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).

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

<table>
<thead>
<tr>
<th>Strain or mutation(s)</th>
<th>β-Galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Long fusion&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.16 ± 0.07</td>
</tr>
<tr>
<td>hns</td>
<td>1.91 ± 0.11</td>
</tr>
<tr>
<td>rssB</td>
<td>1.02 ± 0.10</td>
</tr>
<tr>
<td>hns rssB</td>
<td>1.93 ± 0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells were grown in LB medium at 37°C, and β-galactosidase was assayed as previously described (24). Units are arbitrary activity 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, RpoS<sub>270::LacZ</sub>), SG30059 (hns<sub>kan</sub>), SG30018 (rssB<sub>kan</sub>), and YN534 (hns<sub>kan</sub> rssA<sub>kan</sub>), all derivatives of SG30013 (see Table S1 in the supplemental material).

<sup>b</sup> Cells were grown as described in footnote <sup>a</sup>. The fusion protein is stable. The strains used were strains EM1246 (wild-type, RpoS<sub>270::LacZ</sub>) and YN714 (hns<sub>kan</sub>), 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<sub>kan</sub>-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 sprE<sub>19::cam</sub> or rssA<sub>2::cam</sub>, 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 rssA<sub>2::cam</sub> 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 RpoS<sub>750::LacZ</sub> 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.](http://jb.asm.org/)

(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 (pBAD-RpoS); △, hns strain YN561 (pBAD-RpoS); ○, hns strain YN788 (pBAD-GFP-ssrA). (B and C) Symbols: ■, wild-type strain YN783/pBAD-GFP-ssrA; △, hns strain YN778 (pBAD-GFP-ssrA); ○, hns clpP strain YN792 (pBAD-GFP-ssrA). WT, wild type.
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+/H11001 host, RpoS was 10-fold more stable in an hns mutant than in an hns+/H11001 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+/H11001 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. 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 for A, and RpoS levels were determined by Western blotting as described in the legend to Fig. 1.](http://jb.asm.org/)

![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<sub>DS8P</sub>/pBAD24-RpoS); ○, rssBD58P hns strain YN873 (rpoS::tet hns::kan rssB<sub>DS8P</sub>/pBAD24-RpoS).](http://jb.asm.org/)
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REFERENCES


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