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Quantitative control of the stationary phase-specific sigma factor, σ^S, in *Escherichia coli*: involvement of the nucleoid protein H-NS

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In *Escherichia coli*, recent intensive studies revealed that expression of a certain subset of genes is under the control of the stationary phase-specific sigma factor, σ^S, which is encoded by the *rpoS* gene. Since σ^S functions predominantly under certain growth conditions, its activity and/or cellular content has accordingly to be tightly controlled, however, the underlying molecular mechanism is at present unclear. We previously demonstrated that expression of the *cbpA* gene, encoding an analogue of the DnaJ molecular chaperone, is largely dependent on σ^S function. Here we have found that a mutational lesion of the *hns* gene, which encodes one of the well-characterized nucleoid proteins, H-NS, affects the cellular content of σ^S remarkably and consequently affects the expression of *cbpA*. Enhanced accumulation of σ^S in *hns* deletion cells was particularly observed in the logarithmic growth phase and was demonstrated to result from an elevated translational efficiency of *rpoS* mRNA and also from an increased stability of newly synthesized σ^S. Although H-NS is known to influence the transcription of a number of apparently unlinked genes on the chromosome, in this study we provide a novel instance in which H-NS is deeply implicated in post-transcriptional regulation(s) of the expression of *rpoS*. As to physiological relevance, it was also demonstrated that *hns* deletion cells exhibit an extreme thermotolerance even in the logarithmic growth phase, presumably because of the enhanced accumulation of σ^S.

Key words: cell cycle/*Escherichia coli*/H-NS/sigma factor

Introduction

In *Escherichia coli*, it is well known that expression of a set of genes is specifically induced when cells enter the stationary phase and/or encounter stressful conditions for growth, such as nutrient starvation. Accordingly, they can adapt to, and survive under, harsh conditions for growth. In this context, recent intensive studies revealed that a certain subset of such stationary phase-specific genes is under the control of an alternative sigma factor, named σ^S (for reviews see Siegele and Kolter, 1992; Hengge-Aronis, 1993). The corresponding structural gene, named *rpoS*, was discovered in several laboratories independently and designated *nur*, *katF*, *appR* or *csi2*. For example, the *katF* allele was first identified as a mutant that affects expression of the *katE* gene encoding a stationary phase-specific catalase (HPII). The amino acid sequence, deduced from the *katF* gene, showed significant homology with that of the *E.coli* major sigma factor, σ^{70}. It was therefore reasonably postulated that the *katF* gene product functions as an alternative sigma factor in *E.coli* (Mulvey and Loewen, 1989). This has indeed been demonstrated by a series of *in vitro* transcription experiments, in which a holoenzyme of RNA polymerase was reconstituted with the *katF/rpoS* gene product. The reconstituted RNA polymerase containing the *katF/rpoS* gene product, but not σ^{70}, is able to efficiently trigger *in vitro* transcription from appropriate promoters (Tanaka *et al.*, 1993). Therefore, this new sigma factor is now called σ^S (stationary phase-specific sigma factor) or σ^{38} (sigma factor with a molecular weight of 38 kDa).

Despite the fact that σ^S plays an important role in the transcriptional control of expression of certain genes in the stationary phase and/or under stressful growth conditions, the mechanism underlying the control of its functioning is unclear. Since σ^S functions predominantly under certain growth conditions, as described above, its activity and/or cellular content has accordingly to be tightly controlled. Apparently, the cellular content of σ^S increases in the stationary phase and under conditions of carbon or phosphate source starvation. Several investigators have provided evidence previously, mainly by monitoring the expression of either a transcriptional or translational *rpoS–lacZ* fusion gene, that such control of the cellular content of σ^S appears to be achieved at both the transcriptional and post-transcriptional levels (Mulvey *et al.*, 1990; Lange and Hengge-Aronis, 1991a; Loewen *et al.*, 1993; McCann *et al.*, 1993). However, the conclusions were not always consistent with each other, depending on the growth conditions used. In a rich medium, transcription of *rpoS* mRNA was suggested to increase by 3- to 6-fold during transition from the logarithmic to the stationary growth phase. In a minimal medium, however, expression of σ^S from a *rpoS–lacZ* translational fusion, but not a transcriptional fusion, increases in response to carbon source starvation (~8-fold), suggesting the importance of post-transcriptional control (McCann *et al.*, 1993). Furthermore, Takayanagi *et al.* (1994) recently suggested that the stability of newly synthesized σ^S increases notably in the stationary phase. These results suggest that the mechanism(s) underlying the quantitative control of σ^S under various growth conditions appears to be very complex. In fact, a more comprehensive study addressing these issues by Lange and Hengge-Aronis (1994) demonstrated that *rpoS* and/or σ^S expression is not only transcriptionally controlled, but is also extensively regulated at the levels of translation and protein stability. In this context, the *cya* gene product (for

cAMP synthesis) and the *relA/spoT* gene products (for ppGpp synthesis) were suggested to be involved as effectors controlling σ^S synthesis (Lange and Hengge-Aronis, 1991a; Gentry *et al.*, 1993). It was recently proposed that homoserine lactone is also an inducer for expression of σ^S (Huisman and Kolter, 1994). Nevertheless, no obvious protein factor involved in the regulation of the cellular content of σ^S has been identified to date. Here we provide such a first instance, demonstrating that the nucleoid protein H-NS plays an important role in regulation of the cellular content of σ^S.

We previously demonstrated that expression of the *cbpA* gene encoding an analogue of DnaJ (Ueguchi *et al.*, 1994) is largely dependent on the function of σ^S, thereby its expression can be induced specifically in the stationary phase (Yamashino *et al.*, 1994). During the course of studies on the regulation of the *cbpA* gene, we found that a mutational lesion of the *hns* gene, which encodes one of the well-characterized nucleoid proteins (H-NS), affects the cellular content of σ^S remarkably and consequently affects the expression of *cbpA*. The enhanced accumulation of σ^S in cells carrying an *hns::neo* (Km^r) allele, observed particularly in the logarithmic growth phase, was demonstrated to result from enhanced translation of *rpoS* mRNA and also from increased stability of newly synthesized σ^S. The H-NS protein is a major constituent of the *E.coli* nucleoid and is implicated in the compact organization of the chromosome (Drlica and Rouviere-Yaniv, 1987; Pettijohn, 1988; Pon *et al.*, 1988; Schmid, 1990). Based on recent intensive genetic evidence, this protein appears to influence the transcription of a number of apparently unlinked genes on the chromosome (Göransson *et al.*, 1990; Hulton *et al.*, 1990, and references therein). Here we provide a novel instance in which H-NS is deeply implicated in post-transcriptional regulation(s) of the expression of a certain gene, *rpoS*.

Results

σ^S-dependent expression of the cbpA gene in an hns mutant

It was demonstrated previously that expression of the *cbpA* gene is largely dependent on the function of σ^S. Transcription of *cbpA* is dramatically induced, in a σ^S-dependent manner, either in the stationary phase in a rich medium (e.g. Luria broth) or upon phosphate starvation in a minimal medium (Yamashino *et al.*, 1994). However, when cells were grown in a conventional minimal medium (e.g. M9 glucose), expression of *cbpA* was hardly induced even in the stationary growth phase, as monitored as β-galactosidase activity (Figure 1, open circles), while σ^S was indeed present in these cells (Figure 2, open bar). In this study, we then asked the question why stationary phase-induced and σ^S-dependent expression of *cbpA* is observed only in a rich medium, i.e. not in a minimal medium. We learned from previous literature that such apparently contradictory phenomena were observed for another σ^S-dependent gene, *bolA* (Lange and Hengge-Aronis, 1991b). These results suggested that the mechanism by which expression of certain σ^S-dependent genes is regulated is not as simple as previously thought. As an approach to clarify this problem, in this study we focused our attention on the role of the nucleoid protein H-NS,

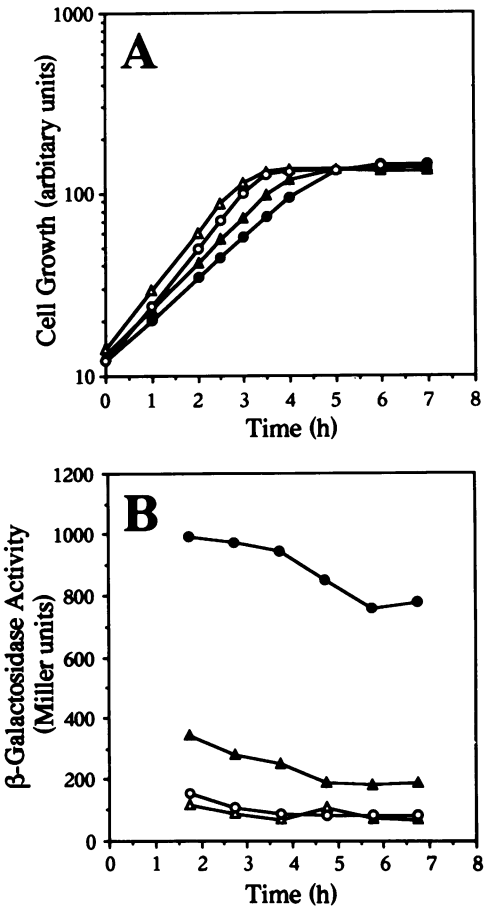

Fig. 1. β-galactosidase activity expressed by the *cbpA−lacZ* operon fusion. Strains GY4 (*hns*$^+$ *rpoS*$^+$; open circles), GY8 (*hns::neo*; closed circles), GY12 (*rpoS*::Tn*10*; open triangles) and GY14 (*hns::neo/ rpoS*::Tn*10*; closed triangles) were grown at 37°C in M9 minimal medium containing thiamine (2 μg/ml), glucose (0.2%) and appropriate amino acids (isoleucine, leucine and valine, 20 μg/ml each). Note that all these strains carry the *cbpA−lacZ* fusion gene. Both the cell growth (**A**) and β-galactosidase activity (**B**) were monitored. Note also that one optical density unit at 600 nm corresponds to 100 arbitrary units.

for two reasons. First, we found that the *cbpA* promoter contains a curved DNA structure (Yamashino *et al.*, 1994), which was suggested to be a preferential target for the DNA binding protein H-NS (Yamada *et al.*, 1990; Owen-Hughes *et al.*, 1992). Furthermore, circumstantial evidence suggested previously that this presumed global regulatory protein may somehow play a role in regulation of the expression of certain σ^S-dependent genes (Kolter and Moreno, 1992; Olsén *et al.*, 1993). In particular, it was proposed that a subset of σ^S-dependent genes, if not all, are derepressed in an *hns* background (Arnqvist *et al.*, 1994).

First of all, *E.coli* cells carrying a *cbpA−lacZ* transcriptional fusion were grown in M9 minimal medium and then β-galactosidase activity was monitored (Figure 1). It should be noted here that we used M9 minimal medium, unless otherwise noted, in order to obtain unified results throughout a series of experiments. In the wild-type background, the expression of *cbpA* was not induced in any growth phase (Figure 1, open circles), as mentioned above. In an insertional inactivation *hns* mutant (*hns::neo*)

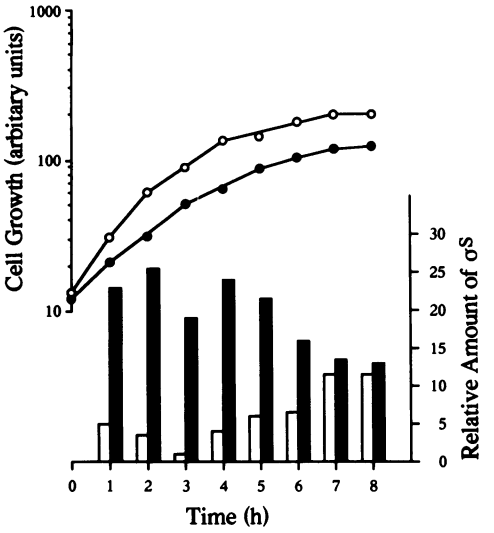

Fig. 2. Cellular content of σ^S. MC4100 (open symbols) and MC4100 *hns::neo* (closed symbols) were grown at 37°C in M9 minimal medium containing thiamine (2 μg/ml), glucose (0.2%) and appropriate amino acids (isoleucine, leucine and valine, 20 μg/ml each). The cell growth was monitored by measuring turbidity at 660 nm (circles). At the times indicated, total cellular proteins were prepared by precipitation with trichloroacetic acid. The samples (10 μg of protein) were subjected to Western blot analysis with an anti-σ^S antiserum. The bands detected on the filter were quantified by densitometric scanning. The amounts of σ^S are expressed relative to that detected for MC4100 cells at 3 h, the value of which was taken as one (bars).

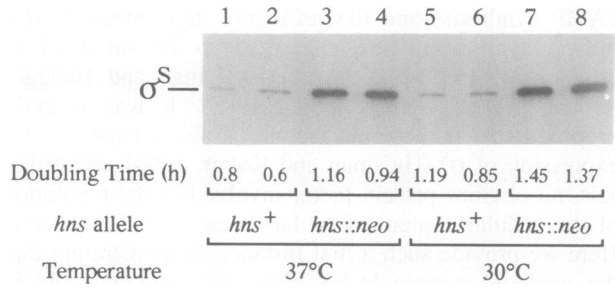

Fig. 3. Cellular content of σ^S of cells grown under different growth conditions. MC4100 (lanes 1, 2, 5 and 6) and MC4100*hns::neo* (lanes 3, 4, 7 and 8) were grown in M9 minimal medium containing thiamine (2 μg/ml) and glucose (0.2%) at either 30 or 37°C. Certain amino acids were further added at 20 μg/ml each (lanes 1 and 5, no supplementation; lanes 2, 3, 6 and 7, isoleucine, leucine and valine; lanes 4 and 8, all 20 amino acids). Total proteins were prepared by precipitation with trichloroacetic acid in the mid-logarithmic growth phase. Samples (10 μg of protein) were subjected to Western blot analysis. Growth rate of cells was estimated by determining the doubling time, as indicated in the figure.

background, however, it was found that the expression of *cbpA* in the minimal medium was strongly enhanced throughout the growth phases (closed circles), although the level decreased slightly in the stationary phase. This enhanced expression of *cbpA* in the *hns::neo* mutant was suggested to be largely dependent on the function of σ^S, since the level of expression of *cbpA* was remarkably reduced in a *hns::neo/rpoS*::Tn*10* background (closed triangles). Essentially the same phenomenon as was observed for the minimal medium was seen when the cells were grown in a rich medium, i.e. the *cbpA* gene was remarkably expressed in Luria broth even in the logarithmic growth phase, as well as in the stationary phase (data not shown; see Yamashino *et al.*, 1994). From these results, it was suggested that H-NS may be involved in the regulation of σ^S-dependent expression of *cbpA*, either directly or indirectly.

Cellular content of σ^S increases in the hns mutant

To explore the above phenomenon further, we examined the cellular content of σ^S in both the wild-type and *hns::neo* background by means of Western blot analysis with an anti-σ^S antiserum. Strain MC4100 (wild-type) and its *hns::neo* derivative were grown in M9 minimal medium and total cellular proteins were prepared at intervals (Figure 2). Each sample was subjected to analysis. In the wild-type cells, the amount of σ^S increased when the cells had entered the stationary phase (Figure 2, open symbols). This is consistent with previous results with regard to the growth phase-dependent occurrence of σ^S (Gentry *et al.*, 1993; Tanaka *et al.*, 1993). In the *hns::neo* mutant cells, however, σ^S accumulated to a significantly higher level even in the logarithmic growth phase (Figure 2, closed symbols). The content of σ^S in the mid-logarithmic growth phase was ~10-fold higher in the *hns::neo* mutant than in the wild-type. The amount of σ^S decreased gradually to more or less the same level as in the wild-type in the stationary phase. In this regard, enhanced accumulation of σ^S in the *hns::neo* background also occurred when the cells were grown in Luria broth (data not shown). It was thus revealed that in the *hns::neo* background, the cellular content of σ^S somehow markedly increases, particularly in the logarithmic growth phase. Comparison of the results in Figures 1 and 2 showed that they are compatible with the idea that accumulation of σ^S in the *hns::neo* mutant results in enhanced expression of *cbpA* (note a good correlation between the cellular content of σ^S and the level of expression of *cbpA*).

H-NS participates in regulation of the cellular content of σ^S

The *hns::neo* mutant used here showed a reduced growth rate relative to the wild-type (see Figure 2). We therefore considered the possibility that the observed accumulation of σ^S in the *hns::neo* mutant was secondary to differences in growth rate. To examine this possibility, we determined the contents of σ^S in cells grown under various growth conditions under which the cells grew at relatively higher or lower growth rates. In order to vary the growth rate, both the wild-type and *hns::neo* mutant cells were grown in M9 minimal medium with or without supplementation with amino acids or at different growth temperatures. As shown in Figure 3, regardless of the growth rate (or the growth conditions), the amount of σ^S was always higher in the *hns::neo* mutant cells than in the wild-type ones. Thus, the possibility mentioned above appears to be unlikely.

In order to further confirm the H-NS-dependent phenomenon in question, the *hns::neo* mutant cells were grown in a minimal medium in which either glucose or phosphate was limited (Figure 4). Under both the growth conditions tested, enhanced accumulation of σ^S was again observed for the *hns::neo* mutant. We thus concluded that a mutational lesion in the *hns* gene results in stable

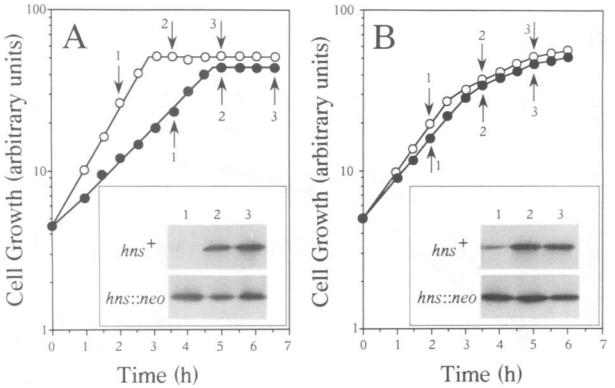

Fig. 4. Cellular content of σ^S under nutrient starvation conditions. Both MC4100 (open circles) and MC4100*hns*::*neo* (closed circles) were grown in glucose-limited M9 minimal medium containing thiamine (2 μg/ml), glucose (0.045%) and appropriate amino acids (isoleucine, leucine and valine, 20 μg/ml each) (**A**) or TG medium containing 0.064 mM KH_2PO_4 (**B**). At the times indicated, total proteins were precipitated with trichloroacetic acid and then subjected to Western blot analysis (10 μg of protein) (insets in the panels).

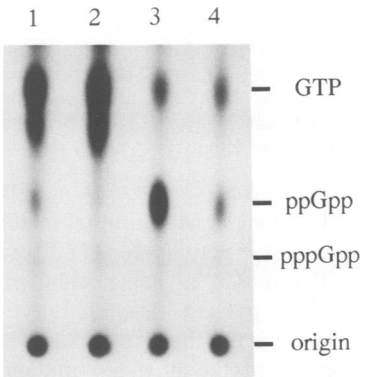

Fig. 5. Cellular content of ppGpp. MC4100 (lanes 1 and 3) and MC4100*hns*::*neo* (lanes 2 and 4) were grown in TG medium containing 0.2 mM KH_2PO_4. Glucose was added at either 0.4 (lanes 1 and 2) or 0.04% (lanes 3 and 4). ^{32}P-labelled nucleotides were analysed by thin-layer chromatography on poly(ethylene)imine–cellulose plates, followed by autoradiography. Other details are given under Materials and methods. Positions of GTP, ppGpp and pppGpp are indicated.

accumulation of σ^S, regardless of the growth conditions for *E.coli* cells.

It was recently proposed that ppGpp may function as a positive signal leading to the accumulation of σ^S (Gentry *et al.*, 1993). Thus, we needed to examine whether or not ppGpp is somehow accumulated in the *hns*::*neo* mutant, which may consequently result in the accumulation of σ^S. Both the wild-type and *hns*::*neo* mutant cells were pulse-labelled with [^{32}P]orthophosphate in TG medium in the logarithmic growth phase. Labelled nucleotides were separated by thin layer chromatography, followed by autoradiography. ppGpp did not accumulate in the *hns*::*neo* mutant, but rather slightly decreased, as compared with the wild-type (Figure 5). When essentially the same pulse-labelling experiment was carried out for cells grown in a glucose-limited minimal medium, ppGpp clearly increased in the wild-type cells, as has been documented previously (Cashel and Rudd, 1987). The same was true for the *hns*::*neo* mutant, although it was observed that the amount

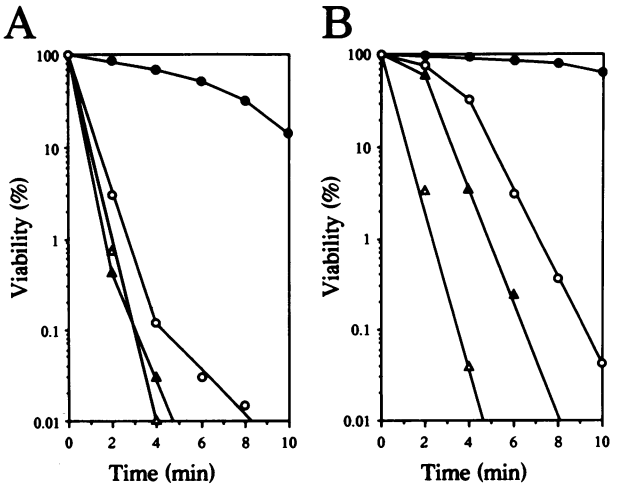

Fig. 6. Thermotolerance of the *hns*::*neo* deletion mutant. Strains were grown at 37°C in M9 minimal medium containing thiamine (2 μg/ml), glucose (0.2%) and appropriate amino acids (isoleucine, leucine and valine, 20 μg/ml each). Cells were harvested either in the mid-logarithmic growth pahse (**A**) or in the stationary phase (overnight culture) (**B**). They were diluted appropriately with saline (0.84% NaCl) and then treated at 55°C for the times indicated. Portions were taken at intervals and then spread on Luria agar, followed by incubation at 37°C for 24 h. Viable colonies were scored and the values were expressed relative to that of each control culture without heat treatment. Strains used were: MC4100 (wild-type; open circles), MC4100*hns*::*neo* (*hns*::*neo*, closed circles), KT1100 (*rpoS*::Tn*10*; open triangles) and MC4100*hns*::*neo rpoS* (*hns*::*neo*/*rpoS*::Tn*10*; closed triangles).

of ppGpp was significanly smaller in the *hns*::*neo* mutant than in the wild-type. We do not know at present why the level of ppGpp is lower in *hns*::*neo* cells than in wild-type ones. In any event, when considering the proposed view that ppGpp functions as a positive signal leading to the accumulation of σ^S, our observations suggest that the enhanced accumulation of σ^S in the *hns*::*neo* background cannot simply be accounted for by accumulation of ppGpp.

These results together are compatible with the idea that H-NS is deeply involved in regulation of the cellular content of σ^S, thereby resulting in the accumulation of σ^S in a growth phase-independent manner in the *hns*::*neo* background. In other words, in the wild-type background, H-NS may function as a negative factor controlling the cellular content of σ^S, particularly in the logarithmic growth phase.

Physiological relevance of the accumulation of σ^S in the hns mutant

It is well known that when *E.coli* cells enter the stationary phase, they become more tolerant to several stressful conditions, such as high temperature (>50°C) and high concentrations of H_2O_2 and NaCl (osmotic solute) in a given growth medium (Siegele and Kolter, 1992; Hengge-Aronis, 1993). In this context, a set of gene products whose expression is dependent on σ^S is crucially responsible for such a phenomenon. If the growth phase-independent accumulation of σ^S we observed in this study is indeed physiologically relevant, then one could expect that the *hns*::*neo* mutant cells should be tolerant to stressful conditions even in the logarithmic growth phase. We examined this possibility and demonstrated that it is indeed the case (Figure 6). Various cell types (wild-type, *hns*::*neo*,

rpoS::Tn*10* and *hns*::*neo*/*rpoS*::Tn*10*) growing rapidly in M9 minimal medium were exposed to high temperature (55°C). At intervals, portions of the cultures were taken and grown on plates at the permissive temperature (37°C). The exponentially growing *hns*::*neo* mutant cells are extremely thermotolerant as compared with the wild-type cells. The *hns*::*neo*/*rpoS*::Tn*10* double mutant was highly sensitive to the high temperature treatment, suggesting that the thermotolerance observed for the *hns*::*neo* mutant was dependent on the function of σ^S. On the other hand, when overnight cultivated cells were subjected to the same experiment, the wild-type cells also became more tolerant to the heat treatment, as expected (Figure 6). The *hns*::*neo* mutant cells were much more thermotorelant in the stationary growth phase. Under these stationary conditions, it was also found that the *hns*::*neo*/*rpoS*::Tn*10* mutant cells were significantly more tolerant to the stress than the *rpoS*::Tn*10* cells were. This can be explained by assuming that certain gene products whose expression is affected by H-NS, but not by σ^S, may also be partially responsible for the observed thermotolerance. In any event, the result strongly suggests that the accumulation of σ^S in the *hns*::*neo* background is physiologically relevant and also that σ^S protein accumulated in the logarithmic growth phase is active from a functional point of view.

Transcription of the rpoS gene in the hns mutant

We then attempted to elucidate the mechanism underlying the accumulation of σ^S in the *hns*::*neo* background. H-NS is known to function as a negative regulator for the expression of certain genes at the transcriptional level (Göransson *et al.*, 1990; Hulton *et al.*, 1990; Ueguchi and Mizuno, 1993). Thus, the most simple explanation would be that H-NS functions directly as a negative regulator for transcription of the *rpoS* gene, whereby σ^S can accumulate in the *hns*::*neo* mutant cells. To examine this possibility, we constructed an *rpoS* promoter–*lacZ* transcriptional fusion on the chromosome. The *rpoS* gene is known to be transcribed from four distinct promoters, named P1–P4. Among them, P2 was demonstrated to be induced in a rich medium in the stationary growth phase (Takayanagi *et al.*, 1994). A 1.4 kb *Cla*I–*Dra*I fragment encompassing all of these four promoters was connected upstream of a promoterless *trpA'*–*'lacZ* gene on pMS434HS (Hirano *et al.*, 1987) and then the resultant construct was transferred into the chromosome using a λ phage vector (see Materials and methods). Such a lysogen carrying the *rpoS*–*lacZ* transcriptional fusion gene was constructed in both the wild-type and *hns*::*neo* backgrounds and designated GY29 and GY30, respectively. These strains were grown in M9 minimal medium and β-galactosidase activity was monitored (Figure 7). In the wild-type (GY29), the transcription of *rpoS* was considered to be more or less constitutive, although the level was relatively low. This result is roughly consistent with previous ones (Lange and Hengge-Aronis, 1991a; McCann *et al.*, 1993). In the *hns*::*neo* mutant (GY30), the β-galactosidase activity was slightly higher (~2-fold) than that in GY29 in the logarithmic growth phase and gradually decreased to a similar level to that observed in the wild-type in the stationary growth phase. We also conducted essentially the same experiment with the use of glucose-limited minimal medium and obtained essentially the same

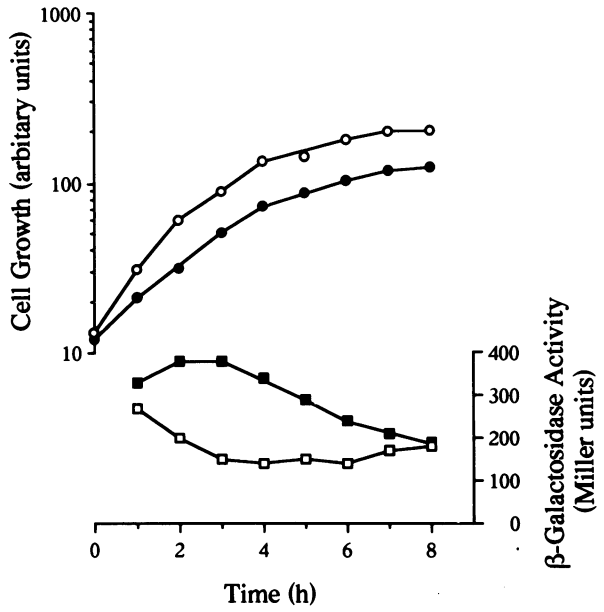

Fig. 7. β-galactosidase activity expressed by the *rpoS*–*lacZ* operon fusion. GY29 (*hns*$^+$; open symbols) and GY30 (*hns*::*neo*; closed symbols), both carrying the *rpoS*–*lacZ* operon fusion on the chromosome, were grown at 37°C in M9 minimal medium containing thiamine (2 μg/ml), glucose (0.2%) and appropriate amino acids (isoleucine, leucine and valine, 20 μg/ml each). Both the cell growth (circles) and β-galactosidase activity (rectangles) were monitored.

results as described for the M9 minimal medium (e.g. the level of β-galactosidase activity for GY30 was only 1.5-fold higher than that for GY29; data not shown). Assuming that the level of β-galactosidase activity reflects the transcription of *rpoS*, the remarkable (>10-fold) accumulation of σ^S in the *hns*::*neo* mutant is hard to explain by a change in the level of *rpoS* transcription (see Figures 1 and 2). In order to gain an insight into the mechanism in question, therefore, regulation at the post-transcriptional level must be considered, as addressed below.

Enhanced translation of σ^S mRNA in the hns mutant

In general, post-transcriptional regulation of the expression of certain genes may be attributed to changes in either the stability or the translational efficiency of mRNA or the stability of the gene product. We thus examined each possibility to further account for the enhanced accumulation of σ^S in the *hns*::*neo* mutant.

First, we determined the level of *rpoS* mRNA. Total cellular RNAs were extracted from cells grown in M9 minimal medium in the logarithmic growth phase. They were then subjected to Northern hybridization analysis. It was revealed that the amounts of *rpoS* mRNA determined for both the wild-type and *hns*::*neo* mutant cells were comparable (Figure 8A). Keeping this fact in mind, we next examined the translational efficiency of *rpoS* mRNA by means of pulse-labelling with [^{35}S]methionine (Figure 8B). Rapidly growing cells in M9 minimal medium were pulse-labelled for 2 min and then proteins were subjected to immunoprecipitation with an anti-σ^S antiserum, followed by SDS–PAGE and fluorography. It was found that σ^S was synthesized at a remarkably higher rate in the

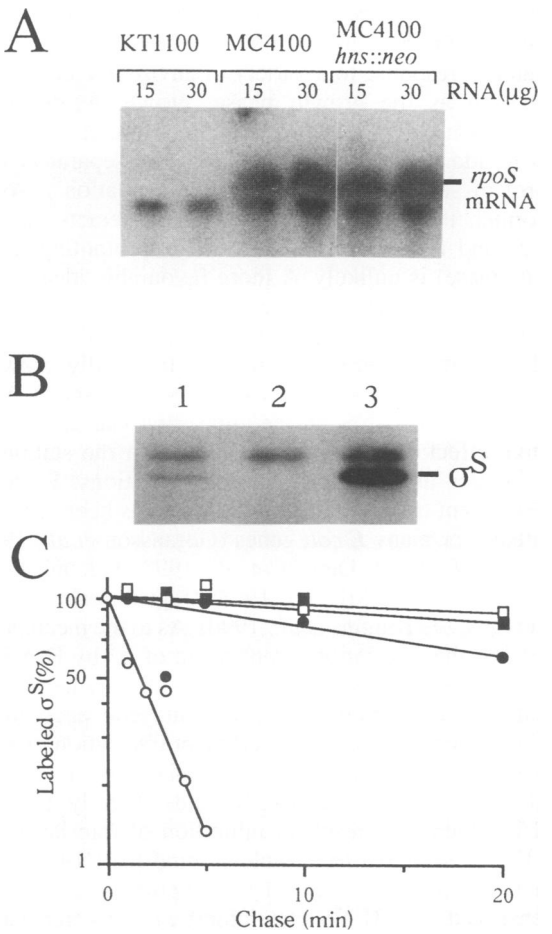

Fig. 8. Accumulation of σ^S mRNA, its translational efficiency and the stability of σ^S. (**A**) Strains were grown at 37°C in M9 minimal medium containing thiamine (2 μg/ml), glucose (0.2%) and appropriate amino acids (isoleucine, leucine and valine, 20 μg/ml each). In the mid-logarithmic growth phase, total RNA was extracted from the harvested cells. Indicated amounts of these RNA samples were applied onto a formaldehyde–agarose gel and then subjected to Northern blot analysis with a randomly radiolabelled 841 bp *Stu*I fragment as a probe (see Materials and methods). Samples analysed were from KT1100 (*rpoS*::Tn*10*), MC4100 and MC4100 *hns*::*neo*, as indicated. (**B**) Strains were grown as described above. In the mid-logarithmic growth phase, 0.3 ml of culture was pulse-labelled with [^{35}S]methionine for 2 min. Labelled proteins were immunoprecipitated with an anti-σ^S antiserum and analysed by SDS–PAGE, followed by fluorography. Lane 1, MC4100; lane 2, KT1100 (*rpoS*::Tn*10*); lane 3, MC4100*hns*::*neo* (*hns*::*neo*). The bands corresponding to σ^S are indicated (the bands seen just above σ^S appear to be contaiminants). (**C**) MC4100 (open symbols) and MC4100*hns*::*neo* (closed symbols) were grown as described above. In either the mid-logarithmic growth phase (circles) or the stationary phase (rectangles), 0.9 ml of cultures were labelled with [^{35}S]methionine for 1 (for the former case) or 2 (for the latter case) min and then chased with non-radioactive methionine. At the intervals indicated, portions (0.2 ml) were taken and the amount of labelled σ^S was analysed as described under Materials and methods. The amount of labelled σ^S was expressed relative to that determined for the sample without chase.

hns::*neo* mutant than in the wild-type (estimated to be ~15-fold by comparing lanes 1 and 3). Essentially the same experiment was repeated with shorter labelling times (1 min and 30 s; note that these periods are shorter than the determined half-life of the σ^S protein, see below), and essentially the same results were obtained (data not shown). Considering the fact that the cellular content of *rpoS* mRNA is nearly the same in both the wild-type and

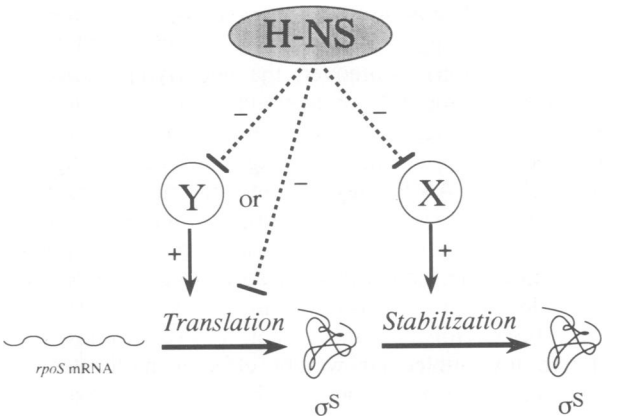

Fig. 9. Possible mechanisms by which H-NS affects the translational efficiency and stability of σ^S. As demonstrated in this study, H-NS affects both the translational efficiency and stability of σ^S. Plausible mechanisms underlying such effects exerted by H-NS are shematically shown. For details, see Discussion.

hns::*neo* backgrounds (see Figure 8A), it is suggested that the translational efficiency of *rpoS* mRNA is notably enhanced in the *hns*::*neo* background.

The rpoS gene product is more stable in the hns mutant

We also examined the fate of the *rpoS* gene product in the *hns*::*neo* background. Rapidly growing cells in M9 minimal medium were pulse-labelled for 1 min with [^{35}S]methionine and then chased with cold methionine. The fate of the labelled σ^S was determined by means of immunoprecipitation, followed by SDS–PAGE and fluorography. The half-life of σ^S was estimated by densitometric quantification of the labelled σ^S on the fluorogram (Figure 8C). It was revealed that the half-life of σ^S in the *hns*::*neo* mutant was estimated to be much (>10 times) longer than in the wild-type. The short half-life of σ^S (~2 min) observed for rapidly growing wild-type cells was consistent with the previous results of Takayanagi *et al.* (1994). The fate of σ^S in cells which had fully entered the stationary growth phase was also analysed by 2 min pulse-labelling (Figure 8C). It was confirmed that σ^S becomes very stable in stationary phase cells in the wild-type, as has been reported previously (Takayanagi *et al.*, 1994).

From these results, we suggest that the *hns*::*neo* mutation results in not only enhanced translation of *rpoS* mRNA, but also enhanced stabilization of the product, σ^S, particularly in rapidly growing cells, thereby resulting in marked accumulation of σ^S even in the logarithmic growth phase. It should be emphasized that in the wild-type background such a situation is attained only at the onset of the stationary phase. From these findings (Figures 7–9), we can now reasonably explain how the *hns*::*neo* mutation results in a marked increase in the cellular content of σ^S in the logarithmic growth phase (Figure 2) and thereby we can also explain why σ^S-dependent *cbpA* expression is remarkably enhanced in the *hns*::*neo* background (Figure 1).

Discussion

Several lines of previous study suggested that the mechanism(s) underlying the quantitative control of σ^S under

various growth conditions appears to be very complex (Lange and Hengge-Aronis, 1994), as mentioned above (see Introduction). Moreover, the underlying molecular mechanism is not fully understood. Lange and Hengge-Aronis (1991a) reported that transcription of *rpoS* was increased in a *cya* deletion background, suggesting that the level of cAMP may be an important parameter. However, recent results of McCann *et al.* (1993) were not consistent with this idea. It was also proposed that ppGpp and homoserine lactone (or its derivatives) are positive signals leading to enhanced accumulation of σ^S (Gentry *et al.*, 1993; Huisman and Kolter, 1994). Besides these putative low molecular weight effector molecules, no protein factor has been reported to be involved in regulation of the cellular content of σ^S. In this study we provide evidence that H-NS plays an important role in the mechanism underlying regulation of the cellular content of σ^S, particularly at the post-transcriptional level.

In the wild-type background, the cellular content of σ^S increases only when cells encounter certain environmental conditions, as mentioned above (Figures 2 and 4). In the *hns::neo* mutant, however, σ^S is accumulated even in rapidly growing cells (i.e. regardless of growth conditions), under which conditions the content of σ^S is normally very low (Figures 2 and 4). The observed accumulation of σ^S in the *hns::neo* mutant is not a secondary effect due to differences in growth conditions and growth rates, as demonstrated (Figures 3 and 4). In general, H-NS is thought to function as a negative regulator for expression of certain genes, either directly or indirectly (Hulton *et al.*, 1990; Ueguchi and Mizuno, 1993). In the case of the *rpoS* gene, however, the effect of H-NS on transcription of *rpoS* mRNA appears to be minimal, if any (Figures 7 and 8A). Rather, the marked increase in σ^S in the *hns::neo* mutant was demonstrated to result from changes in both the translational efficiency of *rpoS* mRNA and the stability of σ^S (Figure 8B and C). However, the presumed involvement of ppGpp appears unlikely (Figure 5). These results are most simply explained by assuming that H-NS is deeply, if not directly, involved as a negative regulator in regulation of the cellular content of σ^S, whose effect is relieved in response to environmental conditions (e.g. upon entry into the stationary phase and/or upon nutrient starvation). In any case, it should be emphasized that H-NS is the first protein factor that has been clearly demonstrated to be involved in regulation of the cellular content of σ^S, as far as we know.

As described above, the mechanism(s) underlying the quantitative control of σ^S under various growth conditions appears to be very complex (see Introduction). Recent comprehensive studies addressing the relevant issues by Lange and Hengge-Aronis (1994) and Takayanagi *et al.* (1994) demonstrated that *rpoS* and/or σ^S expression is not only transcriptionally controlled, but is also extensively regulated at the levels of translation and protein stability. In their studies, H-NS was not taken into consideration. In this study, however, we propose that this nucleoid protein must be deeply implicated in the molecular mechanism(s) underlying the quantitative control of σ^S. How is H-NS able to control both the translational efficiency of *rpoS* mRNA and the stability of σ^S? At present we do not know the underlying molecular mechanism(s). However, several explanations can be envisaged, based on the well-documented fact that H-NS is a DNA binding protein that can function as a global transcriptional regulator in response to a variety of environmental conditions, such as the growth phase, growth temperature, medium osmolarity and medium pH. Here it would be better to address the following two issues separately (i.e. 'control of stability' and 'control of translation'). As to the former, the simple idea that H-NS interacts directly with σ^S and functions as a degradation promoting factor (e.g. protease) is unlikely. A more favourable idea is that H-NS may negatively regulate the transcription of a gene encoding a putative factor which is responsible for the stability of σ^S (Figure 9, factor X). In rapidly growing cells, expression of factor X is severely repressed through the function of H-NS at the transcriptional level, this negative effect of H-NS being relieved in the stationary phase and/or under certain growth conditions. Such H-NS-dependent transcriptional regulation has been reported repeatedly for many *E.coli* genes (Göransson *et al.*, 1990; Hulton *et al.*, 1990; Dersch *et al.*, 1993; Falconi *et al.*, 1993; Ueguchi and Mizuno, 1993; Ueguchi *et al.*, 1993; for a review, see Higgins *et al.*, 1990). As to the mechanism underlying the translational repression of σ^S by H-NS, at least two explanations are possible. H-NS may negatively regulate the transcription of a certain gene encoding a putative factor involved in the efficient translation of *rpoS* mRNA (Figure 9, factor Y). Alternatively, it is also possible to imagine that H-NS binds directly to *rpoS* mRNA, which may result in inhibition of translation. In fact, H-NS is known to be able to bind to RNA as well as to DNA (Lammi *et al.*, 1984). Spurio *et al.* (1992) demonstrated that H-NS can inhibit *in vitro* translation when poly(U) and phage MS2 RNA are used as templates. We are currently examining this possibility. These principal ideas with regard to how H-NS is possibly involved in the quantitative control of σ^S are schematically summarized in Figure 9, although more complicated explanations can be proposed.

Finally, it should be noted that in this study we did not emphasize another intriguing phenomenon, which can be seen in Figures 1 and 2. That is, in the wild-type background, the level of σ^S in the stationary phase appears to be high enough to result in induction of the *cbpA* gene. Nevertheless, induced expression of *cbpA* could not be seen. In the *hns::neo* background, however, expression of *cbpA* was fully induced, while the level of σ^S in the *hns::neo* cells was more or less the same as that in the wild-type cells. These observations suggest that the occurrence of σ^S does not necessarily result in the induction of σ^S-dependent genes. In other words, it is suggested that not only the quantity of σ^S (i.e. abundant or not), but also the state of σ^S (i.e. functional or not) are under the control of certain physiological conditions in *E.coli*. In this context, our observations strongly suggest that H-NS is involved not only in the quantitative control of σ^S, but also in the qualitative control of σ^S function (see Figure 2). Clarification of this interesting issue must await further experimentation.

Materials and methods

Bacterial strains and media

E.coli strains used in this study were all derivatives of MC4100 [*Δ(argF-lac)U169 araD rpsL relA flbB deoC ptsF rbsR*] (Casadaban, 1976). An

insertional inactivation *hns* mutant (*hns::neo*), named MC4100*hns::neo*, was constructed previously (Yamada *et al.*, 1991). A *rpoS*::Tn*10* insertional inactivation mutant, named KT1100, was kindly provided by Dr K.Tanaka (Tokyo University). An *hns::neo rpoS*::Tn*10* double mutant was constructed by means of P1 transduction, in which KT1100 and MC4100*hns::neo* were used as donor and recipient strains respectively. Strain GY4 carrying a *cbpA−lacZ* operon fusion, which is a derivative of MC4100 (Yamashino *et al.*, 1994), was constructed previously. GY8 and GY12 are *hns::neo* and *rpoS*::Tn*10* derivatives respectively of GY4, whereas GY14 is a *hns::neo/rpoS*::Tn*10* derivative. Strain GY29 carrying an *rpoS−lacZ* operon fusion on the chromosome was constructed in this study (see below). GY30 is an *hns::neo* derivative of GY29. Cells were mainly grown in M9 minimal medium (Miller, 1972) supplemented with thiamine (2 μg/ml), glucose (0.2%) and certain amino acids (isoleucine, leucine and valine, 20 μg/ml each). TG medium contains 120 mM Tris−HCl, 0.2% glucose, 2 μg/ml thiamine and certain amino acids (isoleucine, leucine and valine, 20 μg/ml each), in addition to the following salts at the appropriate concentrations; NaCl, KCl, NH_4Cl, Na_2SO_4, $MgCl_2$, $CaCl_2$, $FeCl_3$ and KH_2PO_4. It should be noted that the *hns::neo* mutant used in this study requires the branched chain amino acids for better growth in minimal media, as shown previously (Yamada *et al.*, 1991).

Construction of an rpoS−lacZ operon fusion on the chromosome

Construction of an *rpoS−lacZ* operon fusion on the chromosome of MC4100 was carried out essentially according to the method developed by Hirano *et al.* (1987). Briefly, a 1.6 kb *ClaI−DraI* fragment encompassing all four presumed *rpoS* promoters was purified from pKTF111 (Takayanagi *et al.*, 1994). After treatment with T4 DNA polymerase, this fragment was inserted into the previously blunt-ended *Hin*dIII site of pMS434HS. The resultant plasmid carrying an *rpoS−lacZ* operon fusion gene was designated pGY11. An *E.coli* strain harbouring λpF13 was transformed with pGY11. The phage lysate was prepared from the transformant by UV irradiation. MC4100 was infected with the phage lysate and then blue plaques were selected on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as candidates for lysogens carrying the *rpoS–lacZ* operon fusion. Lysogens thus purified were further scored for the Lac^+ phenotype, then one such lysogen, named GY29, was used in this study.

Assay of β-galactosidase activity

Assay of β-galactosidase activity was carried out according to the method of Miller (1972).

Western blot analysis

Western blot analysis with a polyclonal antiserum raised against σ^S was carried out as described previously (Ueguchi *et al.*, 1993). This antiserum was a gift from Dr Tanaka (Tokyo University). Protein concentration was accurately determined for each sample using a Micro BCA Protein Assay Reagent Kit (Pierce, USA). Immunoblot data was quantitatively analysed with a double beam densitometer (CS-9000; Shimazdu Co. Ltd).

Northern blot analysis

Total RNA was prepared by the hot phenol method (Aiba *et al.*, 1981). Other details are essentially as described by Sambrook *et al.* (1989). A 0.84 kb *StuI* fragment encompassing a coding region of the *rpoS* gene, isolated from pKTF101, was used as a probe, which was afterwards labelled with [α-^{32}P]dCTP (110 TBq/mmol; Amersham International) using a random primer labelling kit (Amersham International).

Measurement of ppGpp in cells

Cells were grown in TG medium (Echols *et al.*, 1961) containing 0.2 mM KH_2PO_4. Glucose was added to final concentrations of 0.4 and 0.04% for standard and glucose starvation growth conditions respectively. In early logarithmic growth phase, [^{32}P]orthophosphate (370 MBq/ml; Amersham International) was added (50 μCi/ml). After 2 (for normal growth conditions) or 3 (for glucose starvation conditions) h incubation, labelled nucleotides were extracted from cells and then analysed by thin-layer chromatography on poly(ethylene)imine−cellulose plates according to the method of Bochner and Ames (1982).

Pulse-labelling and pulse–chase experiments of σ^S

Cells were grown in M9 minimal medium containing thiamine (2 μg/ml), glucose (0.2%) and appropriate amino acids (isoleucine, leucine and valine, 20 μg/ml each). Pulse-labelling was initiated by adding 10 μCi [^{35}S]methionine (37 TBq/mmol; Amersham International) to 0.3 ml of culture. After 2 min labelling, cells were immediately treated with trichloroacetic acid (final concentration 5%). For pulse–chase experiments, 0.9 ml of culture was labelled with 40 μCi [^{35}S]methionine. After 1 (for cells in the logarithmic growth phase) or 2 (for cells at the stationary phase) min labelling, non-radioactive methionine was added to a final concentration of 0.2 mg/ml. Portions (0.2 ml) were taken at intervals and total proteins were precipitated with trichloroacetic acid (final concentration 5%) by incubation for 15 min on ice. Precipitates were collected (10 000 *g* for 3 min) and then washed with ice-cold acetone and finally dissolved in a solution containing 1% SDS, 50 mM Tris−HCl (pH 8.1), 1 mM EDTA. These samples were subjected to immunoprecipitation with an anti-σ^S antiserum, according to the method of Ito *et al.* (1981) with a slight modification, namely 0.1% Lubrol PX (a non-ionic detergent) was used instead of Triton X-100. Labelled σ^S was analysed on SDS−10% polyacrylamide gels (Laemmli, 1970), followed by fluorography (Chamberlain, 1979). Densitometric analysis of the fluorograms was carried out by a double beam densitometer (CS-9000; Shimadzu Co. Ltd)

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