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

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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, 34, 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 (HIIb) 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 fisH 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
using the ΔpoH and ΔhrcA 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 upshift, 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 (*ΔhrcA*) 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 range plasmid was used as the result of plasmid integration (confirmed by EcoRI fragment harboring the *dnaK* promoter). The resulting plasmid was integrated into the chromosome of KN208 (Takara Shuzo, Tokyo, Japan) to replace the *hrcA* gene in pBBR-DnaKJ (34) as a probe and was inserted into the *EcoRI* site of the *hrcA* gene in pBBR-DnaKJ (as described previously (28)). Anti-DnaK and anti-ClpB sera were kindly donated by M. Kohiyama (University of Paris) and C. Squires (Tufts University), respectively. Anti-β-galactosidase antisera was obtained from Organon Teknika-Cappell.

**Construction of plasmids.** To construct pKK232-PpoH-lacZ, most of the cat gene of pKK232-8 (Amersham-Pharocia) was removed and a βgal site was created by PCR using primers (TCTCCAGTTTTTTTCTCC and TCTCCAGCGCAGGC). The *BglII* site was used to insert a *BamHI* fragment containing the *lacZ* region derived from pmC1871 (Amersham-Pharocia) 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-PpoH-lacZ. To construct pUCD-PpoH-lacZ, a broad-host-range plasmid, pUCD2 (7), was digested with SphiI and BamHI, blunted by T4 DNA polymerase, and joined with the PpoH-lacZ fragment excised from pKK232-PpoH-lacZ by *BsrAI* and *SacI*. To construct pTRC99A-SP, a SphiI fragment containing the spectinomycin and streptomycin resistance gene cassette of pUT/Sm (10) was inserted into the *BsaAI* site of pTRC99A (Amersham-Pharocia). To construct pBBR-dnaKJ and pBBR-dnaKL, a 3-kb *EcoRI* fragment containing the entire *dnaK* operon was cloned by using a portion of *dnaK* (34) as a probe and was inserted into the *EcoRI* site of pBRR122 (Mobiltec, Gottingen, Germany). The *dnaK* operon is transcribed in the same direction as the *cat* gene in pBBR-dnaKJ, whereas it is transcribed in the opposite (or reverse) direction in pBBR-dnaKL (as indicated by the final letter of the plasmid designation). To construct pBBR-groESL and pBBR-groE, a 2.5-kb *EcoRI* fragment containing the *groESL* operon (33) was inserted into the *EcoRI* site of pBRR122. The *groESL* operon is transcribed in the same direction as cat in pBBR-groESL but in the opposite direction in pBBR-groE.

**Determination of protein synthesis and degradation rates.** Mid-logarithmic phase cells were labeled with L-[35S]methionine (600 μCi; 100 Ci/ml) with or without a subsequent chase with unlabeled Met (200 μg/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-polycrylamide 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 Hybrid-ECL nitrocellulose membrane filter (Amersham-Pharocia), 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 (GAAGGTGATTCGCCTGCACAATC) and RpoH-R (CCTTATCTATGGTC TGGAAAGCGC) 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).

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

| Strain | Relevant genotype | Source or reference |
|--------|------------------|---------------------|
| GV3100 | CS8-C1 cured of pTiCS8 | 18 |
| GV3101 | GV3100 Rif* | 18 |
| KN201  | *A. tumefaciens* ΔrpoH ΔhrcA | 28 |
| KN207  | KN201 *rpoH* (ΔpoH*“* was integrated into the chromosome) | This work |
| KN208  | KN201 Plac-ΔrpoH (Plac-ΔpoH*“* was integrated into the chromosome) | This work |
| KN209  | KN613 *rpoH* (the chromosomal *rpoH* was replaced by Plac-ΔrpoH) | This work |
| KN214  | KN209 Prc-dnaKJ (the chromosomal dnaKJ was replaced by Prc-dnaKJ) | This work |
| KN613  | GV3101 ΔhrcA | 28 |
| KN614  | KN613 Prc-dnaKJ (the chromosomal dnaKJ was replaced by Prc-dnaKJ) | This work |
| KN615  | KN613 Prc-groESL (the chromosomal groESL was replaced by Prc-groESL) | This work |
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 (*P*poH-lacZ) was introduced into the wild-type strain (*rpoH*<sup>+</sup>) and the ΔrpoH strain. When lacZ expression was examined by measuring β-galactosidase, the ΔrpoH 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<sup>+</sup> 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 α<sup>32</sup> level observed in *E. coli* (27) and other 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 ΔrpoH strain, the resulting strain carrying intact rpoH (PrpoH...
Destabilization of normally stable RpoH during the adaptation phase. Stability of RpoH was then examined during the heat shock response by pulse-labeling cells with [35S]methionine at 25°C, followed by a chase with excess unlabeled methionine at 25°C or after shift to 37°C, and determining the remaining RpoH by immunoprecipitation. Unlike E. coli σ32, the A. tumefaciens RpoH was found to be very stable, with a half-life of about 60 min at 25°C (Fig. 4). When the labeled cells were shifted to 37°C, gradual destabilization was observed after a lag time of about 10 min. After 20 to 30 min, the half-life of RpoH decreased to about 20 min and returned to near the initial stability after about 60 min. The transient destabilization (about threefold) seemed to account for, at least in part, the decrease of RpoH level during the adaptation phase. Thus, both transient induction of RpoH synthesis at the transcription level and subsequent protein destabilization appeared to explain the transient increase in the RpoH level observed upon heat shock at least under the set of conditions employed.

Rapid initial induction of HSP depends primarily on activation of RpoH. The above finding that the induction of HSP (DnaK) precedes the increase in the RpoH level (Fig. 1) suggested that the transcription of heat shock promoters does not

![Graph](image-url)

**TABLE 2.** Autogenous control of RpoH-dependent transcription of *rpoH*

| Strain and plasmid | β-Galactosidase activity |
|--------------------|--------------------------|
| KN613 (*rpoH*)     |                          |
| pUCD-lacZ          | 0.6 ± 0.4                |
| pUCD-P*poH-lacZ    | 1.011 ± 1.44             |
| KN201 (Δ*rpoH*)    |                          |
| pUCD-lacZ          | 1.4 ± 0.5                |
| pUCD-P*poH-lacZ    | 41.4 ± 0.4               |

*Plasmid carrying the promoterless lacZ or lacZ driven by the authentic P*poH* promoter was inserted in the *rpoH* or Δ*poH* strain as indicated, and the resulting transformants were grown in synthetic medium at 25°C to determine β-galactosidase activity. Averages of at least three independent measurements with standard errors are presented in Miller units.
necessarily require the increase in RpoH level. To further substantiate this observation, we analyzed the heat shock response in strain KN208 carrying P\textasciitilde 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

increased RpoH level appeared to cause virtually normal heat shock response in \textit{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 (\textit{\textdegree}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
were comparable.

The activities for the two control strains

constructed using the P

lac

rpoH

possibility, another DnaK-underexpressing mutant was con-

sequence of an increase in the RpoH level. To test this

RpoH level for unknown reasons (see Discussion).

RpoH, although DnaK overexpression failed to reduce the

negatively modulate the synthesis of other HSP including

strongly suggested that the DnaK but not GroE chaperones

dramatically enhanced (Fig. 6B, compare lanes 1 and 2).

DnaK chaperones were reduced, both ClpB and RpoH were

When the

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 level (Fig. 6C). All the results taken together
strongly suggested that the DnaK but not GroE chaperones
greatly 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 con-
structed 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 show-
ing no increase in RpoH as expected (Fig. 6B, compare lanes
3 and 4). Even in this strain, the amount of ClpB was elevated
above 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 sub-
sequent 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 promot-
ers, the reporter plasmid pUCD-PrepoH-lacZ was introduced

![Graph](image)

FIG. 6. Effects of changes in the levels of DnaKJ chaperones or GroESL chaperones on the levels of other HSP during steady-state growth.

Table 4. Effects of altered DnaK or GroE chaperone level on transcription from the rpoH promoter

| Strain | Chaperone level | Relative β-galactosidase activity |
|--------|-----------------|---------------------------------|
| rpoH+ strains |
| KN613(pBBR122)(pUCD-PrpH-lacZ) (control) | Normal | 1 |
| KN613(pBBR122-dnakJ)(pUCD-PrpH-lacZ) | Excess DnaKJ | 0.38 ± 0.05 |
| KN613(pBBR122-groESL)(pUCD-PrpH-lacZ) | Excess GroESL | 0.96 ± 0.27 |
| KN615 Pre-groESL(pBBR122)(pUCD-PrpH-lacZ) | Reduced GroESL | 1.21 ± 0.05 |

Plac-’rpoH strains |
| KN209(pBBR122)(pUCD-PrpH-lacZ) (control) | Normal | 1 |
| KN214 Pre-dnakK(pBBR122)(pUCD-PrpH-lacZ) | Reduced DnaKJ | 10.60 ± 4.55 |

*The pUCD-PrepoH-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 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* σ32 have 36% identity (29). *A. tumefaciens* RpoH and *E. coli* σ32 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 ΔrpoH 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. crescentus* 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 σ 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
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 *rhoH* 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 *rhoH* 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 fsH 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 *rhoH* 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 fsH 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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**TABLE 5. Comparison of regulatory strategies for the heat shock response**

| Heat shock response characteristic | Regulatory strategy in: *A. tumefaciens* | Regulatory strategy in: *E. coli* |
|-----------------------------------|--------------------------------------|----------------------------------|
| 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 |

*E. coli* and *A. tumefaciens* E. coli
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