a recA mutant (Demple et al. 1983). The major lethal effect of H₂O₂ is through DNA damage (Ames et al. 1985, Demple et al. 1986, Yonei et al. 1987). Thus, the dnaK gene product may be a repair enzyme that acts on H₂O₂ induced DNA damage.

In general, there seems to be an overlap between the heat shock response and oxidative stress: (a) hydrogen peroxide treatment causes the expression of five heat shock proteins, including DnaK (Morgan et al.
1986); (b) *E. coli* dnaK and xthA mutants are sensitive to both heat and H$_2$O$_2$ treatments; (c) superoxide dismutase is induced by heat shock in *E. coli* (Privalle and Fridovich 1987); (d) heat shock and H$_2$O$_2$ both cause the accumulation of adenylated nucleotides such as AppppA in *E. coli* and *Salmonella typhimurium* (Lee et al. 1983); and (e) the 5'-nucleotidase activity of the *E. coli* DnaK protein is inhibited by AppppA (Bochner et al. 1986). In addition, the general effects of heat and H$_2$O$_2$ are similar. Both heat and H$_2$O$_2$ cause base alterations resulting in apurinic and apyrimidinic (AP) sites (Massie et al. 1972, Hagensee and Moses 1986, Ripley 1988). Single strand breakage, which is the most commonly reported heat-induced DNA damage, may arise from AP sites as a result of direct hydrolysis of a phosphodiester bond or indirectly as a result of endonuclease attack (Bridges et al. 1969b). The association between heat shock and oxidative stress may be based on the similarity of the DNA damages they produce. That is, because of damage similarity, the heat shock response may be well suited to repair both heat and oxidative damages. Alternatively, the heat shock response may simply protect a cell by rendering
cellular targets, other than DNA, more resistant to heat and $H_2O_2$.

The *dnaK* gene is not required for repair of UV induced DNA damage.

Another DNA damaging agent is UV light. UV irradiation results in several types of DNA damage including: pyrimidine dimers, DNA cross-links, DNA-protein cross-links, and single-strand breaks. Pyrimidine dimers, are the most common UV-induced damage, accounting for approximately 50% of the inactivating events in either bacteriophage T4 or *Haemophilus influenzae* transforming DNA (Bernstein 1981). The *dnaK* mutant is not sensitive to UV irradiation compared to wild type (Fig. 3-5). This implies that if the *dnaK* gene product is a DNA repair protein, it is specific in the type of DNA damage it recognizes. The heat shock response of *E. coli*, therefore, may not involve a general type of DNA repair system, but may only be effective against specific types of damage. Such damages may be formed during both heat and $H_2O_2$ exposure, but not during UV irradiation.

Induction of the heat shock response results in resistance to heat, i.e. thermotolerance. This induced
heat resistance does not depend on reoA, uvrA, or xthA mediated systems, but is highly dependent on the dnaK gene. One function of the E. coli heat shock response could be to specifically repair the type of damage caused by heat and H₂O₂. This hypothetical function may also occur in other organisms exhibiting a heat shock response since the E. coli dnaK gene shows considerable sequence homology with the hsp70 genes of many organisms including Drosophila and humans (Hunt and Morimoto 1985). In support of this idea, it has recently been shown that the presence of a monoclonal antibody against Hsp70 reduced the viability of heat shocked mammalian fibroblasts (Ribowol et al. 1988). This result suggests that the Hsp70 proteins of all organisms may protect cells from the lethal effects of heat, possibly by repairing lethal DNA damage.

Spontaneous DNA damage due to heat or oxidative processes appear to be a serious problem with which organisms must cope (Ames et al. 1985, Setlow 1987). Strong selective pressure to protect against such damages may have led to the high degree of conservation and the ubiquity of the heat shock response.
The \textit{grpE} gene may influence the heat shock response

In \textit{E. coli}, the heat shock response is positively regulated by the \textit{htpR} gene (Neidhardt and Van Bogelen 1981). The protein encoded by \textit{htpR} is a sigma factor which directs transcription from heat shock promoters (Grossman \textit{et al.} 1984). Synthesis of heat shock proteins seems to be essential for the development of thermotolerance (Yamamori and Yura 1982, Plesofsky-Vig and Brambl 1985). Evidence is presented here that one of these heat shock genes, the \textit{grpE} gene, encodes a protein which might control heat shock gene expression. A \textit{grpE} mutant (\textit{grpE280}), while naturally quite heat resistant, is able to become even more resistant to heat if induced for thermotolerance. However, the heat shock response in \textit{grpE280} cells is not completely derepressed, since heat shock proteins are not constitutively expressed in \textit{grpE280} cells, but are simply expressed more rapidly than in wild type (Fig. 3-10).

A \textit{grpE}\textsuperscript{+} revertant behaves identically to wild type in terms of the phenotypic characteristics studied, indicating that a single mutation gives rise to all phenotypes associated with the mutant strain. The \textit{grpE280} mutation not only renders a cell unable to form
a colony at high temperature, and unable to support a phage λ infection, but it also confers resistance to the lethal effect of heat.

The functional role of $\text{grpE}$

Any hypothetical function of the $\text{grpE}$ gene product must be reconciled with the previously reported $\text{grpE}_{280}$ phenotype. The role of the GrpE protein in DNA replication is well documented (Saito and Uchida 1977, Ang et al. 1986). Therefore, it is likely that DNA replication plays a role in the $\text{grpE}_{280}$ phenotype described here. The DnaK protein functions at the level of initiation of DNA replication in both bacteriophage λ and E. coli (Figure 4-1) (Liberek et al. 1988, Sakakibara 1988, Zylicz et al. 1989). In addition, it has recently been shown that purified GrpE protein is able to bind to DnaK in vitro (Zylicz et al. 1987). Thus, GrpE may function in the initiation of DNA replication in concert with DnaK although this has not been directly demonstrated. Further, it has been reported that, in an in vitro λ DNA replication system, the presence of GrpE abrogates the requirement for high levels of DnaK protein, possibly because GrpE stabilizes DnaK or enhances its function (Alfano and McMacken
Figure 4-1. Schematic diagram of in vitro bacteriophage lambda DNA replication. The functions of the various proteins are as follows: O\(^r\), recognition of \(\lambda\) origin of replication (ori); P\(^r\), component of \(\lambda\) replication initiation complex; DnaB, component of \(E.\) coli primosome; DnaG, primase; DnaJ, component of replication complex; DnaK, component of replication complex; Ssb, single stranded binding protein; Pol III, DNA polymerase (Zylicz et al. 1989)

At temperatures lower than 43°C, grpE and dnaK genes are not essential for E. coli growth (Ang et al. 1986, Paek and Walker 1987). At higher temperatures, the GrpE and DnaK proteins might function together, possibly through GrpE stabilization of DnaK, at the E. coli replication origin to allow DNA replication to proceed. When the heat stress imposed upon such a cell becomes excessive, the heat shock response would be induced, helping the cell to survive the heat exposure. In a grpE mutant, initiation of replication may be blocked at high temperature because the DnaK protein is not stabilized at the origin. This blockage of replication may signal induction of the heat shock response, protecting the cell from the lethal effects of heat until the temperature is lowered, at which point DNA replication could proceed (Figure 4-2). In this fashion, a grpE mutant could survive heat stress better than wild type cells because the protective heat shock
Figure 4-2. Schematic diagram of possible events during initiation of DNA replication in *E. coli* at high temperature. A) Wild type cells (grpE+). B) Cells carrying a grpE280 mutation (grpE-). Functions of the various proteins are as follows: DnaA, recognition of *E. coli* origin of replication (oriC); DnaB, component of primosome; DnaC, component of primosome; DnaK, component of replication initiation complex; GrpE, component of replication initiation complex; PolC, DNA polymerase.
response is induced earlier by blocked replication. Under the conditions during and subsequent to H$_2$O$_2$ and UV exposure (i.e. low temperatures), initiation of replication can proceed in grpE$^{+}$ cells because the DnaK, and therefore, the GrpE proteins are not required. Indeed these cells are no more sensitive to H$_2$O$_2$ or UV than wild type cells (Figs. 3-8 and 3-9). In other words, the environmental conditions for which the GrpE protein is required were not present during H$_2$O$_2$ or UV exposure.

The heat shock response of *E. coli* appears to be strongly involved in DNA replication and repair at a fundamental level. Indeed, there is evidence linking the initiation of bacterial DNA replication to the regulation of the heat shock response (see below). Involvement in critical cellular functions such as the initiation of DNA replication could be responsible for the extraordinary evolutionary conservation exhibited by the heat shock response.

Interestingly, it has been found that filamentous growth and motility are linked properties in *Bacillus subtilis*. Mutants which lack autolytic enzyme activity (lyt$^-$) not only grow as filaments, but also lack flagella, implying that autolytic activity is necessary
for motility (Fein 1979, Polley and Karamata 1984). As indicated in chapter III, $\text{grpE}280$ cells are non-motile, while both wild type and $\text{grpE}^+$ revertant cells are motile. Furthermore, the $\text{dnaK}$ mutant utilized in the studies described in chapter III has been shown to form filaments at stressful temperatures, i.e. 37°C (Bukau and Walker 1989). Therefore, the heat shock response may be connected in some way to the mechanism of flagellar morphogenesis.

Regulation of cyclic AMP promotors

In $\text{E. coli}$, cAMP regulates gene transcription through a cAMP receptor protein, CRP (Emmer et al. 1970, Botsford 1981). Binding of cAMP to CRP causes a conformational change in the protein which allows binding to specific promotors. When CRP binds to a promoter, RNA polymerase is able to bind and initiate transcription at a site downstream of the CRP binding site (Botsford 1981). The cyclic AMP-CRP complex does not stimulate transcription by unwinding regions adjacent to the promoter, but does cause changes in DNA structure (deCrombrugghe et al. 1984). A functional CRP protein is essential for cyclic AMP-mediated gene
regulation. The CRP protein is a dimer which is able to bind two molecules of cyclic AMP. The amino-termini of the dimer contain the cyclic AMP binding domain while the carboxyl termini possess a DNA binding domain. Upon binding of cyclic AMP to CRP a conformational change occurs in CRP allowing it to bind to DNA. The cyclic AMP-CRP complex binds to DNA at very specific sites which show fairly conserved consensus sequences. Upon binding to DNA the cyclic AMP-CRP complex causes changes in DNA structure and stimulates the formation of closed complexes between RNA polymerase and promoter DNA by increasing the affinity of RNA polymerase for the promoter, and by blocking competing and overlapping promoter sites (deCrombrugghe et al. 1984).

A hierarchy of CRP binding affinities to different promoter regions may exist and depend upon the degree of symmetry in the DNA of the CRP binding region. This hierarchy of CRP binding would help to explain the observation that different cyclic AMP-regulated genes require different levels of cyclic AMP for activation (deCrombrugghe et al. 1984). This type of regulatory mechanism may function to control heat shock protein synthesis in E. coli.
Regulation of the heat shock response by cAMP

Heat shock protein synthesis in E. coli is regulated by changes in the concentration of the htpR gene product (Straus et al. 1987). This protein is a sigma factor (σ^{32}) which allows RNA polymerase to recognize heat shock gene promoters (Grossman et al. 1984). The htpR gene itself, however, is transcribed by RNA polymerase containing the primary sigma factor of E. coli, σ^{70} (Bloom et al. 1986). During exposure to high temperature (45°C) there is a 3-fold increase in the level of the heat shock sigma factor (htpR) messenger RNA (mRNA) (Skelly et al. 1987). Therefore, some "signal" must direct the primary RNA polymerase to the htpR gene during conditions which induce the heat shock response (i.e. high temperature). Since a cya deletion mutant fails to exhibit heat shock protein synthesis, cAMP might be a signal for htpR transcription.

The htpR gene is the only element of the E. coli heat shock response known to be transcribed by the sigma-70 modified RNA polymerase (Bloom et al. 1986). All other heat shock genes are transcribed by an RNA polymerase modified by the heat shock specific sigma factor (sigma-32) and the promoter elements of these
genes have sigma-32 specific consensus sequences in the -35 and -10 regions. Under normal environmental conditions the htpR promoter is not recognized by the RNA polymerase holoenzyme, and, therefore, the heat shock response remains uninduced. However, given the appropriate inducing signal, the sigma-70 modified RNA polymerase is able to initiate transcription of the htpR gene. If cyclic AMP is indeed associated with the regulation of the E. coli htpR gene, then a CRP binding site should be present in the promoter region of this gene. Further, any presumptive CRP binding site within this region must, necessarily, comply with the rules governing CRP binding sites established in other cyclic AMP-regulated prokaryotic genes.

As described in Chapter III, I have analyzed the 300bp region immediately up-stream of the transcription start site of the E. coli htpR gene and have identified a presumptive CRP binding site. Interestingly, the presumptive CRP binding site is located on the "untranscribed" strand of the htpR promoter and is located 160bp upstream from the transcription start site of this gene (Crickmore and Salmond 1986). As is the case with other cyclic AMP-regulated genes, the -35 and -10 promoter elements of
the htpR gene, 5'TTGCTA3' and 5'TATCGAT3' respectively (with 19bp between), only partially match the consensus sequences established for these elements in E. coli (Crickmore and Salmond 1986).

The presumptive CRP binding site described above for the htpR gene meets all the requirements necessary for cyclic AMP regulation. The characteristics of the presumptive binding site indicate that CRP would have a low binding affinity for this site implying that a high level of cyclic AMP would be required for htpR expression. Furthermore, it appears that the sigma-70 RNA polymerase could not efficiently initiate transcription from the htpR promoter given the poor homologies of the -35 and -10 elements of the htpR promoter and the E. coli consensus sequences for these elements.

The presumptive CRP binding site, as mentioned, is centered 160bp upstream of the transcription start site of the htpR gene. The CRP binding site of the cyclic AMP regulated genes studied to date lie between 36 and 107bp upstream of their respective gene's transcription start sites (deCrombrugghe et al. 1984). This discrepancy can be explained by the fact that at least
four other htpR transcripts are seen in vitro with start sites at 85, 90, 130, and 220bp upstream of the start site mentioned above (Crickmore and Salmond 1986). Transcription from these sites would place the CRP binding site 75, 70, or 30bp upstream or 60bp downstream of the respective start site. Exactly which transcript is preferred during heat shock is unknown, but all transcripts except the one which places the CRP binding site downstream of the start site are compatible with the rules governing cyclic AMP gene regulation.

The final criterion which must be met in order to confirm the role of cyclic AMP in the regulation of the E. coli heat shock response is that adenylate cyclase activity increases upon induction of the heat shock response. Further, adenylate cyclase activity must be relatively high in order that a large enough concentration of cyclic AMP is produced to stimulate transcription for the htpR promoter. Adenylate cyclase activity during heat shock has not been studied. However, intracellular levels of adenosine triphosphate (ATP) during heat shock have been monitored. In two species of Tetrahymena, cellular levels of ATP were seen to decline by 50% when either species was placed at a temperature which strongly induces heat shock protein
synthesis (Findly et al. 1983). An explanation for this dramatic decrease in cellular ATP levels during heat shock may be that ATP is being converted to cyclic AMP through the action of adenylate cyclase.

A fundamental problem in our understanding of the heat shock response is that we do not know how an organism senses a change in its environment, and thereupon selectively expresses genes involved in the heat shock response. Cyclic AMP is essential for expression of many genes in prokaryotes, and is necessary for activation of enzymes and other proteins in eukaryotes. Most importantly, cyclic AMP functions as a "second messenger" within cells, relaying information about the external environment from the cell membrane to the cell cytoplasm.

Because the heat shock response is highly conserved in nature, a common regulatory mechanism controlling this response can be expected. Cyclic AMP mediated gene regulation offers this continuity. In addition, regulation of the heat shock response by a mechanism such as that described above may help in explaining how such a wide range of treatments cause induction of that response.
Figure 4-3. A schematic diagram of possible events leading to a heat shock response in E. coli.
A model for cAMP mediated heat shock gene regulation

Figure 4-3 is a schematic diagram of possible events leading to a heat shock response in *E. coli*. During periods of increased temperature, membrane bound adenyl cyclase (Botsford 1981) may be activated, resulting in an increased intracellular concentration of cAMP. Cyclic AMP would form a complex with CRP which, in turn, would bind to the presumptive binding site in the region upstream of *htpR*. This would increase the affinity of the primary RNA polymerase for the *htpR* promoter, resulting in increased transcription of the *htpR* gene. Thus, increased levels of the heat shock sigma factor mRNA would be produced.

In eukaryotes the effects of cyclic AMP on metabolic processes are mediated through the activity of cyclic AMP dependent protein kinases. When the intracellular concentration of cyclic AMP increases, cyclic AMP-dependent protein kinases respond by phosphorylating other proteins, controlling the activity of essential transcriptional and translational proteins (deCrombrugghe *et al.* 1984). In my model, cyclic AMP would regulate the activity of these kinase and phosphatase enzymes by means of cyclic AMP dependent
phosphorylation reactions. In this way, environmental stimuli could influence gene expression in eukaryotes and induce the heat shock response.

Heat sensitivity of a cya mutant

The failure of the cya mutant to exhibit heat shock protein synthesis, might lead one to expect that this strain would be more sensitive to heat than wild type. However, contrary to expectation, the cya mutant proved to be more resistant to heat than wild type. Furthermore, the cya mutant was competent to become thermotolerant. This suggests that a heat shock-independent pathway which confers heat resistance exists in cya deficient E. coli. This alternate pathway might be constitutively induced in this strain since, without a heat shock response, cya- cells would likely be extremely sensitive to the lethal effects of heat. In support of this hypothesis, it has been observed that some strains of Saccharomyces cerevisiae with reduced activity of the cAMP pathway show an increased resistance to heat treatment (Shin et al., 1987). Furthermore, the acquisition of thermotolerance in yeast occurs in a cAMP-independent fashion (Cameron et al., 1988).
Alternatively, heat shock proteins may be still providing protection to these cells. As can be seen in Figure 3-11, the DnaK protein (MW=70kd) is present in heat treated cya- cells (lanes 4, 6, and 8), possibly at the uninduced level. The DnaK protein is an abundant protein in E. coli normally comprising about 1% of the total mass of protein at 37°C in glucose-rich media (Pedersen et al. 1978, Georgopoulos et al. 1982). In addition the total contribution of heat shock proteins to cell mass under these conditions represents about 4% - 5% (Neidhardt et al. 1984). Therefore, survival of cya- cells may be due to heat shock (dnaK) dependent repair. The results presented here together with those presented by VanBogelen et al. (1987a) suggest that heat shock protein synthesis is neither necessary nor sufficient for the development of thermotolerance in E. coli.

The heat shock response in E. coli, appears to depend on expression of the cya gene. The precise mechanism of this interaction remains to be defined. However, it is likely that the control is at the level of expression of the htpR gene, the positive regulator of the response, rather than at each heat shock gene
individually. This is suggested by the presence of a presumptive CRP binding sequence within the promoter region of the htpR gene.

The heat shock inducing signal

Insight as to the possible function of the E. coli heat shock response can be gleaned from the physical nature of the signal that serves to induce this response. In order to respond to the environment, some type of signal must be recognized by the cell for heat shock induction to take place. The nature of this inducing signal is unknown, however, several possibilities exist. First, it has been observed that denatured proteins induce the heat shock response in frog oocytes (Ananthan et al. 1986). Furthermore the synthesis of heat shock proteins and development of thermotolerance correlates well with the presence of abnormal proteins in Chinese hamster ovary fibroblasts (Lee and Hahn 1988). Therefore, protein denaturation may be a signal for heat shock induction. A second possible inducing signal could be an increase in membrane fluidity due to heat. Membrane fluidity is known to increase as extracellular temperature increases (Ingraham 1987). Since adenyl cyclase is a membrane
bound protein (Botsford 1981), adenyl cyclase activity may be directly affected by changes in membrane fluidity. Indeed, the plasma membrane may be the cellular component most affected by small changes in extracellular temperature, and thus be the one best suited to signal temperature increases. Therefore, the heat shock response may function to protect cellular targets such as proteins and/or the cell wall or membrane.

A third possible inducing signal is heat induced changes in DNA. Either through DNA damage or blocked replication these changes may ultimately be the inducing signal of the heat shock response since agents which produce these effects tend to induce the heat shock response (Neidhardt et al. 1984). Both DnaK and GrpE are essential for *E. coli* DNA replication at high temperature. Another heat shock gene, dnaJ, is utilized during the initiation of replication in bacteriophage (Zylicz et al. 1989). The GrpE protein appears to interact with the DnaK protein at the λ replication origin to somehow stabilize the initiation protein complex. Additional evidence linking the heat shock response to initiation of replication is that a DnaA
binding site lies within the promoter region of the *E. coli* htpR gene (Crickmore and Salmond 1986, Gill *et al.* 1986, Douglas Smith personal communication). This suggests that the signal for heat shock induction may include information concerning the state of chromosome replication. Furthermore, some heat shock proteins in eukaryotes are cell cycle regulated (Bienz and Pelham 1987) implying that, in some cases at least, heat shock gene expression is closely coupled to DNA replication. Thus, the heat shock response may function in response to heat to ensure that DNA replication can proceed.

Many inducing agents of the heat shock response, including heat, are known to cause DNA damage. Furthermore, heat-induced thermotolerance develops even in cells deficient in excision and/or recombinational repair pathways. Therefore, thermotolerance might include a DNA repair mechanism capable of repairing heat induced DNA damage. Thermotolerance itself is dependent on the dnaK gene, which would directly link the heat shock response to DNA repair. The type of damage repaired by the heat shock response may be quite specific. An indication of this specificity comes from cross-protection experiments in which exposure to one heat shock inducing agent gives rise to protection
against a second heat shock inducing agent. Three agents known to induce both the *E. coli* SOS DNA repair pathway and the heat shock response are: H$_2$O$_2$, glucose starvation, and UV (Melechen and Go 1980, Krueger and Walker 1984, Neidhardt *et al*. 1984, Imlay and Linn 1987, Walker 1985). When *E. coli* is treated with H$_2$O$_2$ or is starved for glucose or amino acids, it becomes more resistant to heat (VanBogelen *et al*. 1987a) Jenkins *et al*. 1988). However, when treated with UV, *E. coli* does not develop heat resistance (data not shown). Thus heat induced damages may be similar to those caused by H$_2$O$_2$ and starvation, and cross protection between these treatments develops because the damages can be repaired through the "thermotolerance" pathway. More evidence of this specificity comes from the result that a dnaK deletion mutant of *E. coli* is more sensitive than wild type to heat and H$_2$O$_2$ but not UV (see Results). These results strongly suggest that the heat shock response is induced in response to either DNA damage or blocked replication, and that the primary function of the heat shock response is to maintain the replicative integrity of DNA either through repair of DNA damages or by somehow protecting DNA from DNA damaging agents.
In *E. coli*, there are at least 17 heat shock proteins, and the functions of most of these proteins are unknown. The known functions of heat shock proteins either relate to regulation of the heat shock response (as is the case for the *htpR* gene), or are associated with the replication of bacterial or bacteriophage DNA (as is the case for the *grpE*, *dnaJ*, and *dnaK* genes). Accurate replication of DNA may be the most basic process of life. Involvement in this fundamental process could account for the ubiquity and extensive homology seen in the heat shock response.
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