NOTES

Modes of Regulation of RpoS by H-NS†

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Regulated degradation of RpoS requires RssB and ClpXP protease. Mutations in *hns* increase both RpoS synthesis and stability, causing a twofold increase in synthesis and almost complete stabilization of RpoS, independent of effects on synthesis and independent of phosphorylation of RssB. This suggests that H-NS regulates an RssB inhibitor or inhibitors.

RpoS, a stress or stationary-phase sigma factor (σ^S), is regulated at multiple levels, including transcriptional regulation, translation by multiple small RNAs, and protein stability (for reviews, see references 8 and 12). One of the major regulators of RpoS turnover is RssB (SprE), a response regulator. RssB is necessary for degradation of RpoS, which usually occurs rapidly during exponential growth and slows down during stationary phase or stress responses (for a review, see reference 8). RssB can be phosphorylated by acetyl phosphate in vitro (2). The phosphorylated form of RssB binds RpoS with high affinity and presents it to the ATP-dependent ClpXP protease for degradation in vitro (23). The level of RssB is low and may be limiting for proteolysis under some conditions (18, 19). The specific sensor kinase(s) or phosphatase(s) that can phosphorylate or dephosphorylate RssB has not been identified.

H-NS is an abundant nucleoid-associated protein. The major role of H-NS is as a global transcriptional repressor for a large number of genes (for a review, see reference 5). Surprisingly, H-NS affects both RpoS mRNA translation and RpoS turnover in logarithmic growth; it was previously reported that there is a 10-fold increase in the RpoS synthesis rate, as well as a 10-fold increase in RpoS stability, in *hns* mutants (1, 22). The work described here was undertaken to ask whether these two effects are linked, for instance, by increased synthesis leading to inefficient degradation by swamping (titrating) the degradation machinery. We found that *hns* mutants have a strong effect on RpoS turnover, independent of any effect on RpoS synthesis, contrary to the titration model. The effect of *hns* mutants on RpoS degradation is an effect on RssB activity, leading to RpoS stabilization.

RpoS stability is increased dramatically in an *hns* mutant. To confirm the involvement of H-NS in RssB-mediated RpoS degradation, isogenic strains carrying two different translational fusions of RpoS-LacZ were used. One fusion is a “long fusion,” RpoS₇₅₀::LacZ, carrying 250 amino acids of RpoS, including the RssB interaction site at amino acid lysine-173;

this fusion protein is subject to RssB-dependent ClpXP degradation (24). The other fusion, a “short fusion,” RpoS₄₇₇::LacZ, carries the same upstream region but only 159 amino acids of RpoS; this fusion protein is stable (E. Massé, unpublished data). Cells were grown in LB medium at 37°C, and samples were taken. There was a 12-fold increase in β -galactosidase activity with the long fusion in an *hns* mutant (Table 1). However, the activity of β -galactosidase with the short fusion was increased only twofold in the *hns* cells (Table 1). This result suggests that there was a strong (sixfold) increase in stability and a modest (twofold) increase in synthesis. Consistent with a twofold increase in RpoS synthesis in the *hns* mutant, the same twofold increase was seen in the long RpoS₇₅₀::lacZ fusion in an *rssB hns* double mutant compared to an *rssB* mutant (Table 1).

***hns* effects on RpoS turnover are independent of synthesis signals.** It has been suggested that changes in RpoS levels may be sufficient to lead to titration of RssB under some growth conditions (18). While we saw only a twofold effect of *hns* mutants on RpoS synthesis, we further checked for the effect of *hns* mutants on RpoS turnover in the absence of the normal synthesis signals. RpoS was cloned under pBAD transcriptional control, deleting the normal signals for transcriptional and translational regulation. Two other control proteins, green fluorescent protein (GFP)-SsrA, which is also a substrate of ClpXP (10, 20) but does not require RssB, and LacZ, which is not a substrate of ClpXP, were also cloned under pBAD control. Isogenic *hns*⁺ and *hns* strains carrying a chromosomal mutation inactivating *rpoS* and carrying plasmid pBAD-RpoS or pBAD-LacZ were grown in LB medium at 37°C in the absence of inducer. Under these conditions, the amount of the RpoS was slightly less than the amount from a chromosomal single-copy gene (data not shown). The half-life of the proteins expressed from pBAD was determined by a spectinomycin chase experiment. Cells were grown to an optical density at 600 nm of 0.4 to 0.45, spectinomycin (final concentration, 100 μ g/ml) was added to the cultures to inhibit further protein synthesis, and samples were removed and analyzed by Western blotting. Consistent with the accumulation of the RpoS₇₅₀::LacZ fusion protein (Table 1), the RpoS half-life was increased 10-fold (to 20 min or more) in an *hns* mutant compared to the 2-min half-life in a wild-type strain (Fig. 1A),

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TABLE 1. Accumulation of RpoS-lacZ in *hns* mutants

Strain or mutation(s)	β-Galactosidase activity	
	Long fusion ^a	Short fusion ^b
Wild type	0.16 ± 0.07	0.89 ± 0.11
<i>hns</i>	1.91 ± 0.11	1.58 ± 0.025
<i>rssB</i>	1.02 ± 0.10	ND
<i>hns rssB</i>	1.93 ± 0.01	ND

^a Cells were grown in LB medium at 37°C, and β-galactosidase was assayed as previously described (24). Units are arbitrary specific activity machine units about 25-fold lower than Miller units. This fusion protein is subject to RssB-dependent degradation. The strains used were strains SG30013 (wild-type, RpoS₇₅₀::LacZ), SG30059 (*hns::kan*), SG30018 (*rssB::tet*), and YN534 (*hns::kan rssB::tet*), all derivatives of SG30013 (see Table S1 in the supplemental material).

^b Cells were grown as described in footnote *a*. The fusion protein is stable. The strains used were strains EM1246 (wild-type, RpoS₄₇₇::LacZ) and YN714 (*hns::kan*), a derivative of EM1246. ND, not determined.

which is consistent with previous reports (1, 22). As expected, LacZ was stable with a half-life of more than 20 min in both wild-type and *hns* strains; the amount of LacZ at zero time was the same in both strains, ruling out an effect of *hns* on expression from the pBAD promoter (data not shown). To assess the effect of the *hns* mutation on degradation of an RssB-independent ClpXP substrate, the stability of GFP-SsrA was examined. The SsrA tag renders proteins, including GFP, sensitive to ClpXP-dependent degradation (6, 7). pBAD-GFP-SsrA was induced for 30 min, followed by treatment with spectinomycin as described above for pBAD-RpoS. The GFP-SsrA protein was unstable, with a 1.5-min half-life in a wild-type strain (Fig. 1B and C), which is consistent with data from other labs (6). GFP-SsrA was at least as unstable in an *hns* mutant, with a much lower initial level of accumulation (Fig. 1C). A *clpP* mutant stabilized the protein in the *hns* mutant strain, as expected (Fig. 1B and C). We concluded that *hns* mutants do not

lead to stabilization of all ClpXP substrates and therefore appear to be specific to the RssB-dependent pathway.

***hns* mutant does not decrease RssB amounts or act via phosphorylation levels.** RpoS degradation depends on the response regulator RssB, in addition to ATP-dependent ClpXP in vivo and in vitro (11, 23). The active (phosphorylated) form of RssB is necessary for its maximal activity in RpoS degradation in rapidly growing cells, although the ability of RssB to be phosphorylated is not required for signal transduction (i.e., stabilization in response to carbon starvation [16]). Given that an *hns* mutant did not stabilize another RssB-independent ClpXP substrate (Fig. 1B), the effect on RpoS might have been due to a decrease in either RssB level or RssB activity. We examined the activity of the *rssB* promoter, using an *rssA-rssB'*::lacZ transcriptional fusion (19). Contrary to the prediction of less *rssB* expression, we found a two- to threefold increase in β-galactosidase activity in the *hns* cells compared to the wild-type cells (data not shown). Furthermore, an *hns* double mutant with either *sprE19::cam* or *rssA2::cam*, alleles of *rssB* that modestly increase the expression of RssB and remove it from control by its normal promoter (13, 17), still exhibited high levels of RpoS accumulation, ruling out a *cis* effect of the *hns* mutant not measured by the transcriptional fusion (data not shown). In the *rssA2::cam* strain, RssB levels were shown not to change when *hns* was mutated (data not shown). These results are consistent with previously reported results of experiments directly measuring RssB levels in an *hns* mutant by Western blotting (11). Therefore, lower RssB levels cannot account for the stabilization of RpoS in an *hns* strain.

Using a plasmid with *rssB* under pBAD control, we measured RpoS and RpoS₇₅₀::LacZ expression when RssB was overproduced at a high level in the *hns* mutant strain. Overnight cultures of *hns* mutant strains carrying either a vector or

FIG. 1. RpoS is stabilized specifically in an *hns* mutant. Cells were grown in LB broth at 37°C to an optical density at 600 nm of 0.3 to 0.5; a sample was removed, and 100 μg/ml spectinomycin was added to the remaining culture. Samples were removed at different times, precipitated with 5% trichloroacetic acid in cold conditions, and analyzed by Western blotting. For quantitation, estimates were extrapolated from a series of sample dilutions that showed a linear response on film after scanning with an Eagle Eye II scanner (24), using 3 min as zero time on the graphs (there was a modest increase in signal between the addition of spectinomycin and 3 min). (A) The only source of RpoS is from the uninduced pBAD plasmid, and the levels of RpoS are comparable to those from the chromosome. Symbols: ■, wild-type strain YN559 (*rpoS::tet*/pBAD-RpoS); △, *hns* strain YN561 (*rpoS::tet hns::kan*/pBAD-RpoS). (B and C) Symbols: ■, wild-type strain YN783/pBAD-GFP-ssrA; △, *hns* strain YN788 (*hns*/pBAD-GFP-ssrA); ▣, *rssB* strain YN791 (*rssB*/pBAD-GFP-ssrA); ○, *hns clpP* strain YN792 (*hns clpP*/pBAD-GFP-ssrA). WT, wild type.

FIG. 2. Suppression of *hns* by RssB overproduction. (A) Strains containing an RpoS::LacZ reporter that measures transcription, translation, and protein degradation were grown in LB medium at 37°C with and without 0.02% arabinose (ara); samples were taken at an optical density at 600 nm of 0.4, and β-galactosidase activity was assayed with a SpectraMax plate reader, as previously described (24). The strains used were vector strain YN513 (*hns::kan/pBAD24*) and pBAD-RssB strain YN514 (*hns::kan/pBAD24-RssB*). (B) Samples were removed at the same time as described above for panel A, and RpoS levels were determined by Western blotting as described in the legend to Fig. 1.

the pBAD24-RssB plasmid were diluted into LB media with or without arabinose at 37°C. Samples were taken at mid-exponential phase, amounts of RpoS were measured by Western blotting, and RpoS₇₅₀::LacZ expression was determined by a

β-galactosidase assay. The amounts of both RpoS and RpoS₇₅₀::LacZ were dramatically (10-fold) reduced when RssB was induced in the *hns* mutant (Fig. 2, lane 4). This result, coupled with the rapid degradation of GFP-SsrA in an *hns* mutant, rules out an effect of *hns* mutants on ClpXP activity and suggests that RssB activity was inhibited but not abolished in the *hns* mutant; the inhibition could be overcome by excess RssB.

One major mode of regulating RssB activity is via phosphorylation and dephosphorylation. Possibly the *hns* mutant, by changing the level of expression of a kinase or phosphatase, could affect RssB activity. Therefore, we asked whether phosphorylation was essential for the effect of the *hns* mutant. Asp58 of RssB is the site of phosphorylation (2), but mutant derivatives of RssB carrying substitutions at this position still retain some activity in vivo (15, 16).

We constructed a chromosomal nonphosphorylatable *rssB* mutation, replacing the wild-type allele of *rssB* with *rssBD58P* by recombineering (4); this allele has been shown to have some activity when it is overexpressed, but it cannot be phosphorylated (11). RpoS stability was determined by Western blotting after a spectinomycin chase (Fig. 3). Consistent with our previous results (Fig. 1), in an *rssB*⁺ host, RpoS was 10-fold more stable in an *hns* mutant than in an *hns*⁺ strain (Fig. 3A and B). Also, as previously found for an *rssBD58A* allele (16), RpoS turnover was modestly (2.5-fold) slower in the *rssBD58P* strain than in an *rssB*⁺ strain (Fig. 3). We noted that the effect of changing the Asp58 residue in the chromosome (16; this study) is apparently less dramatic in terms of RpoS stability than it is in experiments in which the mutant form of RssB is expressed from a plasmid, even when expression was at levels believed to be similar to those from the chromosome (11, 14). We do not currently have an explanation for this difference.

In strains carrying the chromosomal *rssBD58P* allele, an *hns* mutation still increased the RpoS half-life threefold, resulting in turnover that was essentially identical to that for the *hns*

FIG. 3. RpoS turnover in *rssBD58P* strains. Strains were grown in LB medium at 37°C, and RpoS turnover was analyzed as described in the legend to Fig. 1. Symbols: ■, wild-type strain YN879 (*rpoS::tet/pBAD24-RpoS*); □, *hns* strain YN881 (*rpoS::tet hns::kan/pBAD24-RpoS*); ●, *rssBD58P* strain YN872 (*rpoS::tet rssB_{D58P}/pBAD24-RpoS*); ○, *rssBD58P hns* strain YN873 (*rpoS::tet hns::kan rssB_{D58P}/pBAD24-RpoS*).

mutant in an *rssB*⁺ host (Fig. 3). This result is inconsistent with the *hns* mutant acting primarily to change the phosphorylation state of RssB. The phosphorylation-independent action of an *hns* mutant confirms and extends the result previously reported for the effect of carbon starvation and phosphate starvation, which also lead to RpoS stabilization independent of phosphorylation (3, 16). ArcB, a histidine kinase, has recently been implicated in phosphorylation of RssB in vivo and in vitro; an *arcB* mutant was found to slow RpoS turnover in a phosphorylation-dependent manner (14). Consistent with the ability of an *hns* mutant to stabilize RpoS in a strain with a nonphosphorylatable *rssB* allele, we also found that an *hns* mutant also increased the half-life of RpoS in the *arcB* mutant (data not shown).

Conclusions. The results presented here show that an *hns* mutant had a profound effect on RpoS stability, independent of any effect of *hns* on synthesis of RpoS or RssB and without leading to stabilization of other ClpXP substrates. Thus, the *hns* effect on RpoS stability is specific to the RssB-dependent degradation pathway. Surprisingly, this effect is also independent of RssB phosphorylation, but it can be overcome by high levels of RssB. Our results suggest that in the *hns* mutant, RssB is present but inactive.

One explanation for these results postulates a protein(s) that is capable of protecting RpoS from degradation, possibly by blocking RssB access to RpoS, since overproduction of RssB can bypass the defect (Fig. 2). If the protein is synthesized only in *hns* cells and is not abundant in *hns*⁺ cells under the growth conditions used here, the effect of *hns* mutants would be explained. What is the role of the proposed H-NS repression of a gene or genes that, in turn, regulate RpoS stability? We predict that, as for other H-NS-repressed genes, H-NS repression is overcome by binding of a positive regulator under specific environmental conditions. This would allow specific stabilization of RpoS under whatever stress condition leads to activation of the specific RssB inhibitor. If in fact H-NS regulation of this putative gene is negative, this supports the idea that rapid degradation of RpoS may be the default situation, and stabilization under specific conditions underlies regulated proteolysis.

Recent results in our laboratory provide a further basis for understanding this phenomenon. A genetic screen led to the identification of a small protein, IraP, that stabilizes RpoS when it is overproduced, apparently by binding to RssB. It too acts even on the nonphosphorylatable form of RssB (3). IraP is not solely responsible for the stabilization of RpoS in *hns* mutants, however, since mutation of *iraP* in an *hns* mutant does not restore rapid RpoS degradation (A. Bougdour, unpublished observations). We propose that one or more functional homologs of IraP are responsible for stabilization of RpoS in the *hns* mutant. One candidate for such a gene, *yhhP*, was identified by Yamashino and coworkers as contributing to the stabilization of RpoS in *hns* strains (21), but this gene has recently been shown to encode a tRNA modification enzyme, suggesting a thus-far-unexplained level of complexity in regulation of RpoS degradation (9).

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