

DnaK Chaperone-Mediated Control of Activity of a σ^{32} Homolog (RpoH) Plays a Major Role in the Heat Shock Response of *Agrobacterium tumefaciens*

KENJI NAKAHIGASHI,[†] HIDEKI YANAGI,[‡] AND TAKASHI YURA^{*}

HSP Research Institute, Kyoto Research Park, Kyoto 600-8813, Japan

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RpoH (*Escherichia coli* σ^{32} and its homologs) is the central regulator of the heat shock response in gram-negative proteobacteria. Here we studied salient regulatory features of RpoH in *Agrobacterium tumefaciens* by examining its synthesis, stability, and activity while increasing the temperature from 25 to 37°C. Heat induction of RpoH synthesis occurred at the level of transcription from an RpoH-dependent promoter, coordinately with that of DnaK, and followed by an increase in the RpoH level. Essentially normal induction of heat shock proteins was observed even with a strain that was unable to increase the RpoH level upon heat shock. Moreover, heat-induced accumulation of *dnaK* mRNA occurred without protein synthesis, showing that preexisting RpoH was sufficient for induction of the heat shock response. These results suggested that controlling the activity, rather than the amount, of RpoH plays a major role in regulation of the heat shock response. In addition, increasing or decreasing the DnaK-DnaJ chaperones specifically reduced or enhanced the RpoH activity, respectively. On the other hand, the RpoH protein was normally stable and remained stable during the induction phase but was destabilized transiently during the adaptation phase. We propose that the DnaK-mediated control of RpoH activity plays a primary role in the induction of heat shock response in *A. tumefaciens*, in contrast to what has been found in *E. coli*.

Most cells and organisms respond to heat or other stress by inducing a set of heat shock proteins (HSP) to cope with accumulation of unfolded and misfolded proteins. Many HSP are molecular chaperones or proteases, and DnaK (HSP70) and GroEL (HSP60) are major ubiquitous chaperones that play crucial roles in promoting protein folding not only under acute stress but also during normal growth and development (5, 12, 13, 15, 24, 48). In addition, these chaperones and their cochaperones have been implicated in the regulation of heat shock response by negatively modulating the key heat shock regulators, such as σ^{32} (RpoH) (11, 37, 42) and HrcA repressor (23) in bacteria and heat shock factors (36) in eukaryotes.

The RpoH protein, homolog of *Escherichia coli* σ^{32} (14, 20, 49), is widely distributed among the α , β , and γ subgroups of proteobacteria (references 29 and 48 and references cited therein). Analyses of several RpoH proteins from members of the α and γ subgroups of proteobacteria, such as *Agrobacterium tumefaciens* and *E. coli*, revealed that they play a major role in regulation of the heat shock response by enhancing transcription of the heat shock genes (8, 14, 28, 31, 47).

In *E. coli*, where the most-extensive work has been done, the induction of HSP is regulated primarily by a transient increase in the σ^{32} level that results from both increased translation of *rpoH* mRNA and transient stabilization of normally unstable σ^{32} (38, 43). Partial melting of secondary structure for the 5'

portion of *rpoH* mRNA activates translation at high temperatures (27, 50), the mRNA itself serving as a built-in thermosensor (25). On the other hand, turnover of σ^{32} catalyzed by ATP-dependent proteases, such as FtsH (HflB) and HslVU (ClpQY) (17, 19, 44), is modulated by the DnaK-DnaJ-GrpE chaperone team (3, 11, 37, 40, 45), presumably reflecting the cellular state of protein folding. In addition, the control of σ^{32} activity plays a major role in response to temperature downshift (39, 41) or in the heat shock response with *ftsH* mutants in which σ^{32} is highly stabilized (40). The DnaK chaperone team also participates in the negative regulation of σ^{32} activity (11, 21, 40, 45). Furthermore, binding of σ^{32} to core RNA polymerase, the initial step for σ^{32} function, markedly stabilizes σ^{32} (4, 19), precluding precise assessment of the contribution of control of σ^{32} activity in the wild-type bacteria. RpoH from other members of the γ subgroup of proteobacteria, such as *Serratia marcescens* and *Pseudomonas aeruginosa*, also appears to exhibit both translational induction and transient stabilization upon heat shock, leading to the increased RpoH level, as in *E. coli* (30).

In the case of the α subgroup of proteobacteria, the mechanisms underlying heat-induced synthesis of RpoH seem to be quite different. First, the 5' portion of *rpoH* mRNA is not predicted to form the secondary structure, unlike the situation in the γ subgroup of proteobacteria (29; also unpublished results), suggesting the lack of translational control. Second, RpoH synthesis in *Caulobacter crescentus* is markedly heat induced by activating its transcription (32, 46) from the RpoH-dependent promoter (47), leading to the increase in RpoH level. Besides, the conserved inverted repeat sequence (CIRCE), a putative binding site for the HrcA repressor in gram-positive bacteria (16, 23), is found in the *groE* promoter region of several members of the α subgroup (2, 32, 35). Recent studies

* Corresponding author. Mailing address: 12 Hazama-cho, Shugakuin, Sakyo-ku, Kyoto 606-8071, Japan. Phone and fax: (81) 75-781-7828. E-mail: tayura@ip.media.kyoto-u.ac.jp.

[†] Present address: Department of Biophysics, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan.

[‡] Present address: Sumitomo Pharmaceutical Co., Niihama 792-0001, Japan.

TABLE 1. *A. tumefaciens* strains used in this study

Strain	Relevant genotype	Source or reference
GV3100	C58-C1 cured of pTiC58	18
GV3101	GV3100 Rif ^r	18
KN201	GV3101 $\Delta rpoH::tetR \Delta hrcA$	28
KN207	KN201 $rpoH^+$ ($rpoH^+$ was integrated into the chromosome)	This work
KN208	KN201 $Plac'-rpoH$ ($Plac'-rpoH$ was integrated into the chromosome)	This work
KN209	KN613 $Plac'-rpoH$ (the chromosomal $rpoH$ was replaced by $Plac'-rpoH$)	This work
KN214	KN209 $Ptrc-dnaKJ$ (the chromosomal $dnaKJ$ was replaced by $Ptrc-dnaKJ$)	This work
KN613	GV3101 $\Delta hrcA$	28
KN614	KN613 $Ptrc-dnaKJ$ (the chromosomal $dnaKJ$ was replaced by $Ptrc-dnaKJ$)	This work
KN615	KN613 $Ptrc-groESL$ (the chromosomal $groESL$ was replaced by $Ptrc-groESL$)	This work

using the $\Delta rpoH$ and $\Delta hrcA$ mutants of *A. tumefaciens* established that RpoH plays an essential global role in the induction of HSP, whereas HrcA plays a restricted role in repressing *groE* expression under nonstress conditions (low temperatures) (28).

In this study, we investigated the mechanism of RpoH regulation in *A. tumefaciens* by examining the synthesis, stability, and activity of RpoH during the heat shock response. Although the RpoH level is transiently enhanced upon temperature up-shift, this enhancement is preceded by, not followed by, induction of HSP such as DnaK. Several lines of evidence suggest that induction of HSP is caused primarily by the DnaK-DnaJ-mediated activation of preexisting RpoH and only secondarily by increased synthesis of RpoH resulting from increased *rpoH* transcription. On the other hand, the decrease in the amount of RpoH observed during the adaptation phase results from both decreased synthesis and destabilization of otherwise stable RpoH. Thus, the α and γ subgroups of proteobacteria appear to have adopted quite distinct strategies in enhancing the RpoH level and HSP synthesis upon exposure to heat stress.

MATERIALS AND METHODS

Bacterial strains. *A. tumefaciens* strains used in this work are listed in Table 1. For many experiments, derivatives of *A. tumefaciens* strain KN613 ($\Delta hrcA$) lacking the HrcA repressor were used to avoid possible complications arising from its effects on expression of GroE and possibly other proteins. The *rpoH* promoter region (see Fig. 3A, line *2) within the 3.5-kb *ApaI* fragment was replaced by *Plac'* (see Fig. 3B), and the *Plac'-rpoH* fusion was inserted into the *SmaI* site of pTWV228 (Takara Shuzo, Tokyo, Japan) unable to replicate in *A. tumefaciens*. The resulting plasmid was inserted in strain KN201 ($\Delta rpoH \Delta hrcA$) by selecting for carbenicillin resistance (100 μ g/ml); a clone that carried *Plac'-rpoH* at the chromosomal *rpoH* region as the result of plasmid integration (confirmed by PCR) was designated KN208. An isogenic strain, KN207, carrying the authentic *rpoH* promoter was constructed by transforming KN201 with pTW228-*rpoH*. The *rpoH*, *dnaK*, or *groE* promoter on the chromosome was replaced essentially as described previously (28); the *ApaI* fragment containing *Plac'-rpoH* was inserted into pK18*mobsacB*, which was then used to replace the *rpoH* gene of KN613, yielding strain KN209. Strains KN214 and KN614 were obtained from KN209 and KN613, respectively, by replacing the *dnaK* promoter (nucleotides -106 to -1 relative to the initiation codon) by the *BstI107-EcoRI* fragment harboring the *trc* promoter, *lacI* repressor, and spectinomycin resistance gene of pTRC99A-SP. Strain KN615 was constructed by inserting the *BstI1071-EcoRI* cassette mentioned above into the *EcoNI* site at nucleotide -31 of the *groE* initiation codon; the terminator sequence within the cassette disrupts transcript from the authentic *groE* promoter. *E. coli* K-12 strain JM109 was used for DNA manipulation.

Growth media. *A. tumefaciens* cells were grown at 25°C in YEB complete medium with constant aeration or on YEB agar as described previously (28). Davis minimal medium (9) supplemented with 0.2% glucose, 2 μ g of thiamine per ml, 50 μ g of 18 L-amino acids (excluding methionine and cysteine) per ml,

and 1/100 volume of YEB was used as minimal medium. Luria-Bertani (LB) broth was used for growing *E. coli*.

Antisera. Antiserum against RpoH was prepared from a rabbit as described previously (28). Anti-DnaK and anti-CipB sera were kindly donated by M. Kohiyama (University of Paris) and C. Squires (Tufts University), respectively. Anti- β -galactosidase antiserum was obtained from Organon Teknika-Cappel.

Construction of plasmids. To construct pKK232-*PrpoH'-lacZ*, most of the *cat* gene of pKK232-8 (Amersham-Pharmacia) was removed and a *BglII* site was created by PCR using primers (TCTCCAGTTTTTCTCC and TCTCCAGCAGCGCACGC). The *BglII* site was used to insert a *BamHI* fragment containing *lacZ* derived from pMC1871 (Amersham-Pharmacia) in frame with the first seven codons of the *cat* gene. The resulting plasmid was digested with *SmaI*, and the *rpoH* promoter fragment (see Fig. 3A, line *1) was inserted before *lacZ* to yield pKK232-*PrpoH'-lacZ*. To construct pUCD-*PrpoH'-lacZ*, a broad-host-range plasmid, pUCD2 (7), was digested with *SacII* and *BamHI*, blunted by T4 DNA polymerase, and joined with the *PrpoH'-lacZ* fragment excised from pKK232-*PrpoH'-lacZ* by *BseAI* and *ScaI*. To construct pTRC99A-SP, a *SmaI* fragment containing the spectinomycin and streptomycin resistance gene cassette from pUT/Sm (10) was inserted into the *BsaAI* site of pTRC99A (Amersham-Pharmacia). To construct pBBR-*dnaKJn* and pBBR-*dnaKJr*, a 3-kb *EcoRI* fragment containing the entire *dnaKJ* operon was cloned by using a portion of *dnaK* (34) as a probe and was inserted into the *EcoRI* site of pBBR122 (MoBiTec, Göttingen, Germany). The *dnaKJ* operon is transcribed in the same direction as the *cat* gene in pBBR-*dnaKJn*, whereas it is transcribed in the opposite (or reverse) direction in pBBR-*dnaKJr* (as indicated by the final letter of the plasmid designation). To construct pBBR-*groESLn* and pBBR-*groESLr*, a 2.5-kb *EcoRI* fragment containing the *groESL* operon (33) was inserted into the *EcoRI* site of pBBR122. The *groESL* operon is transcribed in the same direction as *cat* in pBBR-*groESLn* but in the opposite direction in pBBR-*groESLr*.

Determination of protein synthesis and degradation rates. Mid-logarithmic phase cells were labeled with L-[³⁵S]methionine (600 μ Ci; 100 Ci/ml) with or without a subsequent chase with unlabeled Met (200 μ g/ml) as indicated for each experiment. Portions of labeled cells were treated with 5% trichloroacetic acid, and the resulting precipitates were washed with acetone and suspended in buffer containing sodium dodecyl sulfate (SDS). Samples with equal radioactivity were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) either directly or after treatment with antibody against RpoH or β -galactosidase to determine the synthesis rate of the protein. The intensities of radioactive protein bands were quantified with a phosphorimager.

β -Galactosidase activity. Cells were grown in synthetic medium and assayed for β -galactosidase activity by the standard procedure (22).

Immunoblotting. Immunoblotting of proteins was performed essentially as described previously (28, 30) by using a Hybond-ECL nitrocellulose membrane filter (Amersham-Pharmacia), and detected with specific rabbit antisera by chemiluminescence techniques.

Isolation and analysis of RNA. Isolation of RNA and primer extension analysis were performed as described previously (28). For S1 mapping, primers RpoH-N (GAAGGTGATTTCGCCTGCACAATC) and RpoH-R (CCTTATCTATGGTC TGGACGGC) were used for PCR amplification of the sequence from nucleotides -305 to -10 of the *rpoH* coding segment; then, 10 cycles of single-direction PCR were done with 5'-fluorescein isothiocyanate (5'-FITC)-labeled RpoH-R. The resulting cDNA with the 5'-FITC label was purified by 6% sequencing gel and used for S1 mapping as described previously (1). S1-protected fragments were resolved by 6% sequencing gel and visualized by FMBIO-II fluorescent-image analyzer (Takara Shuzo).

RESULTS

Transient increase in the rate of RpoH synthesis upon heat shock. The level of RpoH in the cell increases transiently upon shifting the wild-type strain of *A. tumefaciens* from 25 to 37°C (28). To analyze the mechanisms underlying this increase, both the amount and synthesis rate of RpoH were examined upon temperature upshift and the time course of RpoH synthesis was compared with that of HSP synthesis. In agreement with the previous results, the RpoH level as determined by immunoblotting increased, peaked at around 15 min, and decreased to a level near the preshift level by 45 min in complete medium (Fig. 1A). Essentially the same increase with a similar time course was observed when synthetic medium supplemented with amino acids was used, except for a somewhat earlier return (35 min) to the preshift level. Under the same conditions (synthetic medium), the synthesis rate of RpoH, as determined by pulse-labeling with [³⁵S]methionine followed by immunoprecipitation, increased more rapidly, peaking at 10 min (sixfold increase), followed by a rapid decrease to a rate near the preshift rate by about 20 min (Fig. 1B).

We then determined the synthesis rate of HSP, such as DnaK, known to be induced transcriptionally from the heat shock promoter (28, 34). As seen in Fig. 1C, induction of DnaK synthesis occurred almost simultaneously with that of RpoH synthesis and preceded the increase in the RpoH level. This was unexpected, because the induction of HSP synthesis should follow the increase in RpoH level if the latter increase were responsible for HSP induction. Thus, in spite of the marked enhancement of RpoH level, the increased level per se does not account for the initial transcriptional activation of heat shock genes, and the synthesis of RpoH appeared to be coordinately regulated with that of DnaK and presumably of other HSP as well.

Autogenous control in transcriptional induction of RpoH.

Analysis of *rpoH* transcription by S1 protection assay revealed that a single transcript starting from T, 108 nucleotides upstream of the putative *rpoH* initiation codon, is markedly enhanced upon heat shock (Fig. 2). This result was confirmed by primer extension analysis (data not shown). The -35 and -10 regions of this promoter contained sequences similar to those of the *groE* and *dnaK* heat shock promoters (28). The region of 49 bp (Fig. 3A, line *1) including the transcription start site was fused to the promoterless *E. coli lacZ*, and the plasmid carrying the fusion (*PrpoH'-lacZ*) was introduced into the wild-type strain (*rpoH*⁺) and the Δ *rpoH* strain. When *lacZ* expression was examined by measuring β -galactosidase, the Δ *rpoH* mutant exhibited much less activity than the wild type did (Table 2). That this is due to RpoH and not to a product(s) of adjacent genes whose expression is affected by the *rpoH* deletion was shown by the finding that introduction of another plasmid carrying only *rpoH*⁺ was sufficient to restore the high β -galactosidase activity (data not shown). These results indicated that *rpoH* transcription from the above promoter depends largely on RpoH itself.

As expected from these results, heat induction of RpoH synthesis was completely blocked when rifampin, which inhibits transcription, was added 1 min before temperature upshift (Table 3). This is in sharp contrast to the rifampin-resistant increase in the σ^{32} level observed in *E. coli* (27) and other

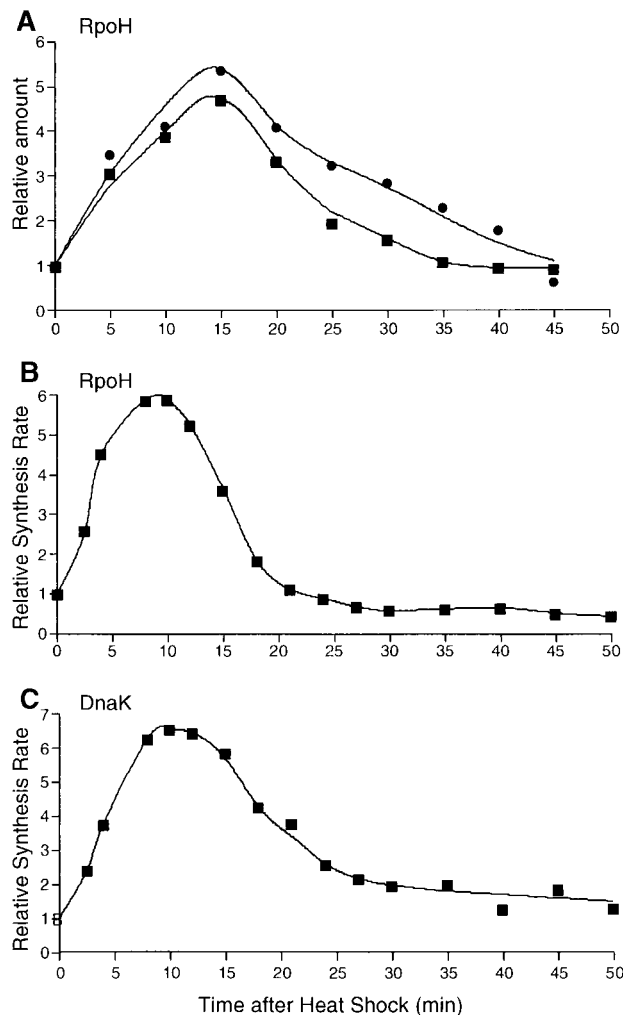


FIG. 1. Transient increases in the level and synthesis rate of RpoH upon heat shock. (A) Cells of wild-type *A. tumefaciens* (GV3101) were grown in complete medium (●) or synthetic medium (■) to the logarithmic growth phase at 25°C and shifted to 37°C at time zero. Samples were taken at intervals, mixed with an equal volume of 2× SDS sample buffer, boiled for 2 min, and analyzed by SDS-PAGE (12.5% polyacrylamide) and immunoblotting with anti-RpoH antiserum essentially as described previously (28). The RpoH band was quantified by densitometry and normalized to the value at 25°C. (B) Log-phase cells grown in synthetic medium at 25°C were shifted to 37°C. Samples taken at the times indicated were pulse-labeled with [³⁵S]methionine for 2 min and treated with trichloroacetic acid, and RpoH was immunoprecipitated, resolved by SDS-PAGE (12.5% polyacrylamide), and quantified as described previously (28). The rate of RpoH synthesis thus obtained was normalized to the value at zero time. (C) Synthesis rate of DnaK was determined by growing and pulse-labeling cells with [³⁵S]methionine as described above for panel B. The cells were treated with trichloroacetic acid and then analyzed by SDS-PAGE (7.5% polyacrylamide). Radioactivity associated with the DnaK band was determined with a phosphorimager and normalized to the value at zero time.

members of the γ subgroup of proteobacteria (30). Finally, when the intact *rpoH* gene or *rpoH* driven by a non-heat shock promoter derived from the *E. coli lac* promoter (*Plac'* [Fig. 3B]) was separately integrated into the chromosome of the Δ *rpoH* strain, the resulting strain carrying intact *rpoH* (*PrpoH*

TABLE 3. Heat-induced RpoH synthesis is inhibited by rifampin^a

Time after heat shock (min)	Rifampin (200 µg/ml)	Relative rate of RpoH synthesis
0	—	1
3	—	3.2
5	—	3.8
3	+	0.6
5	+	0.4

^a Strain GV3100 was grown in synthetic medium at 25°C to mid-log phase and shifted to 37°C with (+) or without (—) rifampin added 1 min before temperature upshift. Samples were taken at the times indicated and labeled with [³⁵S]methionine for 2 min, and immunoprecipitates were analyzed by SDS-PAGE to determine the rate of RpoH synthesis as described in the legend to Fig. 1B. The synthesis rates are presented relative to the value at zero time.

necessarily require the increase in RpoH level. To further substantiate this observation, we analyzed the heat shock response in strain KN208 carrying *Plac'*-*rpoH* in which the RpoH level does not increase upon heat shock. Surprisingly, almost normal induction of HSP such as ClpB, DnaK, and GroEL (Fig. 5A, compare KN208 with KN207) as well as normal accumulation of *dnaK* mRNA (Fig. 5B, compare KN208 with GV3101) was observed, indicating that the increase in RpoH level was not essential for the heat shock response and that the level attained by the *lac* promoter was sufficient for HSP induction. Moreover, addition of chloramphenicol to the wild-type strain 1 min prior to heat shock (>95% inhibition of protein synthesis) did not affect accumulation of *dnaK* mRNA significantly as determined by primer extension analysis (Fig. 5B). Evidently, the transcriptional induction of heat shock genes did not depend on newly synthesized proteins; namely, the preexisting RpoH was sufficient for induction. Thus, RpoH must be somehow activated prior to the enhanced synthesis upon temperature upshift. Such an activation even without

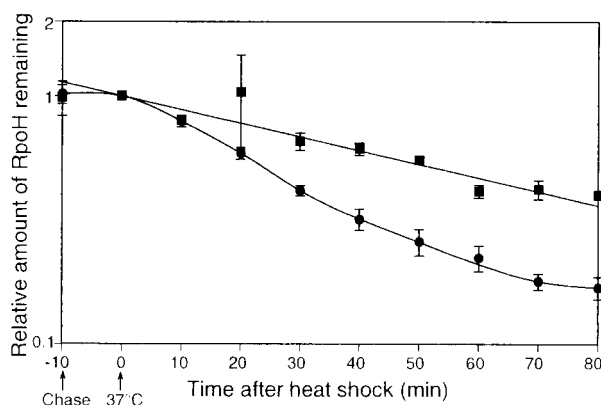


FIG. 4. Transient destabilization of RpoH during the heat shock response. A log-phase culture of wild-type cells (GV3101) grown in synthetic medium at 25°C was divided into two portions, pulse-labeled with [³⁵S]methionine for 2 min, and chased with excess unlabeled methionine. Samples were taken after 3 min and then at 10-min intervals. At the time of second sampling (time zero), one culture was shifted to 37°C (●), whereas the other was kept at 25°C (■). Immunoprecipitates obtained with anti-RpoH antiserum were analyzed by SDS-PAGE. Averages from three experiments are plotted with standard errors (indicated by the error bars).

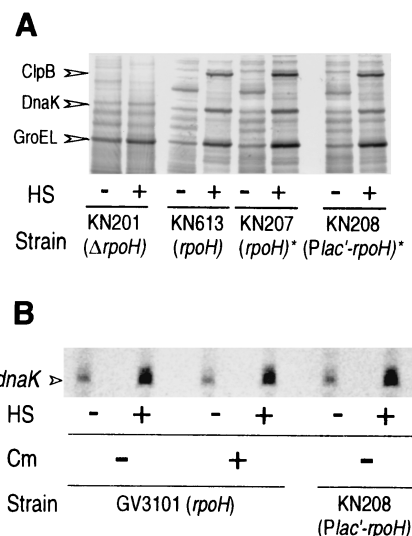


FIG. 5. Neither enhanced RpoH level nor de novo protein synthesis is required for induction of heat shock genes. (A) Induction of HSP was analyzed by pulse-labeling cells with [³⁵S]methionine at 25°C or after heat shocking (HS) the cells (shifting the cells to 37°C for 10 min) and resolving the proteins by SDS-PAGE as described in the legend to Fig. 1C. The positions for ClpB, DnaK, and GroEL are indicated on the basis of the immunoblotting data with specific antisera (28). The results with the wild type (KN613), the $\Delta rpoH$ mutant (KN201), and derivatives of KN201 carrying intact *rpoH* (KN207) or *Plac'*-*rpoH* (KN208) on the chromosome (indicated by an asterisk) are presented. (B) Induction of *dnaK* mRNA was analyzed by growing cells as described above for panel A, and chloramphenicol (Cm) was added (+) to a concentration of 100 µg/ml 1 min prior to temperature upshift as indicated. Samples were taken before (—) and 10 min (+) after heat shock (HS), RNA was extracted, and primer extension was performed using a fluorescence-labeled primer for *dnaK* transcript as described previously (28).

increased RpoH level appeared to cause virtually normal heat shock response in *A. tumefaciens*.

Effects of changes in the cellular levels of DnaK or GroE chaperones on HSP synthesis. To examine the role of chaperones in modulating the RpoH activity, a set of strains that can overexpress or underexpress the DnaK-DnaJ or GroEL-GroES chaperones was constructed. The overexpressing strains were constructed by introducing a multicopy pBBR122 plasmid carrying the *dnaK-dnaJ* or *groES-groEL* operon into strain KN613 ($\Delta hrcA$). Cells harboring these plasmids overproduced the respective chaperones as expected (Fig. 6A). The smaller overexpression of GroEL detected (3- to 5-fold) compared to that of DnaK (ca. 10-fold) may be due in part to the higher basal expression of GroEL found in strains lacking HrcA repressor (28). To determine whether HSP synthesis was affected in these strains, the cellular level of ClpB, a good indicator of the heat shock response, as well as that of RpoH was examined. The level of ClpB but not RpoH was found to be reduced severalfold in strains overexpressing DnaK compared to the control strain carrying vector alone (Fig. 6A, compare lanes 2 and 3 with 1). In contrast, overexpression of GroE (to the extents observed in the present experiments) affected the RpoH or ClpB level only slightly (lanes 4 and 5).

Strains that underexpress the chaperones were constructed by replacing the heat shock promoter of the chromosomal

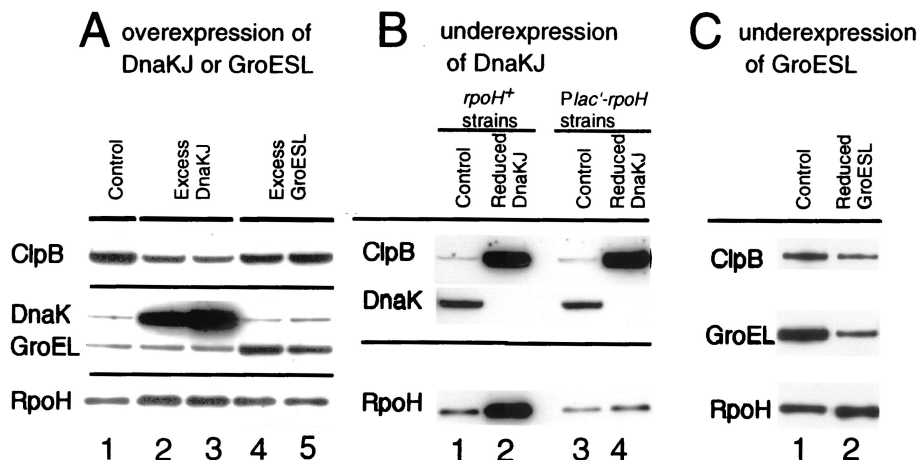


FIG. 6. Effects of changes in the levels of DnaKJ chaperones or GroESL chaperones on the levels of other HSP during steady-state growth. Cells were grown in synthetic medium at 25°C, and proteins were analyzed by SDS-PAGE followed by immunoblotting using specific antisera against *E. coli* ClpB, DnaK, GroEL, or *A. tumefaciens* RpoH as indicated to the left. (A) Overproduction of chaperones by multicopy pBBR122 plasmid carrying the *dnaKJ* or *groESL* operon in strain KN613. Lanes: 1, pBBR122 (control); 2, pBBR122-*dnaKJn*; 3, pBBR122-*dnaKJr*; 4, pBBR122-*groESLn*; 5, pBBR122-*groESLr*. Both DnaKJ and GroESL were expressed slightly more efficiently when the respective operon was inserted in the plasmid in the direction opposite that of the *cat* gene (“r” constructs) than when the operon was inserted in the same direction as that of *cat* (“n” constructs), perhaps due to activity of some uncharacterized promoter(s). (B) Reduced production of DnaK in strains in which the chromosomal *dnaK* promoter was replaced by the *trc* promoter in the *rpoH*⁺ (*PrpoH-rpoH*) or *Plac'-rpoH* background. Lanes: 1, KN613 (*rpoH*⁺ strain [control]); 2, KN614 (*dnaKJ* driven by the *trc* promoter in the *rpoH*⁺ strain); 3, KN209 (*Plac'-rpoH* strain [control]); 4, KN214 (*dnaKJ* driven by the *trc* promoter in the *Plac'-rpoH* strain). (C) Reduced production of GroESL in which the chromosomal *groE* promoter was replaced by the *trc* promoter. Lanes: 1, KN613 (*rpoH*⁺ strain [control]); 2, KN615 (*groESL* driven by the *trc* promoter in the *rpoH*⁺ strain).

dnaK or *groE* operon by the *trc* promoter. The resulting strains produced much lower levels of respective chaperones in the absence of isopropyl-β-D-thiogalactopyranoside. When the DnaK chaperones were reduced, both ClpB and RpoH were dramatically enhanced (Fig. 6B, compare lanes 1 and 2). Again, the reduced GroE expression had little effect on the RpoH or ClpB level (Fig. 6C). All the results taken together strongly suggested that the DnaK but not GroE chaperones negatively modulate the synthesis of other HSP including RpoH, although DnaK overexpression failed to reduce the RpoH level for unknown reasons (see Discussion).

It should be noted, however, that the marked increase in the ClpB level in the DnaK-depleted strain could be a secondary consequence of an increase in the RpoH level. To test this possibility, another DnaK-underexpressing mutant was constructed using the *Plac'-rpoH* strain (KN209) in which the

RpoH level does not increase upon heat shock. The resulting strain (KN214) produced very low levels of DnaK while showing no increase in RpoH as expected (Fig. 6B, compare lanes 3 and 4). Even in this strain, the amount of ClpB was elevated to the level similar to that found in the *rpoH*⁺ background (Fig. 6B, compare lanes 2 and 4), suggesting that the enhanced synthesis of HSP (such as ClpB) resulted from reduced levels of DnaK and not from increased levels of RpoH. The latter pair of strains with *Plac'-rpoH* background were used for subsequent experiments to assess the effects of reduced DnaK level on RpoH activity (see below).

Effects of altered chaperone levels on transcription of heat shock promoters. To determine whether the changes in HSP synthesis caused by altered DnaK levels resulted from altered transcription from the RpoH-dependent heat shock promoters, the reporter plasmid pUCD-*PrpoH'-lacZ* was introduced

TABLE 4. Effects of altered DnaK or GroE chaperone level on transcription from the *rpoH* promoter^a

Strain	Chaperone level	Relative β-galactosidase activity
<i>rpoH</i> ⁺ strains		
KN613(pBBR122)(pUCD- <i>PrpoH'-lacZ</i>) (control)	Normal	1
KN613(pBBR122- <i>dnaKJr</i>)(pUCD- <i>PrpoH'-lacZ</i>)	Excess DnaKJ	0.38 ± 0.05
KN613(pBBR122- <i>groESLr</i>)(pUCD- <i>PrpoH'-lacZ</i>)	Excess GroESL	0.96 ± 0.27
KN615 <i>Ptrc-groESL</i> (pBBR122)(pUCD- <i>PrpoH'-lacZ</i>)	Reduced GroESL	1.21 ± 0.05
<i>Plac'-rpoH</i> strains		
KN209(pBBR122)(pUCD- <i>PrpoH'-lacZ</i>) (control)	Normal	1
KN214 <i>Ptrc-dnaKJ</i> (pBBR122)(pUCD- <i>PrpoH'-lacZ</i>)	Reduced DnaKJ	10.60 ± 4.55

^a The pUCD-*PrpoH'-lacZ* reporter plasmid was inserted in the strains used in Fig. 6; the control and the chaperone-underexpressing strains were also made to carry pBBR122 plasmid. Cells were grown to log phase in synthetic medium at 25°C, and portions of these cell cultures were assayed for β-galactosidase activity and normalized to the value of the respective control. Averages from at least three experiments with standard errors are shown. The activities for the two control strains were comparable.

into the set of strains to examine LacZ expression. Indeed, *rpoH* transcription as determined by β -galactosidase activity was reduced in the DnaK-overexpressing strain but dramatically enhanced in the DnaK-underexpressing strain (Table 4). In contrast, the changes in the GroEL-GroES level did not affect LacZ expression appreciably. These results suggested that the DnaK chaperones serve specifically as a negative modulator of the RpoH-mediated transcription by inhibiting RpoH activity.

We then examined the effects of altered chaperone levels on transcription from heat shock promoters during the heat shock response by pulse-labeling the same set of strains with [³⁵S]methionine followed by immunoprecipitation with anti- β -galactosidase antiserum (Fig. 7). The rates of LacZ expression before the temperature shift agreed well with the β -galactosidase activities in strains overexpressing or underexpressing DnaK chaperones (compare the values at zero time in Fig. 7 with the values in Table 4). The higher or lower LacZ expression in these strains appeared to be maintained during the time period examined. However, when the extent of induction was compared under conditions of various DnaK levels, it was higher with the DnaK-overexpressing strain (11-fold) than with the control (8.7-fold) and was lower with the DnaK-underexpressing strain (2.7-fold) (Fig. 7). Moreover, the induction reached its maximum faster with the DnaK-overexpressing strain than with the control. These results suggested that the DnaK chaperone intimately modulates the transcription of heat shock genes throughout the heat shock response. The data are also consistent with the notion that the pool of free DnaKJ chaperones rather than the total DnaKJ levels is important for the negative regulation of RpoH activity. In contrast, GroE underexpression had no appreciable effects on heat shock induction, whereas GroE overexpression resulted in slightly reduced induction, suggesting a possible subsidiary role of GroE chaperones in regulating the RpoH activity.

DISCUSSION

The amino acid sequences of *A. tumefaciens* RpoH (34 kDa) and *E. coli* σ^{32} have 36% identity (29). *A. tumefaciens* RpoH and *E. coli* σ^{32} recognize similar heat shock promoters and presumably play identical catalytic roles as transcriptional activators of heat shock genes such as *groE* and *dnaK* (28). The regulation of RpoH also appeared to be similar in both species; namely, the cellular level of RpoH increased transiently upon heat shock. However, the mechanism underlying transient increases in RpoH is quite different. In *A. tumefaciens*, the increase occurred at the level of transcription rather than translation and was largely mediated by RpoH itself. This conclusion was based on several lines of evidence. (i) S1 mapping of RNA detected a major heat-inducible *rpoH* transcript initiated at the heat shock promoter (Fig. 2). (ii) *lacZ* expression driven by the *rpoH* promoter was drastically reduced in the $\Delta rpoH$ mutant (Table 2). (iii) Heat-inducible synthesis of RpoH in the wild type was completely inhibited by rifampin, unlike the situation with RpoH in the γ subgroup of proteobacteria (Table 3). (iv) The intact *rpoH* promoter but not the *Plac'* promoter gave rise to the heat-inducible RpoH synthesis (Fig. 3). Similar autogenous control of *rpoH* transcription involving an RpoH-dependent promoter has been reported in *C. cres-*

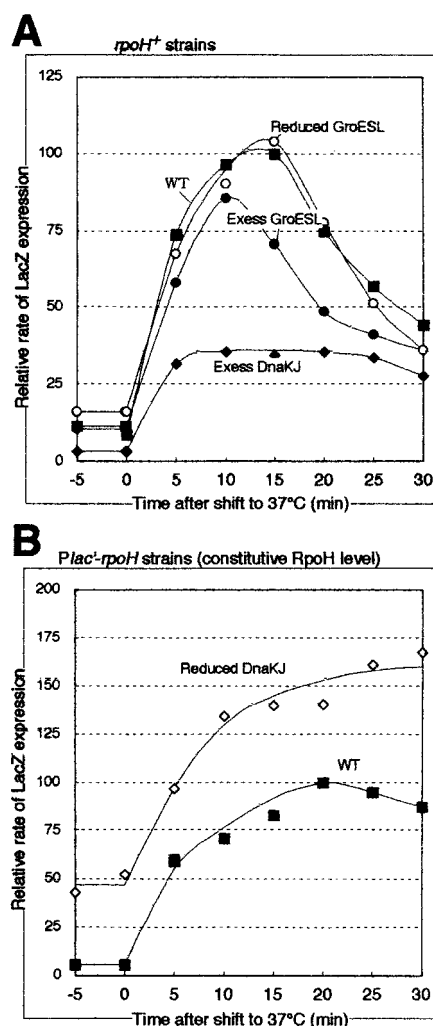


FIG. 7. Time course of change in RpoH activity (LacZ expression from the pUCD-*PrpoH'*-*lacZ* reporter plasmid) during the heat shock response. The same set of strains used in Table 4 were grown in synthetic medium at 25°C and shifted to 37°C at time zero. Samples taken at the times indicated were pulse-labeled with [³⁵S]methionine for 2 min, chased with excess unlabeled methionine for 1 min, and treated with 10% trichloroacetic acid in ice. Radiolabeled *E. coli* σ^{32} - β -galactosidase fusion protein (GF807-FS [26]) was added as an internal reference to each sample containing the same radioactivity, immunoprecipitated with anti- β -galactosidase antiserum, and resolved by SDS-PAGE (7.5% polyacrylamide). The radioactivities of β -galactosidase bands were normalized to that of the reference protein and are shown as percentages of the maximum synthesis rate in the control (wild-type [WT]) strain. (A) Data for the *rpoH*⁺ strain (KN613) carrying pBBR122 (control or WT ■), pBBR122-*dnaKJr* (Excess DnaKJ ◆), pBBR122-*groESLr* (Excess GroESL ●), or KN615 (*P_{trc}*-*groE*) (Reduced GroESL ○) are shown. (B) Data for the pair of *Plac'*-*rpoH* strains, KN209 (control or WT ■) and KN214 (Reduced DnaKJ ◇), are shown.

centus based on both in vivo and in vitro experiments (47). Thus, autogenous transcriptional control of *rpoH* appears to be conserved at least in some members of the α subgroup of proteobacteria.

The control of RpoH level in *A. tumefaciens* differs from that in *E. coli* in another important respect, namely, the control of

TABLE 5. Comparison of regulatory strategies for the heat shock response

Heat shock response characteristic	Regulatory strategy in:	
	<i>A. tumefaciens</i>	<i>E. coli</i>
Induction of RpoH synthesis	Transcriptional level (autogenous control)	Translational level
Control of RpoH activity	Activation during the induction phase	Inhibition during the adaptation phase
Control of RpoH stability	Destabilization during the adaptation phase	Stabilization during the induction phase
Induction of other HSP	Primarily caused by activation of RpoH	Primarily caused by increased RpoH level

proteolytic degradation. RpoH was quite stable in *A. tumefaciens* during steady-state growth at 25°C, and no further stabilization occurred upon shift to 37°C, indicating that stabilization of RpoH does not contribute to the increased RpoH level significantly. Rather, gradual destabilization was observed after a short lag and continued until the RpoH level returned to the preshift level (Fig. 4). This mode of regulation should be contrasted with that found with *E. coli* σ^{32} , which is normally very unstable and transiently stabilized upon heat shock (38, 43). Besides the clear difference in stability under steady-state growth, the change in RpoH stability occurs at different phases of the heat shock response. Although details of the mechanisms for controlling RpoH stability remain unknown, available evidence suggests the involvement of homeostatic mechanism(s) for maintaining the cellular RpoH level within a certain range. For example, replacement of the *rpoH* promoter by *Plac* prevented not only the increase in RpoH level upon heat shock but also the transient decrease during the adaptation phase (Fig. 3C and data not shown). Also, overexpression of DnaKJ chaperones reduced transcription from the *rpoH* promoter (Table 4) but did not reduce the RpoH level significantly (Fig. 6A).

In addition to the distinct regulatory strategies for controlling RpoH levels, the mechanism of induction of heat shock promoters appears to differ strikingly between *A. tumefaciens* and *E. coli*. In *E. coli*, the increased amount of RpoH primarily determines the rate of HSP synthesis through increased transcription from heat shock promoters, although the recent results with *ftsH* mutants suggested potential involvement of activity control as an auxiliary or alternative mechanism (40). In *A. tumefaciens*, a similar increase in RpoH level occurred, but not early enough to explain the induction of DnaK as well as RpoH itself (Fig. 1), suggesting that activation rather than increased level of RpoH is mainly responsible for initial induction of HSP. Consistent with this expectation, the strain unable to enhance the RpoH level upon heat shock exhibited virtually normal HSP induction (Fig. 5A). Moreover, near normal heat induction of *dnaK* mRNA was observed even in the absence of de novo protein synthesis (Fig. 5B). The question then arises, what is the role of transcriptional induction of RpoH upon heat shock? Increasing the RpoH level by introducing extra *rpoH* copies did increase transcription from heat shock promoters, resulting in higher HSP levels and LacZ expression from the *PrpoH'-lacZ* fusion construct (data not shown). It thus appears that increasing the RpoH level provides a subsidiary or fail-safe mechanism in sustaining the increased synthesis of HSP, although it hardly contributes to the initial phase of HSP induction. This mode of regulation in *A. tumefaciens* is in marked contrast with the regulation of σ^{32} in *E. coli*, in which control of activity at the induction phase is

detectable only under special circumstances, as in the *ftsH* mutants where σ^{32} is much stabilized. The differences in regulatory strategy observed in *A. tumefaciens* and *E. coli* are summarized in Table 5.

As for the mechanism of controlling RpoH activity upon heat shock, the activation may occur at any of the steps of RpoH function including binding to core RNA polymerase and transcription by RNA polymerase holoenzyme containing RpoH. The DnaK chaperone machinery appears to play an important regulatory role in this process, since the cellular level of DnaK but not GroE showed a strong negative correlation with the amount of transcription from the heat shock promoters. Direct or indirect inhibition of RpoH activity by DnaK chaperones under nonstress conditions and release of inhibition upon heat stress would be a highly plausible mechanism. If the regulation were to involve direct interaction between RpoH and DnaK-DnaJ chaperones, the mechanism may be similar to the control of activity of σ^{32} postulated for *E. coli* (3, 11, 40, 45). However, possible involvement of other negative factors cannot be excluded. Activity of such factors might in turn be affected positively by DnaKJ, in much the same way that the HrcA repressor of *Bacillus subtilis* is affected by GroESL (23). Such negative factors might also bind and stabilize RpoH, possibly explaining the failure of the DnaK-overexpressing strain to reduce the RpoH level (Fig. 6A).

In any event, the DnaK-DnaJ chaperones with their changing substrate binding activity are the most likely candidates that monitor the cellular state of protein folding and play an important regulatory role in the activity control of RpoH. In view of the difficulty in analyzing the control of activity and stability of σ^{32} in *E. coli* because of the simultaneous involvement of DnaKJ chaperones in both processes, the present system in *A. tumefaciens* may provide a unique opportunity to learn more about the mechanisms of controlling RpoH activity during the early phase of the heat shock response.

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