Ribosomes as sensors of heat and cold shock in *Escherichia coli*

(antibiotics/guanosine tetraphosphate/stringency/temperature)

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ABSTRACT Nearly all cells respond to an increase in temperature by inducing a set of proteins, called heat shock proteins (HSPs). Because a large number of other stress conditions induce the HSPs (or at least the most abundant ones), this response is often termed the universal stress response. However, a careful study of conditions that truly mimic a temperature shift suggested that these proteins are induced in response to a change in the translational capacity of the cell. To test this directly, *Escherichia coli* cells were treated with antibiotics that target the prokaryotic ribosome. Two-dimensional gels were used to evaluate the ability of these drugs to alter the rate of synthesis of the HSPs. One group of antibiotics induced the HSPs, whereas a second group repressed the HSPs and induced another set of proteins normally induced in response to a cold shock. Depending on the concentration used, the induction of the heat or cold shock proteins mimicked a mild or severe temperature shift. In addition, antibiotics of the cold shock-inducing group were found to block high temperature induction of the HSPs. The results implicate the ribosome as a prokaryotic sensor for the heat and cold shock response networks, a role it may serve in eukaryotes as well.

The heat shock response, first described in *Drosophila*, is now recognized as a nearly universal cellular response to a shift up in temperature (1, 2). The components of the response, and the general nature of its induction (increased initiation of transcription of heat shock genes), have been conserved across the boundaries of the prokaryotic, eukaryotic, and archaeobacterial kingdoms, strongly suggesting some important role(s) for the response in cell physiology (1, 2).

Many stress conditions have been reported to induce the heat shock response, leading to the idea that there may be multiple cellular targets, or sensors, that can generate the inducing signal (2). Close analysis of the effects of these stress conditions has revealed that in most instances the response is weak and involves only a subset of the heat shock proteins (HSPs) (3). Furthermore, this weak, partial response constitutes, for most of these stress conditions, a trivial portion of the cell's overall response (3). Only two stress conditions have been reported in *Escherichia coli* that induce the heat shock response exclusively and in a temperature-like manner—treatment with ethanol (3) and depletion of 4.5S RNA (4). Both of these stresses alter the translational capacity of the cell.

Paradoxically, some conditions that decrease the translational capacity of the cell cause repression of the HSPs (5–7). One of these conditions is a shift to low temperature. A shift of *E. coli* from 37°C to 10°C leads to a characteristic response, the most prominent feature of which is the induction of 14 proteins, 13 described previously (6) plus one newly discovered (Fig. 2), several of which are involved in translation (6), a finding that meshes with earlier reports that cold temper-

ature restricts bacterial growth by interfering with an early step in protein synthesis (8–10). Other features of the cold shock response include a continued synthesis of stringently controlled translation proteins and a repression of the basal level of HSPs (7).

The experiments reported here were designed to examine the effects of alteration of the translational capacity of the cell on synthesis of the HSPs. The findings suggest that certain blocks in translation do, indeed, induce exclusively the HSPs and that other translational blocks repress the HSPs and induce the set of proteins induced by a cold shock. We discuss how one cellular target, the ribosome, may generate different signals according to the type of block produced by different stress conditions. The findings implicate the ribosome as a sensor of temperature in controlling at least two global regulatory networks in *E. coli*.

MATERIALS AND METHODS

Bacterial Strain and Growth Condition. *E. coli* strain W3110 was used for the antibiotic experiments. Strain CF1946 (from M. Cashel, National Institutes of Health, Bethesda, MD) is a derivative of W3110 with a deletion of about 85% of *spoT* (substituted by the chloramphenicol resistance gene) and a deletion of about 90% of *relA* (substituted by the kanamycin resistance gene). Cultures were grown aerobically (200 rpm) in water bath shakers (New Brunswick) at the indicated temperature. Cultures were grown overnight in MOPS medium (11) supplemented with 0.04% glucose (which limits cell growth of a carbon source at an OD₄₂₀ of about 1.0). Cultures were diluted 1:100 (to about 10⁶ cells per ml) into glucose-rich MOPS (0.4% glucose, all amino acids except methionine, plus bases and vitamins as described in ref. 12). At an OD₄₂₀ of 0.3 the appropriate amount of antibiotic (see Table 1) was added to the cultures. Antibiotics were purchased from Sigma except for spiramycin, which was a gift from Eric Cundliffe (University of Leicester, Leicester, U.K.).

Temperature shifts were performed by transferring a portion of the culture to another water bath at the appropriate temperature. The 10°C water bath was kept in a 0°C environmental room to maintain a constant temperature of 10°C.

Radioactive Labeling and Two-Dimensional (2D) Gel Electrophoresis. A 1-ml portion of the culture was labeled with [³⁵S]methionine (5 Ci/mmol; 100 μCi/ml; 1 Ci = 37 GBq) at the times indicated and chased for 3 min with 0.167 ml of 0.2 M methionine. Cells were pelleted and washed with cold, unlabeled medium. Extracts were prepared by boiling in SDSBME (from Protein Database, Inc., Huntington Station, NY) and treated with DNARNase (Protein Databases, Inc.). A portion of the extract was precipitated with trichloroacetic acid (TCA) in order to determine the amount of label incorporated into protein. A portion (3 μl) of extract was added to 0.5 ml of ice-cold 5% TCA containing 3 g of methionine per liter and kept on ice for at least 30 min. The precipitate was

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Abbreviations: 2D, two-dimensional; HSP, heat shock protein.
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then collected on a glass fiber filter (Schleicher & Schuell, no. 37-0707) and washed with 10 ml of cold 5% TCA plus 3 g of methionine per liter. Scintillation cocktail (Ecolite +, Research Products International; 10 ml) was added to the filter and samples were assayed for radioactivity. One million cpm were loaded onto each gel (the protein amount varied from 0.5 to 5 μ g). O'Farrell 2D polyacrylamide gels were run as described in refs. 13 and 14 except the first-dimension isoelectric focusing employed BDH 4-8 Ampholines (Hoefer). Kodak XAR-5 film was used to produce autoradiograms. The exposure time was 3–5 days.

RESULTS

Growth in the Presence of Antibiotics. *E. coli* wild-type strain W3110 was grown at 37°C in glucose-rich MOPS. While the cultures were in exponential growth, antibiotics were added at various concentrations (see Table 1). The antibiotics differed greatly in their effect on growth during the first 2 hr after addition. Therefore, in order to compare cultures inhibited to approximately the same extent, concentrations of each antibiotic were chosen that caused approximately 10%, 50%, and 90% inhibition of growth at 30 min after the onset of treatment (results not shown).

Proteins Synthesized During Antibiotic Treatment. Cultures grown in glucose-rich MOPS at 37°C were labeled at three intervals after the drug was added: 0–15, 30–45, and 60–75 min. 2D gels were used to examine the pattern of proteins synthesized.

Two patterns of protein synthesis were observed. One was an induction of the HSPs. This response was observed with a group of antibiotics (H group) that includes puromycin, streptomycin, and kanamycin. This response is virtually indistinguishable from that produced by a shift up in temperature. Only the time course is different. Temperature shifts result in peak induction within the first 10 min (2). Typically the HSPs were not induced during the first 15 min following the addition of any concentration of these antibiotics. The 10% inhibitory concentrations brought about an induction that by 45 min resembled the effect observed during the first 10 min after a shift from 28°C to 42°C (Fig. 1). The 50% inhibitory concentration resulted in an induction similar to that following a shift to 49°C (Fig. 1). The middle concentration of kanamycin and streptomycin caused, by 75 min, massive stuttering of most proteins (multiple isoelectric and molecular weight species observed on 2D gels); some stuttering can be observed with the middle concentration at 45 min or after a shift to 49°C (see Fig. 1 *E* and *F*). The highest concentration caused stuttering by 45 min. The concentration of puromycin that inhibited growth by 90% at 30 min permitted the completion only of proteins of molecular weights less than 30,000 at later times (45 and 75 min). Puromycin had previously been reported to induce some of the HSPs at very high concentration (200 μ g/ml) (3), and recovery from puromycin treatment (200 μ g/ml) also was reported to induce some of the HSPs (3, 22).

The second pattern of protein synthesis was induced by a group of antibiotics (C group) that includes chloramphenicol, tetracycline, erythromycin, spiramycin, and fusidic acid. This pattern consisted of induction of 10 of the 14 cold shock proteins (6), almost complete repression of the HSPs, and a slight increase in the synthesis of ribosomal proteins and other stringently controlled translation factors. All of these responses are among those observed following a shift from 37°C to 10°C. Fig. 2 compares a temperature shift down (37°C to 10°C) with the effects of tetracycline. As seen with the H group antibiotics, changes in rates of synthesis were not observed in the first 15 min with any concentration of the C group of antibiotics. At the lowest concentration of antibiotics a mild cold shock response was observed beginning sometime between 45 and 75 min. At the middle concentration, a cold shock induction similar to that seen upon a shift from 37°C to 20°C was evident by 75 min (results not shown). With the highest concentration of each of the C group antibiotics, a cold shock response similar to that observed upon a shift to 10°C was evident by 45 min (Fig. 2).

Tetracycline Blocks the Heat Induction of the HSPs. Does the induction of cold and heat shock responses by different antibiotics reveal what happens naturally upon shifts in growth temperature? If the ribosome is the natural sensor of high temperature, then prior treatment with a C group antibiotic would be expected to interfere with generation of the heat shock signal.

Accordingly, a culture of strain W3110 growing in glucose-rich MOPS at 28°C was treated with tetracycline (0.8 μ g/ml, a concentration that produces a 70% growth inhibition at 28°C and the lowest concentration that blocked the induction) for 30 min and then shifted to 42°C. A portion of the culture was labeled to monitor the synthesis of individual proteins. As shown in Fig. 3, the HSPs were not induced during the first 10 min after shift to 42°C—typically the period with the highest rate of synthesis of HSPs.

Blockage of the heat shock response was not permanent; increased synthesis of the HSPs was evident 20–60 min after the temperature shift. This gradual accumulation of HSPs would be expected from the reversible nature of the inhibition of translation by tetracycline. The dynamics of antibiotic association-dissociation with an individual ribosome would occasionally free a ribosome to be changed from the antibiotic-induced C configuration to the heat-induced H configuration.

Guanosine Tetraphosphate as a Signal. The nucleotide ppGpp is produced by ribosomes stalled by amino acid starvation, carbon starvation, deficiencies in charged tRNAs, and nutrient shift down (23). This signal appears to be transmitted to the transcriptional apparatus, resulting somehow in an abrupt decrease in the transcription of genes encoding the components of the translational apparatus (23).

Some HSPs are induced by shifting a temperature-sensitive valyl-tRNA synthetase mutant from 28°C to 33.5°C (a semi-permissive temperature that causes a dramatic increase in the level of ppGpp), and this response is RelA⁺ dependent (24).

Table 1. Summary of information about antibiotics used in this study

Antibiotic	Conc., μ g/ml	Response	Occupancy of A site	Ref.
Chloramphenicol	1, 2.5, 10	Cold shock	Aminoacyl-tRNA	15
Erythromycin	40, 100, 500	Cold shock	Aminoacyl-tRNA	16
Fusidic acid (Na ⁺ salt)	0.5	Cold shock	Blocked	17
Kanamycin monosulfate	1, 2, 4	Heat shock	Empty	18, 19
Puromycin dihydrochloride	25, 75, 150	Heat shock	Empty	19, 20
Spiramycin	200, 400, 800	Cold shock	Aminoacyl-tRNA	16
Streptomycin sulfate	2, 4, 10	Heat shock	Empty	17, 19
Tetracycline hydrochloride	0.1, 0.5, 10	Cold shock	Blocked by drug	21

All antibiotics were purchased from Sigma except spiramycin, which was a gift from Eric Cundliffe.

FIG. 1. Proteins synthesized during heat shock or treatment with H group antibiotics. Cultures of W3110 were grown in glucose-rich MOPS medium. A portion (1 ml) of the culture was labeled with [³⁵S]methionine for the times indicated. The 2D gels are shown. (A) The 28°C control culture labeled for 10 min. (B) The 37°C control culture labeled for 15 min. (C) A 28°C to 42°C shift labeled 0–10 min after the shift. (D) Kanamycin (1 µg/ml) treatment at 37°C labeled 30–45 min after addition of the drug. (E) The 28°C to 49°C shift labeled from 0 to 10 min after the shift. (F) Kanamycin (2 µg/ml) treatment at 37°C labeled 30–45 min after the addition of the drug. Boxes have been placed around the HSPs in each panel. Identification of the proteins can be done by comparing this figure with previously published figures (14). Circles have been placed around the cold shock proteins induced by the C group antibiotics (see Fig. 2).

On the other hand, amino acid limitation, by any of a number of means, does not lead to a heat shock response (ref. 3; unpublished results). This result implies that the accumulation of ppGpp is not sufficient to induce the HSPs. Nevertheless, the level of this nucleotide has been reported to

FIG. 2. Proteins synthesized during cold shock or treatment with C group antibiotics. Cultures were grown in glucose-rich MOPS at 37°C and a portion was labeled at the indicated times. The 2D gels are shown. (A) The 37°C to 10°C shift labeled 0–4 hr. (B) Tetracycline treatment (10 µg/ml) labeled 30–45 min after addition of drug. The circled spots are the cold shock proteins that respond to the antibiotic. The cold shock proteins can be identified by comparing this figure with the figure in ref. 6 with the exception of the circled protein in B that has a star next to it. This is a newly identified cold shock protein, B46.5. The arrow in B points to B56.5 (GroEL) as an example of a severely repressed HSP.

increase upon a shift up in growth temperature (25) and to decrease upon a temperature shift down (26, 27) or upon addition of the C-group antibiotics (28). Therefore, the possibility that this nucleotide might serve as signal in the heat and cold shock responses—inducing the former and repressing the latter—seemed a possibility worth reinvestigating.

Since low levels of ppGpp are made in a *relA* mutant, a strain deleted for *relA* and *spoT* (genes encoding the only known ppGpp synthetases) was used (M. Cashel) (23, 29) to determine if ppGpp was necessary for the induction of the HSPs. As shown in Fig. 4, the *relA spoT* mutant was found to have a high level of several of the HSPs during growth at 30°C (compare with strain W3110 at 28°C in Fig. 1A). When shifted to 42°C the double mutant induced the HSPs, but 10

FIG. 3. Failure of tetracycline-treated cells to induce the HSPs after a heat shock. Cultures were grown at 28°C, and a portion was shifted to 42°C 30 min after tetracycline (0.8 µg/ml) was added. A sample (1 ml) of each of these cultures was labeled and the 2D gels are shown. (A) Tetracycline-containing culture at 28°C labeled for 15 min (similar to Fig. 2A except at 28°C and a lower concentration of tetracycline). (B) Tetracycline-containing culture labeled from 0 to 10 min after the shift to 42°C. Control cultures without tetracycline labeled at 28°C without tetracycline and 0–10 min after the shift from 28°C to 42°C looked identical to Fig. 1A and C. Boxes have been placed on the HSPs. One cold shock protein, F10.6, has been circled.

FIG. 4. Induction of the HSPs in a strain deleted for *relA* and *spoT*. Strain CF1946 (deleted for most of *relA* and *spoT*) was grown in glucose-rich MOPS. A portion of the culture was labeled at 28°C, 0–10, 10–20, and 20–30 min after a shift to 42°C, and equal amounts of radioactivity were loaded onto 2D gels. (A) The 28°C sample. (B) The sample labeled 10–20 min after the shift to 42°C. Boxes surround the HSPs.

min later than customary for a normal strain. This delay could be due to the higher "basal" level of HSPs already present at 30°C or it might be related to the temperature-sensitive phenotype of the mutant. At 30°C this strain grew at about the same growth rate as wild-type strains. But at 42°C the growth rate did not increase as it does in wild-type strains, and by 30 min after the shift growth had virtually stopped. It cannot be excluded, however, that ppGpp might help the cells respond quickly to the temperature change, for example, by altering the affinity of core RNA polymerase for particular σ factors, as proposed by Travers (30).

Together these results suggest that the nucleotide, ppGpp, though synthesized on the ribosome in normal cells in a manner consistent with a positive signal for that shock, is neither sufficient nor necessary for induction of the HSPs.

DISCUSSION

Discovering the cellular sensors and the triggering mechanisms for stimulus–response networks is generally a difficult task, and it has been especially so for the heat shock response because of the very large number of conditions that elicit the response. To some extent the analysis has been confused by failure to distinguish between conditions that closely mimic heat and those that lead to only weak induction or induction of only a subset of the HSPs. The nonthermal conditions that induce a normal appearing heat shock response as the major cellular response to stress are conditions known to affect the translational capacity of the cell [ethanol addition (3) and depletion of 4.5S RNA (4)]. Likewise, conditions that have been reported to repress the HSPs—removal of streptomycin from a streptomycin-dependent strain (5), addition of tetracycline (5), addition of glucose to cells inhibited with α -methyl glucoside (5), and a shift from 37°C to 15°C (7)—are conditions that affect the translational capacity or the translational apparatus of the cell.

In this study the intention has been to investigate a number of agents known to inhibit one or another aspect of ribosome function. The results lead to the suggestion that the ribosome may be the primary sensor of conditions that elicit the cold shock and heat shock responses in *E. coli*. The model shown in Fig. 5 expresses in general terms this ribosome sensor model of temperature-related cellular shock responses.

The implications of the ribosome sensor model are that (i) the physical state of the ribosome, or the nature of some product produced by the ribosome, is involved as a signal

FIG. 5. Model for the heat and cold shock regulatory networks. On the far right is depicted a generic diagram of the cellular mechanism for responding to a stimulus (environmental change). The sensor is the cellular process or macromolecule that initially senses the change. It then produces a signal [e.g., synthesis of ppGpp for the stringent response (23)] that is transmitted to the regulator, causing the latter to elicit a response that helps the cells adapt to the stimulus. The return arrow indicates the feedback mechanism to shut down the response once the appropriate behavior is achieved.

linking the environmental stimulus (temperature) and the increased expression of heat shock genes and (ii) one of the functions of the cellular response may be to correct a temperature-imposed dysfunction of translation.

Details of the sensing and signaling mechanism are unknown. Three possibilities may be suggested for the biochemical nature of the ribosome-generated signal.

(i) When the ribosome serves as sensor of conditions that invoke the stringent response, it does so by producing the nucleotide, ppGpp, in conjunction with the two ribosome factors, RelA and SpoT (23, 29). Many of the conditions that induce a heat shock response (including a shift up in temperature) induce a stringent response as well, and, as we have noted (*Results*), at least some conditions that induce a stringent response cause a heat shock response. Nevertheless, the evidence from the *relA spoT* double mutant demonstrates that ppGpp is neither sufficient nor necessary to signal induction of the heat shock (or the cold shock) response. Some small signal molecule, analogous to ppGpp, might be produced by the ribosome in the H or C situation. We have no information about this possibility other than the fact that most of the adenosine and guanosine dinucleotides (AppppA, ApppA, AppppG, etc.) have been ruled out by previous studies (3).

(ii) The state of the ribosome itself might be the signal. This idea has some appeal, because the regulatory molecule for the heat shock response is believed to be σ -32, and a large portion of its increase in level after a shift up in temperature occurs at some posttranscriptional step—i.e., by increased translation of preexisting mRNA for σ -32 (31). This possibility seems open to direct testing *in vitro* and *in vivo*.

(iii) The signal generated by ribosomes in the H situation might be the nature of the proteins they produce. Ribosomes in this situation appear to have clearing of the A site (Table 1) and therefore might have the potential to increase the rate at which amino acids are added to the growing peptide by allowing the elongation factor Tu tertiary complex to enter the A site faster. The aminoglycoside antibiotics are often described as drugs that speed up the ribosome, resulting in a decrease in accuracy (misreading of the mRNA) (16). Conversely, it is possible that the cold shock response is induced when the ribosomes are slowed down or made hyperaccurate. The increase in rate of formation of peptides and the

increased proportion of these peptides that have missense errors may saturate the molecular chaperonins (and other molecules that complex with newly made peptides) and the cellular proteases [in a manner suggested by several studies among prokaryotes (22) and eukaryotes (32)]. The titration of these molecules might be the signal for the heat shock response. This idea unites many features and notions of the heat shock response, including: its inducibility by amino acid analogs (33, 34) and by foreign or denatured proteins (22, 35); the proposed role for ubiquitin or a similar molecule in signaling heat shock (32); and the more recent information that several of the HSPs function as molecular chaperones (36–39) or polypeptide binding proteins (40, 41).

At this point a choice cannot be made among the three alternative signals that might emanate from the ribosome, but the issues are readily addressed experimentally. For example, a recent report (42) indicated that a single nucleotide transition in 16S RNA results in ribosomes that appear to generate a heat shock signal at low temperature.

The ability of a C group antibiotic, tetracycline, to delay the cellular response to a shift up in temperature argues that the ribosome sensor model holds for thermal induction of the heat shock response. There is an alternative model—that ribosomes can generate signals eliciting heat and cold shock responses but that high and low temperature act on some other sensor(s). Given the close mimicry of thermal shifts by the ribosome inhibitors, we are attracted to the elegance and simplicity of the single sensor model.

Our model does not address the phenomenon of partial responses to various stimuli. What about stress conditions that induce only some of the HSPs or induce a large number of other proteins in addition to the HSPs? Does the ribosome participate as one of multiple sensors of these stimuli, or are there complex interactions between different regulatory networks? Studies to understand these interactions will require more intense studies of the stress conditions.

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