Regulation of the Heat Shock Response in *Escherichia coli*

by

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I contributed intellectually, performed all of the experiments included in the paper and wrote the first draft of the manuscript. Christophe Herman provided initial observations for this work and made intellectual contributions. Chi-Zen Lu purified proteins used in this paper.

Chapter 2 is a draft of a paper to be submitted. I suggested the line of experiments that revealed the significance of the mutants, supervised all of the work except the initial screen, and measured the protein binding constants. Takashi Yura performed most of the *in vivo* experiments included in the paper and contributed intellectually. Chi-Zen Lu purified proteins and did the *in vitro* transcription experiments. Mark Poritz performed the initial screen identifying the σ^{32} mutants.

Chapter 3 is a draft of a paper to be submitted. I designed the σ^{32} line of experimentation, and performed most of those experiments. Virgil Rhodius did the Hfq microarrays and σ^{E} experiments. Nidhi Ahuja performed the Western analysis reported in Fig. 4 and part of the β-galactosidase analysis reported in Fig. 3. Emily Witkin did some preliminary adaptation experiments.

To whom it may concern,

I am requesting permission to reproduce the following article as part of my graudate thesis dissertation.

Eric Guisbert, Christophe Herman, Chi Zen Lu, and Carol A. Gross
A chaperone network controls the heat shock response in E. coli
Genes & Dev., Nov 2004; 18: 2812 - 2821

Sincerely,

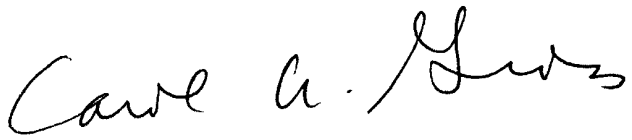
Eric Guisbert

Regulation of the Heat Shock Response in *Escherichia coli*

Eric Guisbert

ABSTRACT

All organisms utilize the heat shock response to sense the level of unfolded proteins in the cell and adjust the level of chaperones and proteases accordingly. The regulation of the heat shock response has been extensively studied, yet there are still unexplained components. I report three contributions to our understanding of this regulation. First, I show that instead of a single chaperone, a chaperone network consisting of at least DnaK/J and GroEL/S is used to sense unfolded proteins. Second, I provide evidence for an unexpected link between activity control and regulated degradation of σ^{32} and suggest that an additional, unidentified factor contributes to the regulation of σ^{32} activity. These results are based on analysis of σ^{32} mutants defective in activity regulation. Third, I demonstrate that Hfq, and potentially small RNA binding partners, contribute to regulation of σ^{32} in at least two distinct ways. Hfq represses DnaK translation, which in turn leads to an increase in σ^{32} activity. Also, Hfq participates in a process that we have termed "long-term adaptation", whereby the activity of σ^{32} recovers to normal levels following long-term chaperone overexpression.



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Prologue

The heat shock response (HSR) is classically defined as the cellular response to temperature increase. A major component of this response is upregulation of a set of proteins, termed heat shock proteins (hsps). These proteins are usually regulated by a single transcription factor, for example, the σ^{32} transcription factor in *Escherichia coli* and Heat Shock Factor (HSF) in eukaryotic cells. Some heat shock proteins, notably chaperones, which help proteins fold, and proteases, which degrade unfolded proteins are a conserved part of the response from bacteria to man. The rapid upregulation of chaperones and proteases during the heat shock response restores an appropriate protein folding environment in the cell, suggesting that maintaining protein folding homeostasis is a primary function of the HSR. Consistent with this idea, many other treatments that destabilize folded proteins or make it more difficult for nascent proteins to fold also activate this response.

A physiological description of the heat shock response

The σ^{32} –mediated heat shock response in *E.* coli has been extensively characterized by examining both temperature upshift and downshift. Following upshift to temperatures $\geq$ 37° but within the growth range of the organism, synthesis of hsps first increases rapidly

during the induction phase of the response, then declines during the adaptation phase of the response and finally achieves a new steady state level (Straus et al. 1987). The initial rapid increase in synthesis allows hsps to rapidly reach the level characteristic of the new temperature. Conversely, following temperature downshift, hsp synthesis abruptly decreases about 20-fold and then gradually increases over the next several doublings until it reaches a new steady state (Straus et al. 1989). Excess hsps are diluted out as cells grow.

Three regulatory loops control the output by altering the level and activity of σ^{32}. First, translation of σ^{32} increases at high temperature. Second, σ^{32} stability is controlled: σ^{32} is rapidly degraded during steady state growth at both low and high temperature, but transiently stabilized after shift to high temperature. Finally, σ^{32} activity is negatively regulated. Activity control is believed to adjust σ^{32} -mediated transcription to a rate appropriate for the level of unfolded proteins present in the cell, leading to transient inactivation of σ^{32} when excess hsps are present. During the induction phase of the hsr, increased translation and transient stabilization of σ^{32} result in a rapid increase in its level, while the high pool of unfolded proteins removes negative regulation of σ^{32} activity. Together, this accounts for the rapid increase in hsp synthesis during the induction phase. During the adaptation phase, activity control is believed to mediate the decline in hsp synthesis that precedes the decline in σ^{32} level. Likewise, following temperature downshift, activity control is believed to mediate the decline in hsp synthesis that precedes the decline in σ^{32} level. The effect of each regulatory loop on response performance has been modeled; these data will be discussed later in the review.

Translational control of σ^{32}

Early studies demonstrated the rate of translation of σ^{32} was controlled by temperature: σ^{32} translation was 5 to 10-fold higher at 42°C than at 30°C (Grossman et al. 1987). A structural transition in *rpoH* mRNA mediates this control: at low temperature, base-pairing within *rpoH* mRNA occludes the Shine-Dalgarno sequence and the translation startpoint of σ^{32} resulting in inefficient initiation of translation; at high temperature, this inhibitory structure is melted so that translation of σ^{32} increases (Morita et al. 1999a; Morita et al. 1999b). The evidence for this view is as follows: (1) destabilizing the predicted inhibitory structure by single base changes increases translation of σ^{32} at low temperature whereas stabilizing the inhibitory structure with single base changes decreases translation of σ^{32} at all temperatures; (2) temperature regulation and the effects of stabilizing and destabilizing mutations can be reproduced *in vitro* by assaying ribosome binding to *rpoH* mRNA. These experiments suggest that translation control responds directly to changes in temperature rather than sensing the cellular folding environment that result from the temperature changes. From this point of view, translational control is an "early warning" system, allowing translation to increase (and decrease) in anticipation of the expected changes in the cellular folding environment.

Regulation of σ^{32} Activity

Control of σ^{32} activity was initially inferred from the fact that under conditions where there is transiently more σ^{32} than necessary, expression of hsps is lower than expected

from the amount of σ^{32} present in the cell (Straus et al. 1989). Thus, activity regulation is observed during the adaptation phase of temperature upshift, after temperature downshift, and when σ^{32} is artificially overexpressed. The "unfolded protein titration model" has been proposed to explain activity control. According to this model, σ^{32} samples DnaK chaperone activity to sense the amount of unfolded proteins in the cell. The DnaK chaperone machine consists of DnaK (HSP70), DnaJ (HSP40), and GrpE. When chaperone levels are abundant relative to unfolded proteins, DnaK feedback inhibits σ^{32} and negatively regulates further chaperone production. Consistent with this idea, overexpression of DnaK inactivates σ^{32} and depleting DnaK leads to accumulation of active σ^{32} (Tomoyasu et al. 1998). The strongest evidence supporting the "unfolded protein titration model" is that expression of unstable proteins that titrate DnaK induces the heat shock response (Tomoyasu et al. 1998). This suggests that σ^{32} is not responding to the total level of DnaK chaperone in the cell, but rather to the ratio of chaperone relative to its unfolded protein substrates. This control circuit allows the cell to continuously monitor its protein folding state and ensure that expression of chaperones is appropriate for the unfolded substrate load.

The molecular mechanism of chaperone-mediated inactivation of σ^{32} is not completely settled. The prevalent model is that the chaperones effectively act as antisigma factors and simply compete with RNA polymerase for binding to σ^{32}. In support of this idea, DnaK binds to σ^{32} *in vitro* and addition of DnaK to an *in vitro* transcription reaction containing purified σ^{32} and RNA polymerase leads to a decrease in σ^{32}-dependent transcription (Gamer et al. 1992; Liberek et al. 1992; Gamer et al. 1996).

Thus far, we have considered only the major Hsp40 family member, DnaJ in mediating activity control. However, *E. coli* has other Hsp40's and one of them, CpbA, is known to be able to mediate activity control *in vivo,* in collaboration with DnaK (Ueguchi et al. 1994; Ueguchi et al. 1995). It is not known to what extent σ^{32} is regulated by DnaK/J verses DnaK/CbpA. Morevoer, CbpA has not been examined *in vitro* in regard to σ^{32} binding or inactivation. Also, CbpA activity can be modulated *in vivo* by the accessory factor, CbpM (Chae et al. 2004). It will be interesting to determine the conditions under which CbpA and CbpM are important in mediating activity control.

There is another potential contribution to the activity control mechanism just described. The DnaK/J chaperone machine requires the GrpE nucleotide exchange factor to exchange ADP for ATP, therefore allowing substrate release and a new round of substrate binding. There is evidence that GrpE can act as a thermosensor (Winter and Jakob 2004). At high temperatures, GrpE activity decreases, leading to a slower ATPase cycle, and which in turn leads to DnaK/J acting more like holdase instead of a foldase. Whether this alteration in the functional properties of GrpE affects activity control has not been investigated.

Degradation control of σ^{32}

σ^{32} is degraded rapidly during steady state growth, exhibiting a $T_{1/2} \sim 1$ min at low temperature (30°), and an even faster rate of degradation ($T_{1/2} \sim 20$ sec) at high temperature (42°) (Morita et al. 2000). In addition, immediately after shift to high temperature, this normally unstable protein is transiently stabilized for a 5 to 10 min period. Currently, some but not all features of this response are understood.

A search for the factors involved in degradation of σ^{32} indicated that FtsH (HflB) is the major protease that degrades σ^{32}. σ^{32} is almost completely stable in cells lacking FtsH, whereas single deletions of other cytoplasmic ATP-dependent proteases have little or no effect on σ^{32} stability (Herman et al. 1995; Tomoyasu et al. 1995). In addition, DnaK is implicated in σ^{32} degradation, as depleting or mutationally inactivating DnaK stabilizes σ^{32} (Straus et al. 1990; Tomoyasu et al. 1998). The involvement of chaperones in σ^{32} degradation means that the "unfolded protein titration model" could explain the transient stabilization of σ^{32} after temperature upshift as well as activity control of σ^{32}. The increased prevalence of unfolded proteins after shift to high temperature would titrate chaperones away from their role in degradation so that σ^{32} would be transiently stabilized. If so, this would allow the rate of degradation to directly monitor the folding status of the cell. Additionally, the increased prevalence of FtsH substrates after temperature upshift could titrate FtsH from degrading σ^{32}, resulting in its transient stabilization, thereby tying degradation rate to FtsH substrate flux. However there is conflicting data about whether FtsH is a limiting component in the degradation reaction (Herman et al. 1995; Tatsuta et al. 1998).

Two groups have investigated the thermal behavior of σ^{32}. Both protease sensitivity and hydrogen-deuterium exchange experiments coupled with mass spectrometry indicate that σ^{32} becomes more unstructured at high temperatures (Rist et al. 2003). In addition, FtsH itself has increased activity at higher temperatures (Herman et al. 2003). This may partially explain the extremely rapid degradation of σ^{32} at high temperatures.

Importantly, the degradation system has not been completely reconstituted *in vitro*. Whereas σ^{32} is degraded very rapidly in vivo, degradation by FtsH *in vitro* is very slow (Herman et al. 1995; Tomoyasu et al. 1995; Herman et al. 2003). Moreover, the DnaK and DnaJ chaperones do not facilitate degradation of σ^{32} *in vitro* (Blaszczak et al. 1999). Investigation of the properties of FtsH revealed that it has a very poor unfoldase activity, both *in vivo* and *in vitro*, so that it essentially waits for proteins to spontaneously unfold before degrading them (Herman et al. 2003). The slow rate of σ^{32} degradation *in vitro* could reflect the time required for unfolding of σ^{32}. Intriguingly, FtsH is a member of the AAA family of proteins, many of which utilize adaptor proteins to modulate their activity (Dougan et al. 2002). For example, degradation of σ^{S} by the AAA^{+} protease ClpXP requires the RssB adaptor protein (Zhou and Gottesman 1998). The putative FtsH adaptor protein(s) missing from the *in vitro* system could provide unfoldase activity and/or recruit chaperones.

σ^{32} structure/function

σ^{32} is a member of the bacterial-specific σ transcription factor family (Gruber and Gross 2003). All σ factors contain binding determinants both for RNA polymerase and for promoter DNA. Binding of a σ factor to RNA polymerase induces changes in both the σ and in RNA polymerase; the resultant holoenzyme is competent to bind to promoters specified by the particular σ factor utilized. Bacteria generally contain a single housekeeping σ factor and several alternative σ factors, which mediate responses to altered environmental conditions. σ factors contain between 2-4 domains, depending on the particular group to which they belong. Housekeeping σ factors are the most complex and contain 4 domains; σ^{32}, a member of the Group 3 σ factors, contains 3 domains: 2, 3, and 4. Each domain carries recognition determinants both for RNA polymerase binding and for promoter recognition. Domain 2 recognizes the -10 region of the promoter and carries the major RNA polymerase recognition determinants whereas Domain 4 recognizes the -35 region of the promoter. There is a reasonable amount of knowledge about the RNA polymerase and promoter recognition determinants in σ^{32}, both as a result of direct studies on σ^{32}, and by extrapolation from studies on other σ factors.

In addition to carrying out the functions common to all σ factors, σ^{32} must encode determinants that allow it to bind to chaperones and to the FtsH proteases, so that its activity and stability can be regulated properly. However, it has proven surprisingly difficult to identify mutations that are specifically altered in its regulatory determinants. One reason for this is that the multiple regulatory loops tend to obscure the true phenotype of such mutations. For example, mutations in σ^{32} that eliminate the binding

site for the FtsH protease would result in accumulation of high levels of σ^{32}, but may not significantly increase σ^{32} activity because activity control would inactivate excess σ^{32}.

The initial search for a region of σ^{32} specialized to carry out regulatory functions focused on the RpoH box (Region C). This region, which spans amino acids 122-144 and is located at the N-terminus of Domain 3, is unique to σ^{32} and its homologues (Nakahigashi et al. 1995). Two peptides from within the RpoH box region bind DnaK (McCarty et al. 1996), but mutating these sites do not lead to defects in σ^{32} regulation (Arsene et al. 1999). A frameshift mutation spanning the RpoH box stabilized σ^{32} (Nagai et al. 1994); later studies showed that this peptide is a substrate for FtsH (Arsene et al. 1999), making this a candidate for the FtsH recognition sequence. However, analysis of the various *rpoH* homologues from *Bradyrhizobium japonicum*, which contains both stable and unstable σ^{32} homologues, indicates that differences in degradation control do not map to the RpoH box (Urech et al. 2000). Thus, there is no strong evidence that the RpoH box is involved either in chaperone or protease binding to σ^{32}. At present, the only known function of the RpoH box is binding to RNA polymerase.

A great deal of attention has been focused on mapping the degradation determinants in σ^{32}. Initial studies showed that C-terminal truncations of 15 or 20 amino acids led to stabilization of σ^{32} both *in vivo* and *in vitro*, but did not affect DnaK/J binding (Blaszczak et al. 1999). However, these truncations had additional vector- encoded amino acids added to their C-termini. When C-terminal truncations of 5, 11, 15 or 21 amino acids without additional vector sequences were analyzed, these proteins exhibited the same

high rate of degradation *in vivo* as wt σ^{32} (Tomoyasu et al. 2001). Moreover, FtsH does not efficiently degrade a peptide derived from the C-terminus of σ^{32}. Another approach to mapping the degradation control region utilized chimeras between *E coli* σ^{32} and *B. japonicum rpoH1*. The σ^{32} encoded by *rpoH1* is 10X more stable than *E. coli* σ^{32}, although the two proteins are 40% identical (Bertani et al. 2001). This work suggests that the main degradation tag lies somewhere between amino acid 36-134.

Several groups have used forward genetic screens to search for σ^{32} mutants with either altered activity or stability (Horikoshi et al. 2004; Obrist and Narberhaus 2005). These screens have converged on a small region within Domain 2 of σ^{32}. Although the precise defect of these mutants is unknown, the analysis performed thus far indicates that these mutants identify a critical regulatory region within σ^{32} that is important for activity and stability control.

Modeling of the σ^{32} regulon

The heat shock response has become an attractive candidate for mathematical modeling due to the complex nature of the system and the large amount of experimental investigation. Multiple mathematical models of the heat shock response in *E. coli* have been developed (Srivastava et al. 2001; El-Samad et al. 2005; El-Samad and Khammash 2006). These models have proven valuable for analyzing the contributions of particular components of σ^{32} regulation, and it is particularly useful for understanding why

regulation of σ^{32} is so complex. Rather than being simply redundant, the two negative feedback loops controlling σ^{32} activity and degradation are proposed to each uniquely contribute to robustness, noise attenuation, and the speed of the response. Additionally, modeling has revealed how the features of the response that are regulated by ambient temperature can contribute to a response that primarily senses unfolded proteins. In particular, modeling has shown how the regulation of σ^{32} translation by ambient temperature contributes to the speed of the response. These models have made important contributions to our understanding of σ^{32} regulation, and when combined with further experimentation, will continue to provide valuable insight.

Extension of the role of σ^{32} by analysis of the regulon

While a majority of known heat shock proteins are chaperones and proteases, the functions of many heat shock proteins remain unknown. Until recently, there was not a comprehensive list of heat shock proteins in *E. coli*. Genome wide analysis of the transcriptional changes during heat shock or σ^{32} overexpression has been done using both filter binding and microarrays (Chuang et al. 1993; Zhao et al. 2005; Nonaka et al. 2006). This analysis, combined with mapping of transcription start sites, has led to a robust model for a σ^{32} dependent promoter which has allowed for genome wide predictions of σ^{32} regulated genes (Nonaka et al. 2006). Many of these proposed σ^{32} binding sites have been verified experimentally using σ^{32}-dependent *in vitro* transcription. Taken together, this has led to a comprehensive description of the σ^{32} regulon in *E. coli* and revealed

important new functions of the heat shock response. Surprisingly, perhaps one fourth of σ^{32} regulon members are localized to the inner membrane, pointing to an unappreciated role of σ^{32} in membrane physiology. Also, there are many links between σ^{32} and RNA metabolism (Korber et al. 1999; Bugl et al. 2000; Korber et al. 2000; Staker et al. 2000; Nonaka et al. 2006). σ^{32} regulon members also play roles in DNA integrity, transcription, central metabolism and substrate transport. In addition, some regulon members are transcription factors suggesting that σ^{32} may act as a master regulator. Together, this data has provided important new clues into σ^{32} physiology that will enrich our understanding of the heat shock response.

Extending the lessons learned in *E. coli* to other organisms and systems

The lessons learned from the *E. coli* heat shock response can be applied to our understanding of heat shock and other stress responses in other systems. The heat shock response is universal, and some heat shock proteins are among the most highly conserved proteins in the cell. While heat shock transcription factors are not conserved, their regulation appears similar in most organisms. The three most widespread heat shock response transcription factors are σ^{32}, HrcA, and HSF. σ^{32} homologues are present in alpha, beta, and gamma proteobacteria (Nakahigashi et al. 1995). While there are a few interesting variations, regulation of σ^{32} homologues is in general very similar to the regulation of σ^{32} in *E. coli*, therefore we will not further discuss this family.

HSF is the eukaryotic heat shock transcription factor. HSF regulation is similar to σ^{32} regulation in many ways. HSF is a transcription factor that positively regulates the heat shock response and is negatively regulated by multiple chaperones, particularly HSP70 (DnaK) and HSP90 (Nollen and Morimoto 2002). As in *E. coli*, a sequestration model has been proposed to explain the mechanism of chaperone regulation of the transcription factor. In addition to chaperone regulation, HSF is regulated at other levels including, but not limited to, oligomerization, phosphorylation, and localization. However, due to the complexities of these regulations, it is often unclear how these components contribute to HSF regulation during stress.

HrcA, a heat shock transcription factor that is widespread in bacteria, also has many similarities to σ^{32} even though it functions as a transcriptional repressor. Chaperones also negatively regulate heat shock gene transcription in HrcA dependent systems, but in these systems the chaperones positively regulate HrcA, which in turn negatively represses heat shock gene transcription (Narberhaus 1999). The proposed mechanism for this regulation is a dependence on chaperones for HrcA folding. In contrast to E coli, it is unclear if there are temperature dependent effects that contribute to HrcA regulation in this system. One interesting unexplored possibility is that HrcA itself can act as a thermosensor. As temperature dependent effects are present in other heat shock responses, it is likely that there are such effects in this system as well.

Chaperones have also been proposed as regulators in other pathways besides the heat shock response. Two examples are the ER stress response in eukaryotes and regulation

of σ^{70} in E. coli. In the ER stress response, a sensor kinase called IRE1 is present in the ER membrane. It is believed that IRE1 senses unfolded proteins in the lumen of the ER through negatively regulated by ER chaperones (an HSP70) (Zhang and Kaufman 2006). Interestingly, there is at least one other example of chaperone regulation of a transcription factor that is not involved in the heat shock response. This example is regulation σ^{70}, the housekeeping sigma factor in E. coli, by HscC, an HSP70 family member (Arifuzzaman et al. 2002). However, there are many unanswered questions in this system including the reason for chaperone regulation.

While much work has been done, further analysis of the heat shock response is critical, especially in light of its contributions to pathogenic states including protein folding diseases, cancers, and ageing. Good progress in understanding some of the main regulators of the heat shock response, yet there are still unknown components. Additionally, as these responses are universal and some components are highly conserved, heat shock responses provides an ideal situation where analysis of the response in simple organisms is likely to be directly applicable to our understanding of the heat shock responses in general.

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Chapter 1: A chaperone network controls the heat shock response in E. coli

Abstract

The heat shock response controls levels of chaperones and proteases to ensure a proper cellular environment for protein folding. In *Escherichia coli*, this response is mediated by the bacterial-specific transcription factor, σ^{32}. The DnaK chaperone machine regulates both the amount and activity of σ^{32}, thereby coupling σ^{32} function to the cellular protein folding state. In this manuscript, we analyze the ability of other major chaperones in *E. coli* to regulate σ^{32}, and we demonstrate that the GroEL/S chaperonin is an additional regulator of σ^{32}. We show that increasing the level of GroEL/S leads to a decrease in σ^{32} activity in vivo and this effect can be eliminated by co-overexpression of a GroEL/S-specific substrate. We also show that depletion of GroEL/S in vivo leads to up-regulation of σ^{32} by increasing the level of σ^{32}. In addition, we show that changing the levels of GroEL/S during stress conditions leads to measurable changes in the heat shock response. Using purified proteins, we show that that GroEL binds to σ^{32} and decreases σ^{32}-dependent transcription in vitro, suggesting that this regulation is direct. We discuss why using a chaperone network to regulate σ^{32} results in a more sensitive and accurate detection of the protein folding environment.

Introduction

Cellular survival depends on maintaining an appropriate environment for protein folding. Chaperones, which assist protein folding, and proteases, which remove misfolded proteins, are among the cellular factors that influence protein folding in vivo (Horwich et al. 1999; Bukau et al. 2000). Environmental factors, including temperature and organic solvents, also influence the internal folding milieu (Hartl 1996). In order to maintain homeostasis for protein folding, cells tightly regulate the expression of chaperones and proteases to compensate for environmental perturbations (Morimoto 1998). This response, called the "heat shock response" because it was identified in relation to heat stress, leads to the induction of the almost universally conserved "heat shock genes", which encode chaperones, proteases, and other stress-related proteins (Herman and Gross 2000). Heat shock proteins are not only important during stress conditions; many are among the most abundant proteins in the cell in all conditions because they have a general role in protein folding (Nollen and Morimoto 2002).

A complex control system regulates expression of heat shock genes, ensuring that they can respond even to small changes in intracellular folding. In *Escherichia coli*, regulation of heat shock genes is mediated by *rpoH*, which encodes the σ^{32} transcription factor (Connolly et al. 1999; Arsene et al. 2000). σ factors are bacterial-specific initiation factors that recruit RNA polymerase to particular classes of promoters (Gruber and Gross 2003). σ^{32} is regulated at multiple levels that use inputs both directly from some stresses and also from the protein folding state of the cell. Increases in temperature have been shown to directly increase translation of *rpoH* mRNA by destabilizing an RNA structural element

overlapping the translation start point (Morita et al. 1999a,b). The protein folding state of the cell regulates both the degradation and activity of σ^{32}. In response to an increased need for protein folding agents (e.g., immediately after temperature upshift), σ^{32} is transiently stabilized (Straus et al. 1987). In response to decreased need for protein folding agents (e.g., during the recovery phase following temperature upshift or immediately after temperature downshift), the activity of σ^{32} decreases (Straus et al. 1989). Additionally, cells use these feedback systems to constantly monitor their folding status during growth under steady-state conditions so that the cellular folding environment remains optimal.

The DnaK chaperone machine, consisting of DnaK (Hsp70 homolog), DnaJ (Hsp40 homolog), and GrpE (nucleotide exchange factor), is implicated in regulation of σ^{32} activity and σ^{32} degradation (Tilly et al. 1983, 1989). Mutations in *dnaK, dnaJ*, and *grpE* all induce the heat shock response through increases in σ^{32} stability and activity (Straus et al. 1990). Also, DnaK overexpression and depletion in vivo leads to changes in σ^{32} activity and degradation (Tomoyasu et al. 1998). Activity control has been replicated in vitro, as DnaK binds directly to σ^{32} and inhibits σ^{32}-dependent transcription in vitro (Gamer et al. 1992, 1996; Liberek et al. 1992). However, the role of DnaK in the regulation of σ^{32} degradation is less clear because it has not been possible to replicate this control in vitro.

The current model for regulation of σ^{32} by the DnaK chaperone machine has been referred to as the "unfolded protein titration model" (Straus et al. 1990; Craig and Gross 1991; Bukau 1993). In this model, unfolded proteins and σ^{32} compete for binding to DnaK, with the DnaK–σ^{32} complex inactive in transcription. In addition, DnaK binding to

σ^{32} facilitates σ^{32} degradation in an unknown way. When unfolded proteins are low relative to DnaK, the inactive DnaK–σ^{32} complex predominates, σ^{32} is rapidly degraded, and heat shock gene expression is low. However, when unfolded proteins are high relative to DnaK, DnaK is titrated away from σ^{32}; the active, stable, chaperone-free state of σ^{32} predominates; and heat shock genes are induced.

A chaperone network controls protein folding in the cell (Buchberger et al. 1996); therefore, it would be surprising if σ^{32} sensed the folding state of the cell by only sampling a single chaperone. Good candidates for additional regulators of σ^{32} are GroEL/S (Hsp60/10 homologs) and HtpG (Hsp90 homolog) because, like DnaK, they are some of the most abundant chaperones in the cell, and they are highly conserved through evolution (Fink 1999). However, previous experiments with GroEL/S and HtpG have not provided evidence that they are involved in regulation of σ^{32}. First, mutations in *groEL* and *groES* that are defective in bacteriophage growth are not altered in heat shock gene regulation (Tilly et al. 1983; Straus et al. 1990). Although those mutations were assumed to be generally defective in GroEL/S function, more recent work has shown that GroEL/S has multiple classes of substrates, and mutations in *groELS* can have differential effects on these classes (Wang et al. 2002). It is important to note that these mutations in GroEL/S did not lead to a defect in cell growth, even though GroEL/S is essential; therefore, we now know that the lack of effect of these mutations on heat shock gene expression does not resolve this question. An additional study examining the long-term effects of GroEL/S overexpression failed to provide evidence that GroEL/S regulates σ^{32} activity (Kanemori et al. 1994). By examining long-term GroEL overexpression, the study does not definitively rule out GroEL/S as a regulator, because it is possible that

other mechanisms can compensate for long-term overexpression. Previous studies with HtpG have given evidence that it can bind to σ^{32} in extracts, yet no experiments have been performed to determine its role in σ^{32} regulation (Nadeau et al. 1993).

In this report, we investigate the role of these two chaperones, GroEL/S and HtpG, in regulation of the heat shock response. We present in vivo and in vitro evidence supporting the idea that GroEL/S, but not HtpG, is used to sense the protein folding state of the cell. We further show that GroEL/S is used together with the DnaK chaperone machine to regulate heat shock gene transcription, and we conclude that a cellular chaperone network regulates the activity of the σ^{32} heat shock factor.

Results

GroEL/S and σ^{32} show a genetic interaction

Our initial evidence that GroEL/S was a regulator of the heat shock response came from identification of a genetic interaction between σ^{32} and GroEL/S. Overexpression of σ^{32} is toxic, resulting in a dramatic reduction in efficiency of plating (EOP) on minimal medium. Toxicity can be alleviated by simultaneously overexpressing a negative regulator of σ^{32} (Herman et al. 1995b). We found that overexpression of σ^{32} alone resulted in an EOP of 1×10^{-4}, whereas simultaneous overexpression of GroEL/S and σ^{32} restored the EOP to ~1. Therefore, GroEL/S can alleviate the toxicity of σ^{32} overexpression, suggesting that it is a negative regulator of σ^{32}.

Overexpression of GroEL/S decreases σ^{32}-dependent transcription

We asked whether the genetic interaction between GroEL/S and σ^{32} was due to the ability of GroEL/S to negatively regulate σ^{32}-dependent transcription. In this experiment, we compared σ^{32}-dependent transcription in cells with and without overexpression of GroEL/S, using a plasmid with the *groELS* operon under the control of an inducible P*ara* promoter (Fig. 1). We assayed σ^{32}-dependent transcription by measuring the accumulation of β-galactosidase from a chromosomal, σ^{32}-dependent *lacZ* transcriptional reporter as a function of cell growth. This "differential rate of β-galactosidase synthesis" measures how σ^{32} activity changes in response to a signal over time, with the slope of the line reflecting the protein synthesis rate of β-galactosidase and therefore σ^{32} activity. Our results indicate that GroEL/S overexpression significantly decreases σ^{32}-dependent transcription, thereby confirming the idea that GroEL/S negatively regulates σ^{32}. Next, we directly compared GroEL/S inhibition with DnaK/J inhibition. When we overexpressed DnaK/J from the same plasmid vector used for GroEL/S overexpression, we found that the eventual extent of inhibition of σ^{32}-dependent *lacZ* reporter expression was approximately the same as that mediated by GroEL/S (Fig. 1). However, whereas GroEL/S overexpression exhibited an immediate inhibitory effect, inhibition by DnaK/J was manifest more slowly. Delayed inhibition was noticed in multiple experiments (data not shown). This effect was not due to a different extent of overexpression of the two proteins. Western blot analysis indicated that both proteins were overexpressed about 10-fold after 1 h of induction (data not shown). We also tested whether HtpG participated in regulation of σ^{32}. In contrast to GroEL/S and DnaK/J, overexpression of HtpG had no

effect on σ^{32}-dependent *lacZ* reporter expression (data not shown), indicating that HtpG, at least alone, does not behave as a negative regulator of σ^{32} in vivo.

Our results indicating that GroEL/S overexpression decreased σ^{32} activity differed from previous results showing no effect after long-term GroEL/S overexpression (Kanemori et al. 1994), leading us to wonder whether cells can adapt to long-term GroEL/S overexpression. To determine the effects of prolonged GroEL/S overexpression, we repeated our previous experiments and re-examined the same cultures the next day. Our results confirmed that σ^{32} activity is not repressed after long-term overexpression of GroEL/S (data not shown), indicating that the cell possesses a mechanism to adapt to long-term GroEL/S overexpression.

Overexpression of GroEL decreases the activity of σ^{32}

The experiments described earlier indicate that overexpression of GroEL/S results in decreased transcription from σ^{32}-dependent promoters in vivo. This decrease could result from a change in the amount or activity of σ^{32}, or from a combination of these two effects. To investigate this issue, we examined the synthesis rate of σ^{32}-dependent proteins, which gives us an instantaneous indication of σ^{32} function. We also determined protein levels using Western analysis, in order to correlate σ^{32}-dependent protein synthesis with GroEL and σ^{32} levels. We overexpressed the *groELS* operon on a plasmid using an inducible P*tet* promoter and analyzed the synthesis of two σ^{32}-dependent proteins, HtpG and DnaK (Fig. 2A; Zhou et al. 1988). Synthesis of both HtpG and DnaK begins to decrease immediately,

exhibiting a four- to fivefold reduction by 10 min following overexpression of GroEL/S. By 10 min after GroEL/S induction, the concentration of σ^{32} had declined less than twofold as measured by Western analysis (Fig. 2B), indicating that GroEL/S must be inhibiting the activity of σ^{32}, as the decrease in its level is insufficient to explain the drop in σ^{32}-dependent protein synthesis. To determine if these inhibitory effects occurred at physiologically relevant levels of GroEL/S, we measured GroEL levels. Western blotting revealed that the amount of GroEL increased approximately twofold at 5 min and fourfold over the course of our experiments (Fig. 2B). We confirmed this result by showing that the synthesis rate of GroEL increases ~16-fold (data not shown), which, when combined with our observed doubling time of 1 h, allows the accumulation of GroEL to be calculated, assuming that it is a stable protein. This estimation of GroEL/S levels is in good agreement with our observed changes in GroEL with Western blotting. A two- to fourfold accumulation of GroEL/S is physiologically relevant, as cells growing at 42°C have two to three times as much GroEL/S as those growing at 30°C (Straus et al. 1990). In summary, GroEL is able to repress σ^{32} activity with an efficiency similar to that of DnaK overexpression in vivo (Tomoyasu et al. 1998). Moreover, the negative regulation of σ^{32} activity following GroEL/S overproduction occurs at amounts corresponding to its normal physiological variation within the cell.

As specificity controls for this experiment, we analyzed the synthesis of RseA, a σ^{E}-dependent protein (De Las Penas et al. 1997), and RpoB, a σ^{70}-dependent protein (Fig. 2C; Barry et al. 1979). The synthesis of these proteins declined only twofold by 10 min after overexpression of GroE/S from the P*tet* promoter, as compared with the four- to

fivefold repression of HtpG synthesis, indicating that GroEL/S has a specific repression component for σ^{32}-mediated transcription. To ensure that the repression of σ^{32} activity was not an artifact of the induction system, we tested two additional induction systems, P*ara* and P*lac*. For each system, overexpression of GroEL/S repressed HtpG synthesis several fold more than RseA synthesis (data not shown), indicating that there is σ^{32}-specific repression of gene expression regardless of the promoter used to overexpress GroEL/S.

The ratio of GroEL/S to substrates is important for regulation of σ^{32}

We showed that increasing the levels of GroEL/S leads to down-regulation of σ^{32} activity; however, the unfolded protein titration model suggests that it is not the total level of chaperone that is important for determining σ^{32} activity, but the ratio of chaperone to substrate. To determine what population of GroEL/S was important for regulation of σ^{32}, we asked whether simultaneous induction of HrcA, a GroEL/S-specific substrate, would reverse the effects of GroEL/S overexpression (Mogk et al. 1997; Reischl et al. 2002; Wang et al. 2002). We found that simultaneous induction of HrcA and GroEL/S led to a reversal of the effects of GroEL/S overexpression alone (Fig. 3). This indicates that the population of GroEL/S that regulates σ^{32} is most likely free GroEL/S not associated with substrates, confirming an important prediction of the unfolded protein titration model.

Depletion of GroEL increases σ^{32} levels

The experiments described earlier show that overexpression of GroEL/S results in decreases in σ^{32} activity; however, if GroEL/S is a regulator of σ^{32}, then decreases in the level of GroEL/S should lead to an increase in σ^{32} activity. A previous report indicated that depleting GroEL/S resulted in stabilization of σ^{32} and therefore increased σ^{32}-dependent transcription (Kanemori et al. 1994). We validated that the activity of σ^{32} increases when GroEL/S is depleted using a strain with the chromosomal copy of *groEL/S* driven by the P*ara* promoter (Fig. 4). Also, we examined whether decreasing the amount of free GroEL/S by induction of a GroEL/S-specific substrate increases σ^{32} activity and whether this depletion also works by increasing the concentration of σ^{32}. We found that induction of HrcA increased both σ^{32} activity and the amount of σ^{32} (Fig. 3; data not shown). By analogy with the effects of GroEL/S depletion, this increase in amount is likely to reflect a decrease in degradation of σ^{32}. Thus, whether the concentration of free GroEL/S is decreased by depletion or titration with an unfolded protein substrate, the result is the same: The amount and activity of σ^{32} increases.

GroEL regulates σ^{32} during stress conditions

Our results with steady-state growth predict that the changes in GroEL/S levels during temperature upshift play a regulatory role in the heat shock response. We tested this idea by comparing the heat shock response of cells whose chromosomal copy of *groEL/S* is driven by the P*ara* promoter with wild-type cells containing σ^{32}-mediated transcription of

groE/S. Previous work established that P*ara groELS* cells have about 80% as much GroEL as wild-type cells growing at 30°C (McLennan and Masters 1998). We validated that number by determining the rate of GroEL synthesis in the two strain backgrounds and showed further that after a shift to 42°C, there is less than a twofold increase in GroEL synthesis from P*ara* (data not shown), indicating that over the short 30 min window of a temperature upshift experiment, P*ara groELS* cells will experience little change in GroEL level. In contrast, the wild-type cells are able to quickly increase the level of GroEL in response to a temperature upshift. We find that the heat shock response in P*ara groELS* cells is altered in two respects (Fig. 5). First, the heat shock response is greater, showing twice as much induction at 2.5 min as wild-type cells, and higher peak expression at 5 min. Second, the shut-off response is delayed compared with wild-type cells, even though all other hsps are present at higher-than-normal levels. This experiment, which has been replicated several times, indicates that even a very small (20%) reduction in the level of GroEL prior to upshift, combined with prevention of GroEL/S accumulation during upshift, leads to a demonstrable increase in the extent and duration of the heat shock response. Together, these effects establish that GroEL/S is involved in negatively regulating heat-shock gene expression during stress conditions.

The data presented thus far argue that the level of GroEL/S is important for σ^{32} regulation in vivo but does not reveal whether this regulation is direct or indirect. This issue is particularly important for chaperones, which may be expected to have indirect effects. We addressed this issue in the next two sections by using in vitro experiments to determine

whether GroEL/S, in the absence of other molecules, can bind to σ^{32} and alter its transcriptional activity.

GroEL binds to native σ^{32}

We examined whether the GroEL subunit of the GroEL/S chaperone machine could bind directly to σ^{32} in vitro using gel filtration (Fig. 6A). In this and all other in vitro experiments performed with chaperones, the chaperone preparation was first cleaned of peptides by incubation with Affi-gel blue beads (see Materials and Methods). When GroEL and σ^{32} were incubated together and then separated on a gel filtration column, approximately one-half of the σ^{32} eluted in a peak coincident with free σ^{32} and the remainder eluted in a higher molecular weight fraction, indicating binding to GroEL. This experiment shows that GroEL can bind directly to σ^{32} in vitro. Two additional experiments indicated that GroEL binds to native rather than misfolded σ^{32}. First, 95% of the σ^{32} preparation was able to bind to RNA polymerase (E), indicating that our preparation was almost completely active. Second, after our initial gel filtration with σ^{32} and GroEL, we isolated the peak containing unbound, free σ^{32} and reanalyzed GroEL binding (Fig. 6B). Analysis of this σ^{32} fraction revealed that its binding characteristics were identical to those of unfractionated σ^{32}, exhibiting partial binding to GroEL and almost complete binding to RNA polymerase. This indicates that our σ^{32} does not exist in two distinct populations, for example, one misfolded and one native, and further, that passage through the gel filtration column did not lead to inactivation or misfolding of our σ^{32} preparation. To determine whether σ^{32} binding to GroEL was similar to misfolded

protein binding to GroEL, we tested whether a substrate-binding mutation in GroEL, GroELY203E, prevented σ^{32} binding. This mutant is believed to affect the normal protein-binding site of GroEL, as it is defective in the binding of several unfolded proteins in vitro and cannot complement a temperature-sensitive *groEL* mutant in vivo (Fenton et al. 1994). We found that GroELY203E binds very poorly to σ^{32} in our gel filtration assay, suggesting that the normal unfolded substrate-binding site on GroEL is used for σ^{32} binding. Taken together, these experiments show that GroEL is able to directly bind to native σ^{32} using its normal substrate-binding site. Assuming that the binding is in equilibrium and that our GroEL preparation is mostly active, the binding constant for this reaction is ~1 μM.

The GroEL/S chaperone machine inhibits σ^{32}-dependent transcription in vitro

GroEL binds directly to σ^{32} in vitro, suggesting that this interaction may be sufficient to inhibit σ^{32} function. We therefore tested whether addition of GroEL to a σ^{32}-dependent in vitro transcription reaction decreased the activity of σ^{32}. Results of a representative transcription reaction are shown in Figure 7A, and the quantified and summarized data for all experiments are shown in Figure 7B. We found that a fivefold molar excess of either GroEL or GroEL/S over σ^{32} inhibits σ^{32}-dependent transcription in vitro approximately threefold (Fig. 7B, cf. columns 1 and 2,3, respectively). Moreover, this inhibition is specific to σ^{32}, as GroEL has no effect on σ^{70}-dependent transcription (Fig. 7B, cf. columns 7 and 8). Inhibition requires binding to σ^{32}, as the GroELY203E-binding mutant does not inhibit σ^{32}-dependent transcription (Fig. 7B, cf. columns 1 and 4). These

two controls allow us to rule out that the ATPase activity of GroEL is causing the inhibition; however, we did an additional experiment to further rule out the possibility that the GroEL ATPase activity was contributing to inhibition of σ^{32}. We showed that GroEL inhibits σ^{32} activity even when reactions are performed with 1.2 mM ATP (six times higher than the ATP concentration normally present in the transcription reaction; data not shown). We conclude that direct binding of GroEL/S to σ^{32} inhibits its transcription activity.

Previous work has shown that the DnaK/J chaperone machine inhibits σ^{32} activity in an in vitro transcription reaction (Gamer et al. 1996). We replicated that result in our system using DnaK/J cleaned of peptides by passage through an Affi-gel blue column (see Materials and Methods; Fig. 7B, cf. columns 1 and 5). We then asked whether the DnaK/J and GroEL/S together further inhibited transcription. We added sufficient DnaK/J and GroEL/S to result in approximately threefold inhibition of activity by each separately and found that, together, they result in close to a ninefold decrease in σ^{32} activity, indicating that these two chaperone machines independently inhibit σ^{32} (Fig. 7B, cf. columns 6 and 5,3).

Discussion

A major challenge for all organisms is to maintain a constant intracellular folding environment. This requires a robust and sensitive stress response that is capable of

responding to small changes in the level of misfolded proteins. Until now, it was believed that the DnaK chaperone machine was the sole sensor of the folding environment in *E. coli*. In this work, we show that every important criterion used to establish the regulatory role of DnaK also establishes a similar role for the GroEL/S chaperone machine. The use of a chaperone network to detect unfolded proteins allows the cellular folding status to be sensed more completely, and, in addition, provides a more sensitive indicator of folding state than is possible by a single chaperone machine.

GroEL/S, like DnaK, is involved in the regulation of both σ^{32} activity and σ^{32} stability. Overexpression of GroEL/S inhibits the activity of σ^{32} in vivo, with efficiency approximately comparable to inhibition resulting from DnaK overexpression. In both cases, inactivation of σ^{32} is direct, as either protein can selectively inhibit σ^{32} transcription in vitro with comparable efficiency. There is also a link between chaperone level and the stability of σ^{32}, with underproduction of either the DnaK or GroEL/S chaperone machines stabilizing σ^{32}. However, the mechanism of this effect is unclear because it cannot be reconstituted in vitro. σ^{32} is degraded very rapidly in vivo (T 1/2 ~1 min) by the FtsH protease (Herman et al. 1995b; Tomoyasu et al. 1995). FtsH degrades σ^{32} very slowly in vitro, and neither the addition of DnaK or GroEL/S separately or together facilitates proteolysis (Blaszczak et al. 1999; C. Herman, unpubl.). Recent evidence indicates that FtsH lacks a robust unfoldase activity, and an additional unidentified factor may allow FtsH to proteolyze σ^{32} by acting as an unfoldase (Herman et al. 2003). It remains to be seen whether the chaperones work together with this factor to promote degradation, or whether they influence degradation by a more indirect pathway. Interestingly, we further show that cells can adapt to long-term overexpression of GroEL/S. This suggests a new

layer of complexity in the regulation of the heat shock response. We show that, in addition to its role in the regulation of σ^{32} under steady-state conditions, GroEL/S is important for proper regulation of σ^{32} during stress conditions.

To our knowledge, this is the first reported example of GroEL/S binding to a folded, active protein. Moreover, our studies indicate that the normal substrate-binding site on GroEL/S mediates interaction between the two proteins. Together, these observations suggest that GroEL/S is able to bind to σ^{32} because some aspect of the structure of native σ^{32} mimics that of an unfolded protein. Although there are no structures of intact, uncomplexed σfactors, a combination of high-resolution structures of individual domains and a low-resolution structure of σbound to RNA polymerase has led to the generally accepted notion that all σfactors consist of domains separated by flexible linkers (Campbell et al. 2002; Murakami et al. 2002). The flexible linkers, a portion of one of the domains or the aspects of σ^{32} that are not conserved among other σs, may be specialized to exist as an unfolded segment to allow σ^{32} binding to GroEL/S and possibly to DnaK. In support of this idea, deuterium/hydrogen exchange followed by rapid proteolysis and mass spectrometry analysis has shown that a large portion of the C terminus of σ^{32} undergoes an unusually fast exchange with the solvent, suggesting that it is either highly flexible or poorly structured (Rist et al. 2003). Such regions could serve as chaperone binding sites. As σ^{32} is specialized to transcribe at lethal temperatures and has been shown to maintain active transcription in vitro at such temperatures (Blaszczak et al. 1995), such unstructured regions would have to exist in concert with a core folded region that allows the protein to maintain activity.

The mechanism by which binding to GroEL inhibits σ^{32} transcriptional activity is unknown. The simplest model is that GroEL binding simply sequesters σ^{32} in its central cavity, thereby preventing it from binding to RNA polymerase. However, the simple sequestration model seems inconsistent with our current data. First, GroEL binding to σ^{32} appears to be much weaker than RNA polymerase binding to σ^{32}, and in the concentrations used in the in vitro transcription experiments, we would not expect GroEL to be able to compete with RNA polymerase for σ^{32} binding. Moreover, sequestration of σ^{32} by GroEL/S may be expected to prevent rapid σ^{32} degradation in vivo, as GroEL normally binds proteins inside its central cavity. However, σ^{32} does not accumulate on GroEL/S overproduction. An alternative model is that inactivation of σ^{32} involves two processes: sequestration followed by release in a different conformation that is transiently unable to bind RNA polymerase but can still be degraded. This model may also help to explain the role of GroEL in regulation of both σ^{32} activity and σ^{32} stability.

The use of two independent sensors of the intracellular folding state has important consequences for the cell. First, the use of a chaperone network is expected to increase the accuracy of the surveillance for this signal-transduction pathway. Even though a single chaperone can sense a significant number of the proteins in the cell, use of both DnaK and GroEL/S allows accurate counting of the folding state for the many substrates that interact preferentially or solely with one of the two machines. In this regard, it is particularly important to sense GroEL/S occupancy, as it is the only essential chaperone and therefore folds dedicated substrates important to cellular viability. Second, the use of a chaperone network is expected to increase the sensitivity of the signal-transduction pathway. Global changes in protein folding, such as the increased misfolding during heat

shock, leads to changes in protein folding for both GroEL-dependent and DnaK-dependent proteins. Sensing the levels of both classes of proteins leads to a much larger signal from a given stress, and, therefore, a much smaller change in global protein folding can be sensed.

Interestingly, there is emerging evidence in eukaryotic cells that a chaperone network may be used to regulate the heat shock response. For example, in *Saccharomyces cerevisiae*, the heat shock response is regulated by the transcription factor Hsf1. Hsf1 has long been known to be under the control of Hsp70 (Baler et al. 1992; Halladay and Craig 1995; Shi et al. 1998), but recent evidence also implicates Hsp90 (Zou et al. 1998). This interesting parallel lead us to analyze the ability of the *E. coli* homolog of Hsp90, HtpG, to regulate σ^{32}, but we observed no regulation in our experiments. This difference between prokaryotes and eukaryotes may be correlated with the physiology of these chaperone systems. In prokaryotes, GroEL/S is one of the most important general protein folding machines, and HtpG plays a poorly understood but presumably less critical role. In contrast, eukaryotes use Hsp90 in many important physiological pathways, whereas CCT/TriC, the eukaryotic cytoplasmic chaperonin, appears to be more specialized, although the extent of specialization is controversial (Feldman and Frydman 2000; Young et al. 2001; Hartl and Hayer-Hartl 2002). Although the two specific sensors of the protein folding environment may be different in *E. coli* and *S. cerevisiae*, it seems likely that the rationale behind having multiple sensors of protein folding has been conserved.

Materials and methods

Strains

Strains used in this study are all derivatives of K-12. All strains were isogenic with JM105, genotype *supE endA sbcB15 hsdR4 rpsL thi Δ(lac-proAB)* (Herman et al. 1995b); C600, genotype *supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21* (Sambrook et al. 1989); or 594, genotype *lacZ-350 galK2 galT22 rpsL179 lacIpoZΔ(Mlu)* λJW2(P*htpG*::*lacZ)* (Herman et al. 1995a). Strain CAG48176was made by standard P1 transduction (Miller 1972) of a P*ara*-groELS (McLennan and Masters 1998; Nielsen et al. 1999) into C600. Transductants were selected for resistance to kanamycin and confirmed by ensuring arabinose-dependent growth.

Media and antibiotics

LB rich medium and M9 minimal medium were prepared as described (Sambrook et al. 1989). M9 medium was supplemented with 0.2% glucose (unless otherwise noted), 1 mM $MgSO_4$, and 2 μg/mL thiamine. Complete M9 minimal medium was also supplemented with all amino acids (40 μg/mL) except methionine and cysteine. When required, media was supplemented with the following antibiotics: 30 μg/mL kanamycin; 20 μg/mL chloramphenicol; 100 μg/mL ampicillin, 50 μg/mL spectinomycin. A final concentration of 0.2% L-(+)-arabinose, 25 ng/mL anhydrotetracycline, and 1 mM IPTG were used as inducers for P*ara*, P*tet*, P*lac*, and P*tac* promoters.

Efficiency of plating

Overnight cultures of strain JM105 carrying either pDS1 (P*tac-rpoH*) (Bahl et al. 1987) or pDS1 and pKV1561 (*Plac-groELS*) (Kanemori et al. 1994) were grown at 30°C in M9 minimal media. Serial dilutions were made and plated on M9 minimal media with and without 1 mM IPTG at 30°C. EOP values were calculated by dividing the number of colony forming units (cfu) in the presence of IPTG by the number of cfu in the absence of IPTG.

β-*Galactosidase assays*

Overnight cultures of strain 594 carrying either plasmid pGro7 (P*ara-groELS*) (Nishihara et al. 1998), or plasmids pGro7, pJDW39 (P*T5/lac-hrcA*) (Wang et al. 2002), and pJM100 ($lacI^q$) (McCarty and Walker 1994) in LB media at 30°C were diluted 1:100 and grown until they reached exponential phase. Cultures were then either used as a control or induced with 0.2% arabinose, 1 mM IPTG, or both. σ^{32} activity was assayed by monitoring β-galactosidase activity from a chromosomal σ^{32}-dependent promoter in strain 594 (Herman et al. 1995b). Samples were taken at various time points to determine σ^{32} activity, and assays were performed as described (Miller 1972).

Pulse-labeling

For GroEL/S overexpression, saturated overnight cultures of strain C600 carrying plasmids pGro7 (P*ara-groELS*), pKV1561 (P*lac-groELS*), or pGro11 (P*tet-groELS*)

(Nishihara et al. 1998) grown in M9 minimal media with all amino acids except methionine and cysteine at 30°C were diluted 1:100 and then grown until they reached exponential phase. For GroEL/S depletion, an overnight culture of strain CAG48176 with the chromosomal *groELS* promoter replaced with P*ara*, grown at 30°Cin M9 minimal media containing all amino acids except methionine and cysteine and having 0.2% fructose as the carbon source and 0.1% arabinose to maintain near wild-type levels of GroEL/S, was diluted 1:100 and then grown until it reached exponential phase. For each time point, an 800-μL aliquot of cells was pulse-labeled for 1 min with EasyTag Expre^{35}S^{35}S protein labeling mix (NEN) followed by a chase with unlabeled methionine and cysteine. Extracts were then TCA precipitated as described in the Western blotting section. Samples were resuspended in 50 μL of 2% SDS and 50 mM Tris (pH 7.5). The extracts were diluted in 750 μL RIPA (50 mM Tris at pH 7.5, 500 mM NaCl, 0.1% SDS, 1% NP-40, and 0.5% sodium deoxycholate) and an aliquot was counted in a scintillation counter. To normalize the samples, we used equal numbers of counts per minute. Immunoprecipitation was done in a total volume of 750 μL containing extract, polyclonal antibodies, 25 μL of a 1:1 suspension of protein A-conjugated Sepharose beads, and RIPA buffer. For HtpG, DnaK, and RseA synthesis, we added an extract containing a labeled truncated version of the protein to use as an internal control prior to immunoprecipitation. The samples were rocked at 4°C for at least 1 h, and the beads were washed three times with 900 μL RIPA. Immunoprecipitated proteins were eluted from the beads with Laemmli sample buffer and boiling. The entire sample was then loaded onto an acrylamide gel, and the proteins were visualized using a Molecular Dynamics Storm 560 PhosphorImager scanning system.

Western blotting

Samples for Westerns (900 μL) were collected and ice-cold TCA was added to a final concentration of 5%. Samples were precipitated on ice for at least 30 min, followed by centrifugation. After TCA was removed, the samples were resuspended directly in Laemmli sample buffer. An equal number of cells were loaded in each lane of the polyacrylamide gels and the proteins were transferred to nitrocellulose. The blots were probed with 1:10,000 dilutions of polyclonal rabbit antibodies, and then probed with 1:10,000 dilution of anti-rabbit horseradish peroxidase-conjugated secondary antibody. Western blots were developed with chemiluminescence and exposed to film. Bands were scanned and analyzed using Alpha Innotech densitometry software (Alpha Innotech).

Gel filtration

The following proteins were purified essentially as described: GroEL and GroELY203E (Fenton et al. 1994), core RNA polymerase (Sharp et al. 1999), and σ^{32} (Gamer et al. 1996). All chaperone preparations were cleaned of misfolded proteins by incubation with Affi-Gel Blue beads overnight at 4°C in the presence of 5 mM ATP and 10 mM $MgCl_2$. Proteins were diluted to the appropriate concentrations in protein-binding buffer (PBB) in a final volume of 500 μL and incubated at 20°C for at least 30 min. PBB contains 100 mM KCl, 0.01% NP-40, 20 mM Tris (pH 7.5), 5 mM MgCl, and 10% glycerol. Samples were then loaded onto a Superose 12 gel filtration column and run in PBB. For σ^{32}, a fraction of the protein used was whole-cell labeled with ^{35}S before the purification. This

was added to the cold σ^{32} before the mixture was added to the binding reaction. Thirty fractions, 1 mL each, were collected and the fractions were counted in a scintillation counter.

In vitro transcription

The following proteins were purified essentially as described: GroES (Fenton et al. 1994), σ^{70} (Sharp et al. 1999), DnaK, DnaJ, and GrpE (Suh et al. 1998). All chaperone preparations were cleaned of misfolded proteins by incubation with Affi-Gel Blue beads overnight at 4°C in the presence of 5 mM ATP and 10 mM $MgCl_2$. Holoenzyme was reconstituted by incubation of core RNA polymerase and σ^{32} or σ^{70} in protein dilution buffer (PDB) containing 20 mM HEPES (pH 7.9), 100 mM KCl, 10 mM $MgCl_2$, 0.1% BME, 10% glycerol, 12 µg/mL BSA, and 0.1% Tween. Additional proteins were added as required and samples were incubated at least 10 min on ice. Transcription was initiated by adding an equal volume of transcription mix containing 20 mM HEPES (pH 7.9), 100 mM KCl, 10 mM $MgCl_2$, 0.1% BME, 50 nM template, 2 mM ATP, 2 mM GTP, 2 mM UTP, 0.1 mM CTP, and 0.4 µL ^{32}P α-CTP (3000 Ci/mmol), and samples were incubated 10 min at 30°C. Linear DNA templates were generated using PCR, for Eσ^{32} transcription, the promoter was P*htpG*, and for Eσ^{70}, P*T7A1* was used. Reactions were stopped by the addition of 10 volumes of transcription stop mix (TSM) containing 20 mM EDTA, 250 mM NaCl, 1% SDS, and 200 µg/mL glycogen. Samples were then phenol extracted to remove proteins from the reaction mixture and the RNA was ethanol precipitated and loaded onto a 6% polyacrylamide gel. As an internal control, a 60-nucleotide, ^{32}P end-

labeled oligomer was added to each reaction. The transcripts were visualized using a Molecular Dynamics Storm 560 PhosphorImager scanning system.

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Figure Legends

Figure 1. Effects of GroEL/S and DnaK/J overexpression on σ^{32}-dependent transcription. An exponential phase culture of strain 594 with a σ^{32}-dependent lacZ reporter and carrying either a plasmid able to overexpress GroEL/S (pGro7) or a plasmid able to overexpress DnaK/J (pKJE7) was grown at 30°C with and without 0.2% arabinose to induce GroEL/S or DnaK/J overexpression. A standard differential rate of synthesis plot is shown. The uninduced (control) strains gave identical results; therefore, for simplicity, we have included the data for only one control strain. This experiment and every other differential rate of synthesis experiment was performed at least three times with similar results.

Figure 2. GroEL/S overexpression results in inhibition of σ^{32} activity in vivo. An exponential phase culture of strain C600 carrying plasmid able to overexpress GroEL/S (pGro11) was grown in M9 minimal media containing all amino acids except for methionine and cysteine at 30°C. Anhydrotetracycline was added at time 0 to induce GroEL/S overexpression. The rate of synthesis of two σ^{32}-dependent proteins, HtpG and DnaK (*A*), and a σ^{E}-dependent protein, RseA, and a σ^{70}-dependent protein, RpoB (*C*), were measured. (*B*) The level of GroEL and σ^{32} were measured using Western analysis. All protein synthesis and Western data shown are the average from at least two independent experiments.

Figure 3. The ratio of GroEL/S to substrates in vivo is important for determining the activity of σ^{32}. An exponential phase culture of strain 594 carrying a plasmid able to overexpress GroEL/S (pGro7) and one able to overexpress HrcA (pJDW39) was grown in LB at 30°C in the absence of inducers (control) or in the presence of 0.2% arabinose to induce GroEL/S overexpression and/or 1 mM IPTG to induce HrcA expression. A standard differential rate of synthesis plot is shown.

Figure 4. Depletion of GroEL/S in vivo increases σ^{32} activity. Strain CAG48176 whose chromosomal *groELS* gene is driven by the inducible P*ara* promoter, was grown in exponential phase at 30°C in M9 minimal media containing all amino acids except methionine and cysteine with 0.2% fructose as the main carbon source and 0.1% arabinose to maintain near wild-type levels of GroEL/S. Depletion of GroEL/S was initiated at time 0 by removing arabinose from the media and adding 0.2% glucose. HtpG synthesis and GroEL levels were analyzed as in Figure 2.

Figure 5. Changing the levels of GroEL/S increases the magnitude and duration of the heat shock response. An exponential phase culture of strain C600 or a derivative having the chromosomal *groELS* gene under control of the inducible P*ara* promoter (CAG48176 was grown at 30°C and subjected to heat shock by increasing the temperature to 42°C. σ^{32} activity was measured by examining the rate of synthesis of HtpG.

Figure 6. GroEL interacts directly with active σ^{32} in vitro. (*A*) Purified ^{35}S-labeled σ^{32} (500 nM) was incubated with GroEL (2 μM), core RNAP (2 μM), or a GroEL-binding mutant, GroELY203E (2 μM), for 30 min at 20°C in protein-binding buffer (PBB). The proteins were then fractionated on a Superose 12 gel filtration column with PBB at 4°C.

Fractions were collected and counted on a scintillation counter to determine the level of σ^{32} in each fraction. (*B*) The free σ^{32} peaks from the GroEL and σ^{32}-binding reaction in A were pooled and additional unlabeled σ^{32} was added to bring the concentration to 500 nM as in *A*. This σ^{32} was then incubated with 2 μM GroEL or 2 μM core RNAP and analyzed as in *A*.

Figure 7. GroEL inhibits σ^{32}-dependent in vitro transcription. Multiround in vitro transcription was performed with holoenzyme containing either σ^{32} ($E\sigma^{32}$) or σ^{70} ($E\sigma^{70}$) (100 nM) incubated with GroEL (500 nM), GroES (1 μM), GroELY203E (500 nM), DnaK (2 μM), DnaJ (400 nM), or combinations thereof. An end-labeled oligo was added to each reaction as an internal control. Transcription reactions were phenol-chloroform extracted, ethanol precipitated, and analyzed on a 6% polyacrylamide gel. (*A*) Representative transcription gel showing duplicate reactions documenting GroEL inhibition of $E\sigma^{32}$. (*B*) Quantification and summary of transcription results from *A* as well as from additional transcription experiments.

Figure 1. Effects of GroEL/S and DnaK/J overexpression on {sigma}32-dependent transcription

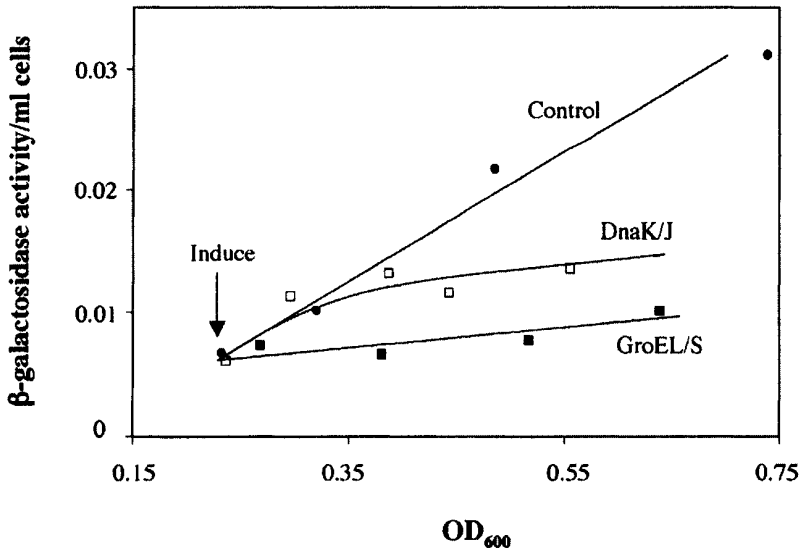


Figure 2. GroEL/S overexpression results in inhibition of {sigma}32 activity in vivo

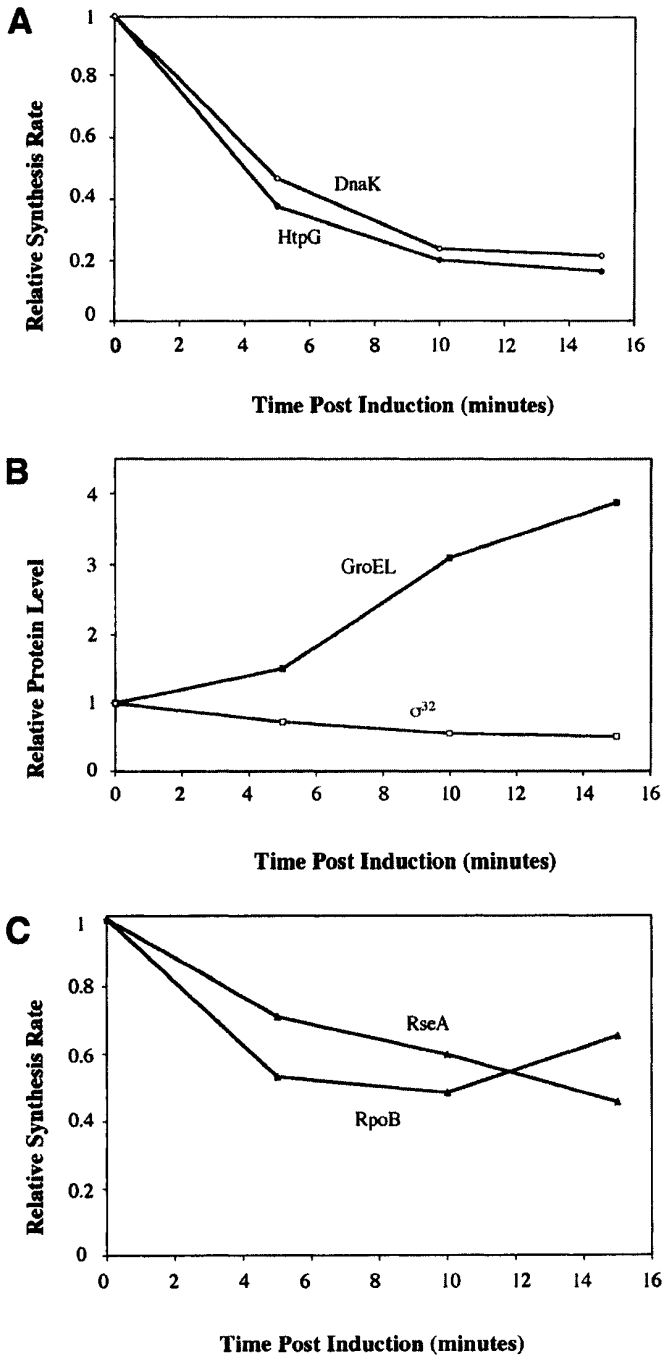


Figure 3. The ratio of GroEL/S to substrates in vivo is important for determining the activity of {sigma}32

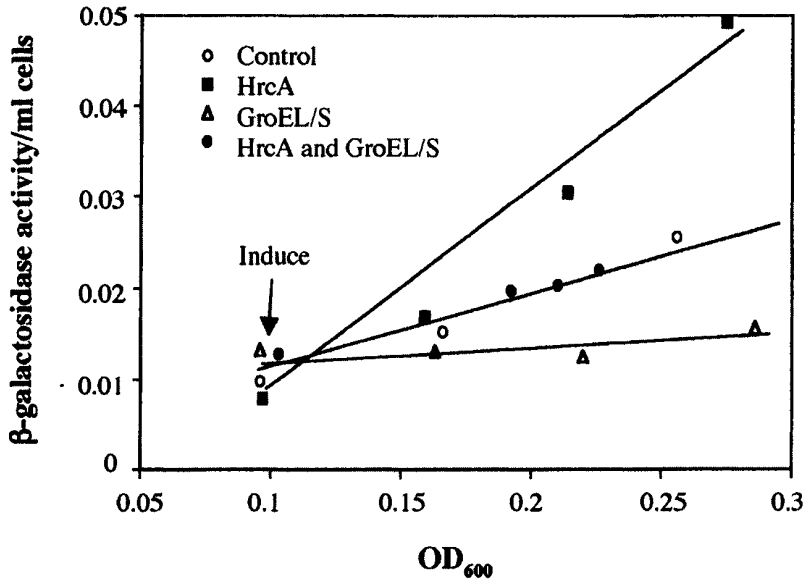


Figure 4. Depletion of GroEL/S in vivo increases {sigma}32 activity

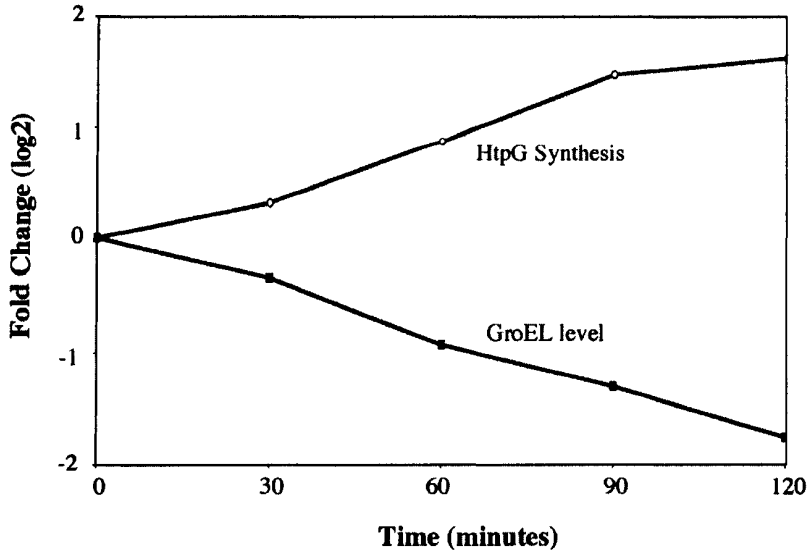


Figure 5. Changing the levels of GroEL/S increases the magnitude and duration of the heat shock response

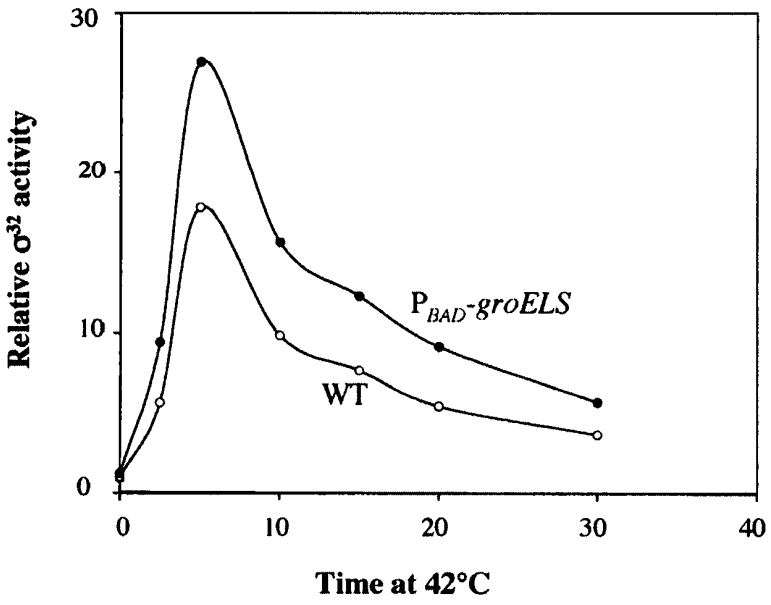


Figure 6. GroEL interacts directly with active {sigma}32 in vitro

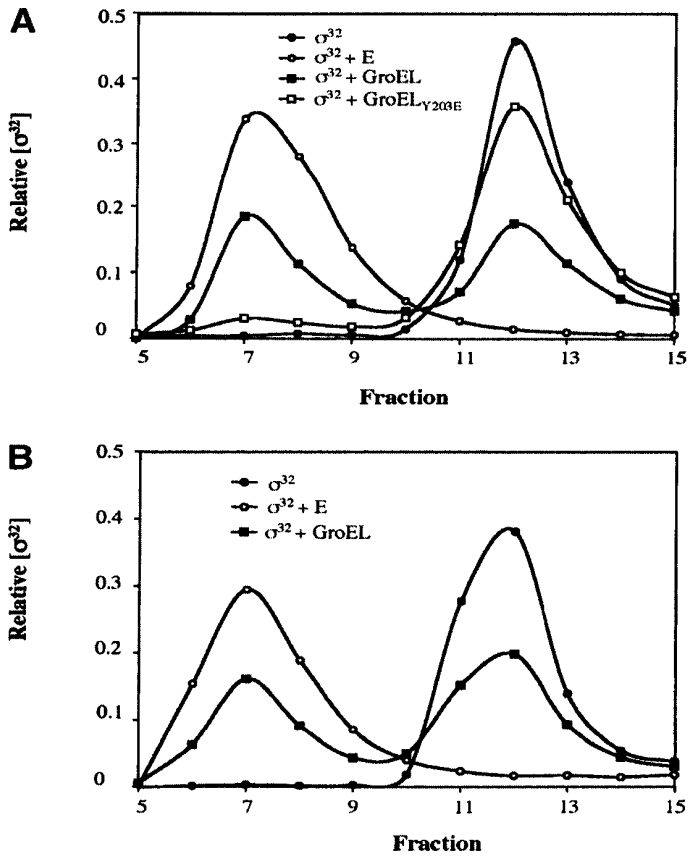
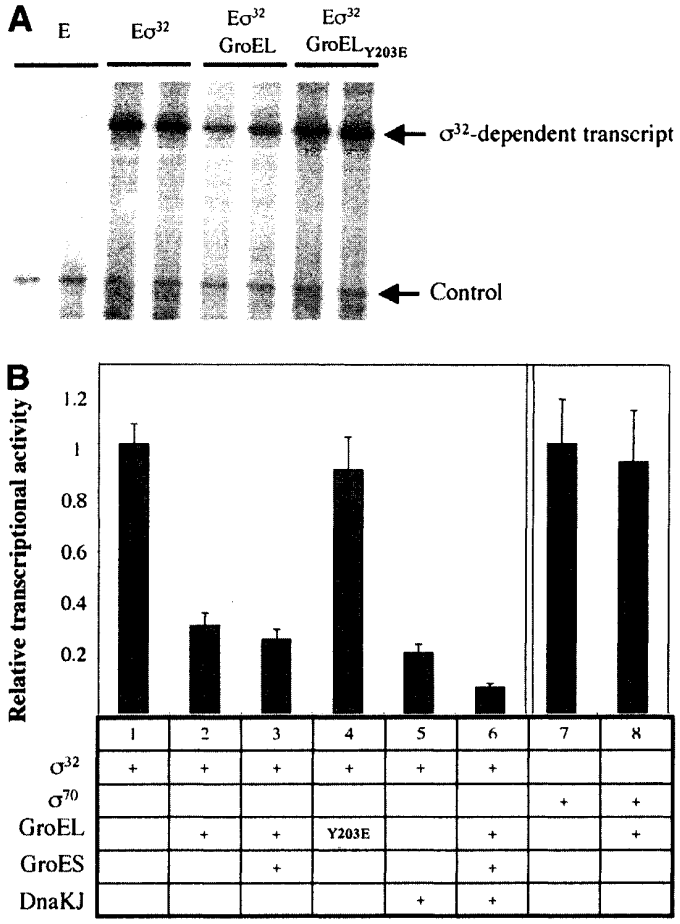


Figure 7. GroEL inhibits {sigma}32-dependent in vitro transcription



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Chapter 2: Insights into regulation of the heat shock response gained by analysis of σ^{32} mutants defective in chaperone-mediated feedback control

Abstract

Induction of chaperones and proteases by heat shock transcription factor is a chief mechanism of protein quality control during normal growth as well as stress response in all organisms. Chaperone-mediated negative feedback control is thought to be a primary regulatory loop that senses the cellular state of protein folding, but how chaperones mediate this control remains unresolved. We here report identification of mutations in the conserved region of bacterial heat shock transcription factor σ^{32} leading to higher activity, stability, and resistance to inhibition by excess chaperones. Most of the mutants obtained were resistant to both DnaK/J and GroEL/S chaperones and showed decreased activation upon depleting chaperones. In contrast, little or no change was found for binding of mutant σ^{32} of strongest phenotype to chaperones or RNA polymerase nor for chaperone-mediated inactivation in an in vitro transcription system. These results suggest that the feedback regulatory loops for inactivation and destabilization of σ^{32} are inter-connected; the mutants are defective in a function downstream of both DnaK and GroEL binding to σ^{32} and required for inactivation and degradation in vivo.

Introduction

The heat shock response is a major homeostatic mechanism for controlling the state of protein folding and degradation in all organisms. In this response, the synthesis of a set of highly conserved heat shock proteins (hsps) including chaperones and proteases are rapidly and transiently induced upon heat stress. Hsps maintain optimal states of protein folding and turnover during normal growth and also minimize cellular damage from stress-induced protein misfolding and aggregation (*e.g.* Bukau and Horwich 1998; Hartl and Hayer-Hartl 2002). Since the level of hsps is primarily controlled by specific heat shock transcription factors, the mode of regulation of these factors is central to our understanding of protein quality control as well as cellular stress responses.

Both prokaryotic and eukaryotic heat shock transcription factors are regulated at multiple levels including negative feedback control mediated by chaperones (Tilly et al. 1983; Straus et al. 1987, 1990; Morimoto 1998; Shamovsky et al. 2006). This negative feedback loop is thought to be the primary sensor of the folding environment of the cell (Craig and Gross 1991; Bukau 1993; Connolly et al. 1999; Nollen and Morimoto 2002). Although some of the players in this feedback loop are identified, little is known about the mechanism(s) by which chaperones negatively regulate their transcription factors (Voellmy 2004; Guisbert et al. 2006). Here, we report transcription factor determinants required for chaperone regulation. We performed these studies in *E. coli*, where the heat shock transcription factor is the alternative sigma factor σ^{32}, encoded by *rpoH* (Grossman et al. 1984; Landick et al. 1984; Yura et al. 1984).

σ^{32} is regulated primarily at the levels of translation, activity and stability (Gross 1996; Yura et al. 2000). Translational control is mediated by an RNA structural element that inhibits translation at lower temperatures but becomes unstructured at high temperatures (Nagai et al. 1991; Yuzawa et al. 1993; Morita et al. 1999a, 1999b), thereby providing a feed-forward loop that responds very rapidly to temperature change. Activity control of σ^{32} is carried out by two chaperone machines whose expression is controlled by σ^{32}: the DnaK (Hsp70), DnaJ (Hsp40) and GrpE machine (Straus et al. 1989; Tomoyasu et al. 1998; Tatsuta et al. 1998) and the GroEL and GroES machine (Guisbert et al. 2004). Each chaperone machine constitutes a negative feedback loop that couples σ^{32} activity to cellular protein folding state. Thus, overexpression of either the DnaK or the GroEL chaperone machine decreases σ^{32} activity, whereas chaperone depletion or overexpression of their protein substrates increases σ^{32} activity (Straus et al. 1989; Kanemori et al. 1994; Tomoyasu et al. 1998; Guisbert et al. 2004). These chaperones are likely to act directly on σ^{32} because they bind to σ^{32} and inhibit its activity in a purified in vitro transcription system (Gamer et al. 1992; 1996; Liberek et al. 1992; Guisbert et al. 2004). A third negative feed-back loop regulates σ^{32} degradation and is mediated by the σ^{32}-controlled FtsH protease with contributions from the two chaperone systems just described: σ^{32} is degraded rapidly in unstressed cells ($T_{1/2}$ = 1 min) but is transiently stabilized in stressed cells (Straus et al. 1987; Tilly et al. 1989; Herman et al. 1995; Tomoyasu et al. 1995). Regulated degradation has not been completely recapitulated in vitro, where degradation of σ^{32} by FtsH is slow and not facilitated by chaperones (Blaszczak et al. 1999; Herman et al. 2003).

To understand the features of σ^{32} that permit feedback inhibition by chaperones, we selected and characterized feedback resistant mutants of σ^{32}. These mutants map to a small conserved region of σ^{32}. Surprisingly, most mutants simultaneously diminished all three negative feedback loops operative in vivo; the most severe mutant essentially eliminated chaperone mediated activity control and degradation by FtsH protease. In striking contrast to the in vivo phenotype, this strong mutant exhibited few defects when tested either for binding or chaperone-mediated inactivation in vitro. The implications of these findings for the mechanism of chaperone-mediated negative feedback control will be discussed.

Results

Isolation of σ^{32} mutants with higher than normal activity

To identify the features of σ^{32} that permit chaperone-mediated negative regulation, we selected mutants expected to be resistant to such regulation. Chaperone accumulation following σ^{32} overexpression triggers a negative regulatory loop that inactivates σ^{32}. We therefore selected for mutants that maintained higher than expected activity when σ^{32} was overexpressed (see Figure 1A). Briefly, a high copy plasmid carrying mutagenized *rpoH* driven from an IPTG-inducible promoter was transformed into cells with two σ^{32}-dependent reporters, one driving expression of *cat* (chloramphenicol resistance) and a

second driving expression of *lacZ*. High expressors were selected on LB-chloramphenicol-IPTG plates and then screened for increased β-galactosidase on indicator plates. Sequencing the entire *rpoH* gene of each mutant revealed 7 independent single base substitutions, most of which were localized around the conserved region 2.1 (Figure 1B). Several other mutants obtained from a slightly different screen (Horikoshi et al. 2004) that mapped in the same region were included for analysis.

We first determined the activity of these plasmid-encoded σ^{32} mutants in a Δ*rpoH* strain carrying the σ^{32}-dependent P*htpG-lacZ* reporter by quantifying the differential rate of β-galactosidase synthesis. Examination of the relative activity of mutant and wild type strains growing at 30°C indicated that all the mutants of region 2.1 or 2.2 exhibit somewhat higher σ^{32} activity than wild type when *rpoH* expression was not induced (1.5 to 2.5-fold; data not shown) and much higher activity when induced with IPTG (2.5 to 5-fold; see Table 1, first column). In addition, these mutants had increased levels of σ^{32} as compared to wild type (data not shown). Thus, these mutants were altered in the activity and/or amount of σ^{32}. Unless otherwise stated, all assays were performed on cells growing at 30°C and σ^{32} activity was calculated from differential rate plots.

Effect of chaperone overexpression on σ^{32}-dependent transcription

We next examined whether the higher activity of σ^{32} mutants correlated with decreased susceptibility to inhibition by excess GroEL/S and/or excess DnaK/J chaperones. To perform this comparison, it was necessary that initial chaperone expression in the mutant and wild type strain was roughly comparable, despite differences in their σ^{32} activity.

Therefore, we placed the chromosomal *groEL/S* or *dnaKJ* operons under control of an inducible σ^{70} promoter, and adjusted expression to the wild type level in all strains. Chaperones were then overexpressed from plasmid-encoded *groEL/S* or *dnaKJ-grpE* controlled by another inducible σ^{70} promoter. As expected from previous studies (Guisbert et al. 2004), the activity of wild type σ^{32} was markedly inhibited by GroEL/S overexpression (~70%) during the time course of 80 to 100 min. In contrast, all Region 2.1 σ^{32} mutants, and one of the Region 2.2 mutants exhibited varying levels of resistance to excess GroEL/S (Table 1). In particular, I54N was essentially resistant to inhibition by GroEL/S overexpression (Figures 2A & B). Quantitatively similar results were obtained when cells were grown and tested at 42°C (Table 1).

The activity of wild type σ^{32} was also inhibited by DnaK/J (or DnaK/J/GrpE) overexpression as expected, whereas I54N mutant was less inhibited (Figure 2C & D). However, the precise extent of inhibition was difficult to quantify: there was an initial lag before inhibition was manifest and as chaperones accumulated, growth was inhibited. The same set of mutants was therefore analyzed by a different DnaK/J overexpression protocol where little growth inhibition was observed. The latter experiments revealed that all the mutants resistant to GroEL/S overexpression were less inhibited than wild type σ^{32} (Table 1). Evidently, these mutants are less sensitive to negative feedback inhibition by both GroEL/S and DnaK/J/GrpE than wild type σ^{32}.

Isolation of the chromosomal I54N and Δ49-52 mutations

We then performed detailed characterization of mutations in the physiologically relevant context of the chromosome, with a goal of comparing the phenotypes of a strong mutant (I54N), with those of a somewhat weaker mutant (A50D). Using *λred-mediated recombination between the parental* rpoH$^+$ *and electroporated oligonucleotides containing the base change for this mutation (see Materials and Methods), we successfully introduced I54N into the chromosomal* rpoH *gene of our standard strain (CAG45146). Assayed in its chromosomal context, I54N-*σ^{32} showed 7-fold higher activity than wild type σ^{32}. We were unsuccessful in introducing A50D, but fortuitously recovered a mutation consisting of a small deletion (A49 to T52) and an E48K substitution, hereafter called Δ49-52 (Figure 1B). *Δ*49-52 had about 4-fold higher activity than wild type σ^{32} and was compared with I54N in most subsequent analyses. These mutants grew almost normally at 30, 37 or 42°C in LB or M9 medium despite the higher σ^{32} activities and hsps produced.

Chromosomal σ^{32} *mutants show marked resistance to excess GroEL/S and DnaKJ chaperones*

We first examined the effect of chaperone overexpression on σ^{32} activity using the differential rate assay described above (Table 1, Figures 2A & B). Consistent with results obtained when the *rpoH* allele was carried on a plasmid, cells expressing

chromosomal I54N were completely resistant to inhibition by excess GroEL/S, whereas cells expressing wild type σ^{32} were strongly inhibited (Figures 3A & B). The Δ49-52 mutant was partially resistant to GroEL/S overexpression (Figure 3C). Both σ^{32} mutants were also more resistant than wild type σ^{32} to inhibition by excess DnaK/J. In these experiments, we overexpressed DnaK/J from the chromosomal *dnaKJ* construct to minimize growth inhibition. Whereas wild type σ^{32} was inhibited 60-70% (Figure 3D), the I54N mutant showed 20-25% inhibition and the Δ49-52 mutant exhibited only slight resistance (Figures 3D-F).

To obtain a more accurate estimation of the effects of chaperone overexpression on σ^{32} activity, we determined the instantaneous rate of synthesis of HtpG, whose transcription is dependent on σ^{32}. Samples were taken at different times after overexpression of either GroEL/S or DnaK/J, and were analyzed using a pulse-chase-immunoprecipitation protocol. The activity of wild type σ^{32} was inhibited about 3-fold by 5 min after induction of GroEL/S and about 10-fold by 10 min (Figure 4A). The Δ49-52 mutant was somewhat resistant to GroEL/S overexpression, exhibiting less than 2-fold inhibition at 5 min and ~5-fold inhibition at 10 min after overexpression. In contrast, the I54N mutant was considerably more resistant to GroEL/S overexpression, exhibiting <30% inhibition over the time course of the assay (Figure 4A). Our previous studies established that this level of inhibition was a nonspecific effect of GroEL/S overexpression, manifest at σ^{70} and σ^{E} promoters, as well as σ^{32} promoters (Guisbert et al. 2004). A similar pattern of inhibition was seen after DnaK/J overexpression although the response was less marked, exhibiting about 3-fold inhibition after 20 min with wild type σ^{32} (Figure 4B). The Δ49-52 mutant was about 2-fold more resistant to DnaK/J

overexpression than wild type σ^{32}, whereas I54N was almost completely resistant (<20% inhibition). Taken together, these results indicate that σ^{32} activity of the "strong" I54N mutant is more resistant to overexpression of both GroEL/S and DnaK/J than the "moderate" Δ49-52 mutant.

The σ^{32} mutants exhibit little response to chaperone depletion

When *E. coli* cells are grown under limited supply of GroEL/S or DnaK/J, the level and activity of wild type σ^{32} increases markedly. We therefore tested whether the I54N and Δ49-52 mutants also respond to chaperone depletion. As in the overexpression experiments, chaperone expression from inducible promoters on the chromosome was adjusted to the wild type level in all strains, and chaperone depletion was initiated by removal of inducer. Whereas the activity of wild type σ^{32} increased 3- to 10-fold following depletion of GroEL/S or DnaK/J over the time course of 2 to 3 hours, the mutants showed little (Δ49-52) or no (I54N) response to depletion during most of this period (Table 2). Thus, the both chromosomal mutants are less sensitive to sudden change (both increase and decrease) in cellular chaperone levels.

Level and stability of mutant σ^{32}

Our experiments quantifying plasmid-encoded σ^{32} indicated that there was more mutant than wild type σ^{32}, suggesting that these mutants might also be altered in degradation control. We therefore quantified the level and stability of the chromosomal mutants.

I54N σ^{32} is much more abundant (10 to 15-fold), and Δ49-52 σ^{32} somewhat more abundant (4 to 6-fold) than wild type σ^{32} (Table 3). Assessment of σ^{32} stability using pulse-chase immunoprecipitation experiments revealed that increased σ^{32} level resulted from increased stability of the mutants, with Δ49-52 showing about a 4-fold increase and I54N exhibiting about a 40-fold increase in stability as compared to wild type (Figure 5). The increase in stability of the mutants is sufficient to explain their increase in level.

We then compared the specific activity (σ^{32} activity/σ^{32} level) of the mutant proteins to that of wild type σ^{32} (Table 3, third column). Setting the activity of wild type σ^{32} as 1.0 (Table 3, line 1), the specific activity of the mutant proteins is very close to that of the wild type (between 0.5-1.0, depending on the mutant and whether the comparison is performed for LB or minimal medium; Table 3, lines 2, 3), even though the levels of the mutant proteins are sufficiently high that they should be feedback inhibited. For comparison, we present the behavior of wild type σ^{32} when its level is increased by absence of FtsH protease (such strains must contain the *sfhC* allele, which suppresses lethality of deleting *ftsH*). As expected from chaperone-mediated feedback inhibition, the specific activity of overexpressed wild type σ^{32} is 10 to 20-fold lower than the specific activity of wild type σ^{32} present at its normal levels (Table 3, line 5), consistent with previous reports (Tatsuta et al. 1998).

Effect of chaperone overexpression on σ^{32} level

To further examine the functional link between chaperone-mediated control of the activity and stability of σ^{32}, we determined how chaperone overexpression affects σ^{32}

level in wild type and mutant strains in M9 medium at 30°C. Upon GroEL/S overexpression, the level of σ^{32} decreased in wild type cells (2-fold after 15 min, and 3-fold after 30 min), consistent with the previous results (Guisbert et al. 2004). In contrast, there was little change in I54N σ^{32} level following GroEL/S overexpression (~30% decrease after 30 min), whereas the Δ49-52 σ^{32} level decreased almost like wild type. Following DnaK/J/GrpE overexpression, σ^{32} level gradually increased (~2-fold after 60 min); however, no such increase was observed with I54N. Thus, I54N σ^{32} appeared to be resistant to changes in stability upon GroEL/S and DnaK/J/GrpE overexpression. Taken together, these results indicate an intimate functional link between chaperone-mediated pathways controlling activity and stability of σ^{32}.

Behavior of the mutants upon temperature upshift

After shift from 30 to 42°C, synthesis of hsps transiently increases about 10-fold as a consequence of increased translation of σ^{32} and its transient stabilization (Straus et al. 1987). In contrast, neither I54N nor Δ49-52 strains exhibited a heat shock response as demonstrated for both strains using the reporter assay for σ^{32}-dependent transcription (data not shown) and for I54N by examining HtpG synthesis (Figure 6). Lack of heat shock response was somewhat unexpected, as the translational response of the mutant remains intact (data not shown). We therefore considered the possibility that the mutants were destabilized at high temperature. Indeed, both mutants were considerably destabilized at 42°C, compared to 30°C, exhibiting $T_{1/2}$'s of 2.5 min (I54N) and 0.5 min (Δ49-52). As a consequence, the σ^{32} level in the mutant strains decreased upon heat

shock instead of transiently increasing (data not shown). Enhanced degradation at 42°C is not a reflection of complete protein unfolding, as the specific activity of the mutant proteins at 42°C (1.0 for I54N and 1.4 for Δ49-52) was similar to that at 30°C (0.5-1.0).

Analysis of I54N σ^{32} in vitro

After demonstrating that the σ^{32} mutants were resistant to chaperone-mediated feed-back inhibition in vivo, we tested our strongest mutant, I54N σ^{32}, for its ability to bind to the GroEL, DnaK, and DnaJ chaperones in vitro (Table 4A). We used fluorescence anisotropy to quantify binding to GroEL and DnaK (see Materials and Methods). Briefly, we bound the chaperones to a fluorescently-labeled substrate and then quantified the ability of unlabeled wild type and mutant σ^{32} to compete with the labeled substrate for chaperone binding. Surprisingly, wild type and I54N σ^{32} bound equally well to both DnaK and GroEL. We measured DnaJ binding using Surface Plasmon Resonance; His-tagged DnaJ was immobilized on an NTA chip and association and disassociation of both mutant and wild type σ^{32} was analyzed. Only a slight decrease was found in binding to DnaJ with the I54N mutant. The in vitro measurements for wild type σ^{32} reported here are consistent with previously reported values (Gamer et al. 1996; Guisbert et al. 2004).

Chaperones are believed to compete with RNA polymerase for binding to σ^{32}. Therefore, another explanation for the mutant phenotype is that the mutant protein binds more tightly to RNA polymerase thereby resulting in chaperone resistance. We therefore measured binding of mutant and wild type σ^{32} to RNA polymerase using fluorescence anisotropy. In these experiments, σ^{32} was fluorescently labeled and we measured the

ability of unlabeled wild type and mutant σ^{32} to compete for binding. Our determined value for binding of wild type σ^{32} is consistent with the previously reported value (Joo et al. 1997); I54N exhibited a slightly lower affinity than wild type for RNA polymerase (Table 4A). Thus, resistance to chaperone-mediated inhibition cannot be explained by increased affinity for RNA polymerase. Additionally, as chaperones compete with RNA polymerase for σ^{32} binding, the slight defect in I54N binding to RNA polymerase may offset the slight defect in DnaJ binding, as the ratio of chaperone/ RNA polymerase binding constants is nearly identical in I54N and wild type σ^{32}.

We next tested the transcriptional capacity of I54N σ^{32} in vitro using a multiround transcription assay conducted at 30°C. Titration of both wild type and mutant σ^{32} (His-tagged and untagged) with a constant level of RNA polymerase revealed that both proteins have similar transcriptional activity in vitro. We then tested chaperone-mediated inhibition of σ^{32}-dependent transcription. Surprisingly, addition of either DnaK/J/GrpE or GroEL/S to the transcription reaction inhibited both wild type and mutant σ^{32} to a similar extent (Table 4B). Addition of either DnaK/J or GroEL alone to the reaction also inhibited mutant and wild type proteins similarly. Finally, a similar extent of inhibition of wild type and I54N σ^{32} was observed with a single-round transcription protocol, or when the reaction was carried out at 37°C or 42°C rather than 30°C.

Discussion

In the present study, we report the characterization of a series of mutations in σ^{32} selected to be resistant to chaperone-mediated feedback inhibition. Our analysis of these mutants revealed three completely unexpected results. First, our mutants were defective in inactivation by both GroEL/S and DnaK/J, yet exhibited no binding defect for either chaperone. Second, in addition to their defects in chaperone-mediated inactivation, our mutants were also defective in regulated degradation. Third, despite a number of attempts, we were unable to replicate the defect in chaperone-mediated inactivation in vitro. We discuss the implications of each of these surprising results below.

The prevalent model for chaperone-mediated inactivation is that chaperone bindings to σ^{32} sequester it from RNA polymerase (Craig and Gross 1991; Bukau 1993). Therefore, when we selected for mutants defective in chaperone-mediated feedback inhibition, we expected to find mutants that were altered in interaction with chaperones or RNA polymerase. The present demonstration that I54N σ^{32} is defective in inactivation without appreciably altering binding to chaperones or RNA polymerase argues against the sequestration model in its simplest form. Instead, these results argue that there is an unanticipated step required for chaperone-mediated inactivation. Since I54N is defective in feedback regulation mediated by both GroEL/S and DnaK/J without significantly affecting chaperone binding, it most probably affects a step downstream of chaperone binding. It is noteworthy that a σ^{32} mutation that decreases RNA polymerase binding still requires DnaK/J for degradation, contrary to the expectation of the above simple model (Tatsuta et al. 2000).

Our finding that all mutants selected for higher activity also exhibited a defect in degradation control was surprising as these pathways were believed to be independent,

linked only by the fact that both utilized chaperones. We do not believe that our selection inadvertently required stabilization of σ^{32}; because σ^{32} was overexpressed, the cells already contained significant amounts of σ^{32} in an inactive form. Importantly a screen that selected only for defects in σ^{32} degradation, without regard to σ^{32} activity, found essentially these same residues (Obrist and Narberhaus 2005). These residues in σ^{32} could affect both inactivation and degradation because a) they have two different functions, one necessary for activity control and one necessary for degradation control; or because b) they have a single function, necessary for both processes. Although we cannot eliminate the first possibility, our data taken together is most consistent with the proposition that these residues have a single, previously unknown function necessary for both pathways. First, based on structural modeling with FliA (Sorenson et al. 2004), which like σ^{32} is a group 3 σ factor, our mutants define a cluster of surface exposed residues (A50, K51, I54 and R91) suggestive of a patch with a single function (Figure 7). The fact that alterations in adjacent residues that are not surface exposed (E48, A49, T52 and L53) have no phenotype (Horikoshi et al. 2004) reinforces the idea that these mutants define a small patch with a crucial function. Second, the fact that the mutational changes are of similar severity for both activity and degradation control rather than exhibiting discrepant effects on the two processes is consistent with the expectations of a model in which the mutations disrupt a single function. Taken together, these results suggest that the mutants identify a region of σ^{32} essential for both activity and degradation control. Furthermore, the single function eliminated by the mutations cannot be explained by disrupting the function of a known regulatory factor. Disruption of FtsH protease is insufficient to explain the mutant phenotype: as demonstrated by Tatsuta et al. (1998),

and reproduced here (Table 3 and data not shown), chaperone-mediated inactivation is operative in the absence of FtsH. Moreover, as described above, the mutants are little affected in chaperone binding, which is the only currently known commonality between inactivation and degradation. These data taken together argue that the pathways regulating activity and stability of σ^{32} are inter-connected and that the mutants are defective in a novel, unknown function linking the two pathways (Figure 8).

Finally, it was quite surprising to find that although I54N-σ^{32} was almost completely resistant to chaperone-mediated inactivation in vivo (Figure 3), it was indistinguishable from wild type σ^{32} in its sensitivity to inactivation by both GroEL/S and DnaK/J in our in vitro transcription system (Table 4B). There are three possible explanations for the discrepancy between in vivo and in vitro results. First, the in vivo phenotype may be a very indirect consequence of the mutational alteration in the protein. We believe that the strength of the phenotypes in vivo, and the fact that selections for both activity and degradation control identify this tight cluster or residues argue against this possibility. Second, the in vitro system may be only partly recapitulating inactivation in vivo. In this respect, the in vitro system may be missing some important conformational change which is manifest in vivo but not in the in vitro conditions that we have explored thus far, or the in vitro system may require an additional factor not currently present. Interestingly, in vitro degradation of σ^{32} by FtsH protease does not mimic degradation in vivo and is believed to be missing at least one factor required in vivo (Herman et al. 2003). It is unclear whether the missing factor is the same one postulated to be missing from our in vitro inactivation system or yet an additional factor. Third, the in vitro inactivation may be an artifact due to a larger excess of free chaperones than are available in vivo. For

example, we use a five-fold excess of free GroEL/S over σ^{32} in vitro, whereas in vivo there are many unfolded proteins that complete with σ^{32} for chaperone binding, so we are unable to determine the actual amount of free chaperones required for inactivation. We are currently unable to distinguish between these three possibilities, but regardless of the reason, it is clear that our current in vitro transcription system is not fully recapitulating inactivation of σ^{32} that occurs in vivo.

In summary, our work casts doubt on two features of σ^{32} regulation that had been thought to be established. First, chaperone-mediated inactivation and regulated degradation of σ^{32} were believed to be independent pathways, linked only by a requirement for chaperones. However, the present results suggest that the two pathways are functionally interconnected in an additional manner. Second, chaperone-mediated sequestration of σ^{32} from RNA polymerase was believed to underlie inactivation. However, the present results indicate that the only mutants thus far defective in this process affect a step not consistent with this simple model. Moreover, these mutants do not recapitulate their inactivation defective phenotype in vitro. All of these results would be explained if the mutants were defective in binding to an unknown factor required for chaperone-mediated inactivation and degradation in vivo. Identification of this factor is crucial for understanding how chaperone-mediated control adjusts the activity of σ^{32} to a level appropriate to maintain protein-folding homeostasis in the cell.

Materials and Methods

Strains

All strains used were derivatives of *E. coli* K-12. Although the σ^{32} mutants were originally isolated in slightly different backgrounds with different *rpoH* plasmids, they were all placed under IPTG-inducible promoter on multicopy plasmids and introduced into strain CAG45146 [MG1655 carrying prophage λJW2(P*htpG::lacZ*)] and an isogenic strain lacking the chromosomal *rpoH*. For chaperone overexpression and depletion experiments, the chromosomal P*ara-groEL/S* (McLennan and Masters 1998) or $P_{A1/lacO-1}$-*dnaKJ lacI*^{q} (Tomoyasu et al. 1998) was transduced into CAG45146 by phage P1 to obtain CAG48239 or CAG48275, respectively. pACYC184-based chaperone expression plasmids pGro11 and pKJE8 (Nishihara et al. 1998) that are compatible with p*rpoH* (pRB11) were used for overexpressing GroEL/S or DnaK/J/GrpE from P*tet* or P*ara* promoter, respectively.

Media and antibiotics

LB medium and M9 minimal medium were prepared as described (Sambrook et al. 1989). M9 medium was supplemented with 0.2% glucose (unless otherwise noted), 1 mM $MgSO_4$, all amino acids (40 μg/ml) except methionine and cysteine, and a vitamine mixture. When required, antibiotics were added to the medium as follows: 100 μg/ml ampicillin, 30 μg/ml kanamycin, 20 μg/ml chloramphenicol. L-(+)-arabinose (0.2%),

anhydro-tetracycline (25 ng/ml) and IPTG (1 mM) were used as inducer for P*ara*, P*tet*, P*lac* and P*trc* promoters, respectively (unless otherwise indicated).

λred-mediated recombination

Synthetic deoxy-oligonucleotides (30 to 70 bp) containing a specific *rpoH* mutation was electroporated into CAG45146 cells carrying pKD46 (*λred*) essentially as described (Datsenko and Wanner 2000; Ellis et al. 2001), and darker colonies were screened on LB agar medium containing X-gal. The *rpoH* mutant candidates were confirmed by β-galactosidase assay, linkage to a nearby tetracycline resistance (Tn10) marker, and by sequencing. The chromosomal *rpoH* mutations thus obtained were then transduced into strain CAG45146 by selecting for the nearby *tet* marker.

β-galactosidase assay

Overnight cultures (LB medium) of strain CAG48239 or CAG48275 with or without chaperone-expressing plasmid were diluted 200- to 500-fold and grown to an exponential phase. Cultures were used either as a control, induced, or depleted for chaperone expression as indicated for each experiment. Samples were taken at intervals, and σ^{32} activity was monitored by measuring β-galactosidase activity expressed from a chromosomal σ^{32}-dependent *htpG* promoter by the standard procedure (Miller 1972).

Pulse-labeling

Cells were grown in M9 medium with all amino acids except methionine and cysteine and pulse-labeled with ^{35}S methionine for σ^{32} synthesis or stability, or with Easy Tag Expre ^{35}S protein labeling mix (NEN) for HtpG synthesis. After a chase with unlabelled methionine and cysteine, cells were precipitated with 5% TCA, washed with acetone, and resuspended in 50 µl of 50 mMTris (pH 7.5)-20% SDS, heated (95°C) for 5 min, and diluted in 750 µl RIPA (50 mM Tris at pH 7.5, 500 mM NaCl, 0.1% SDS, 1% Triton X, and 1% sodium deoxycholate). Immunoprecipitation of samples with equal radioactivities was carried out using a specific antiserum and protein A-conjugated Sepharose beads. The samples were rocked at 4°C for 1 h, and the beads were washed at least 3 times with 900 µl RIPA. The precipitated proteins were eluted from the beads with Laemmli sample buffer and boiled. The entire sample was loaded onto a polyacrylamide gel, and protein bands were visualized using a Molecular Dynamics Strom 560 phosphor imager scanning system. Truncated version of HtpG or σ^{32} was separately labeled and added to each sample before immuno-precipitation as internal reference for quantitation.

Immunoblotting

Cells were mixed with ice-cold TCA at a final concentration of 5%, kept on ice for 30 min, precipitated by centrifugation, washed in acetone, and resuspended in Laemmli buffer. An equal amount of cells (or serial dilutions thereof) were loaded in each lane of

polyacrylamide gels, and the proteins were transferred to nitrocellulose membranes. The blots were first probed with rabbit primary antibodies, and then with anti-rabbit horseradish peroxidase-conjugated secondary antibody, immunoblots were developed with chemoluminescence and exposed to film. Bands were scanned and analyzed using Alpha Innotech densitometry software (Alpha Innotech).

Protein purification

The following proteins were purified essentially as described: RNA polymerase (Sharp et al. 1999), HIS-tagged σ^{32} (Gamer et al. 1996), GroEL, GroES (Fenton et al. 1994), DnaK, DnaJ, and GrpE (Suh et al. 1998). All chaperone preparations were cleaned of misfolded proteins as described (Guisbert et al. 2004). σ^{32} (untagged) was purified by using pQE30-Xa vector, as suggested by the manufacturer (Qiagen). The protein was affinity purified as a HIS-tagged protein, and treated with protease Xa to cleave off the HIS-tag, leaving the intact σ^{32} with no additional amino acid attached. Xa was removed by using Xa removal resin.

Fluorescently labeled σ^{32} was prepared by first purifying HIS-tagged σ^{32} with an L118C mutation from a *slyD* mutant strain to remove a major contaminant (SlyD) highly labeled by the fluorophore. The L118C mutation allows specific labeling at 118 as σ^{32} does not have any endogenous cysteines. Alexa Flour 488 C5-maleimide (Molecular Probes) was used to label σ^{32} as per manufacturers instructions. Briefly, purified σ^{32} was incubated with 10 mM DTT for 30 min at room temperature to reduce any disulfide

bonds. DTT was removed using a PD-10 column, and σ^{32} was coupled to the dye overnight at 4°C, and uncoupled label was removed using gel filtration.

Fluorescence anisotropy

Fluorescence data were collected on an ISS K2 Multifrequency Phase Fluorometer running on Vinci software. For GroEL and RNA polymerase binding assays, labeled σ^{32} was excited at 495 nm and the emission was measured at 522nm. For GroEL, 100nM labeled σ^{32} was used in 50 mM Tris (pH 7.5), 100 mM KCl, 0.002% Tween-20, 0.2 mM βME, and 10% glycerol. For RNA polymerase, 10 nM labeled σ^{32} was used in 50 mM Tris (pH 7.5), 500 mM KCl, 5 % glycerol, 0.05 % NP-40, 0.001 % Tween-20, and 0.01 mM βME. For DnaK binding assays, an N-terminal FITC labeled peptide was synthesized by Tufts Core Facility. The peptide was QRKLFFNLRKTKQ which is a peptide from σ^{32} that has previously been shown to bind DnaK (McCarty et al. 1996?). The peptide was excited at 495 nm and emission was measured at 535 nm. For DnaK, 100 nM labeled peptide was used in 50 mM Tris (pH 7.5), 100 mM KCl, and 10 % glycerol.

Fluorescence anisotropy, r was plotted verses concentration of X, and Kelidagraph was used to fit a curve and calculate the equilibrium binding constant Kd, using the following equation:

$$r = r_f + (r_b - r_f)\left(\underline{(K_d + [P] + [A]) - \{ (K_d + [P] + [A])^2 - (4[P][A]) \}^{-2}} \right)$$

(2[P])

where rf = anisotropy of the free substrate, rb = anisotropy of the complex, [P] = the concentration of substrate, [A] = the concentration of the protein,

Competition experiments were performed using a second, nonfluorescent ligand. This ligand was either unlabeled wild type σ^{32} or unlabeled I54N σ^{32}. The equilibrium binding constant of the second ligand K2 was calculated using the following equation:

$$r = rf + (rb-rf)/[(Kd([L] + K2)/K2[A0])+1]$$

where [L] is the concentration of the competing ligand and [A0] = is the free protein concentration at [L]=0. Derivation of these equations can be found in Vinson et al. (1998).

Surface Plasmon Resonance

Data was collected using a Biacore 1000 and analyzed with BiaEvaluation software. Untagged wild type or I54N σ^{32} was flowed over an NTA chip containing immobilized HIS-tagged DnaJ. The running buffer was 10mM HEPES (pH 7.4), 500 mM NaCl, 50 μM EDTA, and 0.05 % Tween-20. The chip was regenerated by running 10 mM HEPES (pH 8.3), 500 mM NaCl, 350 mM EDTA, and 0.05 % Tween-20, which removed all proteins, followed by immobilization of fresh DnaJ for the next round of binding. Each binding experiment analyzed duplicate samples of at least 4 different concentrations of

σ^{32} run spanning at least 27X change in concentration. Data was analyzed using a 1:1 binding with drifting baseline model using BiaEvaluation software.

In vitro transcription

Multiround in vitro transcription was carried out as reported previously (Guisbert et al. 2004), and single round transcription was done as described (Nonaka et al. 2006).

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Table 1. *Altered activity and response of plasmid-borne mutant σ^{32} to chaperone overexpression*

Strain[a]	Relative rate of β-galactosidase synthesis[b] (30°C)	% Inhibition by chaperone overexpression[c]		
		GroEL/S (30°C)	GroEL/S (42°C)	DnaK/J (30°C)
Wild type	1.0	69	69	75
A50D	5.3	19	21	14
A50T*	3.8	25	--	44
K51E	5.0	23	20	49
I54N	5.3	2	0	23
I54T	2.8	38	29	44

I54A*	6.3	33	--	--
R91P	3.5	29	37	45
R91H	3.8	70	62	52

[a] Derivatives of strain CAG48239 or CAG48275 producing wild type or mutant σ^{32} from pRB11 or similar high-copy plasmid including those reported previously (Horikoshi et al. 2004, indicated by *).

[b] Cultures of CAG48239 derivatives were grown in LB medium at 30°C and plasmid-encoded σ^{32} synthesis was induced by IPTG (1 mM) for 30 min. σ^{32}-dependent transcription was monitored by measuring β-galactosidase and averages from two or more experiments are presented.

[c] GroEL/S was overexpressed from pGro11 carrying P*tet-groESL* by anhydro-tetracycline after prior induction of σ^{32} synthesis for 30 min. DnaK/J was expressed from the chromosomal $P_{A1/lacO-1}$-*dnaKJ lacIq* at the wild type level (5 μM IPTG) or at a great excess (1 mM IPTG). The values are averages calculated from standard differential rate of synthesis plots from 2 to 4 experiments.

Table 2. *Response of the chromosomal σ^{32} mutants to chaperone depletion: Fold increase in σ^{32} activity*

Strain	GroEL/S[a]	DnaK/J[b]
Wild type	3.2	6.3
I54N	1.1	1.2
Δ49-52	1.2	1.8

[a] The σ^{32} mutants carrying P*ara-groELS* on the chromosome were grown in LB medium with 0.2% L-arabinose at 30°C. Exponentially growing cells were collected, washed twice in LB medium, and aerated with 0.2% L-arabinose (control) or 0.2% glucose (GroEL/S depletion). Samples taken at intervals (for 3 hours) were assayed for σ^{32}

activity by measuring β-galactosidase, and differential synthesis rates were analyzed as in Figure 2. The values for typical experiments are shown.

[b] The σ^{32} mutants carrying $P_{A1/lacO-1}$-*dnaKJ lacI*^{q} on the chromosome were grown in LB medium with 5 μM IPTG, washed twice in LB medium, and aerated with 5 μM IPTG (control) or 0.2% glucose (DnaK/J depletion). Samples were taken and analyzed as above.

Table 3. *Summary of activity, level, stability and synthesis of wild type and mutant σ^{32} during steady-state growth at 30°C*

Strain	Activity (A)		Level (B)		(A)/(B)	Stability	Synthesis
	(LB)	(M9)	(LB)	(M9)	(M9)	(M9)	(M9)
Wild type (CAG48238)	1.0	1.0	1.0	1.0[a]	1.0	1.0	1.0
I54N (CAG48302)	6.9	6.8	10	15	0.5	45	1.1

Δ49-52 (CAG48303)	3.8	3.9	4	6	0.6	4	--
sfhC	1.5[b]	--	1.2	1.3	---	---	---
sfhC ΔftsH	3.0[b]	2.3	25	35	0.07	100[b]	---

Wild type and isogenic derivatives were used for comparison. σ^{32} activity was determined by measuring β-galactosidase activity expressed from the P*htpG-lacZ* reporter in LB medium, or by pulse-labeling experiments measuring HtpG synthesis in M9 medium supplemented with amino acids and vitamins. σ^{32} level was determined by immunoblotting. Stability and synthesis were determined by pulse-labeling (with or without chase, respectively), followed by immuno-precipitation.

[a] The wild type σ^{32} level in M9 medium is about one-fourth of that in LB medium.

[b] These values agreed well with the published data (Tatsuta et al. 1998).

Table 4. *Analyses of I54N σ^{32} mutant protein in vitro*

A. *Protein binding*

	Dissociation constants (Kd)			
σ^{32}	GroEL (μM)	DnaK (μM)	DnaJ (nM)	RNA polymerase (nM)
Wild type	2.72 +/- 0.22	5.70 +/- 2.39	39.6 +/- 4.10	5.38 +/- 1.41
I54N	3.83 +/- 0.66	4.38 +/- 1.51	86.6 +/- 21.7	13.64 +/- 4.00

B. *In vitro transcription*

σ^{32}	Relative activity[a]		
	Control	+ GroEL/S	+ DnaK/J/GrpE
Wild type	1.0	0.3 +/- 0.02	0.53 +/- 0.06
I54N	1.0	0.28 +/- 0.13	0.55 +/- 0.07

[a] σ^{32}-dependent transcription of *htpG* was determined essentially as described previously (Guisbert et al. 2004) using purified His-tagged σ^{32}, core RNA polymerase, template DNA, and chaperone proteins as indicated. Multi-round transcription reaction was run at 30°C, and ^{32}P-labeled *htpG* transcript was analyzed by a phosphor imager scanning system. Averages from four measurements with standard deviation are presented.

Figure legends

Figure 1. Selection for σ^{32} mutations on a high-copy *rpoH* plasmid. (A) Plasmid and chromosomal constructs of the parental strain used for selection. (B) Locations of σ^{32} mutants obtained with or without using pMAP1 that carries P*htpG-can* are shown, respectively, above or below the bar, which illustrates nine conserved regions of bacterial σ factor. The Δ49-52 deletion mutant, shown at the bottom, was obtained during transfer of the plasmid-encoded A50D mutation onto chromosome (see text).

Figure 2. Effect of chaperone overexpression on σ^{32}-dependent transcription. Derivatives of strain CAG48239 or CAG48275 carrying the σ^{32}-dependent P*htpG-lacZ* reporter on the chromosome and two compatible plasmids (pRB11 encoding wild type or I54N σ^{32}, and pGro11 encoding P*tet-groESL* or pKJE8 encoding P*ara-dnaKJ-grpE*) were grown at 30°C in LB medium with appropriate antibiotics and other supplements. A pair of cultures with or without chaperone overexpression was then compared, and differential rates of β-galactosidase synthesis are plotted against growth (OD600). (A) and (B), GroEL/S was overexpressed by 25 ng/ml anhydro-tetracycline to 5 to 8-fold of normal (initial) level; (C) and (D), DnaK/J/GrpE was overexpressed by 0.1% L-arabinose to 5 to 6-fold of normal level.

Figure 3. Effect of chaperone overexpression on σ^{32}-dependent transcription in the chromosomal σ^{32} mutants. Wild type and σ^{32} mutants carrying the P*htpG-lacZ* reporter and the P*ara-groESL*/or $P_{A1/lacO-1}$-*dnaKJ* constructs on the chromosome (and pGro11

plasmid for GroEL/S overexpression) were grown and analyzed essentially as in Figure 2, except that DnaK/J was overexpressed from the chromosomal $P_{A1/lacO-1}$-*dnaKJ lacI*^{q} by using 1 mM IPTG added to the cells that had been grown with 5 μM IPTG. (A), (B), and (C), GroEL/S overexpression; (D), (E), and (F), DnaK/J overexpression.

Figure 4. Effect of chaperone overexpression on HtpG synthesis in the chromosomal σ^{32} mutants. Wild type and mutant strains with a chaperone expression plasmid (pGro11 or pKJE8) were grown in M9 minimal medium containing all amino acids except methionine and cysteine at 30°C. At time 0, anhydro-tetracycline or IPTG was added to induce GroEL/S or DnaK/J/GrpE, respectively. Samples were taken at the times indicated and pulse-labeled with ^{35}S-Met-Cys, followed by immunoprecipitation to determine HtpG synthesis. (A), GroEL/S overexpression; (B), DnaK/J overexpression.

Figure 5. Stability of σ^{32} in the chromosomal mutants. Cells were grown in supplemented M9 medium at 30°C, and stability of σ^{32} was determined by pulse-chase experiments using ^{35}S-methionine followed by immuno-precipitation. Residual radioactivity found in σ^{32} after chase with unlabeled methionine is plotted as a function of time.

Figure 6. Heat shock response of wild type and I54N mutant. Cells were grown to a log phase in supplemented M9 medium at 30°C, and shifted to 42°C. Samples were taken at the times indicated and pulse-labeled with ^{35}S-Met-Cys, followed by immunoprecipitation to determine HtpG synthesis.

Figure 7. Structural model for σ^{32} showing that the mutants define a cluster of surface exposed residues. The σ^{32} mutants were mapped onto the structure of FliA (Sorenson et al. 2004) based on an alignment (Clustal V) between σ^{32} and FliA. Shown are A50 in red, K51 in pink, I54 in blue, and R91 in green. Residues A50, K51 and I54 form a close patch in region 2.1, which may interact with R91 located at the tail of region 2.2 (kindly provided by E. Campbell). *(This last sentence could be moved to Discussion.)*

Figure 8. Chaperone-mediated negative feedback control of σ^{32}. Pathways for inactivation and degradation of σ^{32} are depicted either as 'independent' (A) or 'inter-connected' (B). See text for explanation.

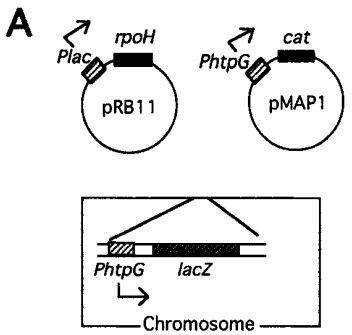


Fig. 1

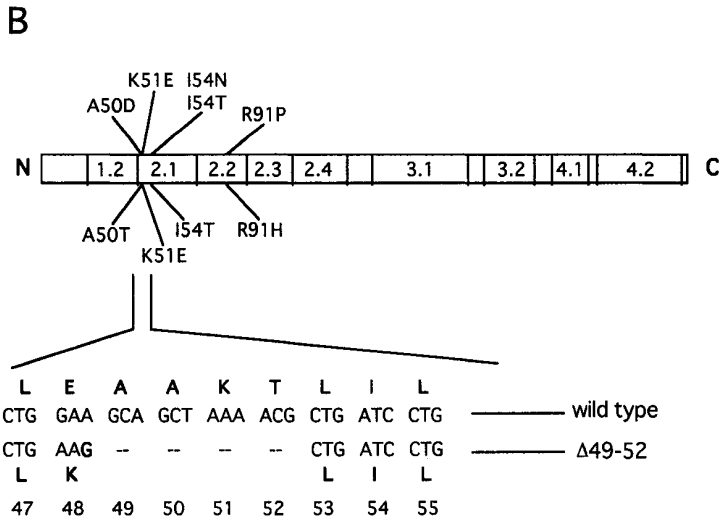
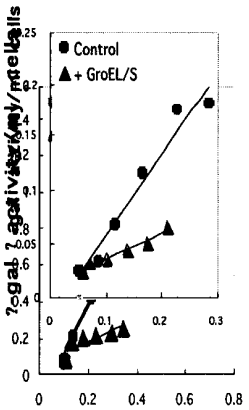


Fig. 2

A wt B I54N



C D I54N

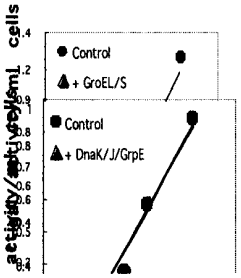
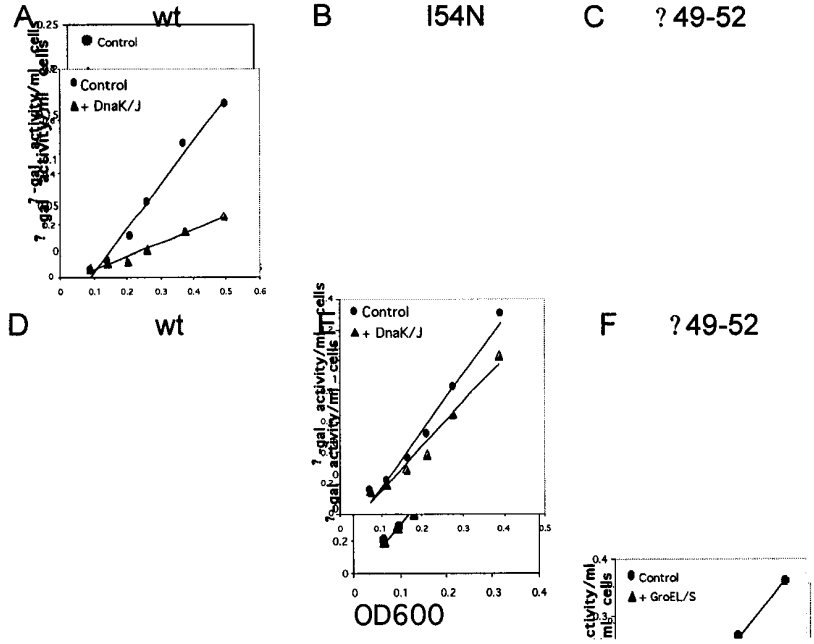


Fig. 3



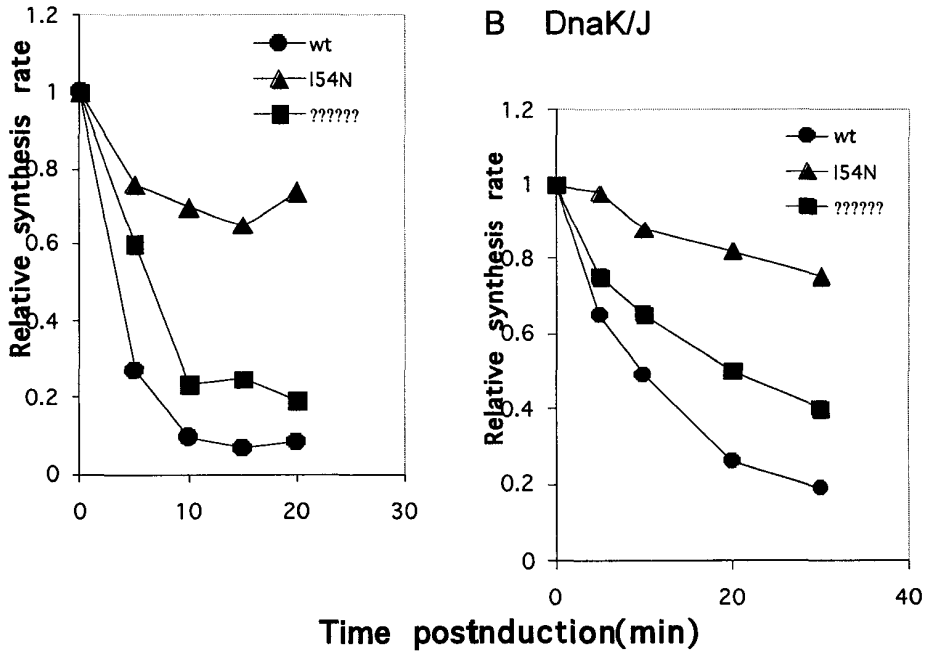


Fig. 5

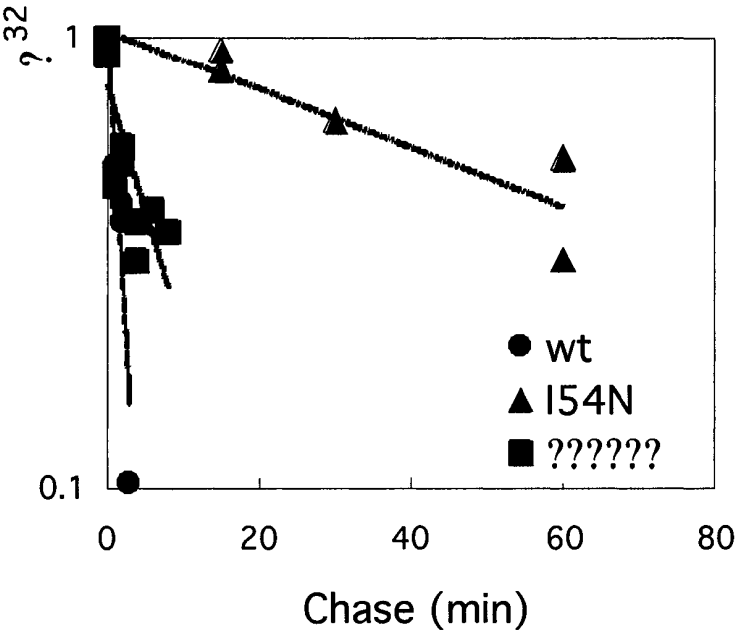


Fig. 6

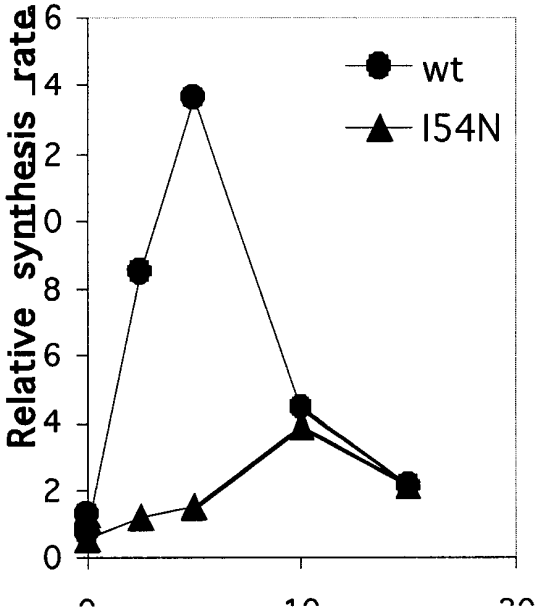
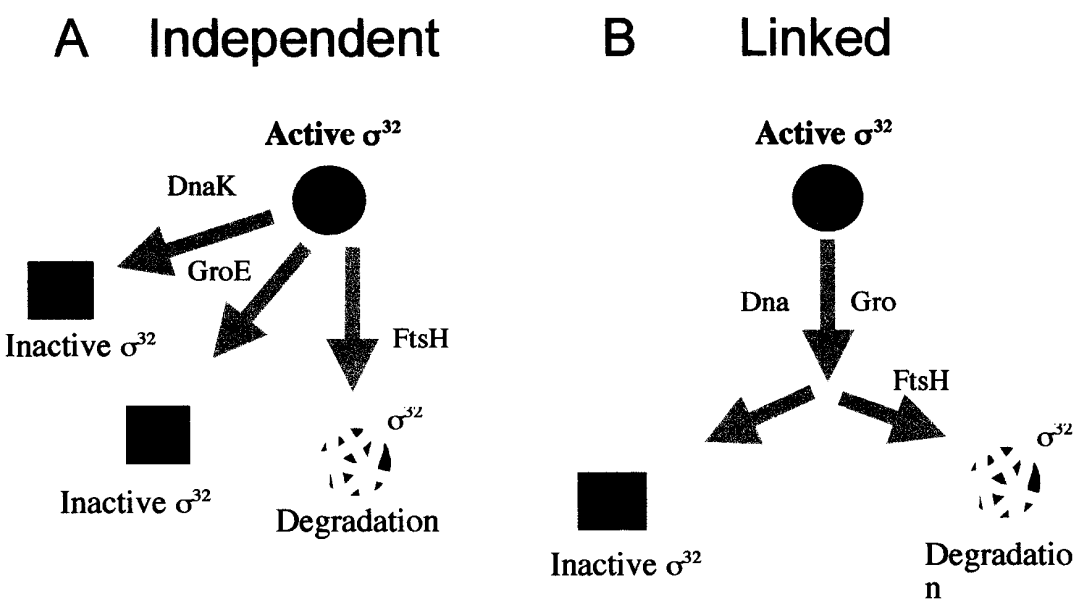


Fig. 7

Fig. 8



Chapter 3: Hfq Modulates the σ^E -mediated Envelope Stress Response and the σ^{32}-mediated Cytoplasmic Stress Response in *Escherichia coli*

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Abstract

We show that Hfq, a small RNA chaperone, regulates *Escherichia coli* stress responses at multiple distinct levels. Cells lacking Hfq: 1) are defective in σ^E-dependent down regulation of outer membrane porin production; 2) exhibit increased translation of DnaK, leading to downregulation of σ^{32} activity; and 3) are defective in long-term adaptation to GroEL overexpression.

The RNA chaperone Hfq, together with its small RNA binding partners, regulates many cellular processes (15), including the σ^S-mediated general stress response. Although its role in the σ^E-mediated envelope stress response and the σ^{32}-mediated cytoplasmic stress response has not been examined in *E. coli*, there are compelling reasons to do so: *Vibrio* cholera *hfq*- cells overexpress the σ^E regulon (2), and Hfq is encoded in the σ^{32} regulon (14). We used microarray analysis to compare RNA expression from MG1655 *kan::hfq* (*hfq*$^-$) and an *hfq*$^+$ control in which *kan* is inserted at

the 3' end of *hfq* (retains Hfq function) to control for polar effects of the insertion (14). We performed 8 independent experiments and observed significant, but weak, induction of 94 transcripts and repression of 175 transcripts (Table S1).

We first examined the effect of Hfq status on the σ^E regulon. In wild-type (wt) cells, upon σ^E overexpression, many genes are upregulated and about 8 operons encoding outer membrane proteins (OMPs) are downregulated (8). Similar to *Vibrio cholera, hfq- E. coli* shows up-regulation of σ^E and its regulon (Table 1A, column 1). Interestingly, *hfq-* cells fail to show the OMP downregulation that is characteristic of higher σ^E activity. The OMP regulation defect is not due to constitutive overexpression of σ^E as Δ*rseA* cells, which also constitutively express the regulon because they lack the antisigma factor, downregulate OMPs (Table 1, columns 1 and 2). Moreover, Hfq is absolutely required for downregulation of these OMPs observed immediately after σ^E overexpression (Table 1A, columns 3 and 4). These results were confirmed by Northern analysis of *ompF* mRNA (data not shown). Together, these data suggest that *hfq-* cells are specifically defective in σ^E-dependent OMP downregulation. This regulation is most likely at the level of mRNA stability since the half-lives of the OMP mRNAs in wt cells are relatively long (1) and their decrease after σ^E overexpression is too rapid to be mediated solely by transcriptional repression (data not shown). We propose that σ^E mediates transcription of small RNA(s) (sRNA) that subsequently decrease the mRNA levels of these down-regulated OMPs. This is an important control feature of the σ^E stress response as σ^E is activated by unfolded outer membrane porins; down-regulation of porin production by σ^E *via* sRNA(s) provides a rapid way to alleviate envelope stress.

We next examined the effect of Hfq status on the σ^{32} regulon. We found that several σ^{32} regulon members are downregulated in the *hfq-* strain (Table 1B). We verified this array data by showing that expression of a σ^{32}-dependent, P*htpG*::*lacZ* reporter was downregulated 2 to 3-fold in *hfq-* cells (Fig. 1, lane 1). Standard β-galactosidase assays were performed at least in duplicate from 2 independent cultures and the results were converted to Miller Units and plotted relative to wt levels. We tested whether Hfq regulated σ^{32} transcription, translation, stability, or activity using *rpoH::lacZ* fusions. A short *rpoH::lacZ* translational fusion (amino acid 1-22 from σ^{32}) is sensitive to altered transcription (5); a long *rpoH::lacZ* fusion (amino acid 1-266 from σ^{32}) is sensitive to altered transcription, translation and stability of σ^{32} (11). We found that neither fusion was affected by the Hfq status of the cell (Fig. 1, lanes 3 and 4), suggesting that Hfq affects σ^{32} at the level of activity control rather than altering σ^{32} transcription, translation or stability.

σ^{32} activity is regulated by the GroEL/S and DnaK/J chaperones (4, 12). These chaperones directly bind to σ^{32} and can inhibit σ^{32} –dependent transcription *in vitro* (3, 4, 6). Therefore a plausible model for general inhibition of σ^{32} activity is upregulation of one of these chaperone machines. We found that in *hfq-* cells GroEL synthesis was downregulated in concert with downregulation of its mRNA using ^{35}S-Methionine pulse-labeling followed by immunoprecipitation to measure protein synthesis rates (Fig. 1, lane 5, (4)). In contrast, synthesis of DnaK was upregulated despite downregulation of its mRNA (Fig. 1, lane 6). This suggests that Hfq decreases translation of DnaK in WT cells

and that two competing effects control DnaK level in *hfq*-cells: 1) transcriptional downregulation due to decreased σ^{32} activity; and 2) translational upregulation due to lack of Hfq. This model predicts that if DnaK transcriptional downregulation were eliminated, the level of DnaK/J would be even higher and σ^{32} activity would be further inhibited in *hfq*- cells. Indeed, when *dnaKJ* is expressed from a P*A1/lac0-1* promoter (13), which is not regulated either by Hfq or σ^{32}, *hfq*- cells exhibit more severe repression of σ^{32} activity than when *dnaKJ* is expressed from its endogenous promoter (Fig. 1, lane 2 compared to lane 1). A plausible model for these effects is that Hfq negatively regulates the translation of the DnaK/J operon, most likely by collaborating with an as yet unidentified sRNA. This is the first report indicating that chaperones are subject to post-transcriptional regulation and that this regulation permits differential chaperone expression under certain conditions.

We next examined the consequences of the chaperone imbalance (increased DnaK/J and decreased GroEL/S) in *hfq*- cells subjected to a temperature upshift. Examination of the synthesis rate of the σ^{32}-dependent HtpG protein using a pulse-chase immunoprecipitation protocol revealed that after upshift from 30° to 42° C, *hfq*- cells exhibited a bigger heat shock response characterized by a larger increase in relative synthesis rates and a longer duration than wt cells (Fig. 2). This indicates that *hfq*- cells, which contain an altered chaperone complement, are compromised for responding to temperature stress.

We also compared the response of *hfq*- and wt cells to GroEL overexpression. Plasmid-encoded *groEL* was overexpressed from an arabinose inducible promoter and σ^{32} activity was measured by examining the β-galactosidase activity from the P*htpG-lacZ* reporter (Fig. 3). We found that both strains are inhibited after 2 hours of chaperone overexpression. We note that measuring σ^{32} activity using Miller units underestimates the amount of σ^{32} inhibition as this measure includes the β-galactosidase accumulated prior to inhibition. Nevertheless, the 2-fold inhibition in the Miller units of wt cells is consistent with previous reports (4). Interestingly, we found that *hfq*- and wt cells differ in their response to long-term (24 hour) exposure to high levels of chaperones. We found that wt cells recover normal σ^{32} activity after extended exposure to chaperones. We term this process "long-term adaptation" and have shown that the process is reversible and independent of the expression system used (data not shown). We ruled out the possibility that adaptation is due to a decrease in the levels of GroEL, DnaK, or the GroEL co-chaperone GroES by measuring protein levels with western analysis (Fig. 4). Equal loading of westerns was confirmed by comparing the desired band with either a nonspecific band or by reprobing with a different antibody. Interestingly, we found that *hfq*- cells are defective in long-term adaptation (Figure 3). Moreover, we found that this defect is likely not due to altered levels of DnaK, as DnaK levels are slightly higher in *hfq*- than in wt cells both before and during adaptation (Figure 4). These results indicate that there is a second, independent effect of Hfq on σ^{32} regulation. Although the mechanism of this effect is currently unknown, the fact that Hfq is involved in this process suggests that a sRNA effect may underlie adaptation.

In summary, we have shown that Hfq is involved in both the σ^E and σ^{32}-mediated stress responses. Hfq normally acts as a sRNA chaperone (10), but the sRNAs involved in these regulatory events have not yet been identified. Interestingly, there is recent evidence that there is also an RNA regulator of the heat shock response in eukaryotes (9). We show that Hfq is required for OMP downregulation upon activation of σ^E. Additionally, Hfq regulates the heat shock response in two ways: it regulates DnaK at the translational level and is required for long-term adaptation of σ^{32} to excess GroEL. This is the first example of differential chaperone regulation in *E. coli*, although other prokaryotes sometimes place GroEL and DnaK under the control of two different transcription factors. Like *E. coli,* many eukaryotes have one transcriptional regulator of temperature inducible chaperones. It is possible that differential post-transcriptional regulation of chaperones also occurs in higher organisms.

We would like to acknowledge Stacy Chen for strain construction. This work was supported by National Institutes of Health (NIH) grants GM57755 and GM32678 (to CAG)

Figure Legends

Figure 1: Effects of *hfq*- on σ^{32} during exponential growth in LB media. Various assays (see text) were used to compare *hfq*- and wt strains, and the effects in the *hfq*- strain are shown relative to wt. Lane 1: σ^{32} activity measured by detirmining the amount of β-galactosidase measured in Miller Units fromσ^{32}-dependent P*htpG-lacZ* reporter. Lane 2: σ^{32} activity (as in lane 1) in a strain in which the endogenous *dnaKJ* promoter is replaced by an IPTG inducible P*A1/lac0-1* promoter. Lane 3: Activity of a short *rpoH-lacZ* translational fusion containing the first 22 amino acids from σ^{32}. Lane 4: Activity of a long *rpoH-lacZ* translational fusion containing most of the σ^{32} coding region (amino acids 1-266). Lane 5: GroEL protein synthesis rates measured using ^{35}S-methionine pulse-labeling immunoprecipitation. Lane 6: DnaK protein synthesis rates measured using ^{35}S-methionine pulse-labeling immunoprecipitation.

Figure 2: Hfq affects the magnitude and duration of the heat shock response. Wt and *hfq*- cells were subjected to a temperature upshift from 30° to 42° C during exponential growth in M9 minimal media supplemented with all amino acids except methionine and cysteine. ^{35}S-methionine pulse-labeling immunoprecipitation was used to measure the synthesis rate of HtpG, a σ^{32}-dependent protein. HtpG synthesis was normalized to the rate at T = 0 for each strain.

Figure 3: Hfq affects long-term adaptation of σ^{32} to GroEL. GroEL was overexpressed from an arabinose inducible promoter at time = 0 in both wt and *hfq*- cells during

exponential growth in LB media. σ^{32} activity was assayed using a P*htpG-lacZ* reporter at 2 hours and 24 hours post induction. β-galactosidase activity is measured in Miller units and then normalized to the activity at t = 0.

Figure 4: The levels of DnaK, GroEL, and GroES are unaffected by Hfq during adaptation. GroEL was overexpressed from an arabinose inducible promoter. The effects of long-term overexpression were examined in both wt and *hfq-* strains using western analysis. Protein levels of DnaK, GroEL, and GroES are indicated at 24 hours post induction.

Fig. 1

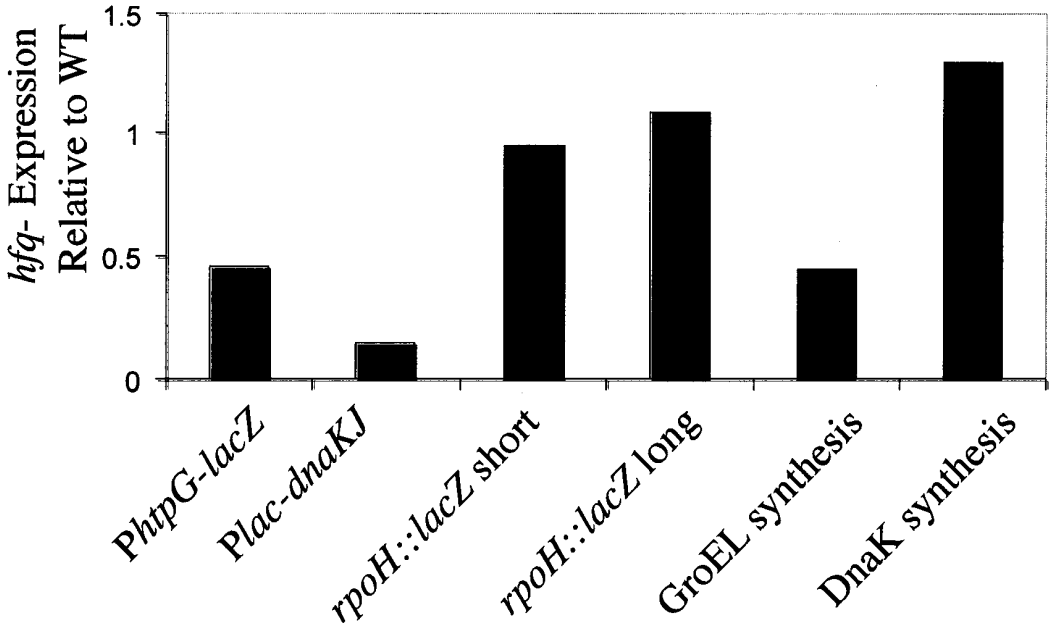

Fig. 2

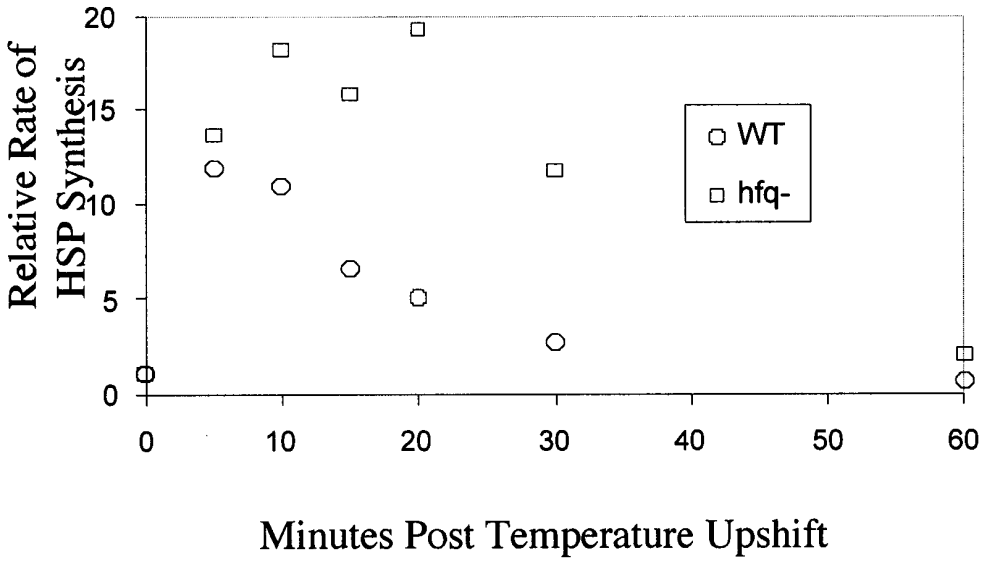

Fig. 3

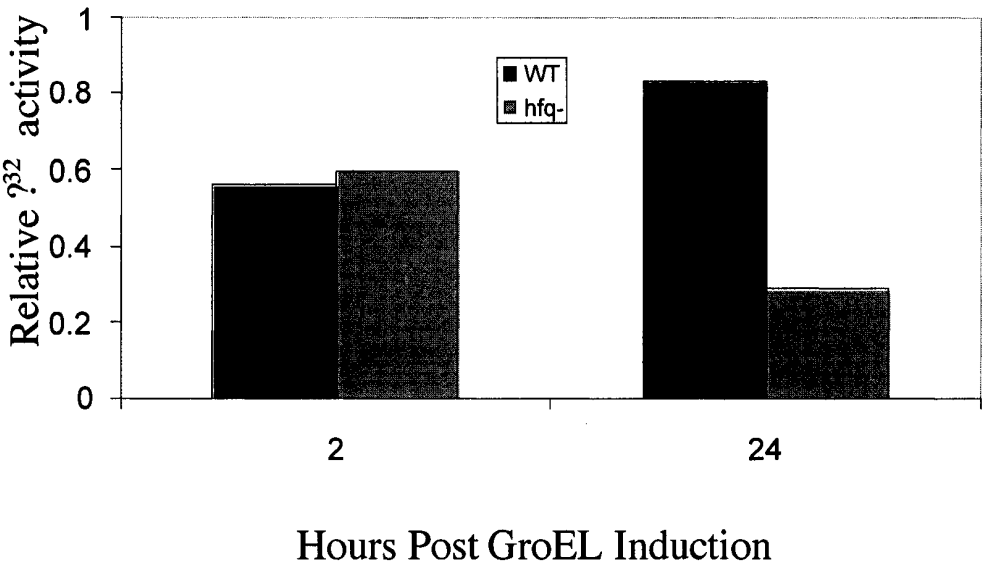

Fig. 4

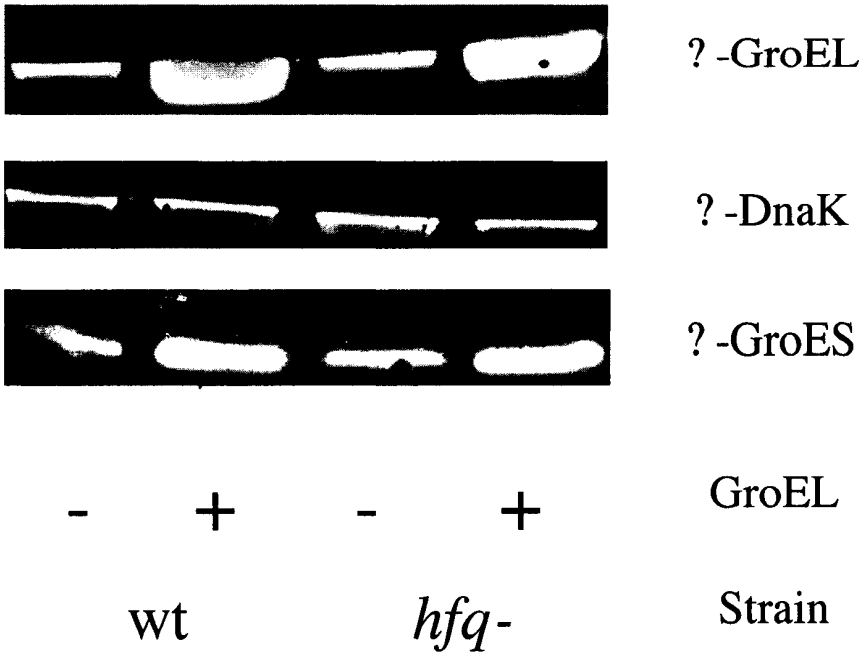

Table 1

Requirements of Hfq on σ^E and σ^{32}-dependent gene expression

A. Effects of Hfq on mRNA levels of σ^E and specific σ^E regulon members				
Gene	**Strain**			
	***hfq*[a]**	***ΔrseA*[b]**	***hfq*⁺ *rpoE* o/exp[c]**	***hfq*⁻ *rpoE* o/exp[d]**
rpoE	2.98	2.64	n/a	n/a
ompA	1.45	0.30	0.25	1.54
ompC	0.93	0.10	0.19	0.88
ompF	1.64	0.06	0.11	1.23
ompX	0.90	0.24	0.32	1.14
b0805	3.09	0.70	0.37	1.28
lpp	1.41	0.59	0.82	1.16
yhcN	1.85	1.10	0.84	1.10
tsx	0.76	0.53	0.40	0.82
htrA[e]	1.78	6.36	8.74	6.94
B/ Effects of Hfq on mRNA levels of σ^{32} and σ^{32} regulon members				
	Strain			
	***hfq*[a]**			
rpoH	1.21			
dnaKJ[f]	0.67			
lon	0.64			

htpG	0.71			
topA	0.77			
hslVU[f]	0.59			
groES groEL[f]	0.48			

Averaged data of mRNA expression ratios from microarray experiments. All strains grown in M9 minimal media + vitamins and amino acids with aeration at 30°C. mRNA samples harvested from mid-log (OD_{450}=0.3) and prepared as described in (8). All microarray expression data is available on the NCBI Gene Expression Omnibus (GEO) website (http://www.ncbi.nih.gov/geo) under the accession code GSE**XXXX**.

[a] MG1655 *hfq*$^+$ *vs* MG1655 *hfq*$^-$; Data average of 8 independent microarrays

[b] MG1655 *vs* MG1655 *ΔrseA*; Data average of 7 independent microarrays

[c] MG1655 *hfq*$^+$ *vs* MG1655 *hfq*$^+$ pLC245. Plasmid encodes P*trc*::*rpoE* (Rhodius *et al.*, 2006); σ^E overexpression induced by addition of 1 mM IPTG at OD_{450}=0.3 and mRNA harvested after 20 min. Data average of 3 independent microarrays.

[d] MG1655 *hfq*$^+$ *vs* MG1655 *hfq*$^-$ pLC245. Induction, sample preparation and data average as [c].

[e] Induced σ^E regulon member.

[f] Data for first gene of operon

Table S1

Genes significantly differentially regulated by microarray analysis of *hfq*$^+$ *vs hfq*$^-$ strains

The Table lists significantly differentially regulated genes from comparisons of *hfq*$^+$ *vs* *hfq*$^-$ strains. 94 genes are classified as significantly induced in *hfq*$^-$ and 175 genes significantly decreased in *hfq*$^-$. Microarrays were performed comparing strains MG1655 *hfq*$^+$ *vs* MG1655 *hfq*$^-$ as previously described in Table 1. Statistically significant genes were identified from 8 independent experiments using the SAM v2.20 software (Tusher *et al.*, 2006; http://www-stat.stanford.edu/~tibs/SAM/index.html). The selected cutoff of delta=1.277 gave the largest number of genes for a False Discovery Rate of 0% at the median percentile. The table is a direct output from the SAM software.

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Epilogue

I have made several contributions to the understanding of σ^{32} regulation. First, I have shown that a chaperone network regulates σ^{32}. Second, I have shown that there is likely to be an unidentified factor that regulates σ^{32} activity control and links activity control with degradation control. Third, I have shown that Hfq modulates σ^{32} activity in at least two distinct ways. While each of these new findings adds to our previous understanding, they also raise many questions and much work remains to be done. Here, I summarize my findings and identify future directions.

A chaperone network regulates σ^{32} activity.

Prior to this work, it was believed that the DnaK chaperone machine was the only chaperone regulator of σ^{32}. I showed that the GroEL chaperone machine is an additional chaperone regulator of σ^{32} using many of the same assays used for DnaK. This observation raises many important questions. For example, how does chaperone regulation by GroEL compare to chaperone regulation by DnaK? It appears that these chaperones regulate σ^{32} in a largely similar manner; however, there are some interesting differences. I demonstrated that overexpression of the GroEL chaperone machine leads to more rapid downregulation of σ^{32} than overexpression of the DnaK chaperone machine. However, these two expression systems are not completely identical and these results cannot be directly compared. The GroEL chaperone machine consists of GroEL and GroES, which are part of a single operon and can easily be co-overexpressed. In

contrast, the DnaK chaperone machine consists of DnaK, DnaJ, and GrpE, and only DnaK and DnaJ are in a single operon. In order to induce the entire DnaK chaperone machine, we artificially place GrpE into an operon with DnaK and DnaJ. This may alter the ratio between DnaK, DnaJ, and GrpE, and influence our results. Therefore, it will be interesting to determine if there really is a kinetic difference in σ^{32} regulation upon chaperone overexpression.

Interestingly, chaperone overexpression may have important differences in their effect on σ^{32} degradation. It was observed that DnaK overexpression leads to stabilization of σ^{32}, which is not observed upon GroEL overexpression (T. Yura personal communication). The consequences or reasons for this effect are unexplored.

Another interesting difference in chaperone regulation by GroEL and DnaK is their affinity for σ^{32} *in vitro*. GroEL binds to σ^{32} with μM affinity. The DnaK protein alone binds with a similar affinity, yet in the presence of the DnaJ cochaperone, DnaK binds to σ^{32} with nM affinity. However, these *in vitro* results may not reflect binding *in vivo* which is governed by an ATPase cycle for both chaperone machines. Neither of these chaperone machines has been extensively characterized for σ^{32} binding with the full chaperone machine in the presence of ATP. Nevertheless, the binding affinities are important because the prevalent model prior to our work was that chaperones inactivated σ^{32} by directly competing with RNA Polymerase for σ^{32} binding.

We have speculated on the consequences of utilizing a chaperone network to sense unfolded proteins, but our hypotheses have not been experimentally investigated. First, we speculated that having two chaperone regulators allows a more sensitive response. Given that the pools of substrates of the two chaperone machines are only partially overlapping, and that stress may cause damage to both pools of substrates, using both chaperones to sense protein damage should allow sampling of more damaged proteins for the same amount of stress, which potentially allows a more sensitive response. Second, we speculated that having two chaperone regulators allows a broader sampling of protein damage. One untested idea in this speculation is that the pool of DnaK substrates may be more sensitive to certain types of damage than the pool of GroEL substrates. It would be interesting to determine if σ^{32} activity was more sensitive to GroEL or DnaK depletion under certain types of stress. We have based some of our ideas on the hypothesis that the two chaperone machines work together based on their additive effects *in vitro* transcription assay. However, since then our results have questioned the validity of this assay. Therefore, it will be interesting to reinvestigate this important issue by examining the effects of the two chaperone machines *in vivo* when they are overexpressed or depleted together.

Identification of a second chaperone regulator raises the important question of whether DnaK and GroEL are the only chaperone regulators of σ^{32}. I have some evidence that a third important chaperone, HtpG (HSP90), is not a regulator of σ^{32}. I overexpressed HtpG from a plasmid, verified overexpression by westerns, and determined that there was no effect on σ^{32} activity. Additionally, I have purified HtpG and observed no effect on a

purified *in vitro* transcription assay. Unfortunately, in neither case was there a positive control demonstrating that HtpG was active since there are no established phenotypes or substrates of HtpG. As DnaK and GroEL are the two major chaperone machines in *E. coli*, it is possible that they are the only regulators of σ^{32}. This can be tested by observing the effects of overexpressing these proteins prior to a temperature upshift. If these are the only two regulators, then simultaneous overexpression of these proteins prior to temperature upshift should virtually eliminate induction of σ^{32}. Similarly, depletion of these two proteins prior to temperature downshift should eliminate repression of σ^{32} activity.

Control of σ^{32} activity investigated through analysis of σ^{32} mutants.

Our identification and analysis of mutations in σ^{32} that reduce inactivation of σ^{32} have provided some interesting results and raised some important questions. We isolated mutations in σ^{32} that prevent inactivation upon σ^{32} overexpression. Presumably, inactivation is mediated by both DnaK and GroEL. Interestingly, we did not isolate any mutants that were specifically defective in either chaperone machine. All of the mutants had defects in regulation by both DnaK and GroEL. However, we did observe differential sensitivities to the two chaperone machines. For example, some of our mutants were more sensitive to GroEL and some were more sensitive to DnaK. However, this effect was small and could be due to experimental variation. We cannot satisfactorily explain why we did not isolate mutations specific for either chaperone machine, except to speculate that because the two chaperones are redundant in regulating

σ^{32} activity, mutations defective in only one machine are unlikely to have a strong phenotype. Similarly, this could explain why we did not isolate mutations that were defective in chaperone binding. It is possible that the chaperones do not bind to the same determinants and that knocking out one chaperone binding may not have strong effects. It would be very interesting to repeat this screen in the absence of DnaK to determine if we could find mutations that were specifically defective in GroEL binding and/or regulation.

Prior to our work, the model for inactivation was that chaperones directly competed with RNA polymerase for σ^{32} binding. This suggests that the chaperones are necessary and sufficient for activity regulation. Interestingly, the mutations that we isolated based on their defect in inactivation are not defective in chaperone binding, suggesting that the simple sequestration model is insufficient to explain inactivation. Whether the chaperones are still sufficient but have activities in addition to binding σ^{32} that are important, or whether there are additional missing factors remains to be determined.

Interestingly, the mutations that we isolated are defective not only in inactivation but also in regulated degradation of σ^{32}. Moreover, a screen selecting for mutations in σ^{32} that were defective in degradation identified mutations in the same residues as our screen. These results suggest that mutations cannot be isolated in σ^{32} that are specifically defective in inactivation or degradation control. Our new model for σ^{32} regulation explains this result by hypothesizing that both pathways require a new intermediate that consists of σ^{32} bound to an unknown factor, and that the downstream pathways may

branch based upon some determinant in this unknown factor. If the downstream decision point does not require determinants on σ^{32}, than it would be impossible to find mutations in σ^{32} specific for a single pathway.

We have found that inactivation observed in the *in vitro* transcription system does not completely mimic inactivation *in vivo*. The two differ because mutations in σ^{32} that eliminate inactivation *in vivo* are inactivated normally *in vitro*. It is possible that the *in vitro* system recapitulates only a portion of *in vivo* inactivation. On the other hand, it is also possible that the *in vitro* inactivation is an *in vitro* artifact. Such an artifact could arise from using chaperone concentrations that are in excess of physiological levels.

Taken together, our results suggest that there is an unidentified regulator of σ^{32} activity and stability. This factor may or may not be the same as the missing factor involved in σ^{32} degradation. It will be important to identify this missing factor and determine how it contributes to σ^{32} regulation. A preliminary strategy that we have used is to identify transposon insertion mutants that increase σ^{32} activity. Several such insertions have been isolated, but none have yet been characterized.

The σ^{32} mutants that we have identified have some interesting properties. As they are chaperone resistant, we hoped to use them to examine temperature upshift effects that are chaperone independent. Unfortunately, we could not do this analysis as the mutations make σ^{32} temperature sensitive. At higher temperatures, these mutants are degraded in an unregulated, DnaK-independent manner (S. Chun, unpublished data). Another

interesting property of these mutants is that the strongest mutant, σ^{32}I54N, contains approximately 8-fold higher chaperone levels than wt. We expected these cells to be sick as expressing high levels of single chaperones causes cell death. This discrepancy may be due to toxicity arising from imbalances in the protein-folding network that arise from single chaperone overexpression. Nevertheless, this high level of chaperones in this mutant is particularly interesting for two reasons. First, induction of chaperone levels is being examined as a therapeutic strategy for a wide variety of protein folding diseases. However, the consequences of enhanced chaperone expression have not been rigorously investigated. Second, induction of chaperone levels is often used as a strategy for enhancing recombinant protein expression and folding in *E. coli*. One common strategy, which has had only limited success, is to overexpress either DnaK or GroEL alone. This strategy results in downregulation of the rest of the σ^{32} regulon, which contains many proteins required for proper protein-folding homeostasis. Use of σ^{32}I54N should eliminate this drawback because the entire σ^{32} regulon is upregulated in this strain. Additionally, further DnaK or GroEL overexpression would not result in inactivation of the σ^{32} mutant. In light of this, I have constructed a BL21(DE3) expression strain containing σ^{32}I54N and collaborated with a lab who used it for the recombinant expression of two *S. cerevisiae* proteins. Unfortunately, σ^{32}I54N did not significantly improve the expression of the two proteins tested. However, this could be due to many reasons and I think it would be interesting to use this strain to see if it enhances recombinant protein expression of other proteins.

Hfq regulation of σ^{32}

My data implicating Hfq as a modulator of σ^{32} activity illuminates two new modes of heat shock regulation. First, I suggest that DnaK translation is modulated by Hfq. This leads to changes in the level of DnaK, which in turn alters σ^{32} activity via the DnaK-mediated negative feedback loop. Therefore, modulation of DnaK translation alters the chaperone balance in the cell. When DnaK translation is increased, the transcription of the rest of the σ^{32} regulon is decreased. Similarly, when DnaK translation is decreased, the transcription of the rest of the σ^{32} regulon in increased. So far, I have observed differential regulation of DnaK and the remainder of the σ^{32} regulon only in Hfq mutant cells; it will be interesting to identify conditions where this regulatory event occurs in wild-type cells. As Hfq normally works with a small RNA to mediate its effects, it is likely that a small RNA regulates this process. Determination of the identity and expression of this small RNA is critical to further study this regulation. To this end, I have established conditions for an initial screen to identify such a small RNA (Appendix 1).

Hfq also modulates long-term adaptation to GroEL overexpression. Long-term adaptation is a phenomenon that I first observed when I identified GroEL as a regulator of σ^{32}. I further characterized adaptation and showed that adaptation is compromised in cells lacking Hfq. I showed that adaptation is not a genetic change and does not depend on the induction system used, and also I showed that neither GroEL, DnaK, nor GroES levels are downregulated during adaptation. However, this is the extent of our

knowledge about adaptation. The mechanisms and consequences, and how Hfq and small RNAs contribute to this regulation remain to be determined.

Summary

In summary, I have many several important contributions to regulation of the heat shock response. I have improved our understanding of how the heat shock response senses unfolded proteins, the components and connections between the major σ^{32} regulatory pathways, and I have also identified new Hfq-mediated regulatory pathways. However, each of these contributions has raised important new questions and research directions. Foremost among these is how the components of the chaperone network contribute to σ^{32} regulation, the identity of a novel regulator of σ^{32} activity, and the identity of small RNA regulators. It is my hope that this work will provide a foundation for much exciting research in the coming years.

Appendix

Hfq, a small RNA (sRNA) chaperone, is a modulator of σ^{32} activity through at least 2 distinct pathways. We have shown that one pathway is likely translational regulation of DnaK. In the presence of Hfq, DnaK translation is downregulated, thereby increasing σ^{32} activity. Hfq modulates translation of other targets along with a small RNA, partially complementary to the target message. It is likely that Hfq utilizes such a small RNA for regulation of DnaK translation. Here, we try to identify this small RNA.

The strategy that we utilized to identify this small RNA is to select for clones from a genomic library on a multicopy plasmid that upregulate σ^{32} activity. We then screened these clones for their effect on σ^{32} activity in the absence of Hfq. Small RNA regulators should only affect σ^{32} activity in the presence of Hfq, but not in its absence.

For the initial selection, we constructed an MG1655 strain containing pMAP1, a plasmid containing a σ^{32}-dependent promoter driving chloramphenicol acetyl transferase, a gene that provides resistance to chloramphenicol. This strain also contained a σ^{32}-dependent lacZ reporter on the chromosome. We transformed this strain with a genomic multicopy library obtained from the laboratory of Philippe Bouloc. This library is a partial Sau3A digest from C600 inserted within the pUC19 BamH1 site. Fragment size is approx 1000-3000, although there are many smaller inserts. The library was successfully used several times, including screens to find small RNAs (Bohn and Bouloc 1998, Douchin 2006). Transformants were selected for growth on 100 μg/ml CAM to select for increased σ^{32}

activity and AMP to select for library plasmid. A small portion of the transformation was plated on AMP alone to estimate the number of transformants.

Approximately 10,000 transformants were analyzed for increased σ^{32} activity. 100 candidates were selected in the initial screen and retested for CAM resistance in liquid cultures. Of the 100 candidates, 22 reached saturation after overnight growth, with the rest reaching saturation after growth for two overnights. All of the fast growing candidates and 25 (approximately one-third) of the slow growing candidates were further tested. Plasmids were purified from the candidates and then retransformed into the initial selection strain or a strain lacking Hfq. σ^{32} activity of these strains was assayed using a liquid β-galactosidase assay. Duplicate samples were assayed from each culture in only a single experiment. The most promising candidates were sequenced from one end only using an M13/pUCR forward primer. This sequence was analyzed using BLAST to determine which genes it contained, in order to determine if there were any known small RNAs in that region of the genome; however, full sequencing analysis was not performed.

Three major classes of candidates were observed in this screen. The first class of candidates induced σ^{32} activity in both wt and *hfq*- cells, with the amount of induction attenuated in *hfq*- cells compared to wt. The strongest candidate of this class contained sequence from *nrdB*, ribonucleoside diphosphate reductase subunit B2. The second class of candidate demonstrated increased σ^{32} activity in wt cells but not in *hfq*- cells. Two fast growing candidates from this class contained sequences from *yniA*, a predicted

kinase, and *topA*, topoisomerase I omega protein I. Two slow growing candidates from this class contained sequences from *rbsA*, a D-ribose high-affinity transport system, and *yraJ*, a predicted OMP. A third class of candidates induced σ^{32} activity more strongly in *hfq*- cells than in wt cells. Three slow growing candidates contained sequences from *potE*, a putrescine-lyase antiporter, *proB*, a gamma-glutamyl kinase involved in proline biosynthesis, and *ilvG*, involved in isoleucine and valine biosynthesis.

We expected that the second class of candidates, which have increased σ^{32} activity in wt cells but not *hfq*- cells, may contain small RNA regulators of σ^{32} that are involved in repressing DnaK translation. Excitingly, one of the candidates contains sequence from *yniA*, which is approximately 2 kilobases from *yniC*, a verified small RNA of unknown function. It will be important to determine if this small RNA is encoded in our candidate and whether this small RNA influences σ^{32} activity. The other three candidates are not nearby known small RNAs.

The third class of candidates, which induce σ^{32} activity more in *hfq*- cells than in wt, was unexpected. This class may contain small RNAs that modulate σ^{32} activity through a pathway distinct from modulation of DnaK translation. Our previous work has shown that small RNAs may regulate σ^{32} during adaptation to long-term chaperone overexpression, and this class of candidates may contain such small RNAs. One of the candidates contains sequence from *ilvL*, which is approximately 3 kilobases from *ilvG*, which is a short sequence known to bind Hfq. *ilvG* is thought to encode a leader peptide

sequence and not a small RNA, although the reason this sequence binds to Hfq is unexplained. The other two candidates are not nearby known small RNAs.

In summary, we performed a screen in order to identify small RNA regulators of σ^{32} activity. We did not reach saturation of the screen for two reasons. First, we needed to analyze 20,000 colonies, twice as many as we started with. Second, we did not assay all of the slow growing candidates that we isolated. Nevertheless, we isolated several candidates that may contain small RNA regulators of sigma32. In particular, one candidate may contain a known small RNA. Repetition of the screen in order to reach saturation and further analysis of these candidates should be very exciting.