Riboregulation in *Escherichia coli*: DsrA RNA acts by RNA:RNA interactions at multiple loci

(*hns*/*rpoS*/antisense/small RNA/Hfq)

RICHARD A. LEASE, MICHAEL E. CUSICK, AND MARLENE BELFORT

Molecular Genetics Program, Wadsworth Center, New York State Department of Health, and School of Public Health, State University of New York at Albany, P.O. Box 22002, Albany, New York 12201-2002

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ABSTRACT DsrA is an 87-nt untranslated RNA that regulates both the global transcriptional silencer and nucleoid protein H-NS and the stationary phase and stress response sigma factor RpoS (σ^s). We demonstrate that DsrA acts via specific RNA:RNA base pairing interactions at the *hns* locus to antagonize H-NS translation. We also give evidence that supports a role for RNA:RNA interactions at the *rpoS* locus to enhance RpoS translation. Negative regulation of *hns* by DsrA is achieved by the RNA:RNA interaction blocking translation of *hns* RNA. In contrast, results suggest that positive regulation of *rpoS* by DsrA occurs by formation of an RNA structure that activates a *cis*-acting translational operator. Sequences within DsrA complementary to three additional genes, *argR*, *ilvIH*, and *rbsD*, suggest that DsrA is a riboregulator of gene expression that acts coordinately via RNA:RNA interactions at multiple loci.

RNA plays a variety of structural, informational, catalytic, and regulatory roles in the cell. RNA functioning as a genetic regulator, a process termed riboregulation (1), by acting either *in cis* or *in trans*, has been demonstrated widely in bacteria (2–4). Recently, riboregulation has been demonstrated in eukaryotes, including nematodes (5), plants (reviewed in ref. 6), and mammals (reviewed in ref. 7).

Various small RNAs carry out diverse functions in *Escherichia coli* (3, 8). As a first example, 10Sa RNA, renamed tmRNA, binds and antagonizes repressor proteins (9). This RNA also acts to remove truncated mRNAs from stalled ribosomes while tagging the abortive translation products for protein turnover (10). Second, OxyS RNA is involved in inducing a cellular response to oxidative stress and protects against DNA damage (11). Third, *micF* RNA regulates OmpF porin production (reviewed in ref. 12). Finally, DsrA, an 87-nt RNA (Fig. 1) first discovered through its regulatory effect on colanic acid capsule synthesis (13), is both a negative and positive regulator of gene expression in *E. coli*. DsrA represses either the function or synthesis of the global transcriptional silencer and nucleoid protein H-NS, allowing expression of H-NS-silenced capsular synthesis genes when DsrA is overexpressed (13, 14). DsrA also acts at a second locus, enhancing the translation of the general stress response and stationary phase sigma factor, RpoS (σ^s) at low temperatures (14). The mechanism of DsrA action at *hns* and *rpoS* has remained elusive.

H-NS and RpoS are apparent antagonists that either repress or activate transcription, respectively, in response to various environmental signals (reviewed in ref. 15). H-NS silences many unlinked genes, several of which are environmental stress response proteins, through binding to curved DNA seemingly independent of sequence (reviewed in ref. 16). In contrast, RpoS

accumulation favors transcription of a set of genes required for growth under stress (reviewed in refs. 15 and 17). Many stress-response genes are both repressed by H-NS and activated by RpoS, and similar kinds of conditions both relieve H-NS silencing and stimulate transcription mediated by RpoS (15, 18). Nutrient deprivation, osmotic shock, heat shock, or low temperature enhance RpoS translation or block its turnover. Of interest, H-NS also represses RpoS, not by silencing transcription but by inhibiting the translation and enhancing the turnover of RpoS (reviewed in ref. 15). H-NS is itself regulated in interesting and complex ways. H-NS negatively regulates its own transcription (19). H-NS transcription also is repressed by the low-abundance StpA protein, an H-NS paralog (intraspecies homolog). H-NS and StpA cooperate to govern certain H-NS-repressed genes (20). Conversely, H-NS transcription is enhanced coordinately with DNA synthesis and is induced by the cold-shock positive regulator CspA and the FIS protein (reviewed in ref. 16).

In this report, we demonstrate that DsrA acts through RNA:RNA base pairing interactions at the *hns* locus to inhibit translation. DsrA activity at *rpoS* also is proposed to occur by RNA:RNA interactions to stimulate translation by a separate mechanism. There is potential for RNA:RNA interactions at three additional loci: *argR*, *ilvIH*, and *rbsD*. Together, these results suggest that DsrA is a riboregulator that acts via specific RNA:RNA interactions to either negatively or positively regulate translational control of a broad range of biological functions.

MATERIALS AND METHODS

Bioinformatics. Complementarity of DsrA to various genes in *E. coli* was determined by using the BESTFIT and FASTA programs within the GCG suite of programs (Genetics Computer Group, Madison, WI). The significance of matching sequences was determined in GCG by Smith–Waterman alignment (21) by using Monte Carlo randomization of sequences and resampling. Matches greater than three SDs above random were considered significant.

Strains, Plasmids, and Culture Conditions. M182 Δ *stpA::tet^r*, M182 Δ *hns::kan^r*, and M182 Δ *stpA::tet^r hns::kan^r* were constructed previously from M182 (Δ [*lacIPOZY*]*X74 galK galU strA*) and are described elsewhere (20). M182 *hns** (M182*hns3-stu*) was constructed from M182 (see below). Strain GJ74 *proU::lacZ Tn10::tet^r* (22) was provided by Henri Buc (Institut Pasteur). RO90 (MC4100 λ RZ5: ϕ [*rpoS379::lacZ*]) (23) was provided by Regine Hengge-Aronis (Univ. of Konstanz). RK33Z Δ *nhA::(lacZ, kan^r)* was provided by Etana Pagan (Hebrew Univ.) (24). Strains M182 *proU::lacZ Tn10::tet^r* and M182 Δ *nhA::(lacZ, kan^r)* were constructed by P1 transduction (25) from GJ74 and RK33Z, respectively. The *proU::lacZ* reporter strain was selected by tetracycline resistance and was screened for cotransduction of the reporter gene on inducing indicator plates as described (22). Plasmid pA is pACYC184 (26). Plasmid pDsrA, also called pDDS164, was provided by S. Gottesman (National

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Institutes of Health) (13). Plasmids pWKS30 (27) and pMAK705 (28) were provided by S. Kushner (Univ. of Georgia). All strains were grown at 30°C in either TBYE or minimal medium (29) except $\Delta hnsA$ strains, which were grown in a buffered medium (24). Antibiotics were used at the following concentrations: chloramphenicol (25 μ g/ml), tetracycline (10 μ g/ml), kanamycin (10 μ g/ml), or ampicillin (200 μ g/ml).

Cloning and Mutagenesis of *hns*. The *hns*^{*} allele was constructed by amplifying the *hns* gene, along with 500 bp of an upstream regulatory region between *hns* and *tdk*, by whole-cell PCR of strain M182 by using Vent Polymerase (NEB, Beverly, MA). To generate *hns*^{*} (*hns3-stu*), external PCR primers W560 (5'-GGCGGCTCTAGATATATGCGTTCTC-CCTTACGAAGCC-3', *Xba*I site in italics) and W71 (5'-ACA-AAAGCTTATTGCTTGATCAGGAAATCGTCGAGGG-3', *Hind*III site in italics) were used together with internal, mutagenic primers W595 (5'-GAGATTACTACAATGAGC-GAGGCCTTGAAGATTCTGAACAACATCCG-3') and W634 (5'-CGGATGTTGTTCAAGAATCTTCAAGGCCTC-GCTCATTGTAGTAATCTC-3'), via recombinant PCR (30), to introduce point mutations together with a *Stu*I site (in italics; see Fig. 2A). Recombinant PCR products were cloned via *Xba*I and *Hind*III into pWKS30 and were verified by restriction analysis and DNA sequencing.

Construction of Chromosomal *hns* Mutations. The *hns*^{*} gene was introduced into the chromosome of M182 at *hns* essentially as described (28). In brief, the 947-bp *Xba*I-*Hind*III fragment, containing the *hns*^{*} gene along with the upstream regulatory region, was subcloned from pWKS*hns*^{*} into the allele exchange vector pMAK705 (28), generating pMAK*hns*^{*}. M182 was transformed with this temperature-sensitive plasmid, and the chromosomal *hns* allele was replaced with *hns*^{*}, resulting in the strain M182*hns*^{*}. Integration of the plasmid into the chromosome at *hns* was selected by growth at the nonpermissive temperature (44°C) on chloramphenicol plates. Candidate integrants were verified by PCR by using primers W778 (within the vector) and W777 (within *tdk*) (primer sequences below). Simultaneous integrant resolution and plasmid curing were achieved by growth at 30°C in the absence of chloramphenicol. These events were verified by checking clones for chloramphenicol sensitivity at 30°C then by PCR of chromosomal DNA with primer pairs W778 (vector) with W71 (within *hns*) and W777 with W70 (within *hns*). Replacement of chromosomal *hns*⁺ with *hns*^{*} was verified by *Stu*I digestion of whole-cell PCR products (W560 and W71) and by Southern hybridization (data not shown). Oligonucleotides used for verification of gene replacement are W70 (5'-AAAGAATT-CAGGACTACAATGAGCGAAGCAC-3'), W777 (5'-GGA-TAACAATTTACACAGG-3'), W778 (5'-CGGAATAGT-AGAAATATAGC-3'), and W71 (above).

Mutations in DsrA. Plasmid pDsrA was used as a template for inverse PCR (31) with *Pwo* DNA polymerase (Boehringer Mannheim). For DsrA^{*H} (*dsrA-H23*), primers W820 (5'-GG-AATTCAGGCCTTGAAGAAATTCGTTACACCAGG-3') and W821 (5'-GGAATTCAGGCCTCTTGCTTAAGCAAG-TTTC-3') introduced a *Stu*I site (in italics). For DsrA^{*R} (*dsrA-R19*), W818 (5'-GGAATTCGTACACGAGGGGT-CTGATGTGTTTCATC-3') and W819 (5'-GGAATTCGTGA-CAGAAGATTTAAGTGCTTCTTGC-3') introduced a *Bsr*GI site (in italics). Additional point mutations were introduced concomitantly outside these sites (see Figs. 2B and 3A). For pDsrA^{*R}, PCR products (linear plasmids) were digested with *Bsr*GI, were ligated, and were transformed into DH5 α . In the case of pDsrA^{*H}, the oligonucleotides were phosphorylated with T4 kinase before PCR, and the PCR product plasmid was blunt-end ligated and was used to transform *E. coli*. Candidates were screened by restriction analysis with *Eco*RI or *Stu*I, and positive clones were digested with *Stu*I and were gel-purified to remove the short linker DNA. These plasmids then were ligated, were used to transform *E. coli*, and

were rescreened by restriction analysis for loss of the linker, which contains *Eco*RI sites. Positive clones were verified by DNA sequencing.

RNA and Statistical Variance Analyses. RNA levels were determined by primer extension as described (29). Primer W166 (20) was used for *hns*, and W578 (*trxA*; 5'-TTTCACCG-GATACGCTTTCG-3') was used as a loading control. Labeled cDNAs were separated on 8% polyacrylamide sequencing gels and were quantified by using a PhosphorImager and IMAGE-QUANT software (Molecular Dynamics). The *hns* RNA signal was normalized to the M182/pA sample for comparison between experiments. Three experiments were performed in duplicate, and the results were subjected to variance analysis with the program SAS (procedure ANOVA). All P values <0.05 were considered significant. The differences between *hns*⁺ and *hns*^{*} RNA levels were considered significant ($P = 0.0044$), as were the differences between *hns*⁺ and Δhns , each containing pA ($P = 0.0055$) and the DsrA-induced differences in a Δhns strain background ($P = 0.0208$). Other variances in *hns* RNA levels were not considered significant (Y. Ding and C. Lawrence, personal communication).

SDS and Isoelectric Focusing Gels and Western Blotting. H-NS and StpA proteins were purified as described (32). Cell extracts were prepared, were diluted in SDS sample dilution buffer, and were separated as described (33, 34), except nonequilibrium tube gels were run with 2% Nonidet P-40 in a pH 3–10 gradient (Bio-Lyte, Bio-Rad), and tube gels were overlaid with 1% agarose (in SDS protein gel running buffer plus bromophenol blue) before running the second dimension. SDS gels are as described (35), except that the running buffer was 25 mM Tris-HCl (pH 8.3), 41 mM glycine, and 1.3% SDS. For Western blot analysis, proteins were transferred to poly(vinylidene difluoride) membranes (Bio-Rad) with a semi-dry apparatus (Bio-Rad) by using an unpublished protocol (Philippe Bertin, personal communication). In brief, the unequilibrated gel was placed on the membrane and was sandwiched between pieces of filter paper soaked in transfer buffer (25 mM Tris base, 20% methanol). Three filters above the gel also contained 40 mM ϵ -amino-n-caproic acid (Sigma); below the membrane was one filter soaked in transfer buffer, then two filters soaked in 300 mM Tris base plus 20% methanol. Proteins were transferred for 40 min at 0.8 mA/cm² of filter. Polyclonal antibodies raised against either StpA or H-NS were prepared previously (36) (A. Zhang and M.B., unpublished data).

RESULTS

DsrA Acts Through RNA:RNA Interactions To Repress H-NS Activity. Computer database searches revealed that the DsrA sequence contains short stretches of sequence complementary to a number of known genes in *E. coli*. The regions of complementarity tend to cluster either in the DsrA central stem-loop (*hns*' and *hns*' alt, *argR*', and *ilvIH*'), or, alternatively, from the first stem-loop to the central stem (*rpoS*' and *rbsD*') (Fig. 1). Several of these regions partially overlap (e.g., *hns*'/*rpoS*'/*rbsD*'; *hns*'/*argR*'/*ilvIH*'). Of particular interest are the regions of complementarity to *hns* and *rpoS* (Fig. 1A), as DsrA has been shown to be involved in the regulation of both of these genes (13, 14). In view of the observed sequence complementarity, and because DsrA antagonizes H-NS, the basis of the regulation of H-NS could be an RNA:RNA interaction between DsrA and *hns* RNA. We therefore modified *hns* by silent mutagenesis, disrupting the putative RNA:RNA interaction while leaving the H-NS protein sequence unchanged (Fig. 2A).

The *hns* gene, along with 500 bp of upstream regulatory sequence (between *hns* and the divergently transcribed *tdk*), was cloned by site-directed mutagenic recombinant PCR (30) to introduce specific mutations in *hns*. The desired mutations introduce a unique *Stu*I restriction site while generating the *hns*^{*} (*hns3-stu*) allele (Fig. 2A). Recombinant PCR products were

FIG. 1. DsrA contains regions of complementarity to different genes in *E. coli*. Computer searches revealed complementarity to DsrA at or near the 5' ends of the coding sequences of five *E. coli* genes. The DsrA nucleotide sequences complementary to indicated genes are highlighted (white letters on a black background) on the predicted structure of DsrA (13). Open arrowheads indicate potential G:U base pairs between DsrA and target sequences. A prime (') after the gene name designates complementarity to the gene. (A) Complementarity to *hns* and *rpoS* demonstrated in this work. Two possible complementary schemes are shown for *hns*. (B) Putative complementarity to *argR*, *ilvIH*, and *rbsD*.

cloned, were screened by *StuI* digestion, and were verified by DNA sequencing. This allele was introduced into the *E. coli* chromosome at the *hns* locus by gene replacement (28). DsrA was overproduced *in trans* from a plasmid, pDsrA (13), with vector pA (pACYC184) serving as a control. The effect of disrupting the putative *hns* RNA:DsrA RNA interaction was assessed by measuring expression of two genes, *proU* (reviewed in ref. 16) and *nhaA* (37), known to be repressed by H-NS. Expression levels of *proU* and *nhaA* were assayed with *lacZ* fusions to these genes (Fig. 2C and D). Strikingly, DsrA relieved repression of the *proU* and *nhaA* promoters in the wild-type strain (6-fold and 3-fold, respectively) but not in the *hns** strain (Fig. 2C columns 1–4 and D).

These results suggest that disruption of the putative RNA:RNA interaction prevents DsrA from regulating H-NS. To verify that this interaction is the essential element of regulation, the DsrA-overproducing plasmid pDsrA was mutated to produce altered DsrA molecules. The first such variant molecule, DsrA^{*H} (produced by the *dsrA-H23* allele), has lost complementarity to *hns* RNA but has gained putative base pairing with *hns** RNA (Fig. 2B). Significantly, β -galactosidase assays with the *proU::lacZ* fusion indicate that, in contrast to wild-type DsrA, DsrA^{*H} is unable to block the wild-type *hns* but is capable of blocking the *hns** allele (Fig. 2C, compare columns 3 and 5, 4 and 6). Thus, restoration of the base pairing between *hns** and DsrA^{*H} reestablished DsrA regulation of H-NS, confirming that this regulation occurs by RNA:RNA interaction. It is noteworthy that this alteration of DsrA does not destroy its functionality (Fig. 2C, columns 5 and 6).

DsrA also Appears To Regulate RpoS by RNA:RNA Interactions. We next chose to investigate whether an RNA:RNA interaction is involved in DsrA regulation of *rpoS*. The region of DsrA complementary to *rpoS* RNA, termed *rpoS'*, lies largely in the first rather than the second stem-loop of DsrA (Fig. 1A) and partially overlaps the *hns'* region (Fig. 1A, compare *hns'* and *rpoS'*). DsrA has been shown to increase RpoS (σ^s) at low temperatures by enhancing its translation in *E. coli* (14). RpoS translation in *Salmonella typhimurium* is governed by a *cis*-acting RNA sequence, with a stable secondary structure that must be disrupted to allow translation (38) (Fig. 3B). To test whether DsrA acts on *rpoS* *in trans* by an RNA:RNA interaction, we

FIG. 2. DsrA acts via RNA:RNA interactions to repress H-NS. (A) Silent mutagenesis of *hns* to disrupt complementarity to DsrA. Five nucleotides of wild-type *hns* were modified by site-directed mutagenesis (arrows) to disrupt the DsrA:*hns* RNA base pairing (vertical lines). The mutations, which are silent in terms of the H-NS protein sequence (below), create a *StuI* restriction site (underlined) in the altered *hns** allele. The initiator methionine codon is indicated (boxed). (B) DsrA mutations that prevent base pairing with *hns* but allow base pairing with *hns**. Mutated nucleotides are shown next to the DsrA structure (arrowheads). (C and D) Assay of H-NS function in strains with and without *hns* RNA:DsrA complementarity. DsrA-induced repression of H-NS transcription silencing activity was assayed in strain M182 by using a *proU::lacZ* (C) or *nhaA::lacZ* (D) reporter gene. β -galactosidase assays were performed as described (25), except that assays on *nhaA::lacZ* used a buffered growth medium (37). All assays were performed in duplicate; values given are the average of three independent experiments. Light columns, *hns*⁺ strains; dark columns, *hns** strains. Plasmids used: –, pA vector; +, pDsrA; *H, pDsrA^{*H} as in B; *R, pDsrA^{*R} as in Fig. 3A.

altered DsrA within its *rpoS'* region (Fig. 3A), as mutations within the *rpoS* RNA structure can lead to enhanced translation levels (38). Although pDsrA induced *rpoS* expression as predicted (14) (Fig. 3C, columns 1 and 2), the mutated DsrA^{*R} (produced from the *dsrA-R19* allele) no longer induced *rpoS::lacZ* translation (Fig. 3C, compare columns 2 and 3). Of interest, DsrA^{*H} (Fig. 2B) has lost the ability to induce *rpoS* expression (Fig. 3C, compare columns 2 and 4). This latter effect may be attributable to one or more of the mutations in DsrA^{*H} changing the proposed DsrA:*rpoS* interaction (Figs. 2B and 3B). Of importance, DsrA^{*R} is still capable of regulating *hns* RNA (Fig. 2C, compare columns 7 and 8 with columns 3 and 4) and therefore has lost functionality specifically with *rpoS* and not with *hns*, consistent with a direct DsrA:*rpoS* interaction. This result, together with the inability of pDsrA^{*R} to stimulate *rpoS* expression, supports the idea that regulation at *rpoS* occurs by an RNA:RNA interaction.

Effects of DsrA-RNA Interactions on *hns* RNA Levels. The location of the DsrA-complementary sequence proximal to the *hns* translation initiation sequences (Figs. 2A and 4A) suggests that DsrA might regulate H-NS production by inhibiting translation, either by occluding the translation machinery or by enhancing turnover of *hns* RNA. Therefore, *in vivo* *hns* RNA was examined by primer extension analysis. The primer used hybridizes upstream of the deleted region in Δ *hns* constructs, enabling quantitation of transcripts from the deletion allele (Fig. 4A) (20).

Changes in cDNAs reflecting changes in *hns* RNA levels produced by DsrA are subtle (<2-fold) but reproducible (Fig. 4B, black arrowhead, and Fig. 4C). In *hns*⁺ strains, DsrA overproduction does not, on average, appear to significantly affect *hns*

FIG. 3. DsrA activates *rpoS* translation: an RNA:RNA interaction model. (A) DsrA^{*R} mutant RNA. Mutations are indicated by arrowheads. (B) Proposed structure of the *rpoS* mRNA translational operator. Bold letters in the bulge loop indicate the Shine–Dalgarno sequence; boxed AUG indicates the initiator methionine codon; outlined letters indicate mutations that confirm secondary structure and enable Hfq-independent translation (adapted from ref. 38 by using additional sequence of *E. coli rpoS* from ref. 48). The sequence of DsrA is given in white letters on a black background, indicating the complementarity to *rpoS* mRNA deduced in this work (Fig. 1A). Vertical lines are base pairs, dots are G:U base pairs. (C) Effect of altered DsrA on translation of *rpoS* mRNA. The reporter gene is a translational fusion between RpoS and LacZ in strain RO90. The RNA produced by the reporter fusion contains the region of DsrA complementarity. All values shown are the average of three independent experiments.

RNA levels (Fig. 4B, compare lanes 1 and 2; Fig. 4C, line 1). In the absence of DsrA overproduction, *hns*^{*} strains have a 50% higher steady-state level of transcript than wild-type strains (Fig. 4B, compare lanes 1 and 3; Fig. 4C, compare lines 1 and 2), which might suggest an enhanced *hns*^{*} transcript stability. However, DsrA overproduction in *hns*^{*} strains, as in *hns*⁺ strains, does not strongly perturb RNA levels (Fig. 4B, compare lanes 1 with 2, 3 with 4; Fig. 4C, line 2). More dramatically, in Δ *hns* strains, the basal level of transcription is increased >2-fold relative to wild type (Fig. 4B, compare lanes 1 and 5; Fig. 4C, line 3), consistent with disruption of *hns* autoregulation (19). Furthermore, a decrease in *hns* RNA caused by DsrA overproduction is manifest only in Δ *hns* strains (Fig. 4B, compare lanes 5 and 6; Fig. 4C, line 3), as considered further in Discussion.

DsrA Effects on H-NS Translation. To quantify H-NS protein levels, it was first necessary to separate H-NS from the similarly sized StpA protein (15.6 kDa and 15.3 kDa, respectively), which also cross-reacts immunologically with H-NS polyclonal sera (Fig. 5A, lanes 1 and 2). Because H-NS levels predominated (Fig. 5A, compare lanes 5 and 6) and the overall level of protein (StpA+H-NS) decreased when DsrA was overproduced (Fig. 5A, compare lanes 3 and 4), a decrease in H-NS protein was suggested.

FIG. 4. Levels of *hns* RNA during DsrA overexpression. (A) Diagram of the *hns* transcript and primer extension product. The *hns* primer W166 (20) hybridizes upstream of the deleted region in Δ *hns* transcripts, enabling quantitation of RNA. +1 TX, transcriptional start; +1 TL, translational start. Size of primer extension product is given in nucleotides beside wavy line, representing cDNA. (B) Levels of *hns* RNA. RNA harvested from mid-log phase cells grown at 30°C was quantified by primer extension (29). Lanes and markers: P, no RNA; 1, M182⁺/pA; 2, M182⁺/pDsrA; 3, M182⁺/pA; 4, M182⁺/pDsrA; 5, M182 Δ *hns*/pA; 6, M182 Δ *hns*/pDsrA. The sequence of pTZ19U was used as a molecular weight marker (lanes G, A, T, and C). (C) Quantitation of *hns* cDNA primer-extension product. Values are normalized to M182⁺/pA. The average of three independent experiments, each performed in duplicate, is shown; error is reported as SD. All six data sets were subjected to variance analysis.

Because resolution by SDS/PAGE is not straightforward, the proteins were separated by two-dimensional isoelectric focusing (33, 34) followed by electrophoretic transfer to poly(vinylidene difluoride) membranes (P. Bertin, personal communication; see *Materials and Methods*). Lysates from cells with and without DsrA overproduction were examined, and proteins were visualized by Western blot analysis by using mixed StpA/H-NS polyclonal sera (Fig. 5B and C). Purified StpA and H-NS proteins served as markers (not shown). H-NS protein levels decreased in response to DsrA overproduction, with a concomitant increase in StpA protein levels (Fig. 5B, compare M182/pA with M182/pDsrA). M182 Δ *hns* was used as a control, showing the increase in StpA protein in the absence of H-NS. The average DsrA-induced decrease in H-NS levels was consistently $\approx$ 3-fold.

DISCUSSION

DsrA Function at *hns* and *rpoS*. DsrA, a small untranslated RNA, acts at multiple loci as an inhibitor or activator of gene expression. DsrA inhibits the synthesis of the predominant nucleoid protein and global transcriptional silencer H-NS (13) and enhances the translation of the stationary phase and major stress response sigma factor RpoS (σ^S) (14). We have demonstrated that DsrA acts via specific RNA:RNA base pairing interactions at the *hns* locus. Disruption of the RNA:RNA base pairing, by altering *hns* RNA to *hns*^{*} through silent mutagenesis, led to loss of DsrA activity whereas restoration of complementarity with DsrA^{*H}

FIG. 5. DsrA decreases H-NS and increases StpA levels *in vivo*. (A) Western blot of purified proteins and extracts fractionated by SDS/PAGE. The proteins were transferred to membranes and were probed with polyclonal antisera raised against H-NS. Lanes: 1 and 2, purified StpA and H-NS proteins, respectively; 3–7, crude lysates from M182/pA (lane 3), M182/pDsrA (lane 4), M182ΔstpA (lane 5), M182Δhns (lane 6), and M182ΔstpAΔhns (lane 7). (B) H-NS and StpA proteins were separated by nonequilibrium two-dimensional isoelectric focusing and were visualized by Western blot analysis with mixed H-NS- and StpA-specific polyclonal antisera. The pH gradient is from ≈ 10 (left) to 5 (right). Cell extracts are from M182/pA, M182/pDsrA, and M182Δhns. (C) A separate experiment depicts H-NS separated by equilibrium two-dimensional isoelectric focusing.

restored function (Fig. 2). Specific mutations in the *rpoS*-complementary region of DsrA to generate DsrA^R similarly prevented DsrA function at *rpoS* (Fig. 3) without destroying the function of DsrA at the *hns* locus (Fig. 2C, columns 7 and 8). These findings support the idea of specific regulatory targeting by different regions of DsrA (Figs. 1 and 6).

Negative Regulation of *hns* by DsrA. H-NS levels *in vivo* were found to be decreased ≈ 3 -fold by DsrA overproduction (Fig. 5B and C). Although it has been reported that H-NS levels remain constant with DsrA overproduction (13), those findings were reported before the understanding that StpA comigrates with H-NS on SDS gels and cross-reacts with H-NS-specific polyclonal antisera. Resolution of these two proteins on two-dimensional gels clearly demonstrates that DsrA overproduction decreases H-NS protein levels *in vivo* (Fig. 5).

The proximity of the RNA:RNA interaction to the translational control sequences of *hns* RNA (Figs. 2A and 4A), together with the results outlined above, suggest a model of translational inhibition by occlusion of translation (Fig. 6 Left). DsrA might block translation by favoring an *hns* RNA secondary structure that occludes translation initiation or by recruiting a factor that inhibits translation. The observation that *hns* RNA levels are not decreased when DsrA is overproduced in wild-type *hns*⁺ cells is consistent with such an hypothesis (Fig. 4). Whereas DsrA overproduction decreases *hns* RNA levels produced from the Δ*hns* allele (Fig. 4B and C), this result must be interpreted cautiously because *hns* mutants are pleiotropic (reviewed in ref.

16) and Δ*hns* strains grow poorly (20). Although we have not eliminated RNA turnover as a possibility, occlusion of H-NS translation is a more parsimonious model.

Any model of DsrA action on *hns* RNA must consider the complexity of H-NS and StpA cross-regulation and autoregulatory loops (20) as well as the effects of other H-NS and StpA activators and repressors such as FIS, Lrp, and CspA (16). The reduction in H-NS translation was accompanied by an increase in StpA levels (Fig. 5B), which can be attributed to decreased transcriptional repression of StpA by H-NS (ref. 20; R.L. and M.B., unpublished data). Furthermore, because H-NS and StpA coordinately regulate some genes while independently regulating others, small perturbations in H-NS levels can cause global changes in gene expression.

Positive Regulation of *rpoS* by DsrA. RpoS translation requires Hfq (HF-I), a putative RNA chaperone (40, 41) and global regulator of transcription (42, 43). Hfq mutants do not support *rpoS* translation (23). The annealing of DsrA to the *rpoS* translational operator region (Fig. 3B) functionally resembles a set of mutations in this region that enable translation in an *hfq* mutant strain (38) (Fig. 3B). Indeed, compensating mutations in *rpoS* that restore structure also prevent translation in the absence of Hfq (38). In light of these considerations, an “unzipping” model is proposed (Fig. 6 Right). DsrA base pairing to the inhibitory strand of the *rpoS* translational operator prevents *rpoS* intrastrand base pairing, freeing the Shine–Dalgarno sequence and enabling translation.

Although negative regulation by DsrA at *hns* and positive regulation by DsrA at *rpoS* appear to be mediated by RNA:RNA interactions at or near translational control regions, these are apparently mechanistically distinct processes. Of interest, the two regulatory modes involve different regions of the DsrA molecule. Whereas the RNA:RNA interaction that results in negative regulation at *hns* leaves the DsrA 5' stem-loop intact, the positive regulation at *rpoS* is proposed to disrupt this structure (Figs. 1–3 and 6). It is tempting to speculate that the 5' stem-loop is the determinant of negative versus positive control, presenting the recognition/binding site for the effectors of negative regulation (Fig. 6).

DsrA RNA as a Riboregulator at Multiple Loci. Cells have evolved numerous systems for sensing and adapting to changes in their environment. Adverse conditions, including nutrient deprivation, temperature and pH changes, and osmotic shock elicit responses to environmental stimuli via genetic regulatory networks called stimulons (44), which can activate one set of genes while repressing others. DsrA may therefore be a stimulon regulator that uses multiple antisense sequences to fine-tune global regulatory responses to stress by conditionally preventing or enabling expression of target genes such as *hns* and *rpoS*.

Regions of complementarity within the DsrA molecule (Fig. 1B) hint that, in addition to *hns* and *rpoS*, three other genes, *argR*, *ilvIH*, and *rbsD*, also may be regulated coordinately in a fashion similar to *hns* or *rpoS*. The regions of DsrA complementarity

FIG. 6. Models for DsrA riboregulation. At the top, DsrA forms RNA:RNA interactions with target transcripts. On the left (–) is a model for translational repression of *hns*. On the right (+) is a model for translational activation of *rpoS*. Black circles represent ribosomes.

within these genes all map near translation start sequences (data not shown). Again, the regions of complementarity can be mapped roughly into two distinct groups: those that lie in the central stem-loop (*hns*, *argR*, and *ilvIH*) and those that lie in the 5' stem-loop and central stem (*rpoS* and *rbsD*). If the location of the region of complementarity within DsrA corresponds to whether regulation at a specific locus is negative or positive, as for *hns* and *rpoS* (Fig. 6), this would predict that *argR* and *ilvIH*, like *hns*, will be regulated negatively by DsrA whereas *rbsD*, like *rpoS*, will be regulated positively.

All three of the remaining DsrA-complementary genes are involved in metabolic pathways in the cell. *IlvIH* is an enzyme that represents a control point in the biosynthesis of isoleucine, leucine, and valine (45). *ArgR* is a well studied regulon repressor that affects pyrimidine, arginine, and polyamine synthesis (46). The *rbs* gene, which is of unknown function, is in a polycistronic operon that encodes a multi-subunit active transport system for ribose (47). Clearly, DsrA could be important for coordinate regulation of these pathways and those controlled by H-NS and RpoS, thereby acting as a global orchestrator of a broad sweep of biological functions.

Note Added in Proof. Results presented independently by Majdalani *et al.* in this issue of the *Proceedings* (49) confirm our model of translational regulation of *rpoS* via RNA:RNA interactions with DsrA.

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