Genet. Res., Camb. (1990), **55**, *pp.* 1–6 *With 5 text-figures Printed in Great Britain*

A *cya* deletion mutant of *Escherichia coli* develops thermotolerance but does not exhibit a heat-shock response

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(*Received* 2 *June* 1989 *and in revised form* 7 *August* 1989)

Summary

An adenyl cyclase deletion mutant (cya) of *E. coli* failed to exhibit a heat-shock response even after 30 min at 42 °C. Under these conditions, heat-shock protein synthesis was induced by 10 min in the wild-type strain. These results suggest that synthesis of heat-shock proteins in *E. coli* requires the *cya* gene. This hypothesis is supported by the finding that a presumptive cyclic AMP receptor protein (CRP) binding site exists within the promotor region of the *E. coli htpR* gene. In spite of the absence of heat-shock protein synthesis, when treated at 50 °C, the *cya* mutant is relatively more heat resistant than wild type. Furthermore, when heat shocked at 42 °C prior to exposure at 50 °C, the *cya* mutant developed thermotolerance. These results suggest that heat-shock protein synthesis is not essential for development of thermotolerance in *E. coli*.

1. Introduction

When cells of *Escherichia coli* are exposed to high temperature, the synthesis of a relatively small number of proteins is induced. This phenomenon is called the heat-shock response, and the induced proteins are known as heat-shock proteins. The heat-shock response is ubiquitous in nature, being found in organisms as diverse as bacteria and man (Neidhardt *et al.* 1984). In *E. coli*, the heat-shock response is positively regulated by the *htpR* gene (Neidhardt & Van Bogelen, 1981; Neidhardt *et al.* 1983). The product of the *htpR* gene is a sigma factor which enables RNA polymerase to recognize heat-shock gene promotors (Grossman *et al.* 1984). 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* is dependent 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 σ^{70} RNA polymerase to the *htpR* promotor during heat shock is unknown.

When *E. coli* receives a brief, sub-lethal dose of heat, it becomes more resistant to lethal heat exposure. This increased heat resistance is known as thermotolerance, and has been observed in many organisms (Lindquist, 1986). There is evidence linking thermotolerance to the heat-shock response. For instance, thermotolerance does not develop in *htpR* mutants of *E. coli* (Yamamori & Yura, 1982). Likewise, in *Neurospora crassa*, when heat-shock protein synthesis is blocked with cycloheximide, thermotolerance is not observed (Plesofsky-Vig & Brambl, 1985). There are also data which suggest that induction of heat-shock genes is not sufficient for development of thermotolerance. When the *htpR* gene is fused to an IPTG-inducible promotor, addition of IPTG results in heat-shock gene expression. In cells containing such a construct, induction of the heat-shock response (with IPTG) does not result in thermotolerance (Van Bogelen *et al.* 1987). Therefore, heat-shock gene expression may not be sufficient for the acquisition of thermotolerance.

In order to better understand the regulation and function of the *E. coli* heat-shock response, it is useful to study how mutations in various genes effect this response. 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 promotors (de Crombrugghe *et al.* 1984). In this paper the effect of a *cya* deletion mutation on heat-shock gene expression is studied. The *cya* mutant fails to exhibit a heat-shock response in that heat-shock

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protein synthesis is not induced. This mutant, however, is able to develop thermotolerance implying that heat-shock proteins are unnecessary for the establishment of thermotolerance.

2. Materials and methods

(i) *Bacterial strains*

E. coli strains used in this study were the generous gift of Richard Friedman. 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 called *cya*$^-$ (Brickman *et al.* 1973; Kiely & O'Gara, 1983).

(ii) *Labelling and electrophoresis*

Overnight cultures of bacteria were grown at 30 °C in Hershey's broth (Steinberg & Edgar, 1962) prior to each experiment. Fresh cultures were prepared by diluting overnight cultures 100-fold into fresh Hershey broth and incubating with shaking at 30 °C. These were grown to a concentration of about 1×10^8 cells/ml; 100 μl of the bacterial culture was pipetted into each of a series of Eppendorf tubes and placed into a 42 °C waterbath for the desired time. Five minutes prior to each time point, 10 μCi [^{35}S]methionine (ICN) was added to the cells. This allowed 5 min for radiolabel incorporation. At each time point, the corresponding tube was withdrawn from the waterbath and rapidly cooled on ice. Cells were collected in a microcentrifuge. The cell pellets were prepared for and the proteins separated on a 12·5% SDS-polyacrylamide gel (Laemmli, 1970). Following electrophoresis, the proteins were fixed in a solution containing 45% methanol and 10% acetic acid. Next, the gel was dried and exposed to film (Kodak X-OMAT RP) for 48 h.

(iii) *Heat treatment*

Bacteria were grown as described above to a concentration of about 1×10^8 cells/ml. One millilitre of each culture was then pipetted into seven individual Eppendorf tubes; one tube for each time point of heat treatment being studied. These tubes were then placed into a 50 °C waterbath for the desired interval of time. At each time point of heat treatment, one tube was withdrawn from the waterbath. The bacteria were immediately serially diluted in M9 salts solution (Adams, 1959, p. 446) and plated on Hershey agar plates using the agar overlay technique (Adams, 1959, p. 450). 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 at 30 °C for 36–48 h. Survival at any given time point was determined as the ratio of colony-forming units after heat treatment to the number of colony-forming units at the zero dose time point.

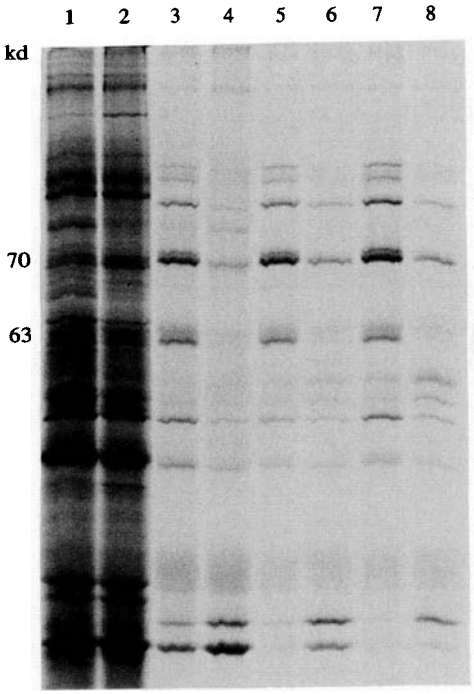

Fig. 1. Comparison of heat-shock protein synthesis in wild-type and *cya*$^-$ *E. coli* treated at 42 °C. Equal numbers of cells were added to each lane. Odd-numbered lanes represent wild type. Even-numbered lanes represent *cya*$^-$ cells. Lanes and times of treatment at 42 °C are as follows: lanes 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.

(iv) *Thermotolerance*

Thermotolerance experiments were carried out as described above for heat treatment except that bacterial suspensions were placed at 42 °C for 15 min immediately prior to 50 °C heat exposure. Survival was determined as above.

3. Results

Wild-type and *cya*$^-$ strains 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. 1). 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 during heat exposure. 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 kDa, respectively. The two most abundant heat-shock proteins in *E. coli* are Dnak and GroEL. These proteins have molecular weights of 69·8 and 62·1 kDa respectively (Neidhardt *et al.* 1984). It is likely, therefore, that the 70 and 63 kDa proteins seen

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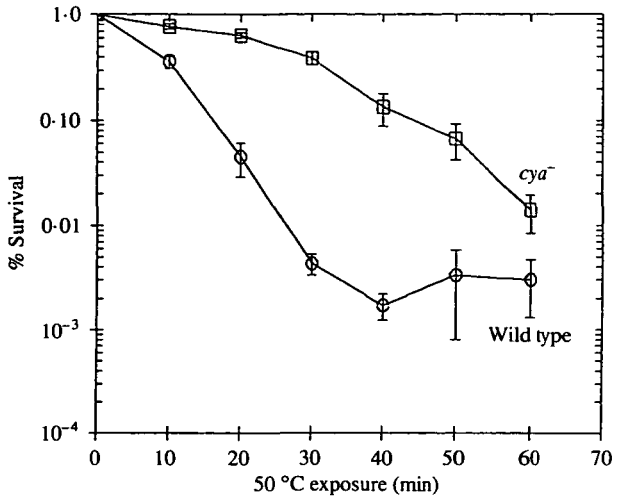

Fig. 2. Sensitivity of wild-type and *cya*⁻ *E. coli* to 50 °C heat exposure. Each point is a mean value based on determinations from at least three experiments for wild type and from at least seven experiments for the *cya*⁻ strain.

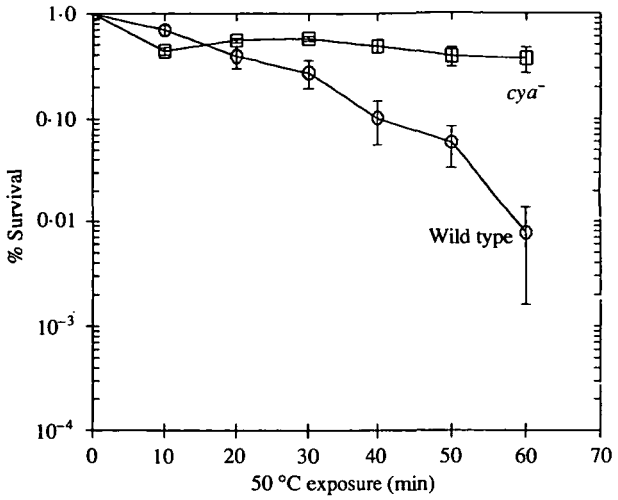

Fig. 3. Sensitivity of wild-type and *cya*⁻ *E. coli* to 50 °C heat exposure following a treatment at 42 °C for 15 min. Each point is a mean value based on determinations from at least seven experiments.

in Fig. 1 are the DnaK 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. 1, 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 70 and 63 kDa proteins are not induced, nor are other proteins whose synthesis is induced in wild type. The 70 kDa 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.

In order to determine whether a *cya* deletion mutation affects 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. 2). 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 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.

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). The *cya* 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.

4. Discussion

In *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 promotor, RNA polymerase is able to bind and initiate transcription at a site downstream of the CRP binding site (Botsford, 1981). Cyclic AMP–CRP binding activates initiation of transcription by increasing the affinity of RNA polymerase for a particular promotor and by blocking competing and overlapping promotor sites. The cyclic AMP–CRP complex does not stimulate transcription by un-

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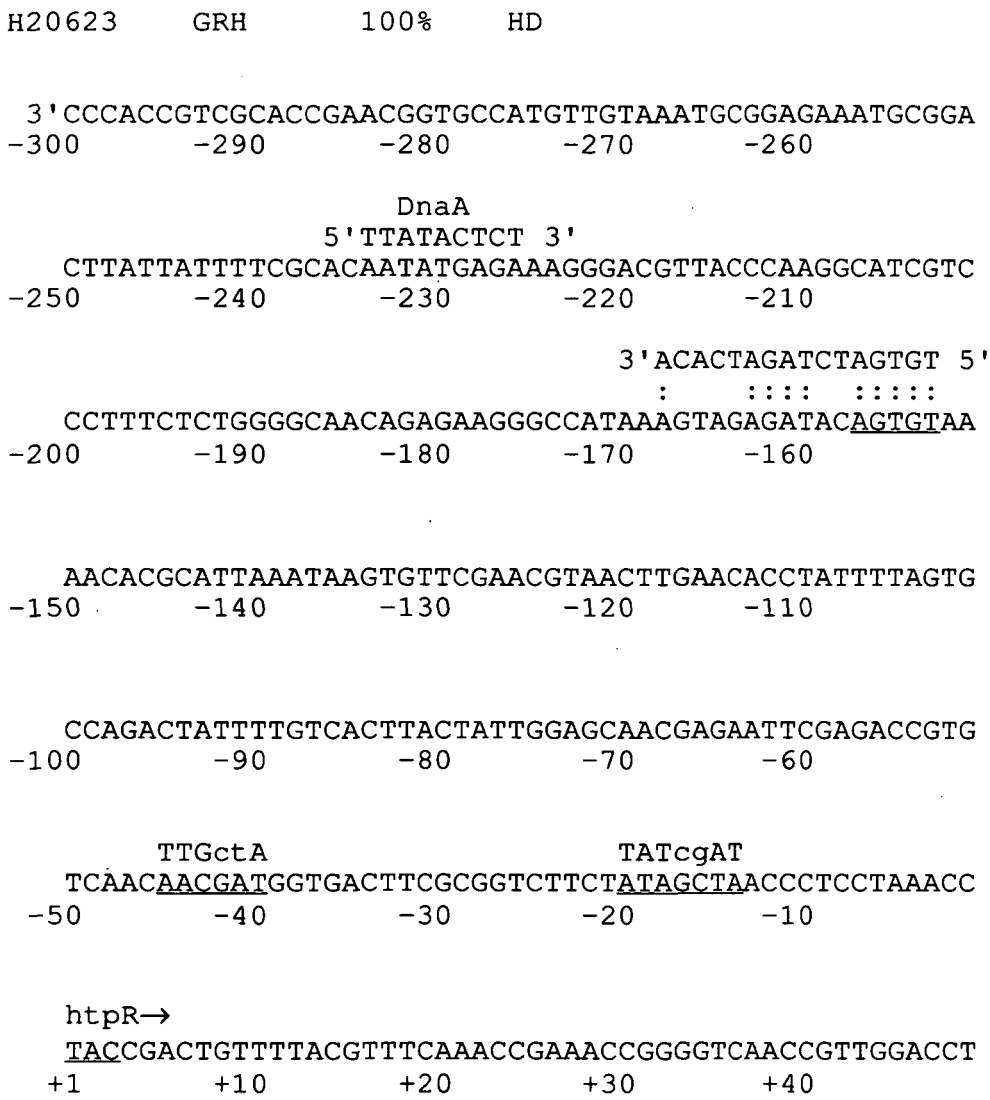

Fig. 4. Sequence of the promotor region of the *htpR* gene of *E. coli* (Crickmore & Salmond, 1986) showing a presumptive CRP binding sequence centred around 160 bp upstream of the start site of transcription. The *E. coli* CRP binding consensus sequence is shown above the presumptive binding site with homologies indicated. The transcription start site is indicated with an arrow. The −35 and −10 promotor sites are underlined and deviations from the consensus appear as lower-case letters. A presumptive DnaA binding site (Crickmore & Salmond, 1986) is also shown. The coding strand is presented.

winding of adjacent regions of the promotor, but does cause changes in DNA structure (de Crombrugghe *et al.* 1984). This type of regulatory mechanism may function to control heat-shock protein synthesis in *E. coli.*

In bacteria, all known effects of cyclic AMP are mediated through the binding of its receptor protein, CRP, to cAMP-regulated promotors (de Crombrugghe *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 promotor region of the *htpR* gene. The requirements for a CRP binding sequence are as follows: (1) the sequence can be on either the 'transcribed' or 'non-transcribed' strand of the promotor DNA; (2) the sequence 5′ TGTGA 3′, 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 TGTGA motif; and (4) a second sequence that follows the 6-bp block (de Crombrugghe *et al.* 1984). The DNA sequence of the *htpR* promotor region (Crickmore & Salmond, 1986) was analysed for the presence of a CRP binding site. A sequence, centred around 160 bp upstream of the start site of *htpR* transcription, meets the requirements for a CRP binding sequence (Fig. 4). This presumptive CRP binding site contains, most importantly, a perfect homology to the 5′ TGTGA 3′ sequence. The 6 bp block 3′ to the TGTGA motif matches well (4 of 6 bases) to the most prevalent bases seen in this region (de Crombrugghe *et al.* 1984). The five bases at the 3′ end of the presumptive CRP binding site (GATGA) do not show great homology (1 of 5 bases) to the most

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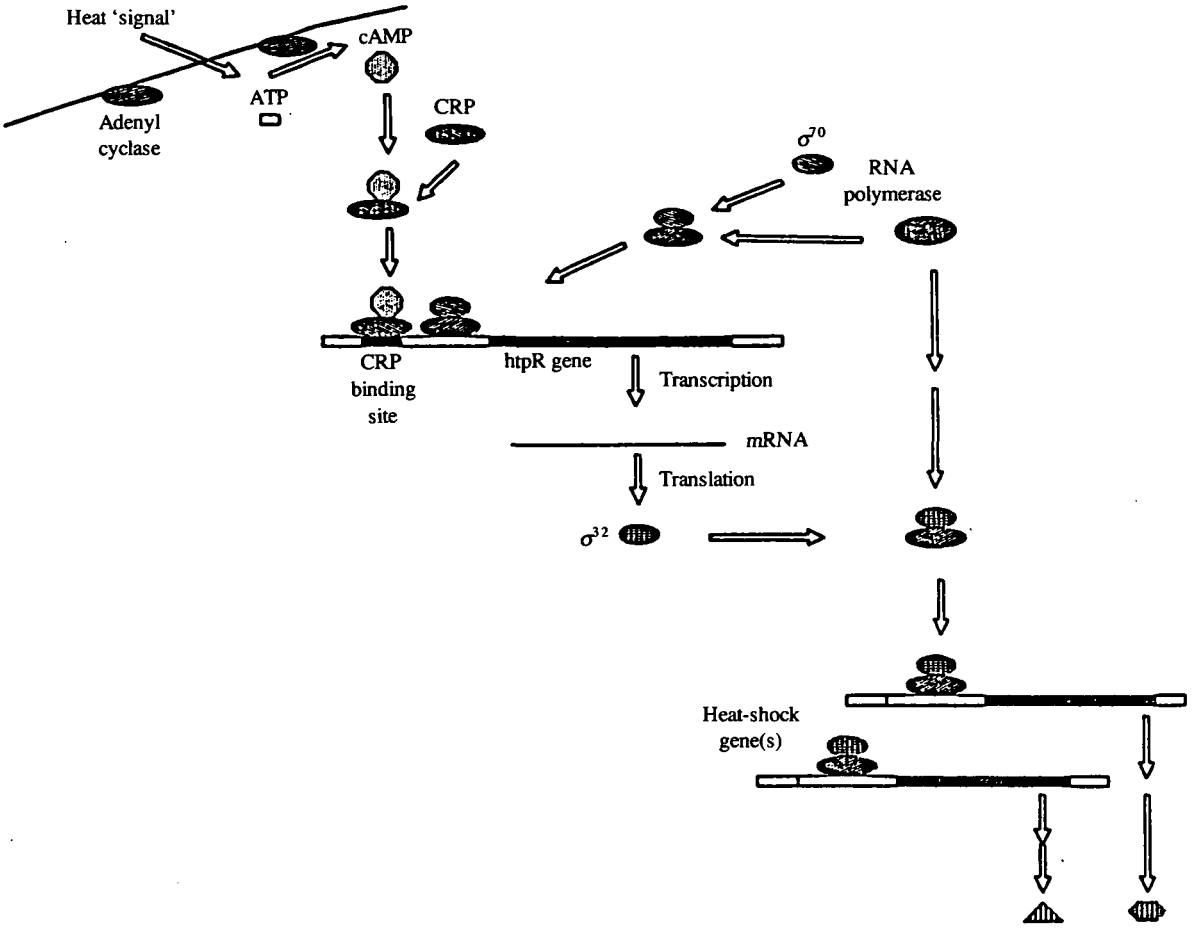

Fig. 5. Schematic diagram of possible events leading to a heat-shock response in *E. coli.*

commonly observed bases (TCACA) 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 (de Crombrugghe *et al.* 1984). One other characteristic of promotor regions of cAMP-regulated genes in *E. coli* is a partial correspondence with the −35 and −10 consensus sequences (de Crombrugghe *et al.* 1984). As can be seen in Fig. 4, these sequences (Crickmore & 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.

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 promotors (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, and because a presumptive CRP binding sequence is located in the region upstream of the *htpR* gene, cAMP might be a signal for *htpR* transcription.

Fig. 5 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* promotor, resulting in increased transcription of the *htpR* gene. Thus, increased levels of the heat-shock sigma factor mRNA would be produced.

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

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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). Most importantly, the results presented here suggest that heat-shock protein synthesis is not necessary for the development of thermotolerance in *E. coli*. This is corroborated by the finding that heat-shock protein synthesis is not sufficient for the development of thermotolerance (Van Bogelen *et al.* 1987).

In conclusion, 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 promotor region of the *htpR* gene. The observation that the *cya* mutant develops thermotolerance implies that thermotolerance is not dependent on the heat-shock response.

I would like to thank Harris Bernstein for his endless dedication and encouragement, and for critical review of this manuscript. I would also like to thank Steve Abedon, Carol Bernstein, Peh Yean Cheah, Paul Hyman, Risa Kandell, and Pat McCreary for thoughtful discussions. This work was supported by NIH grant GM27219-08 to Harris Bernstein.

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