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Modulation of Stability of the *Escherichia coli* Heat Shock Regulatory Factor σ^{32}

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The heat shock response of *Escherichia coli* is under the positive control of the σ^{32} protein (the product of the *rpoH* gene). We found that overproduction of the σ^{32} protein led to concomitant overproduction of the heat shock proteins, suggesting that the intracellular σ^{32} levels limit heat shock gene expression. In support of this idea, the intracellular half-life of the σ^{32} protein synthesized from a multicopy plasmid was found to be extremely short, e.g., less than 1 min at 37 and 42°C. The half-life increased progressively with a decrease in temperature, reaching 15 min at 22°C. Finally, conditions known previously to increase the rate of synthesis of the heat shock proteins, i.e., a mutation in the *dnaK* gene or expression of phage λ early proteins, were shown to simultaneously result in a three- to fivefold increase in the half-life of σ^{32}.

All organisms so far tested respond to a sudden upshift in growth temperature by increasing the synthesis of a set of proteins, a phenomenon called the heat shock response. In *Escherichia coli*, there is a set of about 20 proteins (called the heat shock proteins) whose synthesis is thereby increased (see reference 27 for a review). The amino acid sequences of at least some heat shock proteins in distantly related organisms, including *Drosophila melanogaster* and *Homo sapiens*, are remarkably similar to those in *E. coli* (4, 5, 21), suggesting that the heat shock response is of ancient origin and fundamental importance to cellular physiology. The function of the heat shock proteins, however, is unclear, although it has been shown that they play roles in the assembly and disassembly of macromolecular complexes (GroE [15, 16, 21, 31]), intracellular transport (yeast Hsp70 [7, 9]), transcription (σ^{70} [33]; GroE [38]), proteolysis (Lon [18]), and translation (lysyl tRNA synthetase [37]).

In *E. coli*, heat shock protein synthesis rates peak at about 5 min after a temperature upshift (e.g., from 30 to 42°C) and then decline rapidly to new steady-state levels that are characteristic of the new ambient temperature. Initiation of the heat shock response is regulated transcriptionally. It has been shown that the RNA polymerase core (E) binds to a new initiation subunit, σ^{32} (30), and the resulting holoenzyme, E-σ^{32}, transcribes only heat shock genes (19), which have promoter sequences that differ from those transcribed by E plus σ^{70}, the normal vegetative initiation factor (8). The transcription factor σ^{70} is itself a heat shock protein, so the increase in its concentration after heat shock may contribute to the decline in heat shock protein synthesis. Furthermore, other heat shock proteins, in particular the *dnaK* gene product, contribute to the shutoff, since mutations in their genes prolong the high-level synthesis of heat shock proteins (34). The heat shock response must be tightly regulated in order to allow rapid changes in heat shock protein synthesis rates. Although the level of mRNA from the *rpoH* gene (which encodes σ^{32}) increases after heat shock (11, 12, 36), this increase is insufficient and too slow to be the sole explanation of the rapid effect of heat shock. In this paper, we show that the concentration of active σ^{32} limits the expression of heat shock genes and that the stability of σ^{32} varies in conditions in which heat shock gene expression is modulated. Results similar to some of ours have been reported (3, 20, 32).

MATERIALS AND METHODS

Bacterial strains, phage, and plasmids. The bacterial strains, phage, and plasmids used in the course of this work are shown in Table 1. The *dnaK756* mutation was transduced from strain CG410 into RB791 by P1 cotransduction (25), first selecting for the nearby Tn*10* Tet^r marker and subsequently screening for inability to propagate λ *c*I857 or form colonies at 43°C but ability to propagate λ *c*I857 *dnaK*$^+$ transducing phage.

Construction of the p*tac-rpoH* plasmid. The vector p*tac*12H (p*tac*12 [1] with a *Hin*dIII linker inserted in the *Pvu*II site; obtained from Nina Erwin) was digested with *Hin*dIII and *Pvu*I, and the p*tac*-containing fragment was isolated from a minigel with an NA45 membrane (Schleicher and Schuell). This fragment was ligated with the similarly isolated *Hin*dIII-*Pvu*I fragment of pFN97 (26) that contains the *rpoH* coding sequence with 130 base pairs (bp) of upstream DNA. Two of the *rpoH* promoters are within this fragment (11). Cloning procedures were as published (24) or as recommended by suppliers. Enzymes were purchased from New England BioLabs.

Transformations. Transformations were done as described in reference 24.

Labeling experiments. The procedures used in growing bacteria, in labeling with [^{35}S]methionine (Amersham), in chase experiments, and in terminating incorporation were as described previously (17, 34). In control experiments, termination of label incorporation by the addition of 10 volumes of either ice-cold acetone or ice-cold trichloroacetic acid to 20% (wt/vol) final concentration gave identical results. The experimental results shown were obtained by terminating incorporation with acetone.

Electrophoresis. The procedures for protein gel electrophoresis were as described before (17, 34). Gels were dried

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TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Relevant genotype or phenotype	Source or reference
E. coli		
CG410	C600 *thr*::Tn*10 dnaK756*	This work
RB791	W3110 *lacI*^{q}	Roger Brent
Plasmids		
pFN97	pBR322 *rpoH*$^+$, multicopy	26
pCG178	p*tac*12H, multicopy	1
pCG179	p*tac*12H *rpoH*$^+$, multicopy	This work
Phages		
P1L4	Clear-plaque former	Lucien Caro
λ *c*I857 *cro*27 *S*am7	Overproduces λ early proteins at 42°C	13
λ *c*I857	Clear-plaque former at temperatures above 35°C	Our collection
λ *c*I857 *dnaK*$^+$	Propagates on *dnaK756* bacteria	14

on filter paper and autoradiographed with Kodak XAR-5 film.

Quantitation of protein after gel electrophoresis. Autoradiograms of various time exposures of sodium dodecyl sulfate-polyacrylamide gels were scanned in a 3CS Joyce-Loebl microdensitometer. The response of the machine was linear, and the film darkening was proportional to the amount of sample applied to the gel. A base line was drawn to quantitate the σ^{32} peak by comparison with the GroEL and DnaK proteins, common to all samples. The peaks were then cut out and weighed to measure the relative amounts of protein in the various bands. The values were then plotted against the chase time, and the half-life was estimated from the slope of the decay line.

Results

Overproduction of σ^{32} protein. We constructed a vector with which we could produce additional σ^{32} protein from a regulatable promoter so that the chemical half-life of the protein could be measured directly and the effect of overproducing σ^{32} could be observed. Plasmid pCG179 has the isopropylthiogalactoside (IPTG)-inducible *tac* promoter fused to the *Hin*dIII site upstream of the *rpoH* coding sequences (see Materials and Methods for details of plasmid construction). The *rpoH* promoters P3 and P4 lie within the cloned fragment (11), so some expression of the *rpoH* gene was observed without IPTG induction (Fig. 1, lane f), even in a strain carrying the *lacI*^{q} gene. When IPTG was added to the culture, however, σ^{32} synthesis increased rapidly and massively, with a concomitant increase in heat shock protein synthesis (Fig. 1). (The identity of the σ^{32} and heat shock proteins was verified by two-dimensional gel electrophoresis [data not shown].) Heat shock protein synthesis remained elevated for 60 min after IPTG addition but may have declined relative to σ^{32} synthesis by 120 min after induction. These results support the idea that the σ^{32} concentration is the limiting factor for heat shock gene transcription. Similar results were obtained by Grossman et al. (20), although they observed different kinetics of heat shock protein synthesis in their system.

Half-life of σ^{32} protein. The heat shock response involves rapid changes in the expression of the heat shock genes. Since the σ^{32} concentration appears to be the limiting factor for heat shock gene transcription, it seemed likely that its levels increase and decrease rapidly in response to a temperature upshift.

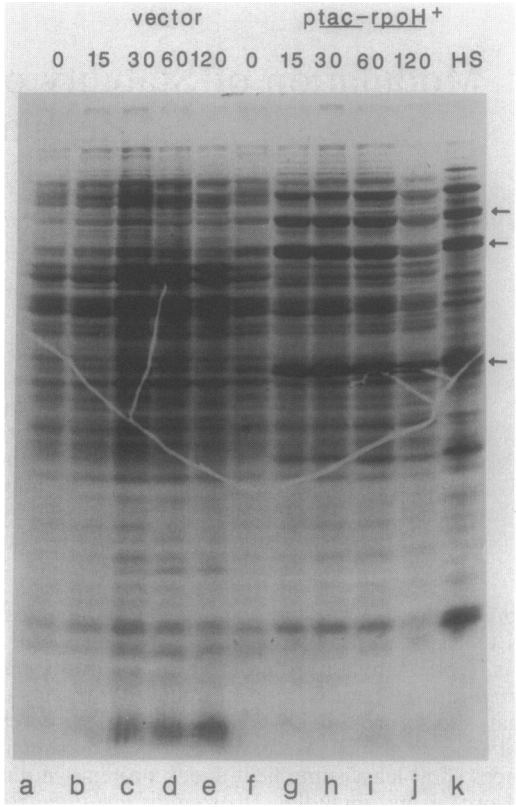

FIG. 1. Overproduction of the σ^{32} polypeptide leads to overproduction of heat shock proteins. Isogenic RB791(pCG178) (vector; lanes a–e) and RB791(pCG179) (*rpoH*$^+$; lanes f–j) bacteria were grown at 30°C to 3 × 10^8 cells per ml. IPTG was added to a final concentration of 1 mM, and the cultures were pulse-labeled with 20 μCi of [^{35}S]methionine per ml from 0 to 1 min (lanes a and f), 15 to 16 min (lanes b and g), 30 to 31 min (lanes c and h), 60 to 61 min (lanes d and i), or 120 to 121 min (lanes e and j). Lane k represents cells grown at 30°C to 3 × 10^8 cells per ml, treated with IPTG for 15 min, shifted to 42°C, and labeled with [^{35}S]methionine from 5 to 6 min after the shift. The lowest arrow marks the position of the σ^{32} protein, and the upper arrows mark the positions of the heat shock proteins DnaK (upper) and GroEL (lower). The numbers above the lanes indicate the times of labeling (in minutes).

Although we showed previously that the level of *rpoH* mRNA rapidly increases about 5-fold after a shift from 30 to 43.5°C, the increase in transcription of some heat shock genes is 20-fold and of similar rapidity, so it seemed likely that other factors contribute to the initiation of the heat shock response. One mechanism by which a protein's concentration can be rapidly adjusted is modulation of its stability. Accordingly, we measured the half-life of σ^{32} produced from the p*tac-rpoH* plasmid during steady-state growth at various temperatures by pulsing cultures for 1 min with ^{35}S-labeled methionine and then chasing with nonradioactive methionine for various times to allow the labeled proteins to decay. We found that σ^{32} was always unstable but was progressively more stable as the growth temperature was reduced (Fig. 2). From the experiment shown in Fig. 2, we measured the half-life of σ^{32} as approximately 0.7 min at 42°C, 1.3 min at 37°C, 1.7 min at 30°C, and 15 min at 22°C. The measured half-lives of σ^{32} at various temperatures varied somewhat from experiment to experiment. However, these values are smaller than those measured by Bahl et al. (3), who found the $t_{1/2}$ to be 8 min at 30°C, and Grossman et

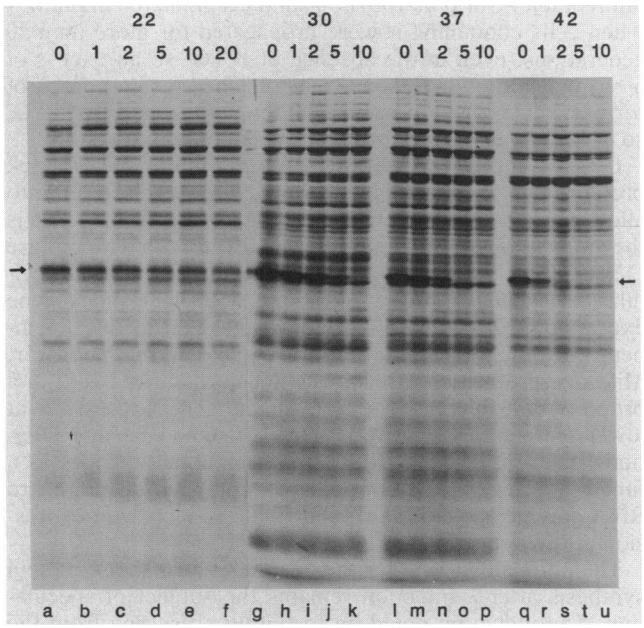

FIG. 2. In vivo half-life of σ^{32} polypeptide is short and varies with temperature. RB791(pCG179) (*rpoH*$^+$) bacteria were grown at 22°C (lanes a–f), 30°C (lanes g–k), 37°C (lanes l–p), and 42°C (lanes q–u) to 3×10^8 cells per ml. IPTG was added, and 15 min later the cells were pulsed for 1 min with 20 μCi of [^{35}S]methionine per ml (lanes a, g, l, and q). The label was chased with excess cold methionine for 1 min (lanes b, h, m, and r), 2 min (lanes c, i, n, and s), 5 min (lanes d, j, o, and t), 10 min (lanes e, k, p, and u), and 20 min (lane f). The samples were collected by acetone precipitation, dried, dissolved in sodium dodecyl sulfate sample buffer, and separated by electrophoresis as described in reference 17. Arrows indicate the position of the σ^{32} protein. Numbers above the lanes indicate the growth temperature and duration (in minutes) of the chase.

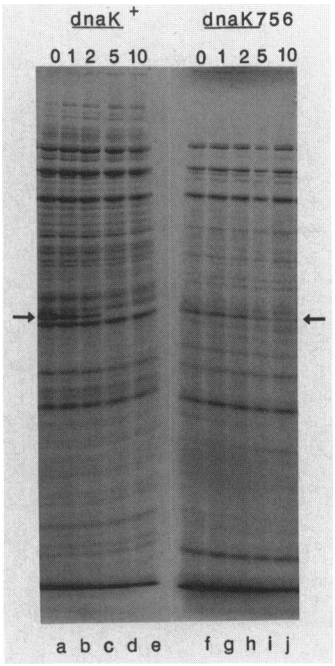

FIG. 3. Effect of *dnaK756* mutation on the half-life of the σ^{32} polypeptide. σ^{32} synthesis was induced in strain RB791(pCG179) as described in the legend to Fig. 1. At 15 min after IPTG addition, the *dnaK*$^+$ (lanes a–e) and *dnaK756* (lanes f–j) isogenic strains were pulse-labeled for 1 min with 20 μCi of [^{35}S]methionine per ml (lanes a and f) and chased for 1 min (lanes b and g), 2 min (lanes c and h), 5 min (lanes d and i), or 10 min (lanes e and j). The samples were prepared and separated by electrophoresis as described in the legend to Fig. 2 and in Materials and Methods. Arrows indicate the position of the σ^{32} protein.

al. (20), who found the $t_{1/2}$ to be 4 min at 42°C, but similar to the half-life found by Straus et al. (32), who used immunoprecipitations to measure the stability of σ^{32} produced from its normal promoters. We do not understand the cause of variability in the σ^{32} half-life measurements.

Effect of the *dnaK756* mutation. The *dnaK* gene encodes a 70,000-M_r heat shock protein that is required for λ DNA replication and bacterial growth at high temperatures (14, 28, 29). The *dnaK756* mutant was previously shown to be defective in the adaptation phase of the heat shock response, wherein the initial rapid rate of heat shock protein synthesis declines to a new rate characteristic of steady-state growth at that temperature (34). In order to test the effect of the *dnaK756* mutation on the intracellular half-life of σ^{32}, the allele was moved into a *lacI*^{q} background by P1 cotransduction with the nearby *thr*::Tn*10* marker. We found (Fig. 3) that the half-life of overproduced σ^{32} (at 42°C) was increased from 45 s in the *dnaK*$^+$ strain to 4.6 min in the *dnaK756* mutant. We confirmed the identity of the stabilized protein by two-dimensional gel analysis. The increased level of σ^{32} protein that should result from this stabilization may be sufficient to allow prolonged high-level transcription of heat shock genes and concomitant overproduction of their products.

It was found that prolonged growth of bacterial cultures of strains carrying both the *dnaK756* mutation and the *rpoH*$^+$ gene on a multicopy plasmid (such as pCG179) resulted in the rapid accumulation of plasmid variants deleted for the *rpoH*$^+$ gene (unpublished results). In the absence of the *lacI*^{q} allele, it was impossible to maintain the pCG179 plasmid in the *dnaK756* background. These results are consistent with the previously demonstrated antagonist roles of the *dnaK* and *rpoH* gene products (34).

Effect of λ early proteins. It was shown previously that production of bacteriophage λ early proteins leads to a mild increase in *E. coli* heat shock protein synthesis (10, 23, 35). We showed that this effect was mediated by the normal mechanism for induction of the heat shock regulon, since the *rpoH165* mutant did not respond to overproduction of λ early proteins made after shifting a λ *c*I857 *cro*27 lysogen to 42°C (35). We measured the half-life of σ^{32} protein in these conditions and found that it increased from 50 s to 4.3 min in the presence of λ early proteins (Fig. 4). Bahl et al. (3) showed that the λ early protein cIII was responsible for stabilization of σ^{32} in their system, although the half-lives that they measured were much longer (8 min without cIII and 32 min with cIII) than ours or that for σ^{32} made from its own promoters in single copy (32).

DISCUSSION

The heat shock response consists of the rapid induction of a set of genes in response to an external stress. Since the level of active σ^{32} limits the transcription of heat shock genes, that level must fluctuate in response to stress. We and others have shown that several mechanisms modulate the σ^{32} concentration in the cell. The levels of the σ^{32} transcripts increase after heat shock as a consequence of mRNA

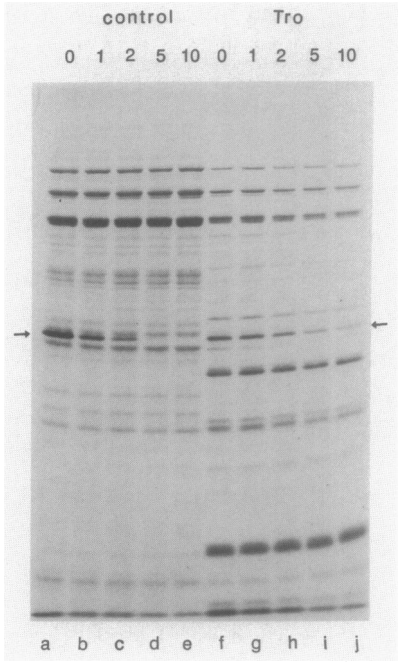

FIG. 4. Effect of the λ Tro phenotype on the half-life of the σ^{32} polypeptide. Isogenic RB791(pCG179) (*rpoH*$^+$; lanes a–e) and RB791(pCG179) (λ *c*I857 *cro*27 *S*am7) (lanes f–j) bacteria were grown at 30°C to 3 × 10^8 cells per ml. At this time, the cultures were shifted to 42°C (time zero). At 15 min, IPTG was added, and at 30 min the cultures were labeled for 1 min with 20 μCi of [^{35}S]methionine (lanes a and f). Following this, the cultures were chased with excess cold methionine for 1 min (lanes b and g), 2 min (lanes c and h), 5 min (lanes d and i), and 10 min (lanes e and j). The samples were prepared and separated as described in the legend to Fig. 2 and in Materials and Methods. Arrows mark the position of σ^{32} protein. Numbers above the lanes indicate the duration of the chase (in minutes).

stabilization (11, 12, 36). In addition, the stability of the protein varies in conditions known to affect heat shock gene expression. Straus et al. (32) also found a transient increase in the $t_{1/2}$ of σ^{32} after heat shock, which is consistent with the transience of the induction of heat shock genes. The observed changes in σ^{32} stability may cause large changes in heat shock gene transcription.

What is the reason for multilevel control of σ^{32} concentration? First, control at several stages allows delicate modulation of a protein's level in the cell. Second, this kind of control can be sensitive to more types of stimuli than a simple regulatory circuit. Third, it is likely that tight regulation is important for cellular viability. There are several lines of evidence supporting the third contention. First, construction of strains containing multicopy plasmids bearing some of the heat shock genes is difficult, and the resultant strains are often unhealthy (unpublished results). Furthermore, the plasmid genes often accumulate mutations, indicating that gross overproduction of heat shock proteins is deleterious to growth. Second, mutations in many heat shock genes are temperature-sensitive lethal or lethal, depending on the nature of the mutation, indicating that their products are required for growth in those conditions (2, 28; unpublished results). These two results suggest that the levels of particular heat shock proteins must be controlled for optimal growth. More direct evidence suggests that skewing the σ^{32} concentration in either the high or low direction should be unfavorable. Our *ptac-rpoH* plasmid accumulated mutations when cells containing it were propagated for more than 40 generations, even in the absence of IPTG, so an excess of σ^{32} is unhealthy for *E. coli* (3; unpublished results). Lack of σ^{32} prevents growth of bacteria at temperatures above 18°C, so σ^{32} plays an essential role at those temperatures (40).

Clearly, the concentrations of σ^{32} and the heat shock proteins must be maintained within defined levels in order to allow normal cellular growth. Further support for this idea is the modulation of heat shock protein levels with temperature (22). It seems clear that at high temperatures, the intracellular levels of σ^{32} protein correlate with heat shock gene expression (32). However, it is possible that at lower temperatures, the σ^{32} levels are not the sole determining factor. Mild overproduction of σ^{32} does not necessarily result in a proportionate increase in heat shock gene expression at lower temperatures (26; this work). It could be that additional factors, i.e., σ^{32} or σ^{70} conformational changes as a function of temperature or ability to compete for binding to RNA polymerase core, also contribute to increased expression at low temperatures.

The rationale, if any, for the turn-on of heat shock protein synthesis after λ infection remains the subject of speculation. The induction could be fortuitous, resulting from the phage's attempt to protect its own unstable proteins (especially cII) from protease attack. Many heat shock proteins are required for λ growth, however, so λ may have tapped into a preexisting regulatory circuit in order to increase their intracellular level. Since cIII mutant phage are viable on *rpoH*$^+$ bacteria, the increase in heat shock protein concentration is nonessential but may provide a limited growth advantage for wild-type phage in nature. However, phage λ does not propagate on *rpoH* temperature-sensitive mutants under nonpermissive conditions (39), indicating that the intracellular concentration of at least a subset of the heat shock proteins is critical for phage growth.

The σ^{32} half-lives that we measured were significantly shorter than those measured by several previous groups (3, 20). Since Straus et al. (32), who studied σ^{32} synthesized from the *rpoH* gene in its normal chromosomal context, found a $t_{1/2}$ similar to ours when the conditions were similar, we presume that our measurements are a better reflection of the in vivo half-life than the longer ones.

The multilevel regulation of the *rpoH* gene and σ^{32} concentration provides a mechanism by which the cell can achieve the correct balance of heat shock proteins.

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