Interactions of the Non-coding RNA DsrA and RpoS mRNA with the 30 S Ribosomal Subunit

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Expression of σ^7, the gene product of rpoS, is controlled translationally in response to many environmental stresses. DsrA, a small 87-nucleotide non-coding RNA molecule, acts to increase translational efficiency of RpoS mRNA under some growth conditions. In this work, we demonstrate that DsrA binds directly to the 30 S ribosomal subunit with an observed equilibrium affinity of 2.8 × 10^7 M^-1. DsrA does not compete with RpoS mRNA or tRNA^Met for binding to the 30 S subunit. The 5' end of DsrA binds to 30 S subunits with an observed equilibrium association constant of 2.0 × 10^6 M^-1, indicating that the full affinity of the interaction requires the entire DsrA sequence. In order to investigate translational efficiency of RpoS mRNA, we examined both ribosome-binding site accessibility and the binding of RpoS mRNA to 30 S ribosomal subunits. We find that this ribosome-binding site accessibility is modulated as a function of divalent cation concentration during mRNA renaturation and by the presence of an antisense sequence that binds to nucleotides 1–16 of the RpoS mRNA fragment. The ribosome-binding site accessibility correlates with the amount of RpoS mRNA participating in 30 S-mRNA "pre-initiation" translational complex formation and provides evidence that regulation follows a competitive model of regulation.

Bacteria employ many different molecular strategies for coping with environmental stresses. To initiate the process of transcribing a specific gene, the multisubunit bacterial RNA polymerase must select and bind to the promoter sequence of the gene or operon (1). There are several different classes of promoter sequences, each recognized by specific RNA polymerase holoenzymes containing appropriate σ subunits (2). The bacteria have evolved so that expression of entire families of proteins under specific growth conditions is initiated by transcription from a shared class of promoter sequences (2). In response to a range of external stimuli, the core RNA polymerase exchanges one type of σ factor for another, shutting off transcription of one family of proteins while initiating expression of another family (2, 3). As a result, the expression of entire gene families can be controlled by affecting the rate and extent of σ factor production (3).

Following exposure to high osmolarity (osmotic upshock), the cytoplasm of Escherichia coli undergoes dramatic changes in the concentrations and composition of its solutes (4–6) resulting in differential expression of approximately 70 genes (5). During osmotic shock, transcription of many of these genes is controlled by RNA polymerase holoenzyme containing the σ^7 subunit, the gene product of rpoS (3, 7, 8).

Exposure of E. coli to high external osmolarity induces an increase in translation of RpoS mRNA by a signaling pathway likely to involve changes in RpoS mRNA structure (8–11). It is predicted that under normal growth conditions, the ribosome-binding site of RpoS mRNA is trapped in a secondary structure that results in repression of translational initiation (see Fig. 1A) (11, 12). Osmotic shock may induce conformational changes in the mRNA that influence the interactions of the mRNA with regulatory factors (3). During osmotic shock, the regulatory factors are expected to increase the single-stranded nature of the ribosome-binding site (the "accessibility" of the ribosome-binding site) and therefore to increase the ability of the 30 S subunit to bind to the mRNA and the fraction of mRNA participating in translational initiation (3, 13, 14).

Three non-coding RNAs (ncRNAs), DsrA, RprA, and OxyS, influence translational initiation of RpoS mRNA. Both DsrA and RprA non-coding RNAs activate translation during osmotic shock, whereas OxyS RNA represses translation during oxidative stress. DsrA is a small, 87-nucleotide, untranslated RNA that stimulates RpoS mRNA translation at low temperatures (15). Its secondary structure is shown in Fig. 1B. DsrA ncRNA has been proposed to enhance translational initiation by base pairing to the 5'-untranslated region of RpoS mRNA (including nucleotides 1–16 shown in Fig. 1A) thereby preventing intramolecular base pairing of the ribosome-binding site (16–18) with other cis-acting mRNA sequences. In this work, we perform the first in vitro studies characterizing the relationship between the accessibility of the ribosome-binding site of RpoS mRNA and formation of translational initiation complexes in vitro.

The mechanism for DsrA translational stimulation is unknown. It has been shown that the effects of DsrA ncRNA on rpoS expression are much greater in the presence of the protein Hfq (11, 19–23). The role of Hfq in the global regulation of gene expression increasingly appears to be that of a facilitator of RNA-RNA interactions that influence rates of mRNA translation and degradation (23–25). The 3' domain of DsrA interacts specifically with Hfq, and it has been proposed that Hfq assists in forming the DsrA-RpoS mRNA hybrid postulated to stimulate translation of RpoS mRNA (21). In this work we demonstrate that DsrA specifically interacts with 30 S ribosomal subunits in vitro. This interaction may be important in the mechanism of rpoS regulation by DsrA.

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1 The abbreviation used is: ncRNAs, non-coding RNAs.
Ribosomal Subunit Interactions with DsrA and RpoS mRNA

EXPERIMENTAL PROCEDURES

RNA Transcripts—RpoS mRNA, DsrA mRNA, and transcripts containing the predicted DsrA binding site (1-34) or the nucleotides 23-87 of DsrA (DsrA-(23-87)) were synthesized from PCR products containing T7 promoter using the Ribomax T7 Transcription kit from Promega (Madison, WI) following the transcription kit protocol. The RpoS PCR products were synthesized from pTE661 (11) (the kind gift of Tom Elliott) using the following primer set: 5'-GGG UCU CUG GUC UGC GCU ACA AUA UUA UCA GAC UUG GUA C-3' and 5'-UCG AAG CUA AGT ATG GCT CCA ACA CAG GGT AGC GAU CUA C-3' for 30 min and loaded into a pre-chilled, 8% non-denaturing polyacrylamide gel. The renatured RNA was immediately placed on ice, and buffer composition was normalized so that all samples had identical concentrations of divalent cation.

In vitro reactions were allowed to equilibrate for 30 min and were then filtered onto prewetted nitrocellulose filters. Fractions were collected and counted on a Packard TriCarb 2000TR scintillation counter. 

Radioactive bands were excised and counted using liquid scintillation. “Fraction bound” refers to the fraction of radiolabeled oligonucleotide in the binary (or ternary) complex band divided by the sum of all the radioactivity in the free and bound bands. At least three independent gels were run for each set of renaturation conditions. Binding constants were determined by fitting the fraction bound to a single site binding isotherm (27) using the software program Sigma Plot (SPSS Science, Chicago).

30 S ribosomal subunits were purified from MRE600 cells grown to A600 of 1 (grown at The Johns Hopkins University) (27). Briefly, frozen, adherent cultures were thawed on ice, resuspended in Buffer A (20 mM Tris-Cl (pH 7.5 at 4 °C), 100 mM NH4Cl, 10.5 mM magnesium acetate, 0.5 mM EDTA, 3 mM 2-mercaptoethanol; 2 mg/ml cells), and lysed by passage through a pre-chilled French press (12,000 pounds/square inch) one or two times. Lysed cells were immediately centrifuged at 30,000 × g for 30 min, and the supernatant was retained. The fraction precipitating between 35 and 70% ammonium sulfate (first addition, 0.196 g of ammonium sulfate per 100 ml of supernatant; second addition, 0.22 g/100 ml) was redissolved in 3.3 ml of Buffer A for each 1 g of cells. The resuspended ribosomes were centrifuged at 52,000 rpm (Beckman T55.2 rotor) for 140 min, and the pellet material was resuspended overnight at 0 °C in Buffer A. Ribosomes were salt-washed one time by centrifugation through an equal volume of Buffer A plus 15% (w/v) sucrose and 500 mM NH4Cl. 30 S subunits were prepared by sucrose gradient sedimentation of ribosomes in Buffer A with 1.1 mM magnesium acetate. 30 S subunits were renatured in Buffer A at 37 °C for 30 min before use.

Binding Assays—Equilibrium titrations were performed by incubating varying concentrations of 30 S ribosomal subunits with a fixed amount of radiolabeled, renatured RNA at 0 °C in Buffer A (27). In competition assays, fixed concentrations of 30 S subunits and radiolabeled RNA were added to increasing concentrations of competitor RNA and incubated in Buffer A at 0 °C. 20-μl reactions were allowed to equilibrate for 30 min and were then filtered onto prewetted nitrocellulose BA85 filters (Schleicher & Schuell). Filters were washed with 200 μl of Buffer A and then counted by liquid scintillation. Binding constants were determined from (minimally) duplicate binding assays assuming a single site binding isotherm (27) and fit with the software program Sigma Plot (SPSS Science, Chicago). Fractional retention values were determined as the counts retained on the filter at saturating 30 S subunit concentrations divided by the total number of counts of radioactive RNA applied to the filter.

RESULTS

DsrA Binds to 30 S Ribosomal Subunits—We examined whether DsrA mRNA interacts specifically with the 30 S subunit by performing a direct binding assay of radiolabeled DsrA

Fig. 1. A, predicted secondary structure of the RpoS mRNA sequence. Secondary structure was predicted using the computer folding algorithm Mfold (45, 46) and has a ΔG = −49.3 kcal/mol. This structure is supported in part by mutational analysis of the helix containing the ribosome-binding site. Positions 17 and 97 are predicted to base pair in this structure. Disruption of this base pair lessens regulation of expression and compensatory mutations at these positions restores regulation of translation (11). B, secondary structure of DsrA mRNA (18).
ncRNA transcript with 30 S subunits in binding buffer at 0 °C. The affinity of the 30 S subunit for the DsrA ncRNA was determined from fits to a single site binding isotherm with an equilibrium affinity to the 30 S subunit, K_{30S}, equal to 2.8 ± 1.4 × 10^{7} M⁻¹ (Fig. 2).

The origin of the interaction between DsrA ncRNA and the 30 S subunit was the focus of our next set of investigations. DsrA binds to the protein Hfq (21, 26), which is associated with ribosomes in _E. coli_ cells (28). Hfq interacts with a variety of RNA and DNA sequences (29, 30). Western blotting analysis using Hfq antisera (the kind gift of Gisela Storz) demonstrated no detectable levels of Hfq in either 30 S ribosomal subunit or 70 S ribosome preparations (data not shown), strongly suggesting that binding of the DsrA to the 30 S subunit resulted from an interaction of the ncRNA with 16 S rRNA or small subunit ribosomal proteins.

To determine which part of the DsrA ncRNA was involved in binding to the 30 S subunit, we constructed two different DsrA fragments. DsrA-(1–34) includes the sequence that forms the first stem loop (nucleotide 1–34) and contains the hybridization sequence to RpoS mRNA (14). DsrA-(23–87) contains the sequences (nucleotides 23–87) that form the second and third stem loops and the Hfq binding domain of DsrA (26). DsrA-(1–34) can bind to 30 S subunits (K_{30S} 0.2 × 10^{7} M⁻¹); however, it has weaker affinity than full-length DsrA (Table I). DsrA-(23–87) shows some affinity to the 30 S subunit, but with our renaturation conditions less than 5% of the total RNA participated in 30 S subunit binding leading to significant error in the quantitative determination of the binding affinity.

**RpoS mRNA-30 S Ribosomal Subunit Interactions**—We measured the affinity of the 30 S subunit to RpoS mRNA using standard nitrocellulose filter binding assays. Binding curves were fit to a single site binding isotherm, and we found that K_{30S} for RpoS mRNA complex formation, was similar to that measured for other mRNAs and was ~2 × 10^{7} M⁻¹ for mRNA renatured in standard renaturation buffer, see Table I for data and Fig. 3 for a typical binding isotherm. The affinity for this mRNA fragment to the 30 S subunit was the same as the affinity measured for longer fragments (data not shown).

The affinities of RpoS mRNA and DsrA noncoding RNA for 30 S subunits were similar to each other. To determine whether they compete with each other for binding to the 30 S subunit, radiolabeled RpoS mRNA was incubated with 30 S subunits and increasing concentrations of either unlabeled RpoS or DsrA competitor RNA. Both the RpoS mRNA and the competitor RNAs were renatured independently as described under “Experimental Procedures.” The labeled RpoS mRNA and 30 S subunits were allowed to equilibrate with competitor RNA and then were filtered. No competition for 30 S subunit binding was observed with the DsrA competitor (Fig. 4A), although, as expected, unlabeled RpoS mRNA was an effective competitor for 30 S subunit binding (Fig. 4B). Similar competition experiments with labeled DsrA, 30 S subunits, and unlabeled tRNA{\textsuperscript{Met}} over the concentration range of 0 to 1 μM indicated tRNA{\textsuperscript{Met}} does not compete with radiolabeled DsrA for binding to the 30 S subunit (Fig. 4C). These results suggest that DsrA does not bind to either the mRNA- or tRNA-binding sites of the 30 S subunit.

**Regulation by Increasing Ribosome-binding Site Accessibility**—Hybridization of DsrA to RpoS mRNA has been postulated to stabilize mRNA conformations that are likely to be more translationally active, thereby inducing translation during osmotic stress (16–18). In order to examine the effect of DsrA hybridization on the extent of 30 S-RpoS mRNA complex formation, we examined whether DsrA RNA could form a complex with RpoS mRNA _in vitro_. We found only a low extent of hybridization of DsrA ncRNA to RpoS mRNA renatured under standard conditions (data not shown). The RNA-binding protein Hfq is necessary for DsrA to affect _rpoS_ translational efficiency _in vivo_ (21). Hfq has been shown to facilitate RNA-RNA interactions and may be required to reduce the effects of competing secondary structures in the DsrA ncRNA and in the RpoS mRNA to allow effective base pairing of the molecules to each other (23, 25, 31).

We designed a DNA antisense oligonucleotide to simulate the effects of hybridizing a smaller region of DsrA to RpoS mRNA. In designing the AS-(1–16) oligonucleotide, we desired...
to maximize the interaction of the oligonucleotide with the mRNA to maximize possible effects on regulation. AS-(1–16) is perfectly complementary to nucleotides 1–16 of the RpoS mRNA structure (shown in Fig. 1A), which is known to hybridize with DsrA in vivo (14). By using these gel mobility shifts, we observed that AS-(1–16) forms a hybrid complex with renatured RpoS mRNA (Fig. 5A, lanes 1 and 2) with \( K_{\text{d}} = 1 \times 10^{-5} \text{M} \) (Table II). We examined whether this antisense oligonucleotide would influence the accessibility of the ribosome-binding site to 30 S subunit binding. Accessibility of the ribosome-binding site was measured by the ability of the RpoS mRNA to hybridize to a radiolabeled DNA oligonucleotide, AS-(101–115), complementary to nucleotides 101–115 of the ribosome-binding site. We renatured the RpoS mRNA in the presence (or absence) of AS-(1–16), and we then probed the accessibility of the ribosome-binding site with AS-(101–115). We used non-denaturing gel electrophoresis to detect hybrid complexes containing AS-(101–115). By kinetically trapping the population of renatured mRNA molecules, we ensured that the differences in the extent of hybridization with the DNA probe resulted from differences in the distribution of structures formed during renaturation. We observed formation of a band that migrates more slowly than the band formed from the RpoS mRNA:AS-(101–115) binary complex (Fig. 5A, compare lanes 5 and 8). This slower migrating band provides evidence for formation of a ternary complex involving RpoS mRNA simultaneously bound to both AS-(101–115) and AS-(1–16) (Fig. 5A, lanes 3–5). Renaturation of RpoS mRNA with AS-(1–16) resulted in increased binding (1.2–1.8-fold increase) of AS-(101–115) to the mRNA (Fig. 5B), demonstrating that the disruption of intra-molecular mRNA-mRNA base pairs involving nucleotides 1–16 of the mRNA increased accessibility of the ribosome-binding site. The affinity of the ribosome-binding site probe AS-(101–115) to RpoS mRNA in the absence of AS-(1–16) was \( K_{\text{d}} = 2.5 \pm 0.6 \times 10^{-6} \text{M} \) and \( 5.9 \pm 2.1 \times 10^{-6} \text{M} \) for RNA renatured in the presence of AS-(1–16) (see Table II). Hybridization of RpoS mRNA to AS-(1–16) increased the RpoS mRNA ribosome-binding site accessibility.

**Effects of an Antisense DNA Oligonucleotide Mimicking DsrA**

**Binding on the Interaction of RpoS mRNA 30 S Ribosomal Subunit Interactions**—The increased accessibility of the ribo-
some-binding site resulting from hybridization of the RpoS mRNA to AS-(1–16) suggested a mechanism of translational induction. In a competitive model of translational regulation by secondary structure, the ribosome-binding site must be unfolded to interact with the 30S subunit (32). If hybridization of DsrA shifts the stability of the mRNA to favor a larger fraction of translationally active molecules, then we would expect to observe that a larger fraction of our total mRNA interacts with saturating concentrations of 30S subunits when the mRNA is hybridized to the DsrA-like AS-(1–16) oligonucleotide than when the mRNA is renatured in its absence. We postulated that the increased accessibility of the ribosome-binding site correlates with an increased fraction of translationally active mRNA. To investigate this possibility, we renatured radiolabeled RpoS mRNA with excess AS-(1–16) (0.2–2.0 μM) and used the hybrid complexes in 30S equilibrium binding assays. We observed that hybridization of RpoS mRNA with an excess of the AS-(1–16) oligonucleotide resulted in an increase in the amount of total mRNA retained on the nitrocellulose filters at saturating 30S subunit concentrations (Fig. 6, A and B). These results suggest that AS-(1–16) hybridization to nucleotides 1–16 of the RpoS mRNA increases the fraction of mRNA that is translationally active. This effect may be very significant in translational induction.

**Divalent Cations Affect Ribosome-binding Site Accessibility and Fractional Retention of RpoS mRNA**—To understand better the effects of changing RNA structure on translational regulation, we examined the effects of altering the concentrations of divalent cations present during RpoS mRNA renaturation on the ribosome accessibility, on the equilibrium affinity constant for 30S subunit binding, and on the fraction of mRNA participating in translational initiation. In all of these experiments, the concentration of divalent cation is varied only during renaturation of the mRNA and is subsequently normalized to identical standard concentrations at 0°C prior to hybridization or binding assays. We have observed that renaturation of the mRNA in the presence of 40 mM Mg2+ alters the extent of AS-(101–115) hybridization to the mRNA relative to the levels measured for renaturation in the absence of Mg2+ (Fig. 7, A and B). Results of several sets of experiments indicate that

\[ K_{(101–115)} = 2.5 ± 0.6 \times 10^6 \text{ M}^{-1} \text{ for RNA renatured without Mg}^{2+} \text{ and } K_{(101–115)} = 0.6 ± 10^6 \text{ M}^{-1} \text{ for RNA renatured with Mg}^{2+} \text{ (see Table II). These results strongly indicate the formation of different final folded structures for the RpoS mRNA when renatured under different conditions, and that the ribosome-binding site is accessible for ribosome binding in a larger fraction of RpoS mRNA when it is renatured in the absence of Mg2+.}

Increasing the amount (from 0 to 40 mM) of Mg2+ cations or physiologically relevant putrescine2+ cations present during RpoS mRNA renaturation results in a slight (~20%) reduction in the binding affinity of the 30S subunits for the RpoS mRNA (see Table I). We observed in our binding assays that when the mRNA is renatured in the presence of increasing concentrations of Mg2+ (or putrescine2+ (not shown)), the fraction of the total mRNA that is retained on the filters at saturating 30S subunit concentrations decreases. A plot of fractional retention of total mRNA versus Mg2+ concentration present during mRNA renaturation buffer is shown in Fig. 6B.

**Fractional retention depends on RpoS mRNA renaturation conditions.** At saturating concentrations of 30S subunits (determined from equilibrium binding isotherms not shown), the fraction of total radiolabeled mRNA bound to the filter (fractional retention) was determined. The fractional retention of the mRNA increases with increasing concentrations of AS-(1–16) added during renaturation (A) and decreases with increasing concentrations of Mg2+ in renaturation buffer (B).

Fractional retention depends upon the fraction of mRNA participating in binding and the efficiency of retaining complexes on the nitrocellulose filters. Because the solution conditions present during 30S subunit-RpoS mRNA complex formation and filtration are identical for all reactions, these differences in filter retention must reflect structural/stability differences in the mRNA induced during renaturation. The divalent cation concentration in the renaturation buffer could affect the partitioning of the mRNA into structures that are either translationally active, participating in 30S binding, or translationally inactive. In this model, the percentage of the total mRNA that binds the 30S subunit is different for mRNA renatured under different conditions. Alternatively, different renaturation conditions may allow the same percentage of the RpoS mRNA to bind 30S subunits but alter some subtle aspect of the mRNA structure that affects the ability of a 30S mRNA complex to “stick” to the filter.

We used competition assays to distinguish between these possibilities. If the renaturation conditions affect the ability of a complex to stick to the filter, then the effectiveness of the competition for binding to 30S subunits with the same concentration of unlabeled RpoS mRNA will be independent of the renaturation conditions of the unlabeled RpoS mRNA. However, if different renaturation conditions yield different percentages of RpoS mRNA competent to bind 30S subunits, then the renaturation condition of the unlabeled RpoS mRNA will determine the extent of competition of a given concentration of unlabeled RpoS mRNA with radiolabeled mRNA for 30S subunit binding. When renaturation affects the partitioning of active and inactive structures, renaturation conditions that increase the concentration of active RpoS mRNA structures (and yield the highest filter retention) also yield the most effective competition. Unlabeled mRNA renatured in standard renaturation buffer more effectively competes with radiolabeled mRNA for binding to 30S subunits than mRNA renatured in renaturation buffer containing 40 mM Mg2+ (Fig. 4B),

**Table II**

| RNA            | Renaturation buffer (RB) | Oligonucleotide binding affinity |
|----------------|--------------------------|----------------------------------|
| RpoS mRNA     | 0 mM Mg2+                | \( K_{(101–115)} = 2.5 ± 0.6 \times 10^6 \text{ M}^{-1} \) |
| RpoS mRNA     | 0 mM Mg2+                | \( K_{(1–16)} = 9.5 ± 4.5 \times 10^5 \text{ M}^{-1} \) |
| RpoS mRNA     | 40 mM Mg2+               | \( K_{(101–115)} = 0.6 ± 10^6 \text{ M}^{-1} \) |
| RpoS mRNA     | 0 mM Mg2+ with AS-(1–16) | \( K_{(101–115)} = 5.9 ± 2.1 \times 10^6 \text{ M}^{-1} \) |
of RpoS mRNA participating in forming 30 S-mRNA “pre-initiation” complexes and provides evidence for a competitive model of regulation.

30 S Subunits Bind the Non-coding RNA DsrA—30 S ribosomal subunits bind to the ncRNA DsrA specifically, with an affinity comparable with that of many mRNA molecules for 30 S subunits. DsrA contains three stem loops (14, 16, 34). The (5') stem loop contains the sequences that hybridize to RpoS mRNA (14, 16, 34). The 3' sequences are necessary for the interaction of DsrA with the protein Hfq (26). Hfq is also involved in regulating translation of RpoS mRNA. We investigated which part of the DsrA molecule was responsible for interacting with 30 S subunits. A weak interaction (K_{ds} \sim \text{mM}) is observed between the 30 S subunit and the first 34 nucleotides of the DsrA sequence that forms the first stem loop. However, low filter retention of 30 S subunit-RNA complexes prevented us from accurately measuring the affinity of 30 S subunits to an RNA transcript containing the 3' end sequences (nucleotides 23–87) of DsrA (data not shown). These results indicate that the full affinity of DsrA for the 30 S subunit (K_{ds} \sim \text{mM}) may depend upon proper folding of secondary and tertiary structure of the entire DsrA molecule.

The translational regulator protein Hfq has been shown to associate with 70 S ribosomes (28) and interacts with DsrA (21, 26). We expected that Hfq might be associated with our 30 S subunits and be involved in DsrA ncRNA binding. Hfq specifically binds a 3' domain in DsrA (26) and has been shown to interact strongly with DNA sequences (29, 30). Western blot analysis using Hfq antiserum demonstrates it is not present at detectable levels in our 30 S subunit preparations and therefore is not responsible for the interaction of the molecules with the 30 S subunit in our in vitro assays.

DsrA binds to 30 S subunits with an affinity comparable with that of RpoS mRNA to the ribosomal subunit. We propose that RpoS mRNA and DsrA ncRNA bind to different sites on the 30 S subunit. In competition experiments between unlabeled DsrA and radiolabeled RpoS mRNA, 30 S subunits and DsrA ncRNA are both in excess over the radiolabeled RpoS mRNA. Because DsrA can bind to both 30 S subunits and to the 5' end of the RpoS mRNA, excess DsrA is required to allow a fraction of DsrA to hybridize the RpoS mRNA and the rest to be available for 30 S subunit binding. If DsrA and RpoS mRNA bind to the same site of the ribosomal subunit, then at high concentrations of DsrA, RpoS mRNA will be displaced from the 30 S subunit. We do not observe this. The extent of RpoS mRNA bound to the 30 S subunit does not decrease even at 400 nM DsrA, suggesting that molecules are not competing for binding at the same site on the 30 S subunit. In similar competition experiments, we observed that tRNA^{34}_{Met} does not compete with DsrA for binding to 30 S subunits, suggesting that DsrA is not binding to the tRNA-binding site in the 30 S subunit either.

The interaction of the mRNA with the 30 S subunit is stabilized in large part by interactions between the Shine-Dalgarno sequence on the mRNA and the anti-Shine-Dalgarno sequence on the 3' end of the 16 S rRNA (32). Additional stabilizing interactions arise through contacts with ribosomal protein S1. We examined whether the DsrA ncRNA had any short regions of complementarity to the 16 S rRNA (nucleotides 606–612) and nucleotides 6–12 of DsrA with nucleotides 634–640 of the 16 S rRNA. The extent of proposed base pairing between DsrA and the 16 S

**DISCUSSION**

Early models of translational regulation of rpoS predicted that changes in mRNA structure resulting from bacterial exposure to stress would influence the accessibility of the ribosome-binding site to ribosomes and therefore translational efficiency (3, 19). The non-coding RNA molecule DsrA has been implicated in translational regulation of rpoS expression (15, 33) as a factor influencing the ribosome-binding site accessibility. In this work, we describe our observation that the non-coding RNA DsrA binds to 30 S subunits. We demonstrate that the ribosome-binding site accessibility is modulated through changes in divalent cation concentration during mRNA renaturation as well as the presence of a DsrA-like antisense oligonucleotide that binds to nucleotides 1–16 of RpoS mRNA. The accessibility of the ribosome-binding site correlates with levels supporting the idea that increasing the concentration of divalent cation in the renaturation buffer decreases the fraction of translationally active mRNA molecules.

**FIG. 7. Renaturation of RpoS mRNA in the presence of 40 mM Mg^{2+} decreases ribosome-binding site accessibility.** RpoS mRNA was renatured in renaturation buffer in the presence or absence of 40 mM Mg^{2+}. The renatured mixture was diluted in renaturation buffer on ice and incubated for 25 min with the AS-(101–115) DNA oligonucleotide probe for ribosome-binding site accessibility. Samples were loaded on an 8% nondenaturing TBE gel. A, representative gel is shown here. Lane 1 shows the AS-(101–115) radiolabeled probe. Lanes 2–6 contain increasing concentrations of RpoS mRNA renatured in standard renaturation buffer without added Mg^{2+}. Lanes 7–11 contain increasing concentrations of RpoS mRNA renatured in standard renaturation buffer containing 40 mM Mg^{2+}. Final RpoS mRNA concentrations are as follows: 0.002, 0.006, 0.018, 0.090, and 0.18 \mu M, respectively. B, quantification of this gel is described by the graph. Bands were excised from the dried gel and quantitated in a scintillation counter. The fraction of RpoS mRNA bound by the AS-(101–115) probe. K=(101–115) = 1.9 \times 10^6 \text{M}^{-1} \text{for RpoS mRNA renatured without Mg}^{2+} \text{and with grey) 40 mM Mg}^{2+} \text{was compared at each RpoS mRNA concentration. We observe that renaturation with added 40 mM Mg}^{2+} \text{decreases the fraction of RpoS mRNA bound by the AS-(101–115) probe. K-(101–115) < 0.6 \times 10^6 \text{M}^{-1} \text{for RpoS mRNA renatured with Mg}^{2+}.}
rRNA in these and several other regions is comparable with the base pairing between Shine-Dalgarne sequences with other mRNA sequences, which may account for the similarity in affinity between 30 S subunits for DsrA and for typical mRNA messages. Although a sequence (or sequences) of the rRNA has not been ruled out in DsrA binding, it is possible that ribosomal proteins specifically interact with DsrA.

Hybridization of RpoS mRNA to an Antisense DNA Oligonucleotide Increases Accessibility of the Ribosome-binding Site—At low temperatures, the presence of the non-coding RNA DsrA increases translational efficiency of RpoS mRNA and is required for full induction of rpoS expression following osmotic shock (15). Hybridization of the ncRNA has been proposed to stabilize a structure of RpoS mRNA that is efficiently translated (14, 16, 18). In the translational induction model, DsrA binds to an upstream RpoS mRNA sequence, preventing specific intra-molecular base pairing interactions in a helix containing the ribosome-binding site. Loss of the intra-molecular base pairs is expected to result in a locally single-stranded ribosome-binding site that is more readily translated than the normal repressed structure.

In vitro work has demonstrated interactions between the non-coding RNA DsrA and a region of the RpoS mRNA that overlaps nucleotides 1–16 in our transcript (14). We observe that the affinity of DsrA for RpoS mRNA was low at 0 °C (data not shown). In vivo it has been shown that regulation by DsrA requires the presence of the protein Hfq. Hfq has been shown to facilitate RNA-RNA interactions and has been implicated in unfolding RNA structures that prevent formation of binary RNA complexes (11, 20–23, 25, 35). Both DsrA and RpoS mRNA are proposed to have extensive secondary structures in the regions that complement each other (14, 16, 34). The interaction of DsrA with RpoS mRNA requires melting out secondary structure in both DsrA and RpoS and, therefore, may be thermodynamically or kinetically unfavorable in the absence of other factors (such as the protein Hfq). Even if DsrA had no secondary structure of its own, it is not perfectly complementary to the RpoS mRNA leader sequence and therefore has a lower affinity for the mRNA than a perfectly complementary sequence would have. In order to make a stronger hybrid complex and to eliminate the requirement for denaturing the DsrA structure, we designed an oligonucleotide, AS-(1–16), that perfectly complements the RpoS mRNA sequence. The effect of increasing intermolecular complementarity and decreasing self-hybridization is that we have made a molecule that hybridizes to renatured RpoS mRNA with an affinity of ~9.5 × 10^6 M^-1 at 0 °C. We used this synthetic DNA oligonucleotide, AS-(1–16), to determine whether its hybridization to RpoS mRNA increases the accessibility of the ribosome-binding site. As expected, we observed an increase in the ability of the AS-(101–115) probe to interact with the ribosome-binding site when RpoS mRNA is hybridized to the AS-(1–16) DNA oligonucleotide.

Ribosome-binding Site Accessibility Correlates Well with the Fraction of mRNA Participating in 30 S Subunit Binding—In determinations of the equilibrium affinity of 30 S subunits for RpoS mRNA, we find that less than 50% of the total RNA interacts with 30 S subunits at saturating concentrations. These data provide evidence for two functionally distinct fractions of RpoS mRNA. One fraction of the mRNA (up to about 50% of the total mRNA in our assays) can bind 30 S subunits to form complexes retained on a nitrocellulose filter or can compete with radiolabeled mRNA to bind 30 S subunits. We designate this fraction of the mRNA to be functionally “translationally active.” The other mRNA fraction is designated “translationally inactive.” It either does not bind to 30 S subunits in the concentration range (0–1.0 μM) we examined or it forms 30 S complexes that rapidly dissociate during the filtration and wash steps of our assay. Controlling the composition of the renaturing buffer of the mRNA can modulate the equilibrium distribution of the two fractions. In the experiments shown here, we have allowed the renaturing mRNA to equilibrate at 42 °C. The rates of interconversion between the “active” and “inactive” forms are greatly reduced by placing the reactions on ice. Differences in the fraction of total mRNA retained on the filter as well as differences in the accessibility to the ribosome-binding site suggest that there are differences in the mRNA structure that persist from renaturation until binding over varied incubation times (15–90 min) indicating that interconversion between different structures is significantly reduced at 0 °C. When the reactions are incubated at 42 °C rather than at 0 °C, we observe a greater extent of hybridization of the RpoS mRNA to AS-(101–116) (data not shown), indicating that at high temperatures, the interaction of AS-(101–115) with the renatured mRNA can shift the equilibrium distribution of mRNA structures. At low temperatures, the mRNA is kinetically trapped so that the inactive form cannot be converted to the active form. Two (or more) populations of RNA structures with different chemical and functional properties is not unusual and has been reported for RNAs as diverse as mRNAs (36) and ribozymes (37).

We observe that renaturation conditions resulting in high levels of AS-(101–115) hybridization to the RpoS mRNA also increase fractional retention of RpoS-mRNA-30 S subunit complexes. These renaturation conditions result in mRNA that is a more effective competitor with radiolabeled RpoS mRNA for 30 S subunits. Renaturation with AS-(1–16) results in a 30–50% increase in the accessibility of the ribosome-binding site and up to an additional 30% of the mRNA participating in binding 30 S subunits determined by fractional retention data. These results are consistent with an interpretation that a ribosome-binding site that more easily interacts with single-stranded DNA is also efficient at 30 S subunit binding and translational initiation.

Divalent Cation Concentration Influences Structure of the mRNA—We have explored whether the accessibility of the ribosome-binding site is modulated by exposure of the mRNA to different divalent cation concentrations. By using the antisense DNA oligonucleotide AS-(101–115) as a probe, we observed that the accessibility of the RpoS mRNA ribosome-binding site is greater when the mRNA is renatured in standard renaturation buffer than when the mRNA is renatured in buffer containing 40 mM Mg^2+. A small reduction in the affinity of 30 S subunits for RpoS mRNA renatured in the presence of 40 mM Mg^2+ or 40 mM putrescine^2+ was observed. Analysis of filter retention data and competition data indicates that renaturing the mRNA in the presence of 40 mM Mg^2+ results in a 30–35% decrease in the amount of the mRNA that is binding at saturating 30 S subunit concentrations relative to that for mRNA renatured in standard buffer. These data again support our conclusion that increased ribosome-binding site accessibility correlates with an increased fraction of mRNA participating in translational initiation events.

Translational Repression and Induction—Translational regulation of rpoS appears to be consistent with a competitive (also called “displacement” (38, 39)) model of regulation. Although small differences are detected in the affinity of both 30 S subunits and AS-(101–115) for the differentially renatured mRNA forms (see Table I), the primary effect of altering renaturation conditions appears to be to change the fraction of the mRNA participating in translational initiation events.

Translational efficiency is evaluated from the rate of protein synthesis and may be proportional to the concentration of a
stable ternary “Initiation Complex” composed of initiator tRNA\(^{30S}\)\(^{Met}\), the 30 S ribosomal subunit, and the mRNA (32, 38, 39). Formation of this stable ternary complex involves two mechanistic steps: 1) the reversible formation of a pre-initiation complex stabilized by base pairing of the conserved Shine-Dalgarno sequence on the mRNA and its complement in the 16 S rRNA, followed by 2) an irreversible conformational change in the pre-initiation complex to form the stable ternary initiation complex, which is stabilized by codon-anticodon base pairing (Fig. 8) (32).

In a competitive model of translational repression, an mRNA structure forms that prevents the 30 S subunit from interacting with the ribosome-binding site of the mRNA (32, 38, 39). In this mechanism, only a fraction of the total mRNA molecules, those with an accessible ribosome-binding site, participate in the formation of a pre-initiation complex, and therefore in translational initiation. Under conditions of thermodynamic control, the distribution between mRNA molecules available to productively participate in initiation is determined by the equilibrium constant between these active and inactive structures (32, 38). We have no evidence that translational regulation is under kinetic control (39), and therefore we are assuming that the simpler model of thermodynamic control applies to repression of rpoS expression. In this model, the level of gene expression, \(E\) (measured in \textit{in vivo} as the relative rate of protein synthesis and \textit{in vitro} as the fraction of mRNA binding 30 S subunits), can be described by Equation 1,

\[
E = \frac{K_{\text{off}}[30S]}{K_r + K_{\text{off}}[30S]} \quad \text{(Eq. 1)}
\]

which relates the value of expression, \(E\), to the equilibrium constant of unfolding the mRNA into an inactive structure (\(K_r\)) (32). In this model, \(K_{30S}\) is the equilibrium binding constant of the 30 S subunit to the active mRNA conformation(s). Our data indicate that the different renaturation conditions (different divalent cations and the presence of AS–(1–16)) result in different \(K_r\) values. Our results suggest that DsrA binding or a decrease in divalent cation concentration in the cell mediated by exposure to high external osmolarity reduces the extent of intramolecular base pairing of the ribosome-binding site in RpoS mRNA. This reduction in \(K_r\) facilitates RpoS mRNA interactions with the 30 S subunit (and/or other regulatory factors) to induce translational initiation.

We applied the thermodynamic competitive model to our \textit{in vitro} fractional retention data using the value of \(K_{30S}\) we measured (2 \times 10^7 M\(^{-1}\)) and a 30 S subunit concentration (8 \muM) expected to be physiologically relevant (32). The analysis suggests that the difference between fractional retention for mRNA renatured in the presence and absence of 40 mM Mg\(^{2+}\) is explained by a value of \(K_r\) in renaturation buffer containing 40 mM Mg\(^{2+}\) that is 1.6-fold larger than \(K_r\) value in standard renaturation buffer and a free energy difference of 300 cal/mol between the RNA samples. (Small differences in our estimates of \(K_{30S}\) (over the range of values observed at low and high Mg\(^{2+}\) concentrations) did not significantly change the differences in \(K_r\) observed.) Hybridization of 0.2 \muM or 2.0 \muM AS–(1–16) to RpoS mRNA results in a destabilization of the mRNA structure by another 470 or 960 cal/mol, respectively, assuming that the affinity of 30 S subunits to the mRNA is not significantly altered by the hybridization (see Table III).

We also used this approach to analyze \textit{in vivo} expression data. Again we assumed that \(K_{30S}\) = 2 \times 10^7 M\(^{-1}\) and that the 30 S subunit concentration is 8 \muM, and that maximal expression, \(E = 1\), corresponded to the most highly expressed variant of rpoS, SD2, as reported in the work of Cunning et al. (11). In \textit{vivo}, expression of the wild-type sequence was reduced to 17% of maximal (i.e. SD2) expression under normal growth conditions and to 41% under osmotic shock conditions (11). Sledjeski and co-workers (21) found that a DsrA mutant decreased expression from wild-type cells grown under normal growth conditions almost 5-fold (to ~4% of SD2 expression).

By using the \textit{in vitro} expression data, we predict that the RpoS mRNA structure (in the absence of Hfq) formed during osmotic stress is more open (less stably folded). We predict that the value of \(\Delta G\) is ~760 cal/mol higher than for the mRNA under normal growth conditions (see Table III). Osmotic stress reduces the intracellular concentration of divalent cations and leads to a change in \(\Delta G\) that is in the same direction as that predicted from \textit{in vitro} data for RpoS mRNA folded in the presence and absence of Mg\(^{2+}\) in the absence of any transacting factors. In the absence of functional DsrA, where rpoS expression is reduced, we predict an increase in the stability of the translationally inactive mRNA conformation of 960 cal/mol from \textit{in vivo} data, similar to our estimates from the \textit{in vitro} data. Although our \textit{in vitro} conditions are quite dissimilar to the \textit{in vivo} cytoplasm, our data suggest that at least part of the osmotic induction of RpoS mRNA translation may be regulated simply by changes in cytoplasmic ion concentration. Binding of DsrA to the mRNA clearly has a similar enhancing effect on the fraction of mRNA participating in translational initiation both \textit{in vivo} and \textit{in vitro}. The effects (in addition to the effects of
other regulatory factors) result in induction of the *rpoS* expression.

Both Hfq and DsrA have been implicated in regulating osmotic induction of RpoS mRNA translation. However, in the absence of the trans-acting regulatory factors Hfq and DsrA, *in vivo* translation of RpoS mRNA can be partially induced by osmotic shock (11, 13). During osmotic upshock, the composition of ions in the cytoplasm of *E. coli* is greatly altered: K⁺ concentration increases 4-fold from 0.2 to 0.8 mM and concentrations of the divalent cation putrescine decrease from 0.05 to 0.01 M (4, 6). Although we are not attempting to reproduce physiological conditions, our experiments demonstrate that reduction in the concentration of the divalent cations putrescine and Mg²⁺ increases the ribosome-binding site accessibility and increases the fraction of mRNA participating in translational initiation. It is plausible that some of the initial translational induction by osmotic shock results from mRNA conformational changes. The effects that we have observed are in the same direction as those predicted to occur during osmotic induction *in vivo*. Divalent cations have been shown, *in vitro*, to affect the secondary and tertiary structures of RNAs over the salt concentration ranges predicted to be relevant *in vivo* (40–43). These conformational changes (affecting both structure and thermodynamic stability of the mRNA) are likely to influence the ability of the mRNA to participate in translational initiation. Although changes in the composition of ions in the cell may affect the structure and stability of other mRNAs *in vivo*, only genes where the rate-limiting step in gene expression is translational initiation caused by an inhibitory mRNA structure will be significantly affected by osmotic shock. Additionally, *rpoS* may be particularly suited to osmotic induction since specific regulatory factors may amplify the effects of mRNA structural and stability changes caused by osmotic stress. van Duin and co-workers have elegantly, and repeatedly, shown that stabilization of secondary structure which sequesters the ribosome-binding site results in decreased translational efficiency (36, 38). Our data support the proposal that the stability and structure of the RNA itself is likely to be modulated as a result of osmotic stress (3) directly influencing translational initiation of the gene.

*Why Does DsrA Interact with 30 S Subunits?—*The function of the interaction of DsrA with the 30 S ribosomal subunit is presently unknown. At 25 °C, full-length DsrA has an extraordinarily long half-life (~2 min at 25 °C), but a truncated form of DsrA isolated from *in vivo* cultures has a half-life of only 5 min (44). The truncated form of DsrA lacks the 5’ sequence that we found binds to 30 S subunits, and we propose that association with the ribosome is responsible for stabilizing DsrA *in vivo*.

RpoS mRNA is tightly folded and requires the action of several trans-acting factors for full translational induction. Base pairing between DsrA and RpoS mRNA is necessary for translational activation by DsrA (14). It has been shown that the effects of RprA, OxyS, and DsrA non-coding RNAs on *rpoS* expression are much greater in the presence of the protein Hfq (9, 11, 20–23). The role of Hfq in global regulation of gene expression increasingly appears to be a facilitator of RNA-RNA interactions that influence rates of translation and degradation of a given mRNA (23–25). Hfq may form a ternary complex with both the ncRNA and mRNA to facilitate their interaction (23), or may transiently alter an RNA structure to promote ncRNA-mRNA interactions (21). The ability of the Hfq protein and an ncRNA to interact with RpoS mRNA, either simultaneously or sequentially, appears to be required in regulating the translation of the wild-type RpoS mRNA sequence. Although one role of the interaction of DsrA with the ribosomal subunit may be to increase the half-life of the ncRNA, we propose that the association of DsrA with the 30 S subunit is mechanistically important in the translational regulation of RpoS mRNA. The interaction between 30 S subunits and DsrA may serve to increase the local concentration of DsrA with Hfq and/or RpoS mRNA. Localizing DsrA to the 30 S subunit may accelerate the interaction of DsrA with ribosome-associated Hfq protein and/or RpoS mRNA, increasing the rate of interactions required for translational induction of RpoS mRNA.

The interaction between DsrA and 30 S subunits may serve to lock the DsrA ncRNA or the 30 S subunit in a conformation that prevents it from prematurely interacting with either Hfq or the RpoS mRNA. The 3’ end of Hfq is required for interactions with Hfq (21). The 5’ end of DsrA binds both RpoS mRNA (14, 17) and weakly to the 30 S subunit. Because Hfq is associated with the ribosome, we speculate that Hfq binds to the 3’ end of the ncRNA and assists in the transfer of the 5’ end of the ncRNA from a binding site on the 30 S subunit to its binding site on the mRNA. DsrA structural changes induced by binding Hfq could decrease the affinity of DsrA to the 30 S subunit and increase the probability of a DsrA-RpoS mRNA interaction. As the ncRNA anneals to the RpoS mRNA, the mRNA unfolds and the ribosome-binding site becomes single-stranded and is immediately stabilized by an interaction with the anti-Shine-Dalgarno sequence of the poising 30 S subunit. As the 30 S subunit clears the ribosome-binding site during initiation, DsrA may re-associate with the 30 S subunit to allow the ncRNA to serve as a catalyst for translational initiation of RpoS mRNA.

Both Hfq and DsrA are required to regulate expression of H-NS protein (14). DsrA binding to hns mRNA decreases the half-life of the hns mRNA significantly (18), possibly as a result of translational repression or through inducing exposure of RNase-sensitive sites. The DsrA sequences involved in binding to the hns mRNA are downstream from the sequences involved in binding RpoS mRNA, suggesting that the interaction of DsrA with the 30 S subunit may have different effects on hns and *rpoS* regulation.

The molecules regulating translational initiation of RpoS mRNA are known, but the choreography of their interactions is just beginning to be characterized. The interactions of DsrA with 30 S subunits must be considered in understanding regulation of translation. Further studies, characterizing the timing and strengths of association of appropriate complexes, may provide insight into the mechanism by which a stress is sensed, and α levels are increased.

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Interactions of the Non-coding RNA DsrA and RpoS mRNA with the 30 S Ribosomal Subunit

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